The Synthesis, Isolation and Evaluation of Novel Anticancer and Antibacterial Therapeutics Derived from Natural Products

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Declaration

No portion of the work referred in this thesis has been in support of an application for another degree qualification of this or any other university, or other institution of learning.

Nicholas Francis Omonga

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Abbreviations

ºC	- Degree Celsius
A-549	- “Human lung carcinoma cells
AGS	- Human Caucasian gastric adenocarcinoma cell
AKT	- Serine/threonine-specific protein kinase
AMPK	- Adenosine 5′-monophosphate-activated protein kinase
B16	- Murine melanoma cell line
Bcl-2	- Apoptosis regulators protein encoded by the Bcl
BCRP	- Breast cancer resistant protein
BMS	- Bristol-Myers Squibb
Capan-2	- Human pancreas adenocarcinoma cells
CC_{50}	- Cytotoxic concentration at $_{50}$%
c-FLIP	- Cellular FLICE (FADD-like interleukine-1β-converting enzyme) inhibitory protein
COX	- Cyclooxygenase
COX-1	- Cyclooxygenase-1
COX-2	- Cyclooxygenase-2
CVB3	- Coxsackievirus B3 enterovirus
d	- doublet
dd	- doublet of doublet
ddd	- doublet of doublet of doublet
dddd	- doublet of doublet of doublet of doublet
DNA	- Deoxyribonucleic acid
DMEM	- Dulbeco’s modified Eagle’s minimum essential medium
DOX	- Doxorubicin
dq	- doublet of quartet
dt	- doublet of triplet
ED_{50}	- Effective dose to produce effect in $_{50}$% of a population
EDTA	- Ethylenediaminetetraacetic acid
eq	- Equivalent
G1	- Astrocytoma cell line
G2/M	- Cell cycle checkpoints in eukaryotes
GMK	- Green monkey kidney cell line
GSTs	- Glutathione-S-transferases
HCT-116	- Human colon carcinoma cells
HeLa	- Human cervix cancer cell line
HepG-2	- Liver hepatocellular carcinoma cell line
HIV/AIDS	- Human immunodeficiency virus /acquired immuno-deficiency syndrome
HIV-1	- Human immunodeficiency virus type 1
HL-60	- Human promyelocytic leukaemia cell line
HPLC	- High performance liquid chromatography
HSV-1	- Herpes Simplex Virus type 1
HT-29	- Human colon adenocarcinoma grade II cell line
Hz	- Hertz
IC_{50}	- Inhibitory concentration for $_{50}$% of viability
IKK	- IκB kinase
IKKβ	- IκB kinase β
IL-2	- Interleukin 2;

iNOS - Inducible nitric oxide synthase
IR - Infrared spectrometry
JNK - c-jun terminal-NH2 kinase
K652 - Human chronic myelogenous leukaemia cell-line
KB - human nasopharyngeal carcinoma
L6 - Rat skeletal myoblasts cell line;
L929 - Murine aneuploid fibro-sarcoma cell line
L929sA - Murine fibro-sarcoma cell line
L-NAME - Nω-nitro-l-arginine methyl ester
LOVO - Human colon adenocarcinoma cell line
LPS - Lipopolysaccharides
LTB4 - Leukotriene B4
m - multiplet
MBC - Minimum bactericidal concentrations
MCF-7 - Breast cancer cell line
McCoy - McCoy's 5A modified medium
MDA-MB-435 - Human breast adenocarcinoma cells
MDCK - Madin-Darby canine kidney cell line
MGC-803 - Human gastric carcinoma cell line
MIC - Minimum inhibitory concentrations
MMP-9 - Matrix metalloproteinase-9
m.p. - Melting point
mRNA - Messenger ribonucleic acid
MRP-2 - Multi-drug resistance protein 2
MRSA - Methicillin resistant *Staphylococcus aureus*
MS - Mass spectrometry
MSSA - Methicillin-sensitive *Staphylococcus aureus*
MTT - 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide
MTS -[3-(4,5-Dimethylthiazole-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt
NCI - National Cancer Institute
NF-κB - Nuclear factor kappa B
NMR - Nuclear magnetic resonance
NO - Nitric oxide
Nrf2 - Nuclear factor 2-related factor 2
NSCLC - Non-Small Lung Cancer
OPN - Osteopontin
p47phox - 47-kilodalton cytosolic subunit of the multi-protein complex known as NADPH oxidase
PABA - Para-aminobenzoic acid
PAF - Platelet-activating factor
PBS - Phosphate buffered saline
PC-3 - Human prostate cancer cell lines
PDGF - Platelet-derived growth factor
PGE2 - Prostaglandin E2
p-gp - Permeability glycoprotein
PI3K - Phosphatidylinositol 3-kinase
PLA2 - Phospholipase A2
PMNs - Polymorphonuclear leukocytes

PPARγ2	- Peroxisome proliferator-activated receptor γ 2
ppm	- Part per million
QR	- Quinone reductase
RAW264	- Murine monocyte/macrophage line derived from ascitic tumour induced with Abelson leukaemia virus
Rf	- Retardation factor
ROS	- Reactive oxygen species
RPR	- Rapid Plasma Reagin
SCF	- Stem cell factor
SGC-7901	- Gastric cancer cell line
SiHa	- Human cervix uteri cancer cell line
SK-28	- Human melanoma cell line
SOD	- Superoxide dismutase
sPLA2	- Secretory phospholipase A2
STAT1	- Signal transducer and activator of transcription 1
SW620	- Human colon carcinoma cell line
Th2	- T helper cells type 2
TLC	- Thin layer chromatography
TNFR1	- Tumour necrosis factor receptor 1
TNF-α	- Tumour necrosis factor-alpha
Topo	- Topoisomerase
TPA	- 12-O-Tetradecanoylphorbol-13-acetate
TRAIL	- TNF-related apoptosis-inducing ligand
Tyrp1	- Tyrosinase-related protein 1
U937	- Human monoblastic leukaemia cell line
PARP	- poly ADP ribose polymerase
CTLA	- Cytotoxic T lymphocytes A
IgG	- Immunoglobulin G
PD-1	- programmed cell death ligand 1
v/v	- volume / volume

Abstract

This study focused on isolation, synthesis, development and evaluation of novel antimicrobial and anticancer agents from natural product. Natural products have found lead applications in the pharmaceutical industry as sources of chemotherapeutics against cancers and infectious diseases. This investigation focussed on isolating sesquiterpene lactones from natural products, and synthesis of flavonoid based anticancer and antimicrobial agents from the flavonoid – chrysin because sesquiterpene lactones and flavonoids are potent anticancer and antimicrobial agents.

In addition to the sesquiterpene lactones – alantolactone (D1) and isoalantolactone (D2), this is the first time costunolide (D3) has been isolated from *Inula helenium*. A series of 8-novel and 10 known Chrysin-derivatives were also synthesised via microwave-assisted O-alkylation of chrysin and other synthetic methods, and their antimicrobial and anticancer activities investigated.

Twenty compounds were screened against eight bacteria and one fungus - *Candida albicans* using Gentamycin and Fluconazole as standard drugs for antibacterial and antifungal studies. 4-((5-Hydroxy-4-oxo-2-phenyl-4H-chromen-7-yl)oxy)butyl acetate (C1), 7-((3,5-dimethylbenzyl)oxy)-5-hydroxy-2-phenyl-4H-chromen-4-one (C2) and 7-(2,4-Dinitrophenoxy)-5-hydroxy-3-phenyl-4H-chromen-4-one (C3) indicated bactericidal inhibition against a broad spectrum of bacteria and *Candida albicans*; *Staphylococcus aureus*, *Escherichia coli, Bacillus cereus* and *Enterococcus faecalis* were the most susceptible bacteria to inhibition by these compounds. MIC of C1 against these microorganisms was 25, 50, 50 and 50 µg / mL, C2: 50, 100, 100 and 100 µg / mL, C3: 12.5, 12.5, 25.0 and 50 µg / mL. MIC values indicated by these compounds were better than those indicated by chrysin and were comparable to those showed by the standard drug – Gentamycin. C1 and C3 also inhibited the growth of the drug-resistant bacteria *MRSA* at doses of 65 and 100 µg / mL respectively. Chrysin derivatives also inhibited the growth of extended spectrum beta lactamase (ESBL) bacteria – *E. coli, K. pneumoniae* and *P. aeruginosa*. C1 and C3 also showed superior antifungal activity against the pathogenic fungus – *Candida albicans* at MIC values of 50 µg / mL respectively compared to the standard antifungal drug – Fluconazole (MIC 125 µg / mL). 7-O-bromochrysin and 7-O-alkylchrysin derivatives of chrysin showed weak antimicrobial activities (MIC ≥ 125 µg / mL) against all bacteria and fungus tested. D1, D2 and D3 also inhibited the growth of *S. aureus* and *B. cereus* at MIC values ≤ 125 µg / mL. The best antimicrobial agent synthesised were C1 and C3. The antiproliferative activities of D1, D2 and D3 were also investigated in hypoxia and normoxia (under hypoglycaemic and hyperglycaemic conditions). In **hypoxia** D1, D2 and D3 elicited a 10 to 55-fold significant antiproliferative activity against leukaemia cell line (K562) in hypoglycaemic conditions with cytotoxicity (IC_{50} values) 0.58, 0.66 and 3.07 µM compared to the antiproliferative activity indicated in hyperglycaemic condition (IC_{50} values – 0.80, 1.18 and 4.77 µM) respectively. In **normoxia** (normal oxygen concentration), the IC_{50} values in hypoglycaemia were 1.80, 1.90 and 6.72 µM for D1, D2 and D3. The IC_{50} values in hyperglycaemia were 2.60, 3.29 and 7.15 µM for D1, D2 and D3 respectively. The antiproliferative activities indicated by D1, D2 and D3 under the conditions stated above were better than those indicated by the positive control – Chlorpromazine hydrochloride (CPZ) with IC_{50} values 32.88, 27.05, 9.36 and 16.21 µM respectively. D1, D2 and D3 were selectively cytotoxic against cancer cell lines but not the normal cell line – BEAS-2B. Anticancer activities against colorectal cancer cell lines (HCT 116 and Caco-2) indicated by C1, C2 and C3 (IC_{50} ≤ 2.0 µM for HCT 116, and IC_{50} ≤ 6.0 µM for Caco-2 cell line), were better than those indicated by chrysin and the positive control drug – (CPZ) – IC_{50} 4.07 and 5.9 µM respectively, and IC_{50} 4.84 µM (chrysin) and 5.17 µM (CPZ) for Caco-2 cell line. C1, C2 and C3 also elicited better anticancer activities against leukaemia (K562) at concentrations of 7.27 µM, 5.58 µM and 8.69 µM; and mesothelioma (Mero-14) cell lines at concentrations of 7.65 µM, 26.31 µM and 8.71 µM respectively, compared to chrysin (IC_{50} 13.13 µM for K562 cell line and

45.99 µM for Mero-14 cell line) and CPZ (IC_{50} 7.38 µM for K562 cell line and 13.42 µM for Mero-14 cell line). Similarly, C1 and C2 also elicited significant cytotoxic inhibition against the triple-negative breast cancer cell line – MDA-MB 468 at IC_{50} values 7.49 and 16.59 µM compared to chrysin.

Anticancer activities elicited by long-chain 7-O-alkylchrysin derivatives (C10, C11 and C12) against HCT 116 cancer cells (IC_{50} 16.4, 17.5 and 18.9 µM) was comparable to those indicated by chrysin and CPZ (IC_{50} 5.9 µM). C16 and C17 also elicited good to moderate cytotoxic inhibition against a broad spectrum of the ten cancer cell lines tested. Interestingly, C1, C2 and C3 were selectively cytotoxic against cancer cell lines but not the normal cell line – BEAS-2B. Chrysin was selectively cytotoxic but CPZ killed both cancer and normal cell lines. D1, D2 and D3 also indicated anticancer inhibition against K562 cell lines during hypoxia and normoxia, and during hyperglycaemia and normoglycaemia. Anticancer inhibition during hypoxia and low glucose for D1 and D2 ($IC_{50} \leq 0.66$ µM) was better than the anticancer inhibition elicited in normoxia and low glucose concentrations ($IC_{50} \leq 1.8$ and 1.9 µM respectively).

In this study, novel anticancer and antimicrobial agents which elicited better bioactivity and solubilities compared to standard antimicrobial agents (Gentamycin and Fluconazole), anticancer positive control agent (Doxorubicin) and chrysin (the parent compound) have been synthesised. Furthermore, hyperglycaemia in normoxic condition was associated with rapid cell proliferation in contrast to hypoxia in hypoglycaemic condition. Compounds isolated in this study were more cytotoxic under hypoxic conditions. Costunolide was isolated for the first time from *Inula helenium*. *In-vivo* cytotoxicity studies of costunolide against leukaemia cell line – K562 in mammalian models is necessary. Some modified chrysin-derivatives also indicated significant cytotoxicity *in-vitro*. *In-vivo* cytotoxicity studies using mammalian model is also required. Synthesised chrysin derivatives were selectively cytotoxic against cancer cell lines but were not toxic against normal cell line at $IC_{50} \leq 100$ µM. From the structure–activity point of view, nature and position of the electron withdrawing and electron donating functional groups on the chrysin core might have contributed to the observed antibacterial and anticancer action.

The observed anticancer activity of alantolactone, isoalantolactone, costunolide, C1, C2, C3, C6, C8 and C10 and the antimicrobial activity of C1, C2 and C3 makes them an attractive drug candidate than chrysin and shows that substitution at the 7-OH position of chrysin could enhance the bioactivity of chrysin.

Chapter 1 Introduction

1.1 Cancer; an Overview

1.1.1 What is cancer?

Cancer is a group of multifaceted systems comprising hematopoietic cells (Jadav & Niper-Ahmedabad, 2014; National Cancer Institute, 2014), mesenchymal stem cells, fibroblast, endothelial cells, infiltrating macrophages and heterogeneous cancer cells etc. (Catalano et al., 2013), which interacts individually or with other cells within a nested complex niche, giving rise to cancer. To understand what cancer is, it is important to understand the role of the cell cycle and apoptosis in cancer.

1.1.1.1 The cell Cycle and Apoptosis

In multicellular organisms, homeostasis is maintained as cells continuously proliferate and die. Many researchers have suggested that this regulation is achieved by coupling the cell cycle processes and apoptosis (programmed cell death), by controlling certain regulatory factors within the cell. Manipulation of the cell cycle could either induce or prevent an apoptotic response. This process (apoptotic response) has been recognised for tumour suppression genes such as cyclin-dependent kinases (Cdks), c-Myc, RB and p53 and their regulators (Pucci et al., 2000). These genes may also act to sensitize cells to apoptosis which if unregulated can result in pathological conditions such as neoplasia (Pucci et al., 2000). Apoptosis is a highly conserved mechanism by which eukaryotic cells commit suicide, enabling the organism to eliminate defective and unwanted cells via an ordered process of cellular disintegration which do not induce an inflammatory response (Fathi et al., 2018). Apoptosis could occur during normal cellular function and in diseased condition thus improper regulation of the apoptotic machinery could contribute to disorders such as autoimmune diseases, AIDS, anaemia, stroke, neurodegenerative disorders, viral infections and cancer (De-Silva & Kim, 2018; Häcker, 2017; Wyllie, 1997). Apoptosis can be triggered by signals such as chemotherapeutic drugs, irradiation, oncogenes, bacterial or viral infection, growth factors, tumour necrosis factor and Fas ligand (De-Silva & Kim, 2018; Wyllie, 1997). Although several stimuli and factors, both pathological and physiological can trigger apoptosis, not all cells will necessarily die in response to the same stimuli. For instance, some corticosteroid hormones may lead to apoptotic death in thymocytes while other cells are not affected or even stimulated (Elmore, 2007). Some cells express TNF or Fas receptors which binds to, and crosslink proteins, leading to apoptosis while others may have a default death pathway which must

be blocked by a hormone or growth factor (De-Silva & Kim, 2018). Necrosis (cell death as a result of disease, failure of blood supply or injury) should not be confused for apoptosis as both processes can occur simultaneously, sequentially or independently (De-Silva & Kim, 2018; Zeiss, 2003). Apoptosis is also associated with the type / degree of stimuli. For instance, at high doses / concentrations, stimuli such as radiation, heat etc., could induce necrosis but at low doses, they can induce apoptosis. Apoptosis is an energy dependent and highly coordinated process that involves the activation of caspases (a group of cysteine proteases) and a complex cascade which initiates apoptosis to the final demise of the cells (Elmore, 2007). Activation of caspases leads to the cleavage of certain proteins such as the poly(ADP-ribose) polymerase (PARP), DNA-dependent protein kinase (DNA-PK), topoisomerases and lamins (Pucci et al., 2000). It has been demonstrated that macrophages play a potential role in promoting apoptosis in some tissues; removal of macrophages in eyes of the rat for instance, resulted in the survival of vascular endothelial cells that normally undergo apoptosis in the absence of macrophages (Wood & Martin, 2017; Diez-Roux & Lang, 1997).

The cell cycle is a process whereby duplication of genetic information and cell division occur to give rise to two daughter cells. To avoid insertion of wrong genetic information and incorporation of errors in the genome, the cell cycle is highly regulated by checkpoints within the cell. The checkpoints detect DNA damage, incompletely replicated DNA and misaligned chromosomes. These checkpoints are mediated by the cyclin-dependent kinase inhibitors (CKIs) in interphase and mitosis (Chao et al., 2017).

Genetic information is transmitted from one generation to another via genomic replication during the S-phase and its segregation to form two daughter cells during the M-phase (mitosis). The S and M-phases of the cell cycle are crucially ordered to allow for the correct duplication of the genome without error (Bertoli et al., 2013). The S-phase occurs before the M-phase and the M-phase does not occur until the S-phase is complete. Two preparatory gaps – G1 (separates M from S) and G2 (between S and M). After differentiation, the cell cycle exits the G1 phase and enters a quiescent state referred to as G0 (Pucci et al., 2000).

The events and timings of the cell cycle are monitored by checkpoints at the G1/S boundary in S-phase and during the G2/M phase (Chao et al., 2017). The checkpoints are stimulated in the presence of signals such as growth factors, damage to mitotic spindle or DNA. When this happens, the cell cycle arrest occurring because of activation

of the checkpoint, enables the cell to repair the damage (Chao et al., 2017). Once the damage is repaired, progression of the cell cycle continues. If the damage cannot be corrected or repaired, apoptosis occurs (Chao et al., 2017). Cell cycle progression (Figure 1.1) is effected by a series of mechanisms involving activation and inactivation of the cyclin-dependent kinase (Cdks) which are enzymes belonging to a well-conserved family of serine/threonine protein kinases and their activity is dependent on the presence of cyclins (enzymes responsible for initiation of certain processes of mitosis).

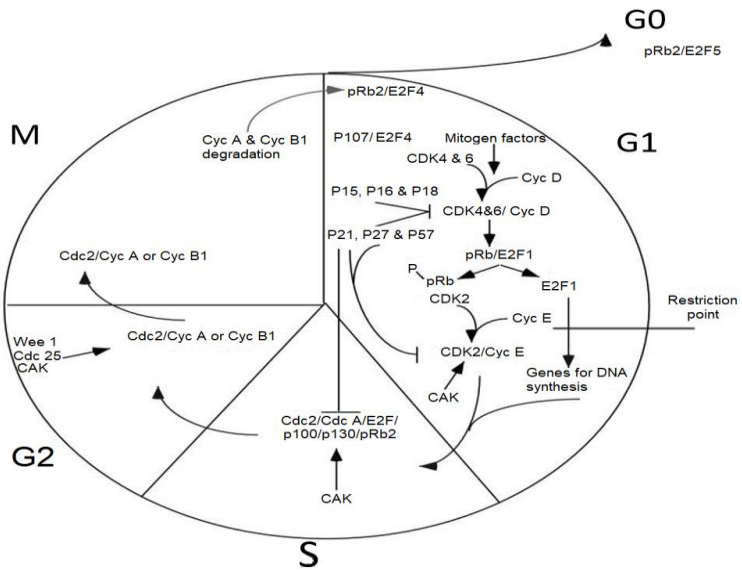


Figure 1.1: Relationship between Cdks and Cdk regulators during the cell cycle (Pucci et al., 2000).

Specific cyclins are abundant in some phases of the cell cycle (where they are needed) and decreases during phases in which they are not needed. During early G1 phase of the cell cycle, Cdk4 and Cdk6 associates with cyclin-D. Phosphorylation of the G1 kinases activates gene transcription necessary for S-phase of the cell cycle (Law et al., 2015). Cyclin D/Cdk complexes are activated during mitosis, allowing the progression of the cell cycle from G0 to G1. On passing the restriction point (R-point), the cells are ready to enter the S-phase which no longer requires the activity of the D/Cdk (Law et al., 2015). Cdk2 is activated by cyclin E during the G1-to-S-phase transition, allowing cyclin A to bind to Cdk2 during S-phase or to Cdk2 in the G2-to-M-phase transition (Law et al., 2015). Cdk-activating kinase (CAK) regulates the Cdk complexes via phosphorylation of the Cdk/cyclin complex (Law et al., 2015). The activity of Cdk is suppressed by phosphorylation of tyrosine and threonine residues whereas the Cdc25 phosphatase family mediates dephosphorylation and subsequent activation of Cdk (MacLachlan et al., 1995). Cdk activity is also regulated by the Cdk-inhibitory subunits (CKIs). The CKIs are classified into two; the inhibitors of Cdk4 (Ink4) and the Cip/Kip families. They provide a tissue-specific mechanism by which progression of the cell cycle is suppressed in

response to intracellular and extracellular signals (Sonawane et al., 2016). The Ink4 consists of four members; p15Ink4b, p16Ink4, p18Ink4c and p19Ink4d. Some of these genes are often deleted or mutated in some human cancers – such as cancers of the lung (Harper & Elledge, 1996; Sonawane et al., 2016). $p21^{Cip1}$, $p27^{Kip1}$ and $p57^{Kip2}$ constitutes the Cip/Kip family which inhibits the Cdks, preventing the transition from the G1-to-S-phase (Harper & Elledge, 1996; Sonawane et al., 2016).

The retinoblastoma protein (pRb) is a tumour suppressor protein which negatively regulates cell growth. This protein is deleted or mutated in certain cancers such as cancers of the prostrate, bone, bladder, breast and lungs (Engel et al., 2015; Riley et al., 1994). The pRb protein also encompasses p107 and pRb2/p130 (Engel et al., 2015). The activities of these proteins are overlapping yet distinct in cell cycle regulation. Phosphorylated form of these proteins actively blocks transcription of genes during the S-phase of the cell cycle. Blockage of gene transcription depends on their ability to bind and actively repress the E2F factors (Engel et al., 2015), which are transcription activators consisting of the DP (Dimerization Partner) family of proteins and the E2F protein (Lam & La-Thangue, 1994). Stimulation of mitogen activates phosphorylation of pRb by G1-phase Cdk (Law et al., 2015), releasing the E2F complex and allowing expression of genes for DNA synthesis (Pucci et al., 2000).

1.1.1.1.1 Mechanisms of Apoptosis

Apoptosis involves a highly complex cascade of energy-dependent molecular events (Figure 1.2). Two pathways; the mitochondrial or intrinsic pathway and the death receptor or extrinsic pathway have been identified.

These pathways are however linked together and influence the activities of one another (Pucci et al., 2000). Another apoptotic pathway involving perforin-granzyme-dependent killing of the cells, and T-cell mediated cytotoxicity has been reported (Igney & Krammer, 2002). The perforin-granzyme pathway induces apoptosis by activating either granzyme A or B. The granzyme A pathway initiates a caspase-independent cell death pathway via single strand DNA damage whereas the granzyme B pathway converges on the execution pathway together with the intrinsic and extrinsic pathways. The cleavage of caspase-3 initiates this pathway, resulting in DNA degradation and fragmentation of the cytoskeleton and nuclear proteins, cross-linkage of proteins, ligand expression for receptors of phagocytic cells and formation of apoptotic bodies which are killed by phagocytic cells (Martinvalet et al., 2005).

Several biochemical modifications exist during apoptosis such as expression of caspases in an inactive proenzyme form which once activated, leads to the activation of other caspases, initiating a cascade of procaspases. Some procaspases can auto-activate or aggregate, leading to a rapid cascade and amplification of the apoptotic signalling pathways (Hengartner, 2000). Caspases are proteolytic in nature, cleaving aspartate residues from proteins (although some caspases are specific to specific amino acids) (Yuan et al., 2016). Once caspases initiation occurs, there tend to be an irreversible commitment towards apoptosis (programmed cell death) (Yuan et al., 2016). Researchers have identified ten major caspases which are classified into initiators (caspases 2, 8, 9, 10), executioners or effectors (caspases 3, 6, 7) and inflammatory caspases (caspases 1, 4, 5) (Yuan et al., 2016; Rai et al., 2005). Caspase-11 (associated with apoptotic regulation and maturation of cytokine during septic shock) and caspase-12 (associated with mediation of endoplasmic-specific apoptotic responses and cytotoxicity by amyloid-β) have been reported. Other caspases such as caspase-13 (a bovine gene) and caspase-14 (whose expression is restricted to embryonic tissues only) have also been reported (Hu et al., 1998; Kang et al., 2002; Koenig et al., 2001; Nakagawa et al., 2000).

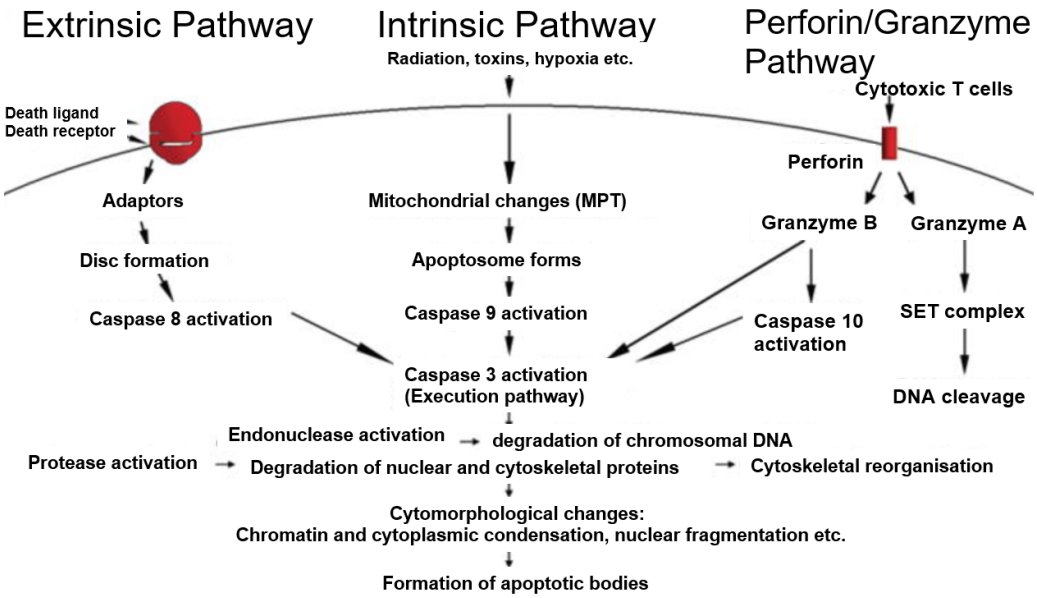


Figure 1.2: Schematic representation of apoptotic events.

The apoptosis pathway comprises of the intrinsic and extrinsic pathway. These pathways require triggering signals for an energy-dependent molecular cascade event. These pathways are activated by initiator caspases – 8, 9 and 10, which in turn activates the executioner caspase-3. Granzyme A operates in a caspase-independent manner during the executioner pathway, resulting in cytomorphological changes such as phagocytosis of the apoptotic bodies by macrophages, neoplastic cells or parenchymal cells; formation of apoptotic bodies and cytoplasmic blebs, chromatin condensation and cell shrinkage (Elmore, 2007).

Flaws with the machinery regulating cell death can give rise to diseases such as cancer, neuro-degenerative diseases (such as Huntington's disease, Alzheimer's disease and Parkinson's disease), ischemia, autoimmune lymphoproliferative syndrome and AIDS (Favaloro et al., 2012), whereas insufficient or excessive apoptosis is responsible for some diseased conditions (Favaloro et al., 2012).

Cancer occur when the mechanisms regulating the cell cycle are dysfunctional with either a decreased removal and/or over proliferation of cells (Favaloro et al., 2012). It has been proven that suppression of apoptosis during carcinogenesis could play an essential role in some cancer development and progression (Favaloro et al., 2012; Kerr et al., 1994). Tumour cells employ several mechanisms to suppress apoptosis such as mutation or down-regulation of proapoptotic protein such as Bax or by expression of antiapoptotic protein such as Bcl-2. Expression of Bax and Bcl-2 is regulated by the p53 tumour suppressor gene (Favaloro et al., 2012). There are evidences that there is an over-expression of Bcl-2 in certain forms of B cell lymphoma and this finding is a strong pointer to the fact that failure of apoptosis gives rise to cancer (Favaloro et al., 2012; Vaux et al., 1988). Evasion of immune surveillance is another method involving apoptosis suppression (Smyth et al., 2001). The death-receptor pathway (perforin/granzyme B pathway) is used by some immune cells such as the natural killer cells and the T-cells to destroy tumour cells (Cheng et al., 1994). Some tumour cells evade immune destruction by decreasing the response of the death receptor pathway to FasL produced by T cells via expression of non-functioning Fas receptor, downregulation of the Fas receptor on tumour cells, expression of Fas ligand on tumour cell surfaces and secretion of soluble Fas receptor that will sequestrate the Fas ligand (Cheng et al., 1994; Elnemr et al., 2001; Liou, 2017; Martinez-Lostao et al., 2015). Fas ligand-mediated counterattack in some tumour cells have been reported (Koyama et al., 2001). This attack depletes the activated tumour infiltrating lymphocytes thus depletion of apoptosis.

Cancers can also result from the alteration of signalling pathways, which leads to dysregulation of apoptosis. The tumour suppressor gene (p53), a transcription factor involved in cell cycle regulation is mostly mutated in tumourigenesis (Mitra et al., 2018). The p53 gene is mutated in over 50 % of all cancers and is responsible for activation of the proteins involved in DNA repair when the DNA is damaged (Mitra et al., 2018). The p53 gene is also involved in regulation of the cell cycle at the G1/S, preventing any further cell cycle activity on recognition of DNA damage and can even initiate apoptosis if the DNA damage cannot be repaired (Mitra et al., 2018). The inability of the p53 gene

regulatory machinery to function properly will result in tumourigenesis (Mitra et al., 2018). Viruses such as the Human papillomavirus (HPV), chemicals and radiation can damage the p53 gene. The Li–Fraumeni syndrome has been reported in patients who inherits only one functional copy of the p53 gene (Mitra et al., 2018) and is characterised by tumour development in early adulthood (Gu et al., 2001).

Another gene associated with tumourigenesis is the ataxia telangiectasia-mutated gene (ATM) which elicits tumourigenesis via the ATM/p53 signalling pathway (Kitagawa & Kastan, 2005; Mitra et al., 2018). Activation of the ATM gene stimulates DNA repair and inhibits the progression of the cell cycle via phosphorylation of p53 (Mitra et al., 2018; Kurz & Lees-Miller, 2004). Phosphorylation of the p53 signals cell cycle arrest at the G1/S checkpoint to allow for DNA repair. This system can be inactivated by epigenetic / somatic alterations and expression of oncogenes such as the HPV, resulting in tumourigenesis (Mitra et al., 2018).

Tumourigenesis also occur from malfunctioning of other cell signalling pathways such as the phosphatidylinositol 3-kinase/AKT pathway. This pathway also regulates other cellular pathways such as cytoskeletal rearrangement, growth and cell proliferation (Vivanco & Sawyers, 2002).

Excessive apoptosis could give rise to many pathological conditions such as cancers, ischaemia, AIDS and neurodegenerative diseases (Favaloro et al., 2012). Inhibition of apoptosis artificially may be key to cancer prevention and therapy. Anti-apoptotic therapy involves stimulation of the PKB/Akt (protein kinase B) pathway, inhibition of Bcl-2 proteins, poly [ADP-ribose] polymerase (PARP) inhibition, caspase inhibition and stimulation of the inhibitors of apoptosis proteins (IAP) (Favaloro et al., 2012). The IAPs are the most important apoptotic regulators because of their involvement in regulation of both extrinsic and intrinsic pathways (Favaloro et al., 2012). In addition to survivin and XIAP (X-linked mammalian inhibitor of apoptosis protein), eight human IAPs have been characterised (Colnaghi et al., 2006; Silke et al., 2002). PARP has a dual role as an enzyme involved in apoptosis, and DNA repair. PARP inhibitors may attenuate inflammation, enhancing the cytotoxicity of anticancer agents pharmacologically (Graziani & Szabo, 2005; Jeswani & Paul, 2017). Veliparib (a new PARP-1 and PARP-2 inhibitor) is used as a chemo-sensitizing drug with additional antitumour activities. This drug accelerates the cytotoxicity of DNA-damaging agents and inhibits DNA repair mechanisms in tumours and has indicated promising results in phase III clinical trials for

metastatic breast cancer, metastatic melanoma, Non-small-cell lung carcinoma (NSCLC), advanced ovarian cancer and triple-negative breast cancer (Jeswani & Paul, 2017).

The problem of cancer has persisted because most cancer treatments cannot target cancer stem cells which are the progenitors of cancers (Pistollato et al., 2015; Zhou et al., 2009). Cancer relapse and increasing resistance of cancer cells to chemotherapy could be traceable to the inability of conventional therapies to destroy cancer stem cells, and therefore the need for novel chemotherapeutic strategies which could target and destroy stem cells (Zhou et al., 2009), and these conventional therapies are often harmful and have life altering side effects. Most conventional drug are poorly absorbed into the cells, and rapidly efflux out of the cells, which is mediated by the ATP binding cassette (ABC) such as glycoprotein P, leading to chemotherapy failure (Hida, et al., 2017; Li et al., 2015). Water soluble drugs on the other hand enter the cell by accompanying transporters and carriers as they move across cell membranes (Dalbeth et al., 2014). This implies that as carriers and transporters uptake into target cells drops, the intracellular drug concentration also drops thus a reduction in drug efficacy (Dalbeth et al., 2014). An increase in expression of target organs and DNA repair is another mechanism where water soluble compounds exert therapeutic effect on cancer cells (Zhang et al., 2017; Liang et al., 2017). Resistance to cancer is associated with regulation of cell cycle, immune responses, programmed cell death, differentiation of cells, DNA mismatch, tissue architecture and replicative aging (Hochberg & Nobel, 2017), and other factors aids the development from host factors and genetic predisposition such as the environment and agents we are constantly exposed to, prolonged infection, age and lifestyle, and this is so because cancer is a complex disease arising from a multifaceted triggers (Catalano et al., 2013; Chen et al., 2017; Merlo et al., 2012; Carillo-Infante et al., 2007).

Cancer relapse occur because stem cells have the characteristics of auto-renewing itself by bypassing the G1/S checkpoint of the cell cycle (Hatfield et al., 2005). This results in development of malignant cancer cells. These cancer cells undergo metastasis, reproduce and become malignant growths (tumour cells) (National Cancer Institute, 2014). Some chemotherapy such as cisplatin, carboplastin etc. can inhibit the growth of some cancer cells such as cancers of the breast, lungs and brain, limiting their growth (Pollyea et al., 2014).

The effects of anticancer agents are potentiated via combinational therapy using liposomes (Hatfield et al., 2005). Liposomes are vesicles of phospholipid bilayers used in

encapsulating both lipophilic and hydrophilic drugs, protecting them from degradation and have been used extensively as nano-carriers for drug delivery (Pandey et al., 2016) and have been successfully used as effective stem cell destruction agents (Hatfield et al., 2005).

The environment a person is constantly exposed to is crucial in cancer incidence. For instance, regular exposure to Bisphenol A (Tse et al., 2017), cigarette smoke, crystalline silica, alcohol and oral contraceptives, as well as exposure to heavy metals, agricultural pesticides and solvents, certain infection and ultraviolet light, has been linked to cancer (Parks et al., 2017).

Viral infection such as hepatitis B, and bacterial infection such as *Mycoplasma pulmonis* can trigger acute localise inflammation which if not regulated by the body could lead to the activation of neoplasm (Carillo-Infante et al., 2007), leading to the release of inflammation causing cells such as isoforms of nitric oxide synthase, nitric oxides and cytokines which are capable of influencing cell proliferation and neo-angiogenesis thus leading to direct DNA damage (Carillo-Infante et al., 2007; Parks et al., 2017).

Genetics is a major cause of cancer as mutation in genetic material occurs spontaneously and is being transmitted from one generation to another. These mutations are often associated with environmental exposure to mutagenic agents and radiation (Stoppa-Lyonnet, 2016).

Chemotherapeutic agents (such as parthenolide) from plants have proven to target cancer stem cell selectively, killing them (Ghantous et al., 2010; Guzman et al., 2005; Pollyea et al., 2014). These findings are very important as it could offer a new insight into the use of plant agents in cancer treatment.

1.1.2 Cancer Epidemiology

Cancer accounts for about 8 million deaths yearly. This equates to about 13% of global mortality from cancer (Cheung-Ong et al., 2013), with colorectal, lung, breast, prostate, stomach and liver cancers accounting for the most commonly diagnosed cancers; these figures are higher in developing countries, especially liver, oesophageal and cervical cancers which are common in developing countries (Merlo et al., 2012). Annually, 1.36 million people are affected by colorectal cancer, which is the 2nd most common cancer in females, and 3rd most common cancer in males and it is treated via local excision, adjuvant chemotherapy, radiation and surgical resection (Moore & Aulet, 2017; Fazeli &

Keremati, 2015; Kim, 2015). In local excision, an area <3cm in diameter is removed and has the advantage of little postoperative complications but a much higher rate of recurrence up to about 21% (Fazeli & Keremati, 2015; Kim, 2015). Colorectal cancer usually results from inflammation in the gut which induces carcinogenic mutagenesis, allowing lymphoid and myeloid infiltration in the colon (Chen et al., 2017). Since colorectal cancer is prevalent in the elderly aged 65 years and above, postoperative chemotherapy became the treatment of choice for colon cancer in 1990s, accounting for 26 % reduction in mortality in patients with stage III colon cancers compared to surgery alone (Kim, 2015). Surgery alongside adjuvant chemotherapy is relatively effective in the treatment of colon cancer but often not applied in patients with colon cancers due to the potential toxicity of the agents to the elderly who are often associated with colon cancers (Kim, 2015). 78% of 55 – 64-year-old received this therapy globally; this figure drops as the age increases with only 11 % of adults aged 85 – 89 years old been given adjuvant chemotherapy (Kim, 2015). Eating habit has been correlated to the prevalence of colorectal cancer; a Mediterranean diet rich in vegetables, fruits, complex carbohydrates, daily consumption of red wine, low consumption of fish and meat is associated with less prevalence of colorectal cancer as these components have chemopreventive effects on colorectal cancers (Farinetti et al., 2017; Wark & Peto, 2017).

Breast cancer occurs when breast tissues, mainly ducts and lobules which are responsible for milk production, develops malignant tumour, with ductal carcinoma in situ (DCIS) accounting for 20% diagnosis of all new cancer cases (Karlsson, 2017; Klevos et al., 2017). Although the exact cause of breast cancer is unknown, risk factors include inheritance of susceptibility genes such as *BRCA2* and *BRCA1*, oestrogen exposure, obesity, alcohol consumption and increasing age (Klevos et al., 2017). The *BRCA2* and *BRCA1* genes located in chromosome 13 and 17 are the key suspects in pathogenesis of breast cancer (Klevos et al., 2017; Lee et al., 2017). Breast cancer is worsened as two-third of women who suffer from breast cancer eventually develops liver metastasis. Others also develop hepatic and bone metastasis (Kenny et al., 2017).

Cancer is a major health concern worldwide, resulting in the death of a quarter of all deaths in many countries. In 2002, cancer accounted for about 7 million deaths worldwide. This is equivalent to 1 in 8 of all deaths. 11 million people were also diagnosed with cancer the same year (Parkin et al., 2005). The number of people diagnosed with cancer is on the increase as the world population keeps increasing and as life expectancy is on the increase and it is estimated that 15 million new cases will be diagnosed every

year by 2020 (Parkin et al., 2005). Of these cancers, lung, breast and colorectal cancers (1.4 million, 1.2 million and 1 million cases) are the most commonly diagnosed cancers. Lung cancer account for 1.2 million deaths, followed by stomach cancer (700,000 deaths) and liver cancer (600,000 deaths) (Parkin et al., 2005). Liver and pancreas cancers have similar mortality rates because of poor prognosis of these cancers. Unlike liver cancer, mortality from breast cancer is much lower due to relatively good prognosis of breast cancer especially in developing countries (Parkin et al., 2005). Incidences and mortality of age-specific cancers such as prostate cancer is falling while other cancers such as lung cancer are on the increase due to increase in cigarette smoking and an increase in mortality rate for cigarette related cancers. On the other hand, death rate from non-smoking related cancers has decreased (Pato et al., 2006).

1.1.2.1 Causes of Cancer

There are multifaceted causes of cancer that can be linked to lifestyle / environment, gene mutation and occupational and environmental carcinogens

1.1.2.1.1 Lifestyle and Environment

Lifestyle and environmental factors responsible for cancer include tobacco, the effects of diet and overweight, reproductive and hormonal factors, viruses, bacteria, and parasites (Karlsson, 2017; Klevos et al., 2017).

1.1.2.1.1.1 Tobacco

Tobacco is a cancer-causing agent (carcinogen). Continuous smoking has been associated with high incidences of lung cancer but remains constant in ex-smokers. The risk increases in people who have been smoking from a young age and continue throughout life. The effect of tobacco smoking is even worse in passive smokers who are constantly been exposed to tobacco smoke (Petra and Wark, 2017). In most developed countries, cigarette smoking among young males was on the increase in the first half of the twentieth century. This led to a surge in the incidence of lung cancer many decades later (Petra and Wark, 2017). Carcinogenic effect of tobacco was previously thought to be restricted to the lung, kidney, bladder, pancreas, and oesophagus. Recently, there are indications that colorectal, myeloid leukaemia, cervix, liver and stomach cancers are also increased by tobacco smoking (Petra and Wark, 2017), thus smoking is an important risk factor for cancer. Smoking causes more death in China where liver cancer is common, while liver cancer is rare in the United States even in smokers (Petra and Wark, 2017).

1.1.2.1.1.2 The Effects of Diet

Cancer epidemiology as a result of nutrition is a complex process. For instance, the risk of cancer in old age may be dependent on diets eaten in early life and not just on current diets. Dietary factors do not show strong and consistent effects that will enable researchers establish them as unequivocal carcinogens. However, consumption of aflatoxin contaminated food and drinking excessive alcohol over a long period have been associated with cancers (Petra and Wark, 2017). During the past two decades, extensive research has indicated that certain diets are associated with cancer development, but opinion still differ on the authenticity of these evidences. In late 20th century, fat and meat where the main focus of nutritional epidemiology as the causes of cancer, while the β-carotene, vitamins A, C, and E, vegetables, fruits and dietary fibre were seen as the protective factors against cancer (Wark and Peto 2017). Recently, researchers are concentrating on processed meat and red meat as risk factors while whole grains (e.g., wheat), vegetables, specific non-nutrient components of fruits, selenium, calcium, vitamin D, B-vitamins and folate are the protective factors (Wark & Peto, 2017). The belief that fatty foods are hazardous, and vegetables and fruits are protective does not seem convincing as previously thought and more mechanisms underlying the role of diet in cancer epidemiology are being investigated (Wark & Peto, 2017). Nevertheless, the role of saturated fat (bad fat) in cancer epidemiology is being reviewed by the World Cancer Research Fund / American Institute for Cancer Research and research findings have indicated that diets rich in n-3 unsaturated fatty acids (present mainly in fishes) is beneficial in preventing cancer (Wark & Peto, 2017). Natural foods rich in antioxidants are beneficial in preventing cancers compared to dietary supplements. For instance, a 12-year randomised trial indicates that administration of dietary supplements of β-carotene did not reduce the risk of cancer, but β-Carotene obtained from dietary carrot proved to be effective in reducing the risk of cancer (Greenwald et al., 2007). Most reports have concluded that a change in the dietary intake from saturated fats to unsaturated fats, fruits and vegetables and dietary fibre might reduce one-third of cancer deaths, and there is a consensus that obesity-associated cancers are avoidable (Wark & Peto, 2017). Another consensus is that cancer is common with obese or overweight people. This evidence is strongest for kidney, gallbladder, endometrium and postmenopausal breast cancers (Calle et al., 1999). Evidence to this is the finding that many obese non-smokers in America are mostly affected by diet associated cancer deaths (Calle et al., 1999). This

finding also indicates that mortality from non-malignant cancers also increase in those who are too fat or too thin. Since obesity is common in Western societies, mortality from weight related cancers may be on the increase as the intake of diets rich in saturated fats increases.

1.1.2.1.1.3 Reproductive and Hormonal Factors

The effect of both exogenous and endogenous hormones and reproductive factors have been linked to ovarian and breast cancers especially the hormonal changes occurring during pregnancy. An important hormone which triggers ovarian and breast carcinogenesis is oestrogen which promotes cell proliferation of both neoplastic and normal breast epithelium (Russo & Russo, 2006). Early child birth, early menopause and late menarche reduces the risk of breast and ovarian cancers (Wark & Peto, 2017). Administration of hormone replacement therapy (HRT) and oral contraceptives is also associated with breast cancer incidence. Administration of HRT is also associated with a transient increase in incidences of endometrial cancer while a combination of progestin and oestrogen HRT increases the risk of breast cancers, but this risk is smaller for endometrial cancers. Administration of oral contraceptives alone is safe, and no ovarian and endometrial incidences have been associated with the use of oral contraceptives (Wark & Peto, 2017). Both HRT and oral contraceptives appears to reduce the risk of colorectal cancer (Wark & Peto, 2017). There is also a correlation between the Western diet and postmenopausal obesity and early menarche which increases the production of endogenous oestrogen. Breast cancer is common in most Western countries (such as Belgium, Denmark and the UK) than in many developing countries (Wark & Peto, 2017).

1.1.2.1.1.4 Viruses, Bacteria, and Parasites

The role of infectious agents in cancer epidemiology globally accounts for about 21 % of all cancers (Parkin, 2006). Over 100 species of human papillomaviruses (HPVs) have been isolated. Analysis of the DNA of sexually transmitted HPVs (constituting a small group) which includes HPV-16, HPV-18 and HPV-45 indicates these viruses are presented in all cervical cancers diagnosed globally (Wark & Peto, 2017). Other HPVs in addition to HPV-16, HPV-18 and HPV-45 have also been isolated in virtually all anogenital cancers globally, and also in cancers of the pharynx and mouth (Wark & Peto, 2017). Vaccines have been developed to curb the effect of some HPVs strains. However, these vaccines are expensive making it difficult for developing countries (with poor cervical

cancer screening facilities) to purchase these vaccines. The effect of these vaccines will be greatest in developing countries (with poor cervical cancer screening facilities), and not in developed countries which have organised cervical screening facilities in place (Wark & Peto, 2017). HPVs vaccines are only effective against some strains of HPVs thus they lack the ability to protect against all HPVs (Wark & Peto, 2017).

The hepatitis B virus (HBV) is also a major agent responsible for hepatic cancer including hepatocellular carcinoma and liver cirrhosis especially in high incidence regions such as the Amazon basin, Sub-Saharan Africa, Southeast Asia and central Asian republics where the carrier rate is over 8 % (Franco et al., 2012). The World Health Organization has estimated that over 2 billion people are infected with HBV and an additional 378 million carriers globally. 620,000 HBV related deaths have been reported globally. It is also estimated that 4.5 million new HBV infections occur yearly globally (Franco et al., 2012). The Mediterranean basin, some Eastern European countries and the Middle East are considered intermediate endemic areas and the carrier rate is estimated at 2 - 8 %. United States, part of South America, Australia and Northern Europe have an HBV prevalence rate of less than 2 % (Franco et al., 2012). Specific co-factors such as malaria (Burkitt's lymphoma in Africa), aflatoxin (liver cancer), smoking (cervix and liver cancers) and salted fish (nasopharynx cancer), in combination with HBV is responsible for cancers stated above (Buell et al., 2005). Therapeutic immunosuppressant causes an increase in incidences of non-melanoma skin cancer and virally induced cancers. Immunosuppressant have also been linked to an increase prevalence of epithelial cancers (Buell et al., 2005). This suggests that unidentified viruses may be responsible for many other cancers as well. *Helicobacter pylori*, a bacterium responsible for chronic gastric bacterial infection has been isolated from stomach cancer, and accounts for about 63 % of stomach cancers and 6 % of all cancers globally. It therefore implies that over 50 % of stomach cancers can be prevented if *H. pylori* is eradicated (Mbulaiteye et al., 2009).

1.1.2.1.2 Occupational and Environmental Carcinogens

Occupational exposure to specific carcinogens such as asbestos and combustion products of coal has caused an increase to certain cancers such as mesothelioma and lung cancers. The effect of occupational exposure to asbestos in the 1940s was only apparent in the 1980s when over 250,000 cases of lung cancer and mesothelioma was reported in Western Europe due to the long latency of the disease (Wark & Peto, 2017).

The tragic effect of asbestos was preventable as the danger associated with its continued use was known by 1960s but because the early warnings from epidemiological data were ignored, mesothelioma became evident in the 1990s (Wark & Peto, 2017). Due to the latent periods of many cancers, laboratory testing of potential carcinogens (such as studies on chromosomal aberration in lymphocytes) should be the first line of action aimed at stopping potentially carcinogenic new agents especially those which may affect the paracrine and endocrine signalling pathways.

1.1.2.1.3 Molecular and Genetic Epidemiology of Cancer

Many cancers are linked to a genetic predisposition or epigenetic cause where gene mutation is passed from one generation to another for example cancer of the breast where defective genes often affect metabolism of carcinogens which could impair apoptosis (programmed cell death), control of the cell cycle, detection and repair of DNA damage (Wark & Peto, 2017).

Family history of people with a particular type of cancer can be compared with those without cancer with the aim of measuring potential risk factors associated with the cancer type. About 34 % of men whose father suffered prostate cancer are twice more liking to develop prostate cancer (Kicinski et al., 2011) while women with female relatives with breast cancer has a two-fold risk of developing breast cancer compared to those without a family history of breast cancer (Kharazmi et al., 2014). Cancer epidemiology therefore tends to increase in regions with family history of cancer.

1.1.3 Cancer Treatment

Several approaches have been used in cancer treatment such as the use of radiation, surgery and chemotherapy (Gregory et al., 1991). The scope of this work is limited to the use of chemotherapy (from phytochemicals) in cancer treatment. This work shall focus on the use of two different approaches to developing phytochemicals for use as anticancer and antimicrobial agents:

i) The extraction of novel compounds from a plant belonging to a group of a well-known sources of bioactive compounds – *Inula helenium.*
ii) The synthetic modification of a well-known flavonoid (chrysin) with promising activities.

The specific aims and objectives shall be discussed in detail in section 1.6.

To discuss cancer treatment, one needs to consider the development and approval of anticancer drugs, a specific or selected anti-cancer agent or drug, methods of treatment, amongst others. To treat cancers, cure through destruction and total removal of malignant cells which usually refuse to respond to some treatments, particularly stem cells (which can regenerate into new cancerous growths) (Hatfield et al., 2005) is required. Radiotherapy and surgery have been used to treat localised cancers; these treatment options cannot be used in treating metastasis in interior organs and tissues (Zimmermann et al, 2014). Metastasis can only be treated by using chemotherapeutic agents which could attack such migrant cells. Unfortunately, chemotherapeutic drugs currently available in the market do not show specificity to tumour or cancer cells as most of them act by either interfering with DNA synthesis or by completely damaging the DNA machinery. By this, they do not only destroy cancerous cells but normal cells as well including cells that are rapidly dividing such as the bone marrow cells responsible for forming blood cells, epithelial cells of the gastrointestinal tract and hair follicle cells (King, 1996; Siddik, 2005). Toxicity from chemotherapeutics affects populations of normal cells. Interestingly, some solid tumours and other malignant tumours like those of breast, lung, rectum and colon have very few dividing cells. Due to this limitation of dividing cells, they are less susceptible or sensitive to chemotherapeutics. One limitation of chemotherapeutic drugs which limits their effectiveness is therefore their toxicities on normal cells. Many drugs have been employed in cancer treatment, and they operate via several mechanisms such as inhibition of M2 macrophages and myeloid-derived suppressor cells, inhibition of microtubule synthesis, simulation of ligands to their receptor etc. (Zimmermann et al, 2014; Zitvogel et al., 2013; Pratt et al., 1994). Available chemotherapeutic agents elicit cytotoxicity and apoptosis by interfering with the mechanism of DNA replication. However, cancer cells have the ability of repairing and renewing their damaged DNA. In addition to this, cancer stem cells can efflux drugs from within the cell environment via transporters known as ATP-binding cassette, rendering protection to these cells from cytotoxic agents (Liu et al., 2008). Because malignant cells can relapse and survive during chemotherapeutic treatment, there is need to find better alternatives to them and compounds isolated from natural products / their secondary metabolites may offer these solutions. Recently, the role of nitric oxide base compounds in cancer chemotherapy has been reviewed and it is believed these compounds offer very promising anticancer properties against tumour cells *in vivo.* However, at low concentration nitric oxide-based compounds are chemostatic, promoting the growth of cancer cells. Nevertheless, specialised delivery system of these nitric oxide-based

compounds into cells is being developed to ensure these compounds gets to their target organs (Vannini et al., 2017).

1.1.3.1 Chemotherapy

The use of drugs to kill cancerous growths or tumours, hindering their ability to grow and divide is known as chemotherapy. It involves administering anticancer drugs systematically into the bloodstream to distribute the drug throughout the body. Chemotherapy involves interference with DNA replication and inhibition of cell growth and division. The fundamental goal of clinical efficiency of any anticancer drug requires such drug to kill malignant cancerous growths *in-vivo* at doses sufficient enough to allow recuperation of critical tissues of the patient such as gastrointestinal tract and bone marrow, enabling such tissues to survive, and enable recovery (Hartner, 2018; Vannini et al., 2017). Chemotherapy can elicit their actions via one or more mechanism of action such as inhibition of DNA synthesis (Vannini et al., 2017; Colvin, 2003), inhibition of DNA elongation (Taşkın-Tok & Gowder, 2014), interference with microtubule functioning (White et al., 2017; Takimoto & Calvo, 2007), by covalently bonding to the phosphate, sulfhydryl, carboxyl and amino groups of important biological molecules (Elzoghby et al., 2015; Malhorta & Perry, 2003). Chemotherapy can be classified base on their mechanism of action into eight groups: anti-tumour antibiotics, antimitotic agents, anti-metabolites, alkylating agents, DNA reactive agents, hormone therapy and immunotherapy. Another class of chemotherapeutic agents are those derived from plants. The different classes of chemotherapeutic agents are discussed below; their history structures, application and potential side effects.

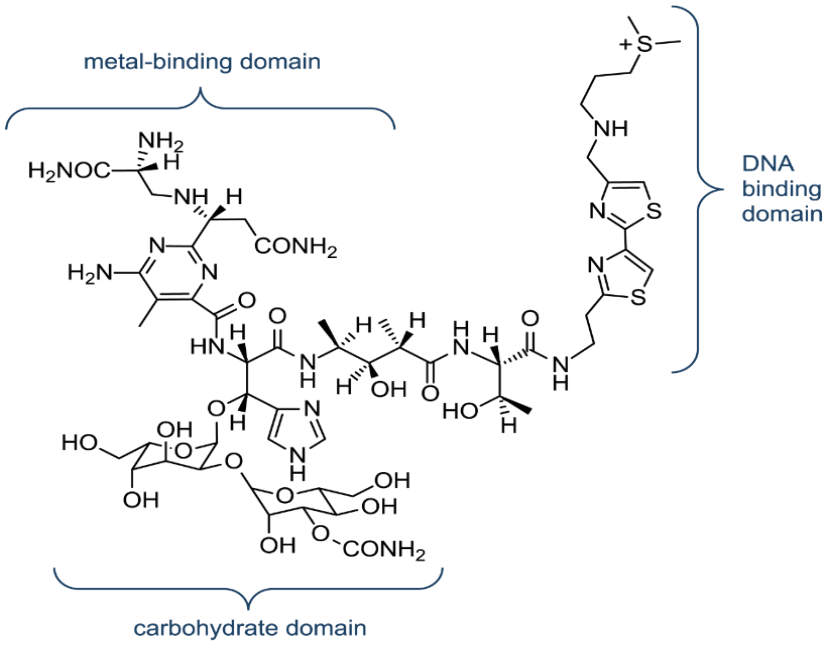
1.1.3.1.1 Anti-tumour antibiotics

Antitumour antibiotics was introduced into the Clinic by Sidney Faber in 1954 when actinomycin D, a cytotoxic drug which he isolated from *Streptomyces* was used in the treatment of Wilm's metastatic tumour in children (Waksman & Woodruff, 1940). The potent nature of actinomycin D paved the way for the development of varieties of antibiotics which exhibited potent biologic actions against malignancies in humans and rats. Mitomycin has been employed in the treatment of clinical cases of myelogenous leukaemia (Crooke & Bradner, 1976), doxorubicin and daunorubicin were used in treating solid tumours and leukaemia (Arcamone et al., 1969). Among these drugs, doxorubicin was the most potent against a range of solid tumours. These drugs elicit their action by binding to the double helix of DNA by inserting their planar rings between base pair stacks

of DNA, leading to distortion of the DNA conformation (Mansilla et al., 2010; Pratt et al., 1994). Bleomycin acts by intercalating DNA at a guanine-thymine and guanine-cytosine sequence, resulting in the formation of reactive oxygen species (ROS) that splits the DNA strand (Mansilla et al., 2010).

1.1.3.1.1.1 Bleomycin

Bleomycin (Structure 1.1) was originally isolated from *Streptomyces verticillus* and occurs naturally as Cu-chelate (Bolzán & Bianchi, 2018), and elicits its anti-tumour activities by binding Fe(II) inside cells, it complex with Fe(II) which involves reduction of oxygen, this leads to a release of –OH radicals which cleaves the DNA, and inhibits incorporation of thymidine into DNA and consists of three domains; metal binding domain, DNA binding domain and carbohydrate binding domain (Bolzán & Bianchi, 2018; Heydari-Bafrooei et al., 2017).



Structure 1.1**:** Structure of Bleomycin showing DNA binding sites (Anticancer compounds, 2009; Murakami et al., 1973)

Bleomycin has been employed to treat several cancer types including vulva, cervix, skin (non-melanoma), and cancers of oesophagus, neck and head, laryngeal, Hodgkin lymphoma, non- Hodgkin lymphoma, and cancer of the testes. It works by binding to cancer cells DNA, inhibition of cell growth and division by producing free radicals as ROS which destroys the DNA. Some of bleomycin advantage is that it produces little or no depression to bone marrow unlike other drugs. It also drains the fluids built up in the body by some cancer types (Shaw & Agarwal, 2004; Pratt et al., 1994). The histidine imidazole

ring is therefore essential for bleomycin activities and is responsible for the scission in the DNA strand (Sergeev & Zarytova, 1996; Murakami et al., 1973). Bleomycin on its own cannot damage dsDNA unless it is complexed to a metal ion.

1.1.3.1.2 Antimitotic Agents

Antimitotic agents (vinblastine and paclitaxel) inhibit the polymerisation mechanics of microtubule via inactivation of microtubule spindle assembly checkpoint (SAC), preventing the transition of the cell cycle from metaphase to anaphase (Masawang et al., 2014). Since antimitotic drugs disrupts chromosome orientation and the formation of spindle, cells remain in senescence-like G1 state or a prolong state of mitotic arrest leading to a subsequent induction of apoptosis (Mitchison, 2012). Microtubules are vital for cell division during mitosis and for correct chromosome segregation. They are formed during interphase (Dumontet & Jordan, 2010). Vinca alkaloid and colchicine alkaloids are examples of antimitotic agents (Takimoto & Calvo, 2007). Other important members include Taxol which causes protracted mitotic arrest of the microtubule dynamics, disrupting it. This leads to a resultant cell death (Olson et al., 2017). During mitosis, microtubule formation is faster compared to its formation during interphase, thus by targeting the microtubules, cancer progression can be halted and apoptosis initiated (Dumontet and Jordan, 2010).

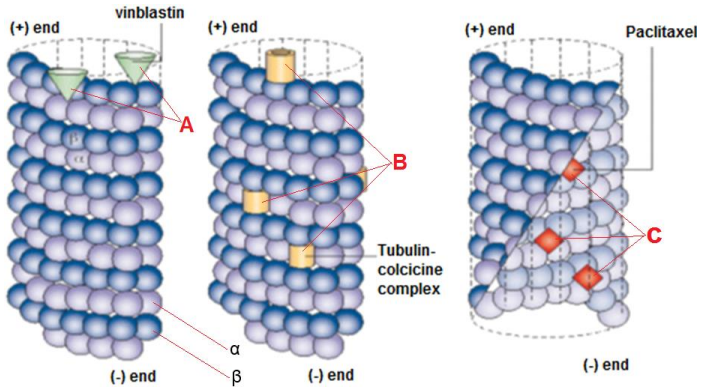


Figure 1.3: Poisoning of microtubules by colchicine leading to mitosis arrest.

The points of inhibition of the microtubule by vinblastine, colchicine and paclitaxel are represented as A, B and C respectively. These drugs elicit their inhibition by either binding to β-tubulin only (vinblastine) or by binding to the interface between α and β-tubulin (colchicine) or by binding to non-toxoid and non-vinca sites (paclitaxel) (Anticancer compounds 2009).

Antimitotic agents inhibit cell division by binding to tubulin and affecting the tubulin microtubule equilibrium which is the precursor for the synthesis of chromosomal proteins, thus preventing the formation of chromosomal proteins. Microtubules within the cells are created by tubulin proteins because without microtubules, cell division cannot occur

(Botta et al., 2009; Olson et al., 2017; Tsvetkov et al., 2011; Zhou & Giannakakou, 2005). Although antimitotic agents have a broad spectrum of activity against various tumour cells especially breast cancer (Masawang et al., 2014), variable clinical responses have been reported. These variable clinical responses are associated with Tumour-associated macrophages (TAMs), which are responsible for suppression of the duration of mitosis arrest induced by Taxol in breast cancer, promoting early mitotic slippage, decreased p53 activation, a decrease in phosphorylation of histone (H2AX) and cancer cell viability at interphase (Olson et al., 2017).

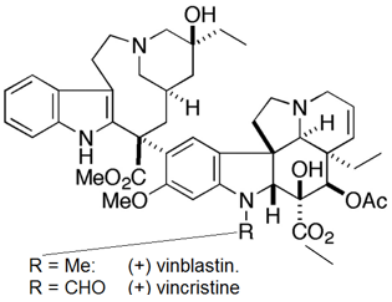
Homeostasis of the cell requires the birth of new cells, and death of old cells. Aged, aberrant or infected cells must be killed and / or removed from the cellular environment in an efficient manner which will not disturb the homeostasis of the cell. TAM receptor tyrosine kinases are therefore responsible for this task. The TAM receptors consist of three receptor proteins – Tyro3, Axl, and Mer which are activated by the binding of the protein-S and Gas-6 proteins which dimerizes, sending a “eat-me” signal which activates phosphotidylserine (Nagata et al., 2010). Activation of phosphotidylserine triggers phagocytosis (eating-up of death cells) (Nagata et al., 2010). When phosphotidylserine is masked, apoptotic cell phagocytosis is inhibited (Balasubramanian & Schroit, 2003). TAMs are therefore responsible for phagocytosis of aged cells by the process of apoptosis.

Apoptosis and Taxol-induced DNA damage is increased by TAMs, increasing the efficacy of Taxol in clinical trials. This is so because the cytotoxic effect of Taxol is suppressed by TAMs, partly through non-autonomous modulation of mitotic arrest in cancer cells (Olson et al., 2017). Targeting TAM-cancer cell interactions therefore increases the efficacy of Taxol.

Colchicine extracted first from Colchicum plant blocks the polymerization of microtubules by binding to β-tubulin (Figure 1.3) resulting in a tubulin-colchicine complex which is one of the two tubulin proteins which makes up the main composition of microtubule. This way, colchicine poisons the tubulin which is required for mitosis to occur, arresting mitosis as a result of spindle poisoning. This inhibition of mitotic spindles has been employed in genetics studies (Dalbeth et al., 2014).

Antimitotic agents are divided into two groups according to their mechanism of actions as either microtubule stabilizing agents or microtubule destabilizing agents. Destabilizing agents inhibits microtubule assembly (polymerization) and binds to either the taxoid-

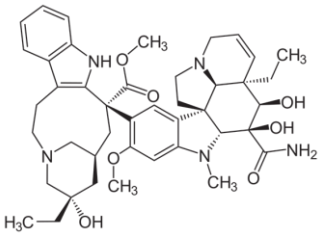
binding domain or the vinca domain (Jordan & Kamath, 2007; Kavallaris, 2010). Drugs which binds to the vinca domain which is located at the interface between α and β-tubulin (Figure 1.3 - B) are called vinca alkaloids and include eribulin, vindesine, vinorelbine, vincristine, vinflunine etc. (Liu et al., 2014). Drugs which binds to colchicine domain include combretastatin-A4, dolastatins, cryptophycins etc. (Attard et al., 2006). Drugs which binds to colchicine domain enhances polymerisation of microtubules, stabilise microtubules and prevent depolymerisation of of Ca^{2+} Which is subsequently disassembled.



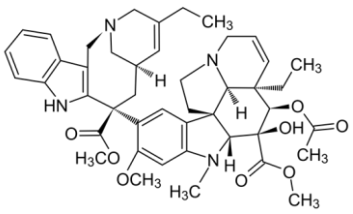
Structure 1.2: structure of vinblastine and vincristine
(Anticancer compounds, 2009).

Vinblastin, taxane and many other drugs belonging to these groups binds to β-tubulin (Figure 1.3 – A) (Altmann, 2001). Other drugs bind to overlapping non-taxoid and non-vinca sites (Figure 1.3 – C) on drug resistant βIII-tubulin-tubulin isotype. These classes of drugs are mainly microtubule-stabilizing drugs eg paclitaxel, laulimalide and peloruside A (Kanakkanthara et al., 2012). The mechanism of action of paclitaxel and peloruside A include arresting the cell cycle at G2/M phase of the cell cycle, resulting in cell death (Kanakkanthara et al., 2012). These compounds are difficult to efflux from the cell by efflux pumps thus making them superior antimitotic agents compared to vinca alkaloids and taxane which can be easily effluxed from the cell (Pryor et al., 2002; Yang et al., 2016). Vinblastine and Paclitaxel are the most common vinca alkaloids whose mechanism of action involves inhibition of microtubule spindle formation (Figure 1.3) and a total arrest of replication (van de Velde et al., 2017). Tubulin assembly is blocked by vinca alkaloids, preventing aggregation of the mitotic spindle, an inability of chromosome segregation prior to cell division thus leading to aggregation of cells in the G2/M phase of the cell cycle (van de Velde, 2017). Vinblastine and Vincristine are used in the treatment of tumours of the brain, breast, liver, lung, kidney, neuroblastomas, sarcomas, lymphomas and acute lymphoblastic leukaemia, and act by binding to β-tubulin, stabilizing the mitotic spindle and preventing its separation; this is key to the preservation of genetic information which is transferred from generation to generation (Aboubaker et

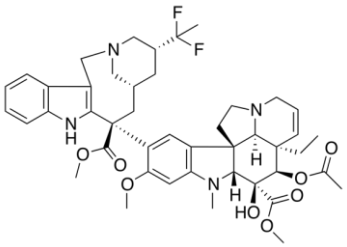
al, 2017; van de Velde et al., 2017). Structurally, vincristine (R=CHO) and vinblastine (R=CH_3) - Structure 1.2 are identical except for the substitution of –CHO for CH_3 on the lower indoline nitrogen. The difference in structural groups attached to the indoline nitrogen results in difference in toxicity and clinical activities. Vincristine is used mainly in the treatment of acute lymphocytic leukaemia in children and often results in peripheral neuropathy at doses > 5.0 mg/m². Vinblastine is used primarily in the treatment of Hodgkin's disease and elicits toxicity at the bone marrow at doses > 6.0 mg/m² (Voss et al., 2009).



Structure 1.3: Vindesine



Structure 1.4: Vinorelbine



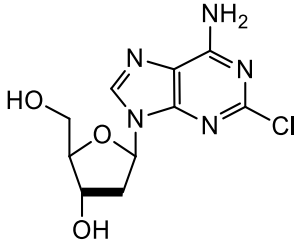
Structure 1.5: Vinflunine

(Scholar 2007).

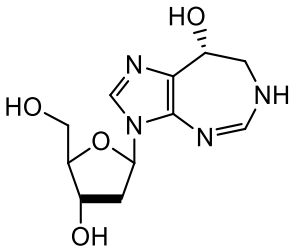
The need to reduce the unwanted side effect of vinca alkaloid has led to the production of other semi-synthetic vinca alkaloids with clinical importance including vinorelbine, vindesine and vinflunine (Structure 1.3 – 1.5) (Aboubakr et al., 2017).

1.1.3.1.3 Anti-metabolites

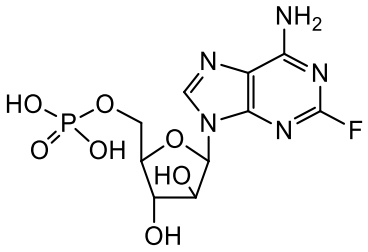
Antimetabolites comprise of a large group of anticancer compounds which are structural analogues of natural metabolites involved in RNA and DNA synthesis but differ in such a manner as to interfere with their metabolism. Antimetabolites include analogues of purine such as cladribine, pentostatin, fludarabine phosphate, 6-thioguanine and 6-mercaptopurine (Structure 1.6 – 1.10), antagonists of folic acid such as methotrexate (Structure 1.11), and analogues of pyrimidine such as gemcitabine, cytarabine, 5-fluorodeoxyuridine and 5-fluorouracil (Structure 1.12 – 1.15) (Bolzán & Bianchi, 2018; Scholar, 2007). Antimetabolites act by disrupting the synthesis of nucleic acids by substituting for the natural metabolite or by interfering with the production of an important nucleotide metabolite (Bolzán & Bianchi, 2018; Scholar, 2007).



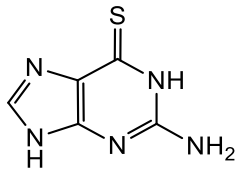
Structure 1.6: Cladribine



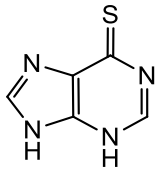
Structure 1.7: Pentostatin



Structure 1.8: fludarabine phosphate



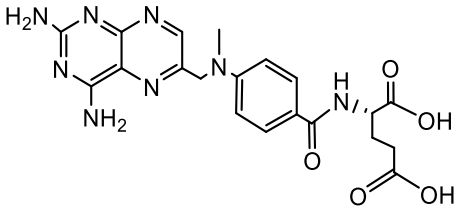
Structure 1.9: thioguanine



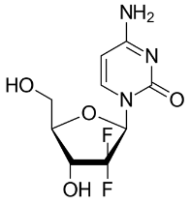
Structure 1.10: mercaptopurine

Structure 1.6 – 1.10: Antimetabolites in clinical practice.

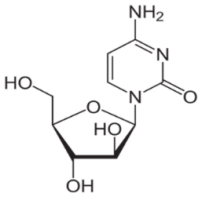
Normal cells of the gastro intestinal track and the bone marrow are often exposed to toxicities from antimetabolites as they are effective mainly in the S phase but not the G0 phase and are very effective in cancers with high growth fractions (Malhorta & Perry, 2003; Scholar, 2007). Severe toxicities and even toxic deaths (2 patients) have been reported for antimetabolites (Dogan et al., 2012).



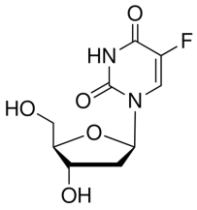
Structure 1.11: structure of methotrexate – a folic acid inhibitor



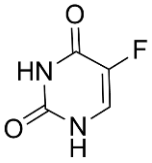
Structure 1.12: Gemcitabine



1.13: Cytarabine



1.14: 5-Fluorodeoxyuridine



1.15: 5-Fluorouracil

Pyrimidine antagonist
(Bolzán & Bianchi, 2018; Scholar, 2007).

Gastrointestinal malignancies, osteosarcoma, dermatitis and mucositis are some adverse effects reported in a certain clinical trial in Turkey (Dogan et al., 2012). In another phase II clinical trials, four deaths were reported when high doses (375 mg/m2) of antimetabolites were administered (Ferreri et al., 2015). There is an increased demand for metabolites in rapidly proliferating cells; the pyrimidine, purine and folate therefore come into prominence as they are the building block of DNA. On attainment of a certain dose (300 – 700 mg/m^2) (Ferreri et al., 2015), antimetabolites will not kill any more cells irrespective of any increase in dosage; however, 5-Fluorouracil is an exception because 5-Fluorouracil in addition to other mechanism of action, acts as an inhibitor of thymidylate synthase, blocking thymidine synthesis, which is a nucleoside used in DNA replication (Avendaño & Menéndez, 2015; Malhorta & Perry, 2003). Antimetabolites are closely similar in structure to purine and pyrimidine bases and act by preventing synthesis of

normal cellular metabolites. The structures of antimetabolites commonly employed in cancer treatment are listed in Structure 1.6 – 1.15.

Figure 1.4 is a schematic representation of inhibition of primers used in DNA synthesis. Folic acid is a requirement for the synthesis of dihydrofolic acid. The main source of folic acid used by humans is obtained from the diet. Another pathway involves the conversion of para-aminobenzoic acid (PABA) to dihydropteridinesyre, which is then converted to folic acid. This step is inhibited by antibacterial sulphonamide, which is also a rate limiting step in this reaction (Hu et al., 2017). Sulphonamide is bacteriostatic implying it does not kill bacteria but stops its growth, and oftentimes sulphonamide is administered alongside with Trimethoprim which is bactericidal and can kill bacteria (Hu et al., 2017).

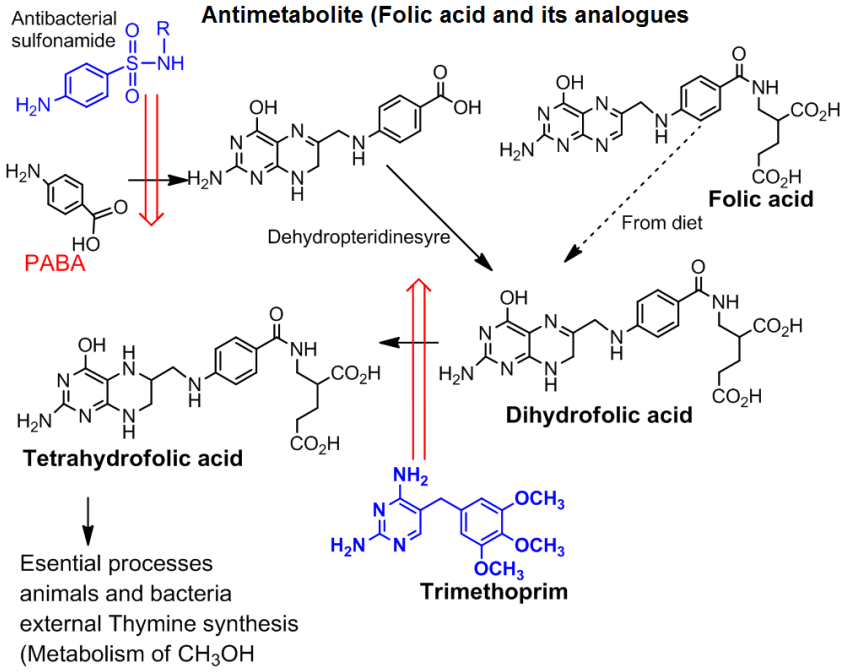


Figure 1.4: Inhibition of primers used in DNA synthesis

Antibacterial sulphonamide inhibits the conversion of PABA to dehydropteridinesyre which is the precursor for dihydrofolic acid synthesis. The second inhibition occurs at the stage where dihydrofolic acid is converted to tetrahydrofolic acid. This step is inhibited by trimethoprim (Anticancer compounds, 2009).

Dihydrofolic acid is then converted to tetrahydrofolic acid essential for DNA synthesis in animals and bacteria. Trimethoprim inhibits the conversion of dihydrofolic acid to tetrahydrofolic acid. Most antimetabolites also interfere with DNA synthesis at this point, inserting mismatch nucleotides into the DNA (Anticancer compounds, 2009). Nucleoside analogues act as antimetabolites via the following mechanisms: incorporation of DNA or RNA leading to misreading of the base sequence (Scholar, 2007), inhibition of enzymes involved in purine / pyrimidine biosynthesis (Bolzán & Bianchi, 2018), inhibition of DNA

kinase (Scholar, 2007; Bolzán & Bianchi, 2018), inhibition of DNA polymerases (Bolzán & Bianchi, 2018; Takimoto & Calvo, 2007).

1.1.3.1.4 Alkylating agents

Alkylation involves replacement of an atom of hydrogen by an alkyl groups, forming cross-linkages in the DNA strand. This leads to carcinogenic, mutagenic, and cytotoxic effects elicited by these compounds. Alkylating agents therefore refers to a group of diverse anticancer agents which covalently binds a substituted alkyl or an alkyl group to the contents of a cell. Once bound to cells, they form carbonium ion intermediates due to their strong electrophilic nature or they may pair with the target molecule, forming transition complexes. Strong covalent linkages result from alkylation of alkylating agents with nucleophilic groups such as carboxyl or sulfhydryl groups (Bolzán & Bianchi, 2018; Scholar, 2007). Clinically, alkylating agents are known to effect cell division and DNA synthesis. Even though alkylating agents elicits their anticancer activities in all phases of the cell cycle, their toxicities and therapeutic effects are linked to interference with cells undergoing rapid proliferation (Scholar, 2007). Alkylating agents include dacarbazine, altretamine, triethylenemelamine, thiotepa, temozolamide, streptozotocin, procarbazine, mitomycin C, melphalan, mechlorethamine, ifosphamide, cyclophosphamide, chlorozotocin, chlorambucil, semustine, carmustine (BCNU) and busulfan (Scholar, 2007; Sharma et al., 2018). These class of chemotherapeutic drugs binds to DNA, preventing correct DNA replication and possess chemical groups which can permanently form covalent bonding with nucleophilic site on the DNA. They react with proteins which bond to the double helix of DNA, inserting an alkyl group to some or all the double helix of DNA. This hinders the linking up of proteins, leading to DNA strand breakage, and eventual cell death (Bolzán & Bianchi, 2018; Takimoto & Calvo, 2007). Alkylating agents can both be used in treating cancers as well as in inducing cancers. This is because alkylating agents are carcinogenic, teratogenic and cytotoxic (Sharma et al., 2018) and most alkylating agents are associated with bone marrow and gastrointestinal toxicities. Toxicities to other organs may also occur. It therefore implies that alkylating agents should be use when the benefits outweigh its side effects.

Alkylating agents are categorised into seven major groups:

a) Nitrogen mustards: ifosfamide, estramustine, cyclophosphamide, chlorambucil, melphalan, mechlorethamine (Sharma et al., 2018; Takimoto & Calvo, 2007; Taşkın-Tok & Gowder, 2014).
b) Nitrosoureas: streptozocin, lomustine (Colvin, 2003; Takimoto & Calvo, 2007).

c) Alkyl sulphonates: busulfan (Sharma et al., 2018; Takimoto & Calvo, 2007).

d) Platinum complexes: oxaliplatin, cisplatin, carboplatin (Takimoto & Calvo, 2007).

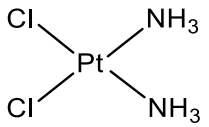
e) Non-classic alkylators: temozolomide, procarbazine, dacarbazine, altretamine (Sharma et al., 2018; Takimoto & Calvo, 2007).

f) Aziridine: thiotepa (Avendaño & Menéndez, 2008; Takimoto & Calvo, 2007).

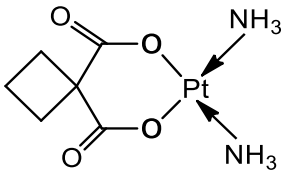
g) Triazene and related compound (Marchesi et al., 2007; Sharma et al., 2018).

1.1.3.1.5 DNA Reactive Agents

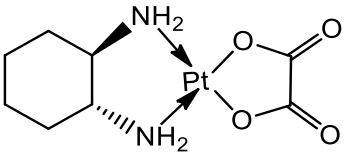
Platinum based agents offer distinctive possibilities for designing chemotherapeutic agents for cancer therapy. The wide spectrum of reactivity, accessible redox states, geometries, range of coordination numbers, ligand and intrinsic property associated with the cationic metal ion, kinetic and thermodynamic potentials enables this metal to offer distinctive biological action against cancer cells. An important DNA reactive agent – cisplatin and its derivatives carboplatin and oxaliplatin (Structure 1.16 – 1.18), elicits their cytotoxic activity via the formation of DNA-drug-adduct. This adduct prevents DNA replication and apoptosis and this property is exploited in the treatment of several cancer types including cancers of the testis, bladder, neck, head and ovaries. 90% cure rate has been reported for these cancers (Brabec et al., 2017; Cheung-Ong et al., 2013).



Structure 1.16: Cisplatin



Structure 1.17: Carboplatin



Structure 1.18: Oxaliplatin

(Brabec et al., 2017).

On entering the cell, cisplatin becomes activated by displacement of its chlorine atom by water molecules, energising it to becoming a strong electrophile which then reacts with nucleophiles including the atoms donating nitrogen on nucleic acid base residue. Intra-strand is the most prevalent DNA-adduct induced by cisplatin, where the platinum atom pairs to the imidazole ring at N7 position of two-guanine adjacent to each other and this accounts for about 60 – 65 % of all adducts. In other cases, where one guanine and adenine are involved, about 20% of these adducts are formed (Brabec et al., 2017). Mitochondrial DNA also seems to be a target for cisplatin (Shu et al., 2016). Apart from intra-strand DNA-adducts, inter-strand and monofunctional adducts have also been hypothesised as responsible for the toxicity of cisplatin (Brabec et al., 2017; Dasari &

Tchounwou, 2014; Johnstone et al., 2014; Millard & Wilkes, 2000), which conflicts with other reports (Pinto & Lippard, 1985).

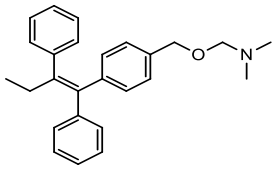
Cisplatin has been hypothesised to induce thermal cytotoxicity which sensitizes tumour cells to cisplatin (Dasari & Tchounwou, 2014). The reaction of cisplatin with DNA is thought to be through the formation of increased inter-stand cross-linkage in the DNA adduct which results in an increased inter-strand cross-linkage of 2.4% at 25°C and 11.5% at 45°C. This increased cisplatin-DNA inter-strand cross-linkage is enhanced in negatively supercoiled DNA compared to linear DNA, an indication that the DNA topology also influences the amount of inter-strand cross-linkages (Brabec et al., 1992; Heymann et al., 2014; Malina et al., 2000). Cisplatin elicits its action via apoptosis and necrosis. In necrosis, the cytosol swells, losing the integrity of the plasma membranes. Once the plasma membranes integrity is lost, cisplatin gushes into the cytosol at concentrations >800 µM. At concentrations ~ 8.0 µM, apoptosis results, if cells are exposed to cisplatin over several days leading to the shrinkage of the cell, DNA fragmentation and chromatin condensation (Tuorkey, 2016). When therapeutic treatments are carefully managed, apoptosis occur (Tuorkey, 2016). In mammalian cells, apoptosis follows two main pathways: extrinsic and intrinsic pathways. The extrinsic pathway requires binding of ligands to tumour necrosis factors (TNF-α), leading to activation of caspase 3, 7 and 9, which in turn activates the death domain proteins associated with the death receptors. This leads to cell death (Tuorkey, 2016). In intrinsic pathway, modulation of the mitochondrial pathway potential leads to the release of cytochrome C and the modulation of apoptotic gene. Bax, a pro-apoptosis gene is then upregulated with Bcl-2, an anti-apoptotic gene, which is down-regulated simultaneously. This mechanism leads to cell proliferation (Dasari & Tchounwou, 2014; Tuorkey, 2016).

There is increased resistance to platinum-based drugs due to epigenetic and genetic factors on the one hand, and poor delivery of the drug into its target organ, leading to increased rate of metabolism, poor bioavailability and increase rate of excretion out of the body, thus limiting the amount of drug reaching the target organ (Brabec et al., 2017). Tumour cell resistance also occurs when either or all of the following occur: the DNA base repair mechanism is activated such that upregulation of genes (such as BRCA1 and BRCA2) involved in drug-induced DNA repair occurs, failure of the cell cycle check-points, alteration of drug cellular target, enhanced by-pass of DNA adducts, increased levels of detoxification through increased cellular thiol production, and increase tolerance to DNA damage (Brabec et al., 2017; Cheung-Ong et al., 2013). Multidrug resistance to

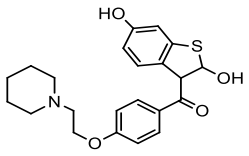
structurally similar drug can also occur when cells exhibit cross-resistance to a particular drug (Brabec et al., 2017). Carboplatin (Structure 1.17) was developed because its six-membered ring conjugation gives it advantage by increasing its half-life from ~ 3.6 hours to over 30 hours and providing aqueous stability to the platinum base drug, reduces the side effect of carboplatin - such as neuropathy (dysfunction or disease of one or more peripheral nerves, typically causing numbness or weakness) but does not eliminate the resistance observed in cisplatin (Brabec et al., 2017; Dasari & Tchounwou, 2014). The problem of cisplatin resistance is overcome by oxaliplatin which although binds to the same site of DNA, and forming the same adduct like cisplatin; however, they are differentially recognised by repair proteins (Brabec et al., 2017).

1.1.3.1.6 Hormone Therapy

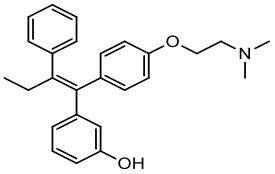
Hormone therapy is a non-toxic and effective cancer therapy option for prostate cancer and involves binding the hormone therapy to progesterone and oestrogen receptors. Testosterone and oestradiol are controlled by the gonadal-pituitary-hypothalamic pathways with oestradiol produced by conversion of peripheral adrenal androgens by aromatase in postmenopausal women, while the ovaries in premenopausal women, produces oestradiol (Tuorkey et al., 2016). Treatment of prostate cancer and breast cancer in premenopausal women is usually achieved by castration (Tuorkey et al., 2016), while aromatase inhibitors or selective oestrogen receptor modulators such as tamoxifen (Structure 1.19) are used in postmenopausal cancers (Tuorkey et al., 2016). Prior to radiotherapy or radical surgery, hormone therapy is often used to shrink the size of the cancers or to prevent or minimise recurrence (Abraham & Staffurth, 2016). Patients with metastatic disease or locally advanced cancers usually respond positively to hormone therapy, although cancer relapse especially those involving ‘castrate-refractory’ diseases have been reported (Abraham & Staffurth, 2016). Tamoxifen is structurally similar to oestrogen and binds at the receptor sight of oestrogen. It possesses both anti-oestrogenic and oestrogenic effects depending on the tissue involved. In mammary tissues, it is anti-oestrogenic and for this reason, it is used in breast cancer treatment and prevention, while on uterine epithelium, it is pro-oestrogenic (Shagufta & Ahmad, 2017). Therefore, tamoxifen is called selective oestrogen receptor modulator as women treated with tamoxifen have experienced incidences on endometrial carcinoma (Abraham & Staffurth, 2016).



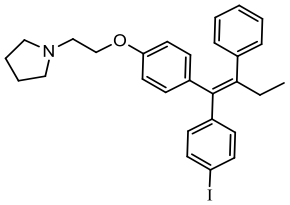
Structure 1.19: Tamoxifen



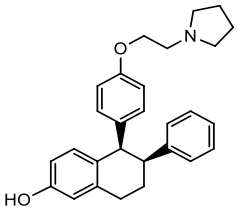
Structure 1.20: Raloxifen



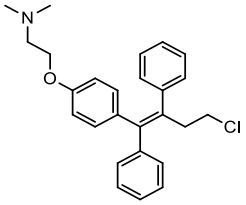
Structure 1.21: Droloxifene



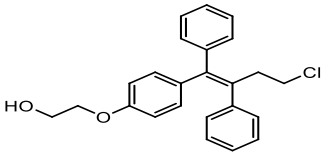
Structure 1.22: Idoxifene



Structure 1.23: Lasofoxifene



Structure 1.24: Toremifene



Structure 1.25: Ospemifene
(Shagufta & Ahmad, 2017).

For this reason, derivatives of Tamoxifen such as ospemifene, toromifene, lasofoxifene, idoxifene and droloxifene (Structure 1.19 – 1.25) have been developed and are currently undergoing clinical trials (Shagufta & Ahmad, 2017).

1.1.3.1.7 Immunotherapy

Monoclonal antibodies have been employed in treating cancer over the past decade for many types of cancers. The use of these antibodies in cancer treatment is known as immunotherapy. Immunotherapy also encompasses distinct tactical treatments including immunocytokines, enzyme inhibitors, immunomodulating checkpoint inhibitors and vaccines (Redman et al., 2017). Sipuleucel-T vaccine has received approval for the treatment of prostate cancer in the US (Redman et al., 2017), while checkpoint inhibitors have found modern application in the treatment of Hodgkin's lymphoma, urothelial carcinoma, non-small cell lung cancer, kidney, and melanoma cancer (Balar et al., 2017; Chan et al., 2017; Rosenberg et al., 2016). Sipuleucel-T vaccine is a novel personalised vaccine which work by programming each patient's immune system to seek out foreign bodies (cancer) and attack it. This vaccine is not just a preventive vaccine but also a therapeutic vaccine due to its immunostimulant abilities (Redman et al., 2017). These antagonist monoclonal antibodies elicit their antitumour activity acting as immunomodulators which targets regulatory molecules on the surfaces of cells, potentiating antitumour immunity. The check-point widely studied includes programmed death ligands-1 (PD-L1), programmed cells death proteins-1 (PD-1), and inhibitor of cytotoxic T lymphocyte associated proteins-4. These checkpoints regulate the activity of

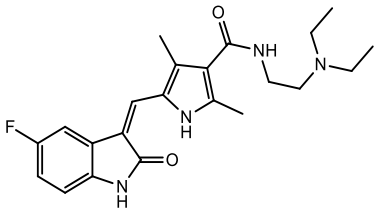
the T-cells (Balar et al., 2017; Chan et al., 2017; Rosenberg et al., 2016). When these molecules are blocked, interactions in the tumour microenvironment and other immune compartments are affected. This further dampens natural inhibitory functions of the immune system, resulting in cell death. Although only Sipuleucel-T vaccine has been approved for the treatment of prostate cancers, other immunotherapeutic agents currently undergoing clinical trials with good success include, Olaparib – (a PARP inhibitor), Ipilimumab, Pembrolizumab, Nivolumab, ProstAtak, DCVAC/PCa, PROSTVAC (PSA-TRICOM) (Redman et al., 2017).

Immunotherapy relies on the ability of these agents to induce an immune response, enabling the body to identify tumour growths as foreign bodies. Identification of tumour cells as foreign bodies could elicit phagocytosis, activation of T-cells and other cellular mechanisms which will eventually lead to tumour cell death (Balar et al., 2017; Chan et al., 2017; Redman et al., 2017). The safety of monoclonal therapeutic antibodies and its efficacy depends on the target antigen accessibility and abundance, allowing the binding of these agents to special targets. These antigens are often exclusively on the surfaces of the tumour cells and are usually homogenous in nature, allowing limited antigen secretion, allowing the immunotherapeutic agents to target cancer cells (Balar et al., 2017; Redman et al., 2017).

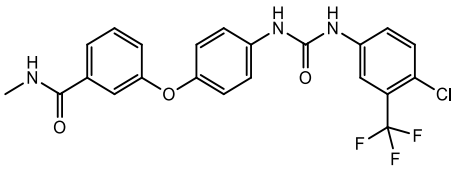
1.1.3.1.8 Protein Kinase Inhibitors

A variety of cellular activities are involved in post-translation modification (PTM) of which phosphatases and protein kinases are the most widely studied. Phosphatase and protein kinases are enzymes involved in dephosphorylation and phosphorylation reactions and genomic analysis indicates encoding of these enzymes by the human genome in a magnitude of between 3 and 5 % of these enzymes. Phosphorylation of proteins allows conformational changes which either activates or deactivates the enzyme complex in single or complex enzyme binding sites and these conformational changes are responsible for regulating the protein kinases when phosphorylation and dephosphorylation of protein kinases occur (Singh et al., 2017). Normal cellular functioning requires phosphorylation and the malfunctioning of this key metabolic function may lead to the onset of cancer which may lead to mutation of genes responsible for proliferation and normal cell growth. Mutation of these genes may disrupt the homeostasis of proteins leading to impairment of signal transduction (Reggi & Diviani, 2017; Singh et al., 2017). By targeting protein kinases, cancer growth can be inhibited and destroyed by

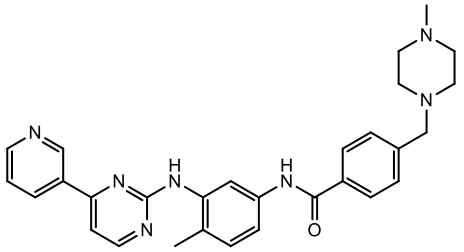
small molecules such as sunitinib, sorafenib, imatinib, and gefitinib (Structure 1.26 – 1.29).



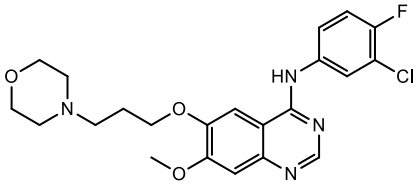
Structure 1.26: Sunitinib



Structure 1.27: Sorafenib



Structure 1.28: Imatinib



Structure 1.29: Gefitinib

(Singh et al., 2017).

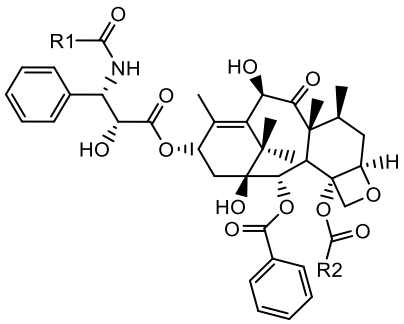
Phosphorylation regulates vast cellular functions including cell signalling, apoptosis, differentiation and cell growth in healthy cells. Cancer results when phosphorylation malfunctions, releasing oncoproteins which are linked to cancer growth (Singh et al., 2017). Oncoproteins are used in marking tumour cells. Signalling pathways such as Cyclin-dependent kinases, Cadherin-catenin complex, MAP kinase, Tyrosine kinase etc. are important cell cycle players involved in phosphorylation-dephosphorylation cascades and deregulation of this process leads to cancerous growths (Singh et al., 2017) and tyrosine kinases encompasses majority of oncoproteins of which MAPK cascade, and Bcl2 family plays a role in cancer progression and development. Bcl2 cascade proteins act as anti-apoptotic proteins as well as apoptotic proteins, with cyclins regulating the cell cycles, whereas the catenin- cadherin complex controls cell adhesion (Reggi & Diviani, 2017; Singh et al., 2017). When phosphorylation is altered in any or all of these pathways, cancer results and by targeting tyrosine kinase, drugs inhibiting ERK, PI3K, and MEK signalling pathways can be used to treat cancers (Reggi & Diviani, 2017; Singh et al., 2017). Crizonib has been approved for treatment of NSCLC cancer treatment while lorlatinib, entrectinib, cabozantinib and ceritinib are undergoing clinical trials for treatment of lung cancer (Roskoski 2017), thus crizotinib is classified as a type I tyrosine ROS1 protein kinase inhibitor (Roskoski 2017). Drugs which can target phosphorylation pathways offer new hope as potential anticancer therapies.

1.1.3.1.9 Anticancer Agents from Plants

Natural products are an important resource of novel drugs, drug analogues and important chemical entities. This is more so because of the need to discovering anti-infectious and anticancer agents. This has continued to present new leads of which many have been successful in clinical trials. Interestingly, plant and other natural products are used in pharmaceutical preparations and pharmaceutical care of which various agents derived from plants are successfully used in treating cancers. Currently, several classes of cytotoxic plants derived natural products have been characterised and are currently being investigated for development and improvement of novel drugs (Shah et al., 2013; Twilley & Lall, 2018). These plant products can be classified mainly into four groups namely taxane diterpenoids, epipodophyllotoxin lignans, vinca alkaloids, and camptothecin alkaloids (including quinolone).

1.1.3.1.9.1 Taxanes

Taxanes were isolated from *Taxus brevifolia.* They act mainly by disrupting the equilibria between microtubules and free tubulin by causing a shift in the assembly position but not by disassembling them, as is the case with other microtubule antagonists (Takimoto & Calvo, 2007; Twilley & Lall, 2018).



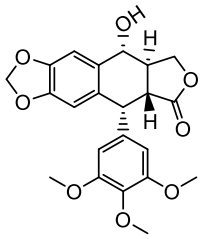
Structure 1.30: structure of Taxol
(Anticancer compounds 2009)

Paclitaxel (Taxol) was the first taxane discovered. No natural compound has been successfully applied in treatment of cancer like paclitaxel. It is the first anti-tumour agent known to promote the over-proliferation of microtubules which are essential elements in cancer chemotherapy. It has been used in Non-small-cell lung carcinoma (NSCLC), breast and ovarian cancer treatment (Twilley & Lall, 2018). Analogues of Paclitaxel which have elicited better anticancer activity have been synthesised such as docetaxel (Twilley & Lall, 2018). In 2009, Taxolog (Milataxel) was successful in phase II trials for colorectal tumour, however, its high toxicity at doses above 35 mg/m^2 is of clinical concern

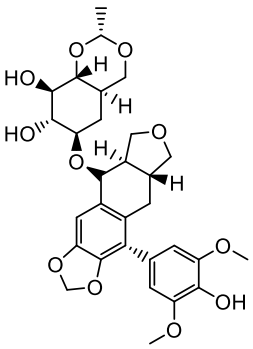
(Ramanathan et al., 2008). Several taxanes and their metabolites are currently undergoing clinical trials with the aim of assessing their tolerance, safety and their continuous daily dosage especially if they advance to phase II clinical trials in patients with solid tumours at advanced stages; an example of such drug is Tasetaxel BMS-275183, BMS-184476 (Heath et al., 2011; Plummer et al., 2002). RPR 109881A, a taxane, which possess a cyclopropyl ring, had advantage over docetaxel as it elicited cytotoxicity against docetaxel sensitive tumour cells (Gelmon et al., 2000). Ortataxel is potent against tumours resistant to docetaxel and paclitaxel, as well as in NSSLC taxane resistant tumours (Beer et al., 2008), taxanes therefore appears to offer promising results as chemotherapeutics with minimal toxicities. Carbazitaxel and docetaxel downregulates the survival signal of ERK1/2 in a manner not disturbing nuclear translocation of the androgen receptor (AP), an indication that other pathways other than the AP pathways are responsible for toxicities induced by these drugs (Mang et al., 2017).

1.1.3.1.9.2 Podophyllotoxin Lignans and Its Analogues

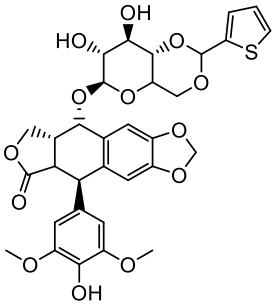
Podophyllotoxin, a lignin derivative and an inhibitor of microtubule assembly was isolated from *Podophyllum peltatum*.



Structure 1.31: Podophyllotoxin



1.32: Etoposide



1.33: Teniposide

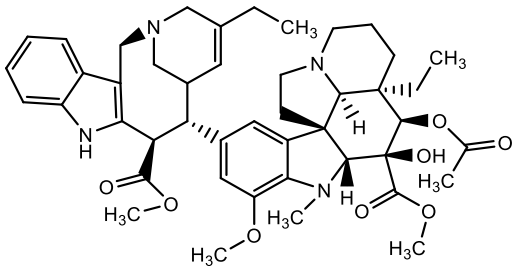
Podophyllotoxin lignans and analogues
(Merzouki et al., 2012).

Teniposide and etoposide elicited cytotoxicity by reversibly binding to tubulin in a manner different from the lead compound, by inhibiting topoisomerase II enzyme (Srivastava et al., 2005). The discovery of teniposide and etoposide has opened the door to much research aimed at synthesizing analogues of podophyllotoxin. This is important because podophyllotoxins have poor solubility, are easily inactivated in bio-systems, and cells become easily resistant to podophyllotoxins. Many compounds with better activities than podophyllotoxin have been synthesized and tested (Kamal et al., 2014; Khazir et al., 2014; Merzouki et al., 2012).

1.1.3.1.9.3 Vinca Alkaloids

Vinca alkaloids were discovered in 1950s in Madagascar periwinkles (*Catharanthus roseus*). Four vinca alkaloids which have been used in cancer treatment include vindesine, vincristine, vinorelbine, and vinblastine (Pratt et al., 1994; Twilley & Lall, 2018). The first compounds isolated – vincristine and vinblastine, are used in treating several cancers, and have been subject to several biochemical and pharmacological investigations (Twilley & Lall, 2018). Vinca alkaloids in clinical use are classified mainly into four groups: vindesine, vincristine, vinorelbine, and vinblastine (Moudi et al., 2013). Previously, it was considered that vinca alkaloids acted mainly by inhibiting tubulin polymerization into microtubules, blocking mitotic spindle formation, and resulting in cells death due to metaphase arrest (Jordan et al., 1991b; Panjawatanan et al., 2014). Vintafolide has produced promising clinical results as a potent anti-tumour agent in the treatment of ovarian cancer (Leamon et al., 2014). It consists of folic acid, a di-sulfide, hydrophilic peptide-spacer, and desacetyl-vinblastine hydrazide (DAVLBH) – a cytotoxic drug. A comparison of vintafolide and other folate-targeted vinca alkaloids such as vindesine, vinorelbine, vinflunine, and vincristine proved the potency of Vintafolide, compared to other drugs (Leamon et al., 2014).

Several clinical trials on vinca alkaloids are currently ongoing including hydravin (anhydrovinblastine) with clinical trials for treatment of lung metastatic sarcoma. By 2003, stage-I clinical trials revealed a 21 mg/mL recommended dose (Ramnath et al., 2003). Because of the limitation of lead vinca alkaloids compounds (such as an increased anti-tumour spectrum, and increased safety), amino acids and derivatives have been grafted into vinxaltine and vintripol to overcome these limitations, with successful phase III clinical trials for vinorelbin in patients with carcinoma of the breast (Budman 1992).



Structure 1.34: Structure of vinca alkaloid
(Anticancer compounds, 2009; O'Boyle et al., 2011)

Vinblastine prevents angiogenesis by attacking the mitotic spindle which shuts-down mitosis, and blocks the cell cycle (Tsvetkov et al., 2011), leading to cell death. Agents

that target tubulin elicit cytotoxicity by either destabilising or stabilising microtubules by disrupting its dynamics, arresting mitosis, and inducing apoptosis (Botta et al., 2009). Microtubules are very sensitive such that even slight alteration of the dynamics of the microtubules has the potential of affecting the mitotic spindle, arresting cell division and causing eventual cell death (Zhou & Giannakakou, 2005). Experimentally, three binding sites have been discovered namely vinca alkaloids, colchicine and taxoid binding sites (Botta et al., 2009). Targeting these binding sites could be future targets for cancer chemotherapy. Vinca alkaloid binds to free tubulin (Figure 1.5) forming vinca-bound tubulin dimer aggregates to form paracrystalline aggregates, leading to cell cycle arrest.

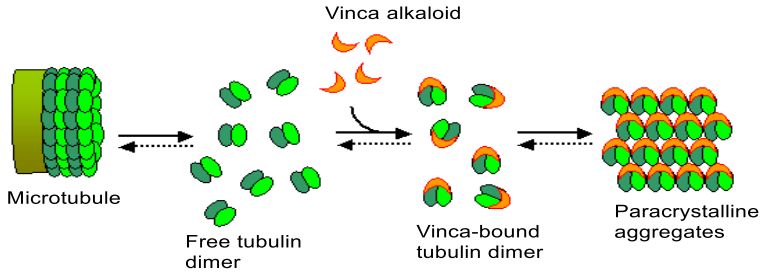


Figure 1.5: metaphase arrest by vinca alkaloids via suppression of microtubule dynamics.

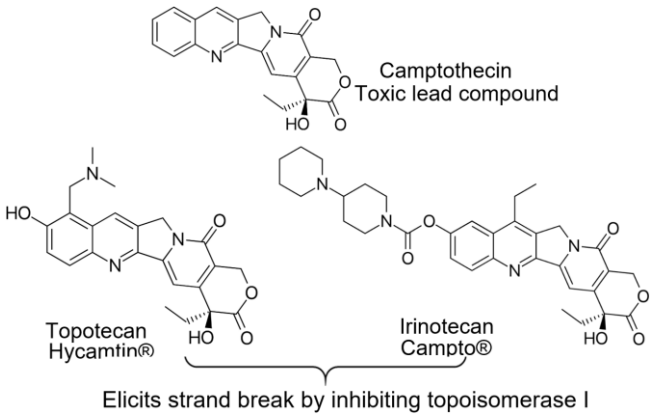
Vinca alkaloids (such as vinblastine) binds to free tubulin dimers (consisting of α and β tubulin) forming vinca-bound tubulin dimer. These dimers aggregate thus bringing about cell cycle arrest in cancer cells (such as breast, Hodgkin lymphomas etc.), including cancers of the germ cell (Anticancer compounds 2009).

Vinca alkaloids are known for their side-effects of anaemia and reduced resistance to infection but finds application in the treatment of tumours of germ cells, breast cancers, Hodgkin lymphomas (Tsvetkov et al., 2011; Zhou & Giannakakou, 2005). Vinca alkaloids such as vincristine bind strongly to tubulin dimers in a reversible manner. It does this by binding to a dimer at one end, poisoning it, dissociating from it, and binding to another end, poisoning it. By this poisoning, the dimers cannot re-assemble to form microtubules, resulting in cell death (Figure 1.5) during the G2-M transition and G1 phase (Lobert & Puozzo, 2008; Simoens et al., 2006).

1.1.3.1.9.4 Camptothecin

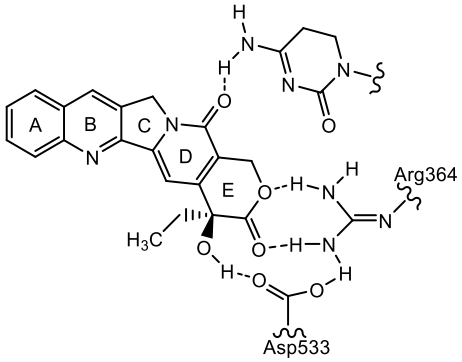
This group of compounds where characterised from a Chinese tree called *Camptotheca acuminata*. It is an inhibitor of nucleic acid biosynthesis and an inducer of DNA strand breaks. It elicits cytotoxicity by inhibiting topoisomerase-I in the DNA (Efferth et al., 2007; Twilley & Lall, 2018). Preliminary clinical trial of camptothecin shows promising anticancer activities but also adverse toxicities, lactone instability, reversal of interaction between drug and its targets, drug reactions and low solubility (Beretta et al., 2012). Due to these disadvantages, several semisynthetic analogues have been synthesized to overcome this

challenge, of which topotecan (hyacamtin), irinotecan (Takimoto & Calvo, 2007), and belatecan have been given approval for use as chemotherapeutic drugs. Recently, a conjugate of comptothecin and artusunate elicited a better anticancer activity, and lower toxicities *in vivo* than camptothecin (Li et al., 2015). These drugs are employed in the treatment of NSCLC, colorectal and ovarian cancers (Oberlies & Kroll, 2004; Twilley & Lall, 2018). Camptothecin bind to DNA topo-I complex, giving rise to a stable complex. This inhibits re-ligation of DNA and eventual DNA damage and apoptosis (Yikrazuul, 2009).



Structure 1.35: Camptothecin and its analogues
(Anticancer compounds 2009)

The E-ring interacts with the enzymes at three different positions with the hydroxyl group forming hydrogen bonds at position 20 with aspartate-533 of the enzyme, and the lactone pairs with arginine-364 (Structure 1.36). The topo-I DNA complex is stabilised by the interaction of cytosine with the D-ring, forming H-bond. The H-bonding takes place between the amino group present in pyrimidine ring of cytosine and carbonyl groups on the D-ring at position 17 (Adams et al., 2006; Beretta et al, 2013; Redinbo et al., 1998).



Structure 1.36: Binding of Camptothecin to DNA and topo-I
(Yikrazuul, 2009).

Other semisynthetic camptothecin analogues such as Karenitecin have been successful in clinical trials against melanoma in phase II trials (Daud et al., 2005). Stage II trials with keranitecin are successful with acceptable toxicity levels (Munster & Daud, 2011).

Namitecan, a hydrophilic derivative of camptothecin is another chemotherapeutic drug offering promising results due to improved cytotoxic potency like topoisomerase I inhibition, stability of the complex where DNA cleavage occurs, enhanced accumulation within the cell, and a distinct sub-cellular localisation (Beretta et al., 2013; Beretta et al., 2012), stability of the lactone ring with favourable pharmacokinetics (Beretta et al., 2012) and cytotoxic efficacy in a variation of tumour xenograft of humans including tumours resistant to irinotecan, and topotecan, especially of carcinomas of the squamous cells (Beretta et al., 2013; Beretta et al., 2012). It is currently undergoing clinical studies. 9-Aminocamptothecin elicited potent activities in pre-clinical trials but has not replicated same as a clinical anti-tumour agent. Although it elicited mild activities against colon and lung cancers, this was not clinically useful and for this reason, it was discontinued in 1999 (Khazir et al., 2014; Twilley & Lall, 2018) as it lacked potency against glioblastoma (Farray et al., 2006). Gimatecan has entered trials for treatment of solid tumour at advanced stages, as they poison DNA topoisomerase-IB, inhibiting the relaxation of supercoil DNA (Prada et al., 2013; Zhu et al., 2009). Further studies on camptothecin has led to the discovery of exatecan mesylate (Ajani et al., 2005), EZN-2208, TP-300 (Patnaik et al., 2013), and other derivatives which aims at overcoming the limitations of lead camptothecins such as poor solubility, toxicity, amongst others.

Several other anticancer agents derived from natural products have been isolated and they include: curcumin, betulinic acid, flavopiridol, omacetaxine mepesuccinate, combretastatin (Jabeena et al., 2014) and colchicine (Anticancer compounds 2009).

Combretestatin A4 (Figure 1.6) is a natural cis-stilbene, isolated from the South African bushwillow tree (*Combretum caffrum*) in 1989 (Cirla & Mann, 2003; McNulty et al., 2015).

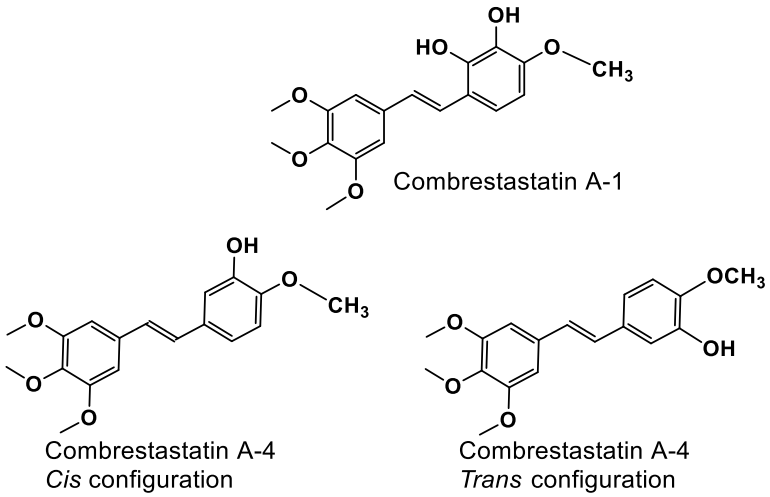


Figure 1.6: Structure of combretastatin A-1 (up) and A-4 (down) (Grisham et al., 2018)

Combretestatin A4 and colchicine belong to a class of compound known as colchicinoids. They are potent microtubule targeting agent (MTA) and have been reported to inhibit cancer cell proliferation *in-vitro* and can destroy newly formed vasculature *in-vivo,* as found in tumour environments. They are therefore called vascular disrupting agent (VDA) (Parkins et al., 2000; Penthala et al., 2015). Phosphate derivative of combretestatin A4 – combretastatin-A4-phosphate (CA4P) has shown an ability to regress tumour vasculature in a variety of cancer cells because it is more soluble than combretestatin A4 and has the advantage of being tolerated better than combretastatin A4 as it does not suppress the immune system; a characteristic of combretastatin A4 (Siemann et al., 2009; Young & Chaplin, 2004). Despite this advantage, CA4P is a poor monotherapy. Nevertheless, the FDA has given it a fast-tracked status for treatment of platinum-resistant ovarian cancer (Cooney et al., 2006; Nagaiah & Remick, 2010). Clinical research examining the potency of CA4P as an adjuvant therapeutic agent has been concluded (Nagaiah & Remick, 2010; Rustin et al., 2010) and the final stages of clinical trials is ongoing (Grisham et al., 2018). It has been reported that CA4P is safe with less side-effect except mild hypertension when taken for prolonged periods (Grisham et al., 2018). Combretestatin A-4 elicits maximum bioactivity when the aromatic rings are in cis orientation (Figure 1.6). However, this compound could easily isomerise spontaneously to inactive trans-stilbene (Figure 1.6) under the influence of protic media, heat or light (Tron et al., 2006). Medicinal chemists are focussing on synthesising derivatives of CA4 with conformational rigidity and to improve the improved pharmokinetic profile by incorporation of the ethylene linker into a ring system, which prevents spontaneous isomerization (McNulty et al., 2017; McNulty et al., 2015). They have been used against leukaemia, colon and lung cancers (McNulty et al., 2015; Penthala et al., 2015). Combretastatin, curcumin, and betulinic acid are being investigated in several clinical trials against several cancer cells (Dark et al., 1997; Goel, Kunnumakkara, & Aggarwal, 2008; Nathan et al., 2012).

Some phytochemicals isolated from plants are important anticancer agents with application in the treatment of several cancers and tumours. A summary of phytochemicals in clinical use or in clinical trials are presented in the (Table 1.1) below.

Table 1.1: Phytochemicals in clinical use or in clinical trials

Anticancer agent	**Isolated or derived from:**	**Compound activity**	**Research and clinical development**	**Reference**
Sulphorap-hane	Isotiocynanate in cruciferous vegetables Br assica	Induces phase 2 detoxification enzymes; inhibits tumour growth in breast cancers; antiproliferate effects	Clinical trials with oral administration of cruciferous vegetable preparation with sulphoraphane	(Gamie et al., 2017; Pledgie-Tracy et al., 2007; Cornblatt et al., 2007 & Heiss et al., 2001).

Paclitaxel (Taxol)	Taxane; *Taxus brevifolia L*	Microtubule disruptor; block mitosis; induce apoptosis; microtubules are polymerized and stabilized.	In clinical use; Phase I-III clinical trials; early treatment settings; non-small lung cancer, breast cancer, ovarian cancer, Kaposi sarcoma. Nanoparticles	(Cragg & Newman, 2005; Heiss et al., 2001; Pezzuto, 1997).
Epipodophyllotoxi	*Podophyllum peltatum L*.;	Pro-apoptotic effects; cell cycle interference	Lymphomas and testicular cancer trials	(Solowey et al., 2014; Unnati et al., 2013
Vincristine	*Catharanthus roseus G. Don*; Vinca alkaloids	Anti-mitotic; microtubule inhibitor; bind to β-tubulin; microtubule stabilizers or destabilizers; pro-apoptotic properties and induce cell cycle arrest; anti-tumour activity	Lymphomas, sarcomas and leukaemias; in clinical use; combination trials	(Solowey et al., 2014 ; Amin et al., 2009.
Vinblastine			Testicular cancer, Hodgkins disease and lymphoma; in clinical use; combination trials	(Solowey et al., 2014; Amin et al., 2009
Vinorelbine			Non-small cell lung cancer; single and combination trials; Phase I-III	(Solowey et al., 2014; Jordan & Wilson, 2004)
Vindesine			Clinical trials for acute lymphocytic leukaemia	(Solowey et al., 2014 & Amin et al., 2009)
Vinflunine			Clinical trials for activity against solid tumours; Phase III clinical trials	(Unnati et al., 2013 & Jordan & Wilson, 2004)
Pomiferin	Isoflavonoid isolated from *Maclura pomifera;*	Pro-apoptotic effects; DNA fragmentation; inhibits oxidative damage of DNA. Cytotoxicity of cancer cells	Growth inhibition in six human cancer cell lines: ACHN (kidney), NCI-H23 (lung), PC-3 (prostate), MDA-MB-231 (breast), LOX-IMVI (Melanoma), HCT-15 (colon)	(Amin et al., 2009 & Son et al., 2007)
Epigallacotechin-3-gallate	Catechin; green tea	Antioxidant; decrease DNA damage from oxidative stress; anti-proliferative effects	Clinical trials in prostate cancer treatment; Phase I clinical study for oral dose administration	(Amin et al., 2009; Raza & John, 2005 & Hakim et al., 2003).
Combretastatin A-4 phosphate	Water-soluble analogue of combretastatin;	Anti-angiogenic; vascular shut-down of tumours; tumour necrosis	Early trials; mimics developed; clinical and preclinical trials	Cragg & Newman, 2005)
Roscovitine	Derived from *olomucine.*	Inhibition of cyclin dependent kinases	Phase II clinical trials in Europe	Cragg & Newman, 2005)
Flavopiridol	Synthetic flavonoid derivative	Anti-inflammatory; tyrosine kinase activity; growth inhibitory effects	Phase I and Phase II clinical trials in solid tumours, lymphomas, leukaemias	Cragg & Newman, 2005 & Newcomb, 2004)
Noscapine	Opium poppy (*Papaver somniferum*)	Antiproliferative properties; microtubule interfering; inhibits tumour growth and progression	Phase I and II clinical trials; limited solubility; research into alternative administration of drug using analogues and nanotechnology.	(Chen et al., 2015; Jyoti et al., 2015; Henary et al., 2014)

(Greenwell & Rahman, 2015).

Sulphoraphane (Fahey et al., 2017; Gamie et al., 2017), Taxol (Zhang et al., 2014), Eppodophyllotoxin (Hande, 1998), Vincristine (Solowey et al., 2014) are in clinical use.

Newly approved anticancer drugs listed in Table 1.2 are being recommended by physicians for the treatment of different cancers. However, some of these drugs are under investigation in phase IV clinical trials with good results. Table 1.2 describes important patents on new chemotherapeutics (Jeswani & Paul, 2017).

Table 1.2: Patent information on different cancer therapeutics

S/N	Patent Publication Number	Inventors	Publication Year	Patent Description in Brief
1	CA 2598759 C	Baxter International Inc., Baxter Healthcare S.A., Berthold Roessler	2013	Preparation of film-coated tablet of trofosfamide with mixture of different film former in order to prevent environment contamination and drug hydrolysis
2	EP 0866712 A1	Nancy M. Gray	1998	Method of use of (−)-fotemustine
3	US 8,846,077 B2	David M. DeWitt	2012	Efficient and high-yielding method of preparation of nanoparticle using 96 well plate for different drugs including nedaplatin
4	EP 2244714 A1	Ronald A. Martell, David A. Karlin	2010	Combined delivery of picoplatin and bevacizumab for treatment of colorectal cancer
5	WO 2015107534 A1	Sougata Pramanick, Vikram Shamrao Gharge,	2015	Preparation of lyophilized and polymorphic forms of treosulfan
6	US 5,935,569 A	Andrzej Mackiewicz, Stefan Rose-John	1999	Vaccines prepared by combination of genetically modified autologous and/or allogeneic cancer cells for improving the body immunogenicity
7	EP 2852413 A4	Steven J. Sucheck, Katherine A. Wall, Sourav Sarkar	2016	Method for incorporating lipid-bound xenoantigen onto the target antigen and then encapsulating into liposomes
8	WO 2014145642 A2	Shyam S. Biswal, Anju Singh	2014	Preparation method and composition of nuclear factor erythroid-2 related factor-2 (Nrf2) inhibitors
9	WO 2014079709 A1	Laurent Gros, Patrice Dubreuil, Alain Moussy, Stéphane Audebert,	2014	Method of intervention comprising analogs of (deoxy)nucleoside or (deoxy)nucleotide in conjugation with small molecule inhibitor/activator for combined treatments
10	US 20,070,093,449 A1	Peter De Porre, Willy Dries, Marc Francois	2007	Preparation method for IV administration of tipifarnib for patients who cannot take oral dose
11	WO 2013130407 A1	Mary Matsui, Daniel Yarosh	2013	Compositions of compounds that inhibit Cu-ATPases, ATP7A and ATP7B
12	US 8,822,500 B2	Vlad Edward Gregor	2014	Use of different small molecules to inhibit anaplastic lymphoma kinase for prevention against hematological and other cancers
13	WO 2014011489 A2	Jeffrey Molldrem, Anna Sergeeva	2014	Method of presenting monoclonal antibody for HLA-A2- restricted peptide PR-1 recognition and their use in diagnosis and treatment thereof

14	EP 2956484 A2	Harry M. Meade	2015	Method of modification of glycosylation models and production of associated antibodies
15	EP 2101747 B1	Andrew P. Feinberg, Andre Levchenko, Dan L.	2015	Inhibition of signal by loss of imprinting of the insulin-like growth factor II gene for decrease in frequency of neoplasia
16	WO 2010022243 A1	J. Silvio Gutkind, Panomwat Amornphimoltham,	2010	Administration of mammalian target of rapamycin inhibitor for prevention of head and neck squamous cell carcinoma

(Jeswani & Paul, 2017).

1.2 Infectious Disease.

Infectious diseases are disorders caused by pathogenic organisms such as parasites, fungi, viruses or bacteria. Some microorganisms live normally in our bodies and some are even helpful and harmless but under certain conditions, they inflict pain and cause diseases to their hosts. Symptoms of infectious diseases vary depending on the causal agent, and they include: diarrhoea, coughing, sneezing, muscle ache, fatigue, fever etc. (Sanchez & Doron, 2017).

Infection is often not synonymous with disease as most infections do not lead to diseases whereas others do lead to varying diseases which could vary in its severity in the affected hosts, and a manifestation of clinical signs and symptoms is often evident in this case. Although several factors contribute to virulence of infectious agents, and the activities occurring between the hosts – virus is a key contributory factor and a determinant of the virulence of infections. Virulence is often associated with the pathogenicity or non-pathogenicity of an infectious agent. Virulence is therefore a relative term explaining or comparing two variables; for instance, one could say bacteria A is more virulent than bacteria B or bacterial strains A is more virulent in humans than in monkeys. The term virulence and pathogenicity refer to the ability of a microorganism to cause diseases in the hosts.

For a microorganism to cause disease, the host must first be infected by the organism after which they spread within the organism, damaging target tissues and may invade the totality of the organism. However, some infections remain localised. After infecting their host, microorganisms are propagated / transmitted to individuals susceptible to them via excretion or secretion into surrounding environments, taken up by a vector or individuals within the vicinity or from one family member to another living within a close range.

Microorganisms enter the body via different routes such as the gastrointestinal tract, the respiratory tract, the skin and other routes such as body fluids.

In this study, we shall concentrate on isolating and developing antimicrobial agents from natural product sources (plant) which could potentially kill bacteria and fungi.

1.2.1 Microbial Infection

Development and identification of specific chemical or biological medicine to prevent, control, or treat patients suffering from microbial infections which causes disease was a

major breakthrough in medical science. This breakthrough was short-lived as the onset of antibacterial resistance emerged, making it difficult to treat certain infections.

Bacterial infection has claimed millions of lives throughout history and many have died globally from diseases caused by bacteria such as tuberculosis – caused by *Mycobacterium tuberculosis* (Clark et al., 2017), black- death or bubonic plague which is caused by, and *Yersinia pestisbacteria* (Gonzalez & Miller, 2016) as well as many other bacterial infections which have devastated humans for centuries. There is a need to develop measures that can curtail the rampant spread of infection and mortality. This includes; provision of portable drinking water, increased sanitation and hygiene, improved education on subjects (especially on disease prevention and control) and most importantly the development of antibiotics to combat these infections. The discovery and introduction of antibiotics has offered many health benefits including decrease in death rate from bacterial infections (Clark et al., 2017; Gonzalez & Miller, 2016; MacGowan & Macnaughton, 2017).

Despite the many benefits of antibiotics, many bacterial strains have developed antibacterial resistance to current therapies. As antibiotic resistance increases, the development of new antibiotics is drying up. With just over eighty years of antibiotics use, most infections which were easily treated are now difficult to treat. Antimicrobials have not only gained prominence in the treatment of bacteria alone but also in many other areas of medical practices such as immunosuppressive treatments (MacGowan & Macnaughton, 2017), which depend on antibiotic prophylaxis and its effectiveness in treatment of infective complications. Resistance to antibiotics therefore is a serious health concern as antibiotic use correlates with antibiotic resistance (MacGowan & Macnaughton, 2017). There is therefore a need to develop new antimicrobials to replace the already depleting ones.

1.2.1.1 Bacterial infection

Bacteria causing infection can be classified into Gram-positive and Gram-negative bacteria depending on the bacterial surface morphology and their differential abilities to stain to the Gram dye. Among bacterial organism, the Gram-positive organisms are the leading cause of infectious diseases and nosocomial blood infections in both the industry and clinics globally (Paterson & Bonono, 2005; Sanchez & Doron, 2017). In the 20th century, a major breakthrough was the discovery of antibiotics which have been used in treating diseases and through upgrades of the existing antibiotics, to treat pathogenic

microorganisms via successful clinical trials. However, many of these antibiotics have been abused and used wrongly, giving rise to antibiotic resistance which has become a major concern in public health. The pressure caused by resistant microorganisms such as vancomycin-resistant *Enterococci*, *Klebsiella pneumoniae, Streptococcus pneumoniae* and *methicillin-resistant Staphylococcus aureus (MRSA)*, have created a huge predicament and medical challenge in the treatment of infectious diseases with a consequence of an increased clinical expenses and failures. Paterson & Bonono (2005) reported the increased incidences of antimicrobial resistance posed by these drug-resistant bacteria.

1.2.1.2 Basic Anatomy of Bacterial Cell

Nearly every genus of bacteria possesses a rigid, carbohydrate-containing structure that surrounds the bacterial cell. This structure is called cell wall. The genus *Mycoplasma* and few exceptions do not have cell walls. The tough rigid structure protects the bacterium from damage by encircling it. Despite the rigidity of the cell wall, small molecules can easily pass through to the cell membrane thus acting as a coarse filter which exclude large molecules. Maintaining the shape of the cell and prevention of cell lysis (bursting) because of osmotic pressure is the primary function of the cell wall (Kapoor et al., 2017). Peptidoglycan is a complex molecule composed of alternating units of N-acetylmuramic acid (NAM) and N-acetylglucosamine (NAG) cross-linked by short peptides. This allows the cell wall to be tough and rigid yet allowing the movement of particles.

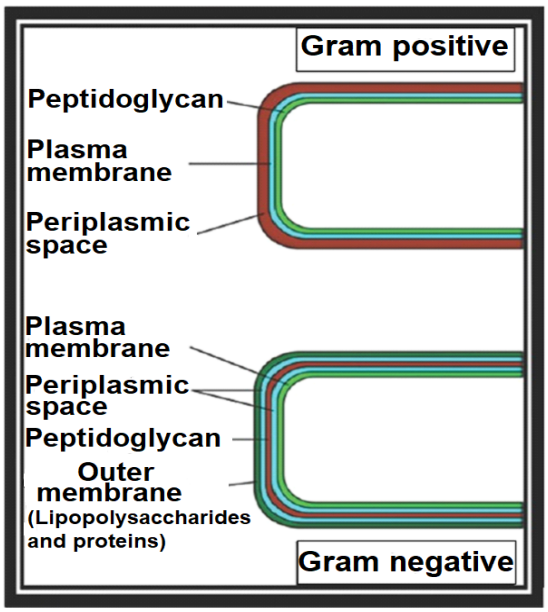


Figure 1.7: Structure of bacterial cell envelope

Gram-negative bacteria possess the outer membrane consisting of liposaccharides and proteins. This is lacking in the Gram-positive bacteria (Kapoor et al., 2017).

The Gram-positive bacteria consists of cytoplasmic membrane surrounded by a rigid and tough mesh known as the cell wall. Gram-negative bacteria consist of a thin cell wall surrounded by a second lipid membrane known as the outer membrane (OM). The space between the cytoplasmic membrane and the OM is called the periplasm (Figure 1.7). Gram-positive bacteria lack the OM, which provides additional protection to the Gram-negative bacteria and prevents many substances from penetrating the bacteria. Despite the role of the OM, porins (channels present in the bacterial membrane) allows the entry of molecules such as drugs (Hauser, 2015). The cell wall gives bacteria their shapes and prevent it from mechanical and osmotic stress. Ions are prevented from leaking out or flowing into the cell by the cytoplasmic membrane (Kapoor et al., 2017).

Example of Gram-positive bacteria used in this study include *Bacillus cereus, Staphylococcus aureus, MRSA, Enterococcus faecalis* and *Pseudomonas fluorescens*; and Gram-negative bacteria – *Pseudomonas aeruginosa, Klebsiella pneumonia, Escherichia coli* (Kappoe et al., 2017). These bacteria were carefully selected in such a manner that the antibacterial activity of compounds tested in this study can be investigated against Gram-positive and Gram-negative bacteria, and to investigate the antibacterial activities of compounds to be tested against extended-spectrum β-lactamase (ESBL)-producing bacteria (such as *Escherichia coli, Klebsiella pneumoniae* and *Pseudomonas aeruginosa*), bacteria associated with food poisoning (*Bacillus cereus*, *E. coli*), bacteria associated with nosocomial infection (*MRSA)* (Paterson & Bonomo, 2005). These bacteria are a major health concerns especially the ESBL as discussed below.

1.2.1.2.1 Gram-negative Bacteria

A serious threat to global public health is the emergence of antibiotic-resistant pathogens (Boucher et al., 2009). In the year 2009, the United States Infectious Disease Society (IDSA) launched an initiative known as the 10 x ‘20 initiative. This initiative is a global commitment aimed at developing 10 novel antibiotics by 2020 (IDSA, 2020) to treat infections caused by “ESKAPE” pathogens (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa,* and *Enterobacter* species) (Mo et al., 2019). This initiative has been applauded by the World Health Organization (WHO) who has listed *carbapenem-resistant Enterobacteriaceae (CRE)*, *A. baumannii* and *P. aeruginosa* as multidrug-resistant (MDR) Gram-negative pathogens which requires urgent public health attention. The American

Food and Drug Administration (FDA) have approved four clinically active drugs against Gram-negative pathogens including plazomicin (June 2018), meropenem / vaborbactam (MER/VAB) (August 2017), ceftazidime /avibactam (CAZ/AVI) (February 2015) and ceftolazane/tazobactam (C/T) (December 2014) (Mo et al., 2019). Several other antibiotic candidates are being developed against Gram-negative bacteria - cefepime/VNRX-5133, eravacycline, cefiderocol, aztreonam/avibactam and imipenem/cilastatin/relebactam (Mo et al., 2019).

1.2.1.2.1.1 *Escherichia coli*

Escherichia coli dwell mainly in the intestine of humans and animals; the pathogenic strains can cause bloody diarrhoea – such as the diarrheagenic *E. coli* (DEC) (Rehman et al. 2018), vomiting and severe abdominal cramp. It is one of the major causes of food poisoning. It is a rod-shaped, facultative, anaerobic Gram-negative bacterium (Vogt & Dippold, 2005). *E. coli* cause diseases mainly via the faecal-oral transmission route. Another strain of *E. coli* is the extended-spectrum β-lactamase (ESBL)-producing *E. coli*. ESBL are enzymes that confers antimicrobial resistance to most β-lactam antibiotics such as monobactam aztreonam, cephalosporins and penicillins (Ben-Ami et al., 2009). Hospitals and community-acquired ESBL producing *E. coli* are prevalent globally as their identification in clinical laboratories can be challenging (Ben-Ami et al., 2009). Carbapenems have been reported to be the best antibacterial agents for treatment of ESBL-producing organisms (Papp-Wallace et al., 2011). Beta-lactamases are enzymes that open the beta-lactam ring, inactivating the antibiotic (Ben-Ami et al., 2009).

1.2.1.2.1.2 *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a rod-shaped Gram-negative bacterium which is a multi-drug resistant pathogen. It is an opportunistic bacterium which causes ventilator-associated pneumonia and other nosocomial infections and sepsis disorder. It is pervasive in those whose resistance to disease has been destroyed and it is the root cause of mortality and morbidity in cystic fibrosis (Fine et al., 1996). Its ability to grow at 25 – 37 °C and even at 42 °C enable *P. aeruginosa* to be distinguished from other *Pseudomonas* species (Prince, 2012). It causes disease in both plants and animals, including humans especially in patients with cystic fibrosis, cancer and those with poor immunity (eg. AIDS patients) (Prince, 2012). The increased prevalence of antibiotic resistant bacteria has continuously challenged modern medicine. As an opportunistic pathogen, *P. aeruginosa* is a major cause of multidrug resistant diseases, especially in

hospitalized and immune-compromised individuals (Gholizadeh et al., 2017). Recently, World Health Organisation (WHO) has identified *P. aeruginosa* as a priority pathogen due to the threat of drug resistant diseases (Skariyachan et al., 2018) and the ability of this bacterium to form biofilm. Biofilm formation worsens severe infections especially in cystic fibrosis patients (Rodriguez-Sevilla et al., 2018). Biofilms are microbial communities (usually adherent to surfaces) encapsulated in a complex of extracellular matrices composed mainly of different lipids, extracellular DNA, proteins and polysaccharides (Flemming et al., 2016). Clinical consequence of biofilms includes wide range of drug-resistance (Kafil & Mobarez, 2015). Bacterial cells inside the biofilms show unmistakable features from their planktonic partners, making them 1000-overlay more prominent than planktonic cells (Hall et al., 2018; Mah et al., 2003). Diminished helplessness of biofilms permits biofilm-based diseases to hold on regardless of anti-microbial treatment (Aghazadeh et al., 2016; Hall and Mah, 2017). A few hereditary systems have been found to be responsible for formation of biolfilms in *P. aeruginosa*, in which some are very much understood, while others are poorly comprehended (Hall et al., 2018).

1.2.1.2.1.3 *Klebsiella pneumonia*

Klebsiella pneumonia is a non-motile Gram-negative, facultative, lactose-fermenting rod-shaped bacterium. Although it is present in the intestine, skin and mouth as a normal flora of these tissues, it can damage the lungs (alveoli) if inhaled. They are resistant to most drugs (Arnold et al., 2011). It belongs to *Klebsiella* genus of *Enterobacteriaceae* and it is the most important clinical member of this group and the third microorganism commonly isolated in blood cultures of patients with sepsis (Xu et al., 2011). It is present in immunocompromised humans and affects mainly the liver and other organs, blood (sepsis), urinary tract and lung (Williams et al., 1990). It is an opportunistic pathogen frequently encountered in hospitals globally and the bacterium is developing resistance to multiple antibiotics including the β-lactams and the cephalosporins and are associated with high mortality rates especially in immunodeficient individuals (Stahlhut et al, 2012). Mortality reported for *K. pneumonia* in studies from Asia (44.82%), Europe (50.06), South America (46.71) and North America (33.24) (Xu et al., 2017) indicates alarming death rates globally from *K. pneumonia* with Europe having the highest mortality rate. Globally, the mortality rate of patients with blood stream infections is 54.30 %, and 13.52 % for those with urinary tract infection. However, the mortality rate for patients with urinary tract infection admitted to the intensive care unit (ICU) was significantly higher (43.13 %) compared to patients not admitted to ICU. The mortality rate for patients admitted to ICU

with bloodstream infection remains high (48.9) (Xu et al., 2017), an indication that *K. pneumonia* is a serious nosocomial health problem. This rate is believed to be significantly higher in hospitals than in reported data (Kanoksil et al., 2013). From January 2007 to December 2008, a large outbreak was reported in a Greek university hospital with 50 patients out of which 34 were in ICU. 18 were infected while 32 were colonised by KPC-2-producing *K. pneumoniae*. 37.5 % mortality rate was reported for non-ICU and 58.8 % for ICU (Souli et al., 2010). The New Delhi multiple DNA resistant strains (NDM-1) accounted for most of the cases reported in India and has been isolated from a French patient who has never travelled to India (Arpin et al., 2012) and this strain has now been a global concern, causing epidemics in Asia, Europe and North America. Another *K. pneumonia strain* – the ST11 first reported in France in 1997, is commonly isolated in Brazil, Spain, Portugal, Norway, Netherlands, South Korea, Hungary and China (Andrade et al., 2011).

Table 1.3 summarizes selected clinical trials of newer antimicrobial agents against MDR Gram-negative bacteria.

Table 1.3: Selected clinical trials of newer antimicrobial agents against MDR Gram-negative bacteria

Study (year)	**Design**	**Indication**	**Study drugs**	**Results (Drug versus comparator)**
Ceftazidime/avibactam (CAZ/AVI)				
RECAPTURE (Wagenlehner et al., 2016)	Phase III, R, DB, Non-Inferiority	cUTI/acute pyelonephritis	CAZ/AVI 2.5 g q8hrs vs. doripenem 500 mg q8hrs, up to 10 or 14 days	-Patient-reported symptomatic resolution at 5 days (70.2% vs. 66.2%) -Combined symptomatic resolution/microbiological eradication at TOC (71.2% vs. 64.5%) -Microbiological eradication at TOC (77.4% vs. 71%)
REPRISE (Carmeli et al., 2016)	Phase III, R, open-label	cUTI or cIAI due to ceftazidime-resistant Gram-negative pathogens	CAZ/AVI 2.5 g q8 hrs vs. BAT, for 5–21 days	-Clinical cure at TOC (91% vs. 91%)
RECLAIM (Mazuski et al., 2016)	Phase III, R, DB, Non-Inferiority	cIAI	CAZ/AVI 2.5 g q8 hrs (+ metronidazole 500 mg q8hrs) vs. meropenem (1 g q8 hrs) for 5–14 days	-Clinical cure at TOC in mMITT population (81.6% vs.85.1%) -Clinical cure at TOC in MITT population (82.5% vs. 84.9%) -Clinical cure at TOC in CE population (91.7% vs. 92.5%)
REPROVE (Torres et al., 2018)	Phase III, R, DB,	HABP or VABP	CAZ/AVI 2.5 g q8hrs vs. meropenem 1 g	-Clinical cure at TOC in mCITT population (68.8% vs. 73%)

	Non-Inferiority		q8hrs, for 7–14 days	-Clinical cure at TOC in CE population (77.4% vs. 78.1%)
Ceftolozane/tazobactam (C/T)				
ASPECT-cUTI (Wagenlehner et al., 2015)	Phase III, R, DB, Non-Inferiority	cUTI	C/T 1.5 g q8 hrs vs. levofloxacin 750 mg IV q24hrs for 7 days	-A composite of microbiological eradication and clinical cure in mMITT (76.8% vs. 68.4%)
ASPECT-cIAI (Solomkin et al., 2015)	Phase III, R, DB, Non-Inferiority	cIAI	C/T 1.5 g q8 hrs (+ metronidazole 500 mg q8hrs) vs. meropenem 1 g q8hrs for 4–14 days	-Clinical cure at TOC in MITT population (83% vs. 87.3%) -Clinical cure at TOC in microbiologically evaluable population (94.2% vs. 94.7%)
Meropenem/vaborbactam (MER/VAB)				
TANGO-I (Kaye et al., 2018)	Phase III, R, DB, Non-Inferiority	cUTI/acute pyelonephritis	MER/VAB 4 g q8 hrs vs. piperacillin-tazobactam 4.5 q8 hrs up to 10 days	-Clinical cure or improvement or microbiological eradication at EOIVT (98.4% vs. 94%)
TANGO-II (Kaye et al., 2017; Wunderink et al., 2017)	Phase III, R, open-label	Known or suspected CRE pathogens, cUTI/acute pyelonephritis	MER/VAB 4 g q8hrs vs. BAT, for 7–14 days	-Clinical cure at EDT (64.3% vs. 33.3%) -Clinical cure at TOC (57.1% vs. 26.7%) −28-day mortality (17.9% vs. 33.3%)
Plazomicin				
EPIC (Cloutier et al., 2017)	Phase III, R, DB	cUTI/acute pyelonephritis	Plazomicin 15 mg/kg q24hrs vs. MER 1 g q8hrs (± levofloxacin po 500 mg q24hrs), for 7–10 days	-Composite cure in mMITT population at TOC (81.7% vs. 70.1%)
CARE (McKinnell et al., 2017)	Phase III, R, open-label	bloodstream infection or HABP/VABP	Plazomicin 15 mg/kg q24hrs vs. colistin 300-mg lading dose; 5 mg/kg/	-All-cause mortality at day 28 in mMITT population (11.8% vs. 40%)
Imipenem/cilastatin/relebactam (IMI/REL)				
Lucasti et al. (Lucasti et al., 2016)	Phase II, R, DB Dose-ranging study	cIAI	IMI (500 mg) + REL (125 mg or 250 mg) q6hrs vs.	-Clinical response rates at DCIV (98.8% [REL 125 mg] vs. 96.3% [REL 250 mg] vs. 95.2%)
Sims et al. (Sims et al., 2017)	Phase II, R, DB Dose-ranging study	cUTI/acute pyelonephritis	IMI (500 mg) + REL (125 mg or 250 mg) q6hrs vs. IMI (500 mg q6hrs)	-Microbiological response rate at DCIV in ME population (98.6% [REL 125 mg] vs. 95.5% [REL 250 mg] vs. 98.7%)
Cefiderocol				
APEKS-cUTI (Portsmouth et al., 2017)	Phase II, R, DB Non-inferiority	cUTI/acute uncomplicated pyelonephritis	Cefiderocol (2 g) q8 hrs vs. IMI (1 g) q8 hrs, for 7 to 14 days	-The composite of clinical or microbiological response at TOC in MITT population (72.6% vs. 54.6%)

				-Clinical response rate in MITT population (89.7% vs. 87.4%)

(Mo et al., 2019)

R = randomized; ME = microbiologically evaluable; DCIV = discontinuation of IV therapy; EDT = end of therapy; CRE = carbapenem-resistant Enterobacteriaceae; clinically evaluable = CE; EOIVT = end of IV therapy; mCITT = clinically modified intention-to-treat; VABP = ventilator-associated bacterial pneumonia; mMITT = microbiologically modified intention to treat, HABP = hospital-acquired bacterial pneumonia; BAT = nest available therapy; cIAI = complicated intra-abdominal infection; TOC = test of cure; DB = double blind; cUTI = complicated urinary tract infection.

1.2.1.2.2 Gram-positive Bacteria

1.2.1.2.2.1 *Staphylococcus aureus*

Staphylococcus aureus are species of bacteria which can cause various types of diseases. It is a coccal Gram-positive bacterium found on the skin, respiratory tract and nose, and is responsible for food poisoning, abscess, skin and soft tissue infections, wound and respiratory infections. It is also responsible for toxic shock syndrome, cellulitis, impetigo, and boils (sinusitis) (Cole et al., 2001; Kluytmans et al., 1997). Over one hundred and seventy-nine *Staphylococcal* strains have been isolated in humans (Xu et al., 2011), and among all species isolated, *S. aureus* has been a leading health concern in the industry, public and the community and has caused a negative impact on the economy and medical sectors of many nations (Sanchez & Doron, 2017). *S. aureus* commonly colonises healthy humans. *Staphylococcus aureus* usually colonise areas such as the nose, throat, genitals, rectum and the perineum of humans (Sanchez & Doron, 2017). It is estimated that 20 to 50 cases / 100,000 population per year are affected by *S. aureus* bacteria globally, with a mortality rate of 10 % and 30 % (Val-Hal et al., 2012). This accounts for a greater number of deaths compared to deaths from viral hepatitis, tuberculosis and AIDS combined. The number of deaths recorded from invasive *S. aureus* was double (25 – 50 %) globally prior to the introduction of penicillin (van-Hal et al., 2012).

1.2.1.2.2.2 *Enterococcus*

Enterococci are a dominant group of bacteria in the intestinal flora of animals and humans. It is a Gram-positive bacterium (classified previously as *Streptococcus faecalis*). It is a commensal bacterium which is resistant to most antibiotics used clinically and it is implicated for urinary tract infection, pelvic and abdominal abscess (Sharifi et al., 2013).

Enterococcus is associated with nosocomial infections due to its intrinsic resistant mechanisms against antibacterial agents as it rapidly acquires antibacterial resistance (Kilbas & Ciftci, 2018). *Enterococcus* have also developed resistance to vancomycin (vancomycin-resistant *Enterococci*). Two species of these bacteria has been identified: *Enterococcus faecalis* and *Enterococcus faecium*. Again, integrons have been associated with *Enterococcus faecalis* and is essential to the resistance of this bacterium to some antimicrobial agents (Deng et al., 2015; Sanchez & Doron, 2017). Many factors are responsible for the increase incidence of enterococcal infections such as extensive use of broad-spectrum antibiotics, long intensive treatments for haematology–oncology patients, increasing numbers of immunocompromised patients and the spread of multi-resistant enterococci (Devrim, Gülfidan et al. 2015). *Enterococci* has a natural ability to acquire, accumulate and share extrachromosomal elements encoding antibiotic resistance genes or virulence traits thus their increasing importance as nosocomial pathogens (Klibi, Gharbi et al. 2006) as they do not just cause hospital infections but also seen increasingly in community-related infections (Aguş et al., 2006), thus their increasing importance as bacterial specie.

1.2.1.2.2.3 *Bacillus cereus*

Bacillus cereus is a rod-shape, aerobic, Gram-positive haemolytic bacterium. Its toxins cause vomiting, nausea and diarrhoea. It causes food poisoning especially from fried rice (Tewari & Abdullah, 2015). It is also known as a mesothelic microorganism because it can grow at temperature range of 10 – 50 °C (with an optimum growth temperature of 28 – 37 °C). Only few strains can grow below 7 °C or above 45 °C. *B. cereus* grows at an optimum pH ranging from 6.0 – 7.0 but can still grow at pH range 4.3 – 9.3. Its spores survive drying and freezing and are moderately heat resistant. Other strains require heat activation for spores to germinate and grow (Tewari & Abdullah, 2015). *B. cereus* can also grow in the intestinal tract of insects and animals, decaying matter, vegetables, water, and soil (where toxins can persist) (Garofalo et al., 2017; Osimani et al., 2017). Two enterotoxins namely non-haemolytic enterotoxin (NHE) and haemolysin BL (HBL) have been identified in *B. cereus*. These enterotoxins combine to manifest their pathogenicity (usually 8 – 16 h after ingestion) (Kumari & Sarkar, 2016). The NHE is the cause of food poisoning while the haemolysin BL possesses dermo-necrotic and haemolytic activities and can cause accumulation of fluid in rabbit ileal loops (Ghelardi et al., 2002) but their mechanism of action is not fully understood (Tausch et al., 2017). It

has been reported also that the ATCC 10876 strain harbours both the HBL and NHE genes (Sastalla et al., 2013). Nevertheless, infections caused by *B. cereus* are self-limiting and have short durations in their host and oftentimes do not present post illness treats (Sastalla et al., 2013). In European Member States, 287 cases of *B. cereus* outbreaks have been reported out of a total of 3073 cases (with 8 % hospitalisation) in 2014. This figure remained relatively constant in 2015 (291 outbreak out of 3131 cases), with 3 % hospitalization rate. Other outbreaks (4 out of 17 cases) in Norway and France (15 outbreaks) in the same year were also reported (EFSA and ECDC., 2016). These outbreaks were traced to *B. cereus* toxins in mixed foods with almond based food accounting for 2 outbreaks and cereal-based food (8 %) (EFSA and ECDC., 2016).

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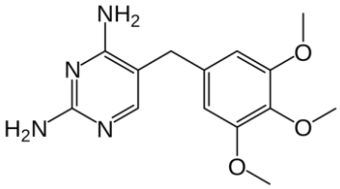
1.2.2 Antibacterial Drugs

Antibacterial drug is a term referring to a group of drugs that includes antiprotozoal, antifungals and antibacterial (Vardanyan and Hruby 2016). The term also includes antibiotics (produced by microorganisms). The discovery of sulphonamide was a significant milestone event in microbiology due to its activity against both Gram-negative and Gram-positive bacteria (Vardanyan & Hruby, 2016). It should be noted that the anticancer activity of sulphonamide has been presented in section 1.1.3.1.3. This section therefore focuses on the antibacterial activity of sulphonamide.

The use of antimicrobial agents in treating infectious diseases could be traced to the year 1910 when herbs and mercury salts were used for this purpose. In the 20th century, salvarsan - (hydroxyaniline derivative of arsenic) obtained by screening substances used in the dye industry in the year 1910. The next antibacterial agent was sulfa drugs in 1930s. On discovery the sulfa drugs acts by inhibiting the folate pathways. Pyrimidine class of compounds were optimized in 1960s and used to screen a variety of bacteria, and trimethoprim (Structure 1.37) indicated good antimicrobial activity (Silver, 2011). However, the discovery of penicillin from *Penicillium notatum* in 1928 by Alexander Fleming brought about a revolution in antibacterial agents as penicillin was the most effective treatment against *Staphylococcus* and other Gram-positive bacteria (Silver, 2011; Singh & Barrett, 2006). Penicillin was purified in 1940.

For nucleic acids to be produced, p-aminobenzoic acid is required by certain bacteria for the synthesis of dihydrofolic acid (used for nucleic acid synthesis). p-Aminobenzoic acid is a key enzyme in folic acid synthesis. Sulphonamides (an antimetabolite), substitutes

for p-aminobenzoic acid resulting in inhibition of enzymes needed for synthesis of purine bases (Vardanyan & Hruby, 2016).



Structure 1.37: trimethoprim (Silver, 2011).

Sulphonamides are among the oldest and widely used antibacterial and they inhibit a vital enzyme (dihydropteroate synthetase) which converts p-aminobenzoic acid to dihydrofolate which is a precursor for tetrahydrofolate formation. Tetrahydrofolate is required for the *de novo* synthesis of purines, thymidylate and some amino acids (such as glycine and tryptophan). Inhibition of folate synthesis in bacteria leads to inhibition of bacterial growth (Vardanyan & Hruby, 2016).

Quinolones are synthetic, broad spectrum bactericidal antibacterial agents which inhibits the DNA gyrase (topoisomerase II), an enzyme required for the replication of the bacteria. Blocking this enzyme leads to inhibition of DNA replication and transcription thus inhibition of bacterial growth (Vardanyan & Hruby, 2016). This unique mechanism of bacterial growth inhibition by the quinolones birth the synthesis of the nitrofurans (synthetic broad-spectrum antibacterial drugs) characterised by the 5-nitro-2-furanyl scaffold (Vardanyan & Hruby, 2016). The mechanism of actions of the nitrofurans is not fully understood but the nitrofurans inhibits bacterial enzymes especially those involved in carbohydrate synthesis (Vardanyan & Hruby, 2016), thus inhibition of key bacterial enzymes by the quinolones will inhibit bacterial growth and reproduction.

1.2.2.1 Classes of Antibacterial Drugs

Antibacterial drugs are classified into the following classes: Sulphonamides such as co-trimoxazole (Bactrim) and trimethoprim (Proloprim). Other classes of antibiotics include the: aminoglycosides such as gentamycin (Garamycin) and tobramycin (Tobrex), tetracyclines such as tetracycline (Sumycin, Panmycin) and doxycycline (Vibramycin), fluoroquinolones such as ciprofolxacin (Cipro), levofloxacin (Levaquin), and ofloxacin (Floxin), macrolides such as erythromycin (E-Mycin), clarithromycin (Biaxin), and azithromycin (Zithromax), cephalosporins such as cephalexin (Keflex) and penicillin such as penicillin and amoxicillin (Kapoor, Saigal et al. 2017, Ullah and Ali 2017). Antibacterial agents are also classified base on their mode of action into those involved in: i) Inhibition

of cell wall synthesis, ii) inhibition of protein synthesis, iii) inhibition of key metabolites necessary for survival of pathogen, iii) disruption of cellular homeostasis by disrupting the permeability of bacterial cell membrane, iv) alteration of nucleic acid synthesis and v) inhibition of bacterial nucleic acid synthesis – Figure 1.8**.** (Vardanyan & Hruby, 2016; Kapoor et al., 2017).

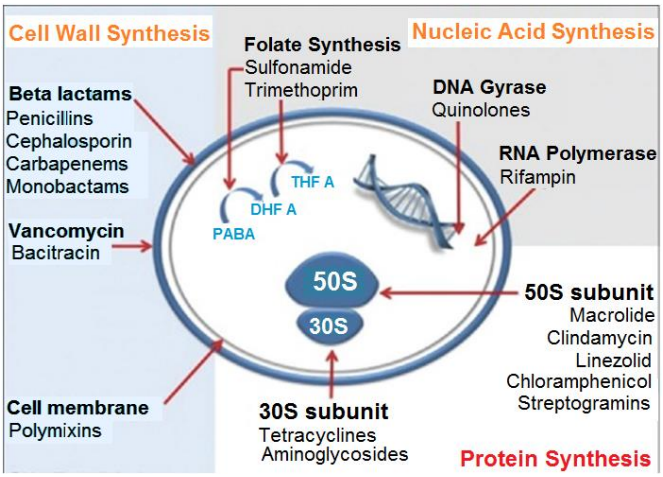


Figure 1.8: Mechanism of action of antibiotics.

Diagram is an illustration of the classes of bacterial agents involved in inhibition of cell wall synthesis (β-lactams, vancomycin), nucleic acid synthesis (folate inhibitors, inhibitors of DNS gyrase, and inhibitors of RNA polymerase) and protein synthesis. Inhibitors of protein synthesis are classified into inhibitors of the 50S and 30S sub-units respectively. The inhibitor of folate synthesis – sulphonamide, inhibits the conversion of para-aminobenzoic acid (PABA) to dihydrofolic acid (DHF A); trimethoprim inhibits the conversion of DHF A to trihydrofolic acid (THF A) (Kapoor et al., 2017).

1.2.2.1.1 Inhibition of bacterial cell wall synthesis

Antibacterial agents involved in inhibition of cell wall synthesis include i) the cephalosporins – 1st generation – cephalexin, cephazolin, 2nd generation – cefoxitin, cefuroxime, 3rd generation – ceftriaxone, cefotaxime, 4th generation – cefepime, ii) penicillin: these class of bacteria are grouped based on the relationship between these class of drug and its interaction with the bacterial enzymes thus; a) penicillin with beta-lactase inhibitors – amoxy-clavulanate, b) peniclinase susceptible with activity against Gram-negative bacilli – piperocillin, amoxil , ampicillin, c) penicillinase resistance – oxacillin, methicillin and d) penicillinase susceptible – pen V and G (Vardanyan & Hruby, 2016; Ullah & Ali, 2017), iii) carbopenems — meropenem, imipenem, iv) Glycopeptide derivatives — bleomycin, teichoplanin and vancomycin (Ullah & Ali, 2017).

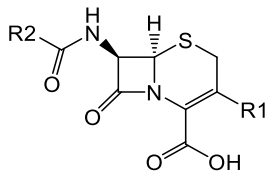
Most antimicrobial agents target the synthesis of bacterial cell wall. However, the composition of bacterial cell wall differs from specie to species. By targeting the cell wall, antimicrobial agents can kill bacteria whose cell wall is susceptible to these antimicrobials. The polymer murein also known as peptidoglycan is an important constituent of the cell

wall. Its location and quantity in a bacterial cell wall differ between Gram-positive and Gram-negative bacteria. Murein consists of repeating units of N-acetylmuramic acid (NAM) and N-acetylglucosamine (NAG) disaccharide subunits. These disaccharide chain crosslink with peptide side chains of neighbouring murein units, offering mechanical strength to it, and allows for diversity in the biochemical nature of the cross-linkages between and within bacterial species (Krishnamoorthy et al., 2017; Mandal et al., 2017; McDermott et al., 2003), a diversity exploited by antimicrobial agents.

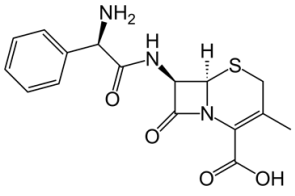
Biosynthesis of peptidoglycans proceeds in four stages:

i) Precursor synthesis in the cytoplasm (Vardanyan & Hruby, 2016).
ii) Transportation of precursors across the cytoplasm (Vardanyan & Hruby, 2016; Krishnamoorthy et al., 2017).
iii) Insertion of glycan units into the cell wall (Vardanyan & Hruby, 2016).
iv) Maturation and linking of transpeptIdes (Krishnamoorthy et al., 2017; McDermott et al., 2003).

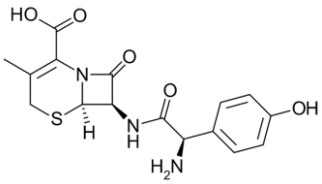
Bacitracin and D-cycloserine inhibits peptidoglycan synthesis at stage 1 and 2 while glycopeptides and β-lactams inhibit bacteria at stage 3 and 4. β-lactam are the most common bacterial cell wall inhibitors and include cephalosporins and its derivatives such as cefelexin and cefadroxil (Structure 1.38 – 1.40) and penicillin and its derivatives such as cloxacillin and ampicillin (Structures 1.41 – 1.43, which binds to transpeptidases forming antibiotic-enzyme covalent complexes and generate mature molecule of peptidoglycan. For β-lactams to exert antibiotic effect on Gram-negative bacteria, they must pass through porins, which are proteins in the cell wall before reaching their targets known as penicillin-binding proteins (PBPs) (McDermott et al., 2003).



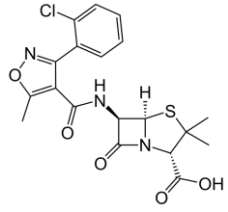
Structure 1.38: Cephalosporin



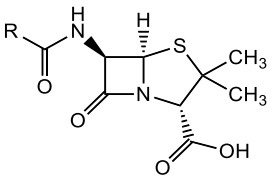
Structure 1.39: Cefalexin



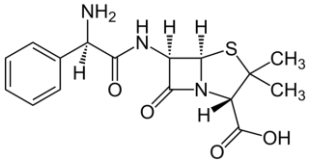
Structure 1.40: Cefadroxil



Structure 1.41: Cloxacillin



Structure 1.42: Penicillin



Structure 1.43: Ampicillin

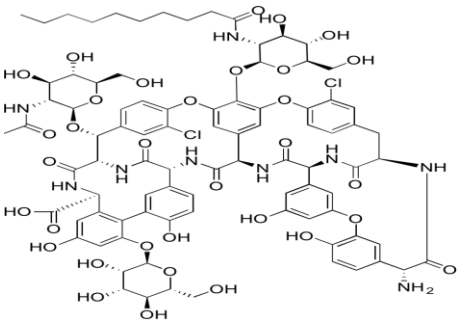
(Krishnamoorthy et al., 2017).

The ability of antibacterial agents to bind to cell wall transpeptidases depends on the agents and PBP involved. Others inhibits the formation of cell walls which easily lyse under increased osmotic pressure.

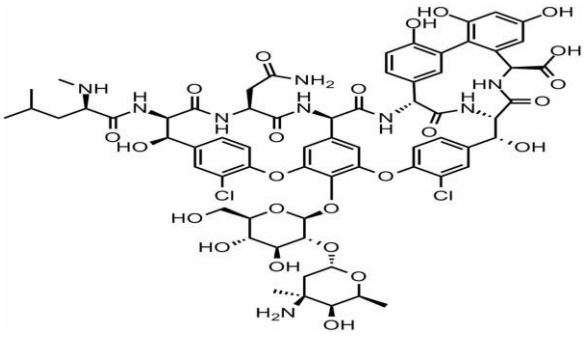
Some bacteria develop resistance to β-lactams via one or more mechanisms such as:

i) A change in the porins of cell walls which leads to limitation of movement of the antibiotic across the osmotic membrane (Krishnamoorthy et al., 2017; McDermott et al., 2003).
ii) Inactivation of the antimicrobial agent by one or more β-lactamase (McDermott et al., 2003; Vardanyan & Hruby, 2016).
iii) Mutation in the targeted PBP (Vardanyan & Hruby, 2016; Krishnamoorthy et al., 2017).
iv) Decreased drug affinity (D'Costa et al., 2011).
v) Active efflux of antimicrobials mediated by energy-dependent pumps, out of the cell (D'Costa et al., 2011).

Gram-negative bacteria exhibit resistance to drugs by the production of β-lactamase, which hydrolyse the β-lactam ring, and inactivate the drug.



Structure 1.44: teicoplanin
(Marrubini et al., 2019)



Structure 1.45: Vancomycin
(Drennan et al., 2018)

Although many drugs have been developed to overcome β-lactamase-induced drug resistance, over time, more β-lactamase isoenzymes have been produced and have been able to induce drug resistance in Gram-negative bacteria (Mandal et al., 2017). Another group of antimicrobials that are inhibitors of cell wall synthesis are the glycopeptides, example teicoplanin (Structure 1.44) and vancomycin (Structure 1.45) which are large heterocyclic complexes consisting of heptapeptide backbone, where substituted sugars attaches to the heptapeptide backbone (McDermott et al., 2003). Activity of glycopeptides are often limited to Gram-positive bacteria and not Gram-negative bacteria as their large

size prevents them from passing across the outer membrane of Gram-negative bacteria which are intrinsically resistant. *Staphylococcus* and *Enterococci* are the most resistant organisms to glycoproteins (McDermott et al., 2003). Beta-lactam antibiotics target the penicillin binding proteins (PBPs) (Kapoor, 2017). The β-lactam ring mimics the D-alanine portion of peptide chain that normally binds to the PBP which in turn interacts with the β-lactam ring (Figure 1.9). This interaction prevents the synthesis of new peptidoglycan due to unavailability of PBP (Kapoor et al., 2017).

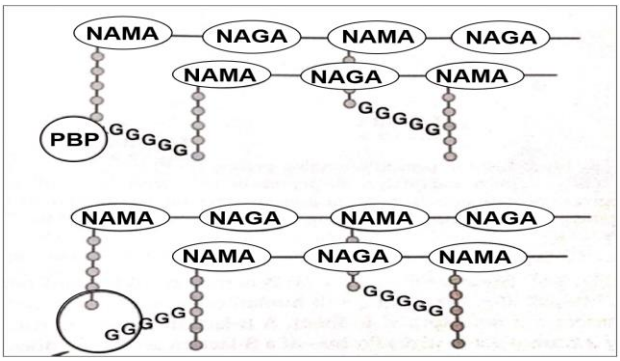


Figure 1.9: Mechanism of action of β-lactam antibiotics

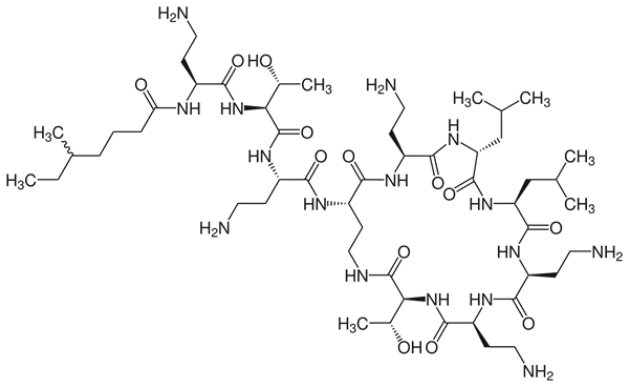
The penicillin-binding-protein (PBP) is the target of β-lactam antibiotics. The β-lactam antibiotics ring mimics the D-alanine portion of peptide chain [link to N-Acetylmuramic acid (NAMA) and N-Acetylglucosamine-6-phosphate (NAGA)] that normally binds to the PBP which in turn interacts with the β-lactam ring. This interaction prevents the synthesis of new peptidoglycan due to unavailability of PBP (Kapoor et al., 2017).

Absence of the PBP leads to lysis of the bacteria – (Džidić et al., 2008; Kapoor et al., 2017). A detail description of the mechanism of drug resistance by β-lactam is presented in section 1.2.2.2 (below).

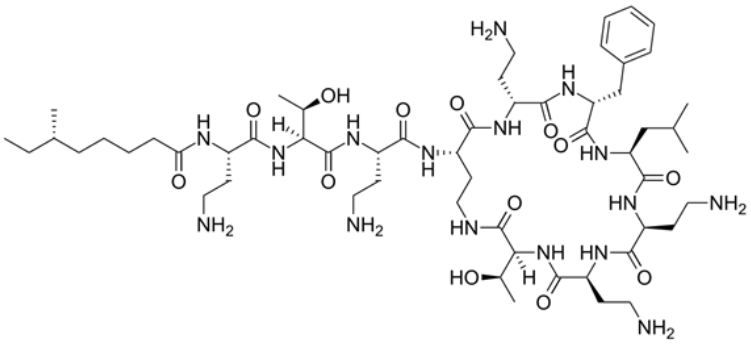
1.2.2.1.2 Inhibition of key metabolites necessary for survival of pathogen

Inhibition of key metabolic steps necessary for bacterial growth and reproduction such as heterotrophic metabolism, respiration, fermentation, electron transport and oxidative phosphorylation, bacterial photosynthesis, anaerobic respiration and the nitrogen cycle, can cause damage to the bacteria, killing it (Jurtshuk, 1996; Mandal et al., 2017). One key element required for metabolic processes to occur in bacteria is hydrogen transfer. Bacteria require ATP and vitamin B complex as coenzyme for redox reactions, so they can grow and multiply. *Thiobacillus thiooxidans* for instance requires inorganic salts and sulphur to grow and multiply. Inhibition of one or more metabolites necessary for bacterial growth will therefore lead to bacteria death (Jurtshuk, 1996; Mandal et al., 2017). Some antimicrobials also interrupt the methylerythritol-4-phosphate, a precursor for the synthesis of isoprenoid. Isoprenoids are key components of the electron transport chain,

or as components of quinones, sterols, hormones containing gibberellin, or components of membranes (Jurtshuk, 1996; McDermott et al., 2003). Some antibiotics inhibit essential cellular processes required for the survival of pathogens. Trimethoprim and sulphonamides disrupt the folic acid pathway; a step necessary for production of precursors required for DNA synthesis. Trimethoprim targets dihydrofolate reductase, sulphonamides binds to and target dihydropteroate synthase. These enzymes act at the rate limiting steps required for folic acid production in bacteria. Humans lack the ability to produce this important vitamin – folic acid (Serrano-Amatriain et al., 2016).



Structure 1.46: Colistin (Fiaccadori et al., 2016)



Structure 1.47: Polymyxin
(Serrano-Amatriain et al., 2016).

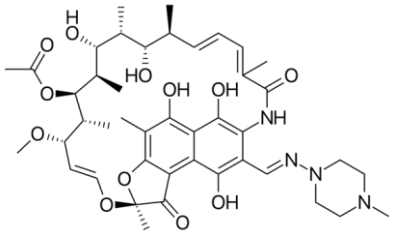
1.2.2.1.3 Disruption of cellular homeostasis by disrupting the permeability of bacterial cell membrane

Cell membrane is a barrier that regulates and segregates the extracellular and intracellular flow of substances and when damaged or disrupted, leakage of essential solutes necessary for the survival of the bacteria occurs. The ability of bacteria to keep out foreign agents from permeating bacterial membranes is necessary for its survival because these bacteria are exposed to extreme conditions of the host environment such as extreme pH, hypertonic environment, as well as the body defence agents. Without protection, the bacteria will experience stress and die. Because cell membranes exist in both prokaryotes and eukaryotes, these classes of antibiotics are poorly selective and

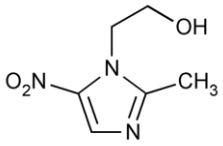
could be toxic to mammalian tissues. Clinically, they are mostly applied topically (Serrano-Amatriain et al., 2016).

1.2.2.1.4 Alteration of nucleic acid synthesis

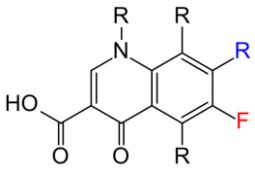
For replication to occur in living cells, RNA and DNA play important roles. Some antibiotics such as rifampicin, metronidazole and quinolones (Structure 1.48 – 1.50) bind to components involved in RNA and DNA synthesis. Others resemble the substrate used in DNA synthesis. This way, they bind to and inhibit RNA and DNA synthesis leading to bacteria death (McDermott et al., 2003). Generally, antimicrobials involved in disruption of RNA and DNA synthesis act by interfering with nucleic acids – e.g. rifamycins (Vardanyan & Hruby, 2016). Antibacterial agents which target DNA synthesis could thereby kill bacteria, preventing the outspread of bacterial infection. Antibacterial agents involved in inhibition of bacterial nucleic acid synthesis include: i) the quinolones – 1st generation (not available), 2nd generation (norfloxacin, ciprofloxacin), 3rd generation (moxifloxacin), ii) rifampicin, iii) pyrimidine derivatives – trimethoprim, iv) metronidazole and v) sulfonamides – Sulfisoxazole, Sulfamethoxazole (Vardanyan & Hruby, 2016).



Structure 1.48: Rifampine



Structure 1.49: Metronidazole



Structure 1.50: Quinolone

(Gao et al., 2019).

Some modifications have been made to the first-generation antibacterial agents involved in inhibition of the bacterial nucleic acid synthesis such as the quinolone. The insertion of fluorine at the C6 position is perhaps important finding which paved way for the development of fluoroquinolones whose antibacterial activity was much better than those of quinolone (Boteva and Krasnykh 2009). Flumequine was the first fluoroquinolone synthesised. It was in use until ocular toxicity was reported. Newer agents with improved antibacterial activity were made such as sitafloxacin, sparfloxacin and grepafloxacin. These agents were discontinued due to reports of significant phototoxicity (Castro, Navarro et al. 2013). The 4th generational quinolones still maintains its broad-spectrum antibacterial activities against both Gram-positive and Gram-negative bacteria, yet it also

inhibits the growth of anaerobic bacteria (Vardanyan & Hruby, 2016). Their hepatotoxicity has been a major drawback to its use (Vardanyan & Hruby, 2016) and should therefore be used as a last resort in treating life-threatening infections.

1.2.2.1.5 Inhibitors of protein synthesis

The information in bacterial DNA is used to synthesise the messenger RNA (m-RNA) in a process known as transcription (Figure 1.10 below). The ribosome then synthesizes proteins from the mRNA, a process known as translation (Kapoor et al., 2017). Translation of mRNA into proteins is catalysed by the cytoplasmic factors and ribosomes (Kapoor et al., 2017). The bacteria contain the 70S unit which is composed of the 30S and 50S ribosomal sub-units (Yoneyama & Katsumata, 2006). By targeting the 30S and 50S sub-units, antimicrobials inhibit bacterial growth and development (Johnston et al., 2002).

Aminoglycosides (AG) are inhibitors of the 30S sub-unit and are positively charged. They attach to the negatively charged OM leading to the formation of large pores which allows penetration of antibiotics inside the bacteria (Kapoon et al., 2017). The primary target site is the ribosome. For AG to enter the ribosome, an energy dependent active bacterial transport mechanism is required, allowing AG to pass through the cytoplasmic membrane.

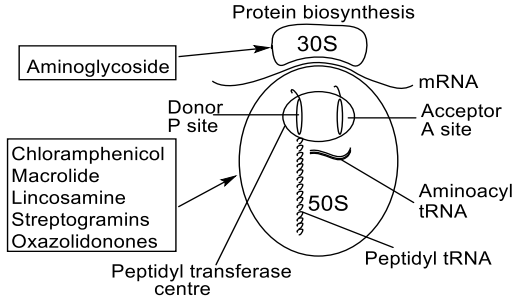


Figure 1.10: Site of action of protein biosynthesis inhibitors.

Transcription of DNA into mRNA which is then translated into protein. This process occurs at the 70S unit (consisting of 30S and 50S sub-units) in the ribosome. Inhibition of the 30S or 50S units leads to inhibition of bacterial growth. Aminoglycosides and tetracyclines inhibits the 30S unit; Chloramphenicol, macrolide, lincosamides, streptogramins and oxazolidonones inhibits the 50S unit (Kapoor et al., 2017).

This process requires oxygen (Kapoon et al., 2017). AG therefore works best in aerobic conditions and indicates poor activity against anaerobic bacteria (Kapoon et al., 2017). Antibiotics acts synergistically with AG thus inhibiting the synthesis of cell wall (such as glycopeptides and β-lactam).

Hydrogen bonds enables the interaction between the 16S rRNA and AG. This binding leads to misreading and premature termination of mRNA translation (Kapoon et al., 2017).

Antibacterial agents involved in inhibition of protein synthesis include: i) lincomycins – clindamycin, ii) chloramphenicol, iii) Tetracyclines – Tetracycline, Doxycycline, iv) macrolides – Erythromycin, Clarithromycin, Azithromycin and v) aminoglycosides – Gentamicin, Tobramycin, Amikacin (Vardanyan & Hruby, 2016; Ullah & Ali, 2017).

Derived from the bacterium *Streptomyces venezuelae*, chloramphenicol (CAM) is a broad-spectrum antibacterial agent which inhibits protein synthesis and was first introduced in 1940s (Ehrlich et al., 1947). Due to the suppression of the bone-marrow and aplastic anaemia caused by systemic administration of CAM, its use has been hampered by its toxicity (Feder-Jr et al., 1981). This limitation has restricted the use of CAM to topical administration in treatment of ear and eye infection. The antibacterial activity of CAM has been preserved due to its limited use enabling it to exhibit antibacterial activity against the *MRSA* bacterium (Fayyaz et al., 2013).

Despite the antibacterial activities of antibacterial drugs, intrinsic resistance is common in certain groups of bacteria due to their biochemical makeup. Gram-negative bacteria therefore contain pumps which efflux macrolides out of the cells as this agent is slowly absorbed into the cell thus, they develop intrinsic resistance to macrolides (Krishnamoorthy et al., 2017). Other bacteria may lack the target sites of some drugs, inhibiting the drug activity. Acquired resistance results from the ability of bacteria to develop biochemical mechanism aimed at dealing with a particular drug being administered constantly. They do this via enzymes that can modify the activity of the antibiotic before it reaches the target site. Several drug modifying enzymes which can modify antibacterial agents have been identified such as the β-lactamases which hydrolysis cephalosporins and penicillin (Krishnamoorthy et al., 2017; Mandal et al., 2017). Cell susceptible to antimicrobial agents are easily killed by these agents.

1.2.2.2 Mechanisms of Antimicrobial Resistance

1.2.2.2.1 Prevention of accumulation of antimicrobials either by decreasing uptake or increasing efflux of the antimicrobial from the cell

Drugs can be transferred by diffusion through the lipid bilayers, porins and by self-uptake into a bacterial cell. Porins are located in the OM of Gram-negative bacteria, allowing

quinolones and β-lactams (small hydrophilic molecules) to cross into the cell. A decrease in porin numbers across the OM leads to decreased entry of fluoroquinolone (FQ) and β-lactam antibiotics (Kapoor et al., 2017). The low OM permeability is responsible for the acquired resistance of *P. aeruginosa* to all antibiotic classes (Kapoor et al., 2017).

Efflux pumps are membrane proteins in bacteria that are responsible for exporting antibiotics out of the cell thus reducing the intracellular concentration of antibiotics (Džidić et al., 2008). Efflux pumps ensures antibiotics are pumped out of the cells at a similar speed to the rate of entry of the antibiotics into the cells, preventing the antibiotics from reaching its target (Wise, 1999). Unlike porins which are present in OM, efflux pumps are present in the cytoplasmic membrane (Wise, 1999). With exception of the polymyxin, all classes of antibiotics are susceptible to the action of efflux pumps which can act specifically to certain antibiotics (Lambert, 2002). Most efflux pumps can pump a wide range of unrelated antibiotics (multidrug transporters), including the FQ, tetracyclines and macrolides and therefore contribute to multidrug resistant organisms (Dzidic et al., 2008).

Modification of target molecules is another way bacteria reduces the potency of antibiotics. Mutation of bacterial gene could prevent binding of antibiotics thus eliciting a massive alteration in the activity of the antibiotics that could either decrease uptake or increase efflux of the antimicrobial from the cell (Kapoor et al., 2017).

i) Altered cell wall precursors: glycopeptides such as teicoplanin and vancomycin can inhibit cell wall synthesis in Gram-positive bacteria by binding to the D-alanyl-D-alanine residues of peptidoglycan precursors, changing it to D-alanyl-lactate thus preventing the cross-linkage of glycopeptides, thus development of bacterial resistance (Dzidic et al., 2008).
ii) Alteration in the 30S or 50S sub-units of the ribosome (Figure 1.10) leads to resistance of drugs that affects protein synthesis such as the AGs, chloramphenicol, tetracycline and macrolides (Lambert, 2002). Streptogramin B, lincosamides, macrolides and chloramphenicol bind to the 50S ribosomal sub-unit to suppress protein synthesis (Tenover, 2006). *E. faecalis* and *Enterococcus faecium* strains are resistant to teicoplanin and vancomycin (Giedraitiene et al., 2011).
iii) Alteration in penicillin-binding protein (PBP): PBP modification confers resistance to Gram-positive bacteria while β-lactamase production confers resistance to Gram-negative bacteria (Kapoor et al., 2017). Mutation of the PBP reduces affinity to β-lactam antibiotics. The resistance of *Streptococcus pneumonia* to penicillin and *E.*

faecium to ampicillin is by this mechanism (Alekshun & Levy, 2007; Džidić et al., 2008). Resistance of *S. aureus* to oxacillin and methicillin is linked to the integration of the staphylococcal cassette chromosome mec – a mobile genetic element, into the chromosome of *S. aureus*. This element contains the mec A resistant gene (Alekshun & Levy, 2007; Džidić et al., 2008; Hiramatsu et al., 2001).

iv) Mutation of the RNA polymerase confers resistance to rifampicin (Kapoor et al., 2017).

v) Mechanisms protecting the bacterial ribosomes also imparts resistance to tetracyclines (Kapoor et al., 2017).

vi) Mutation of topoisomerase IV (coded by genes par C and par E) and DNA gyrase (coded by genes gyr A and gyr B) leads to FQ resistance (Kapoor et al., 2017; Kim et al., 2002).

1.2.2.2.2 Inactivation of Antibiotics

Three main enzymes are responsible for antibiotics inactivation such as β-lactamases, aminoglycoside-modifying enzymes, and chloramphenicol acetyltransferases (AACs) (Dockrell et al. 2004).

β-lactamases hydrolyses the amide and ester bonds of nearly all β-lactams e.g., carbapenems, monobactams, cephalosporins and penicillin (Dockrell et al., 2004). About 300 β-lactamases have been identified and are classified using two classification systems; Bush–Jacoby–Medeiros (functional) and Ambler (structural) (Alekshun & Levy, 2007). Below is a description of the Ambler classification system:

i) Class A β-lactamases are a class of β-lactamases also known as the penicillinase and are susceptible to clavulanic acid (Dockrell et al., 2004; Rice et al., 2003). Class-A β-lactamases commonly encountered are the penicillinases belonging to the *Enterobacteriaceae* and are designated as SHV-1 and TEM-1; they elicit little or no activity against cephalosporin (Rice et al., 2003). These β-lactamases are progenitors of extended-spectrum β-lactamases (ESBL) whose amino acid composition has been substituted allowing hydrolysis of most cephalosporins. They are resistant to third generation cephalosporins (e.g., ceftriaxone, cefotaxime ceftazidime), cefoperazone, ceftazidime, ceftriaxone, aztreonam, but are sensitive to methoxy-cephalosporins e.g., carbapenems and cephamycin. They are also inhibited by inhibitors of β-lactamases such as tazobactam, sulbactam and clavulanic acid (Jacoby & Munoz-Price, 2005; Ma et al., 2005).

ii) **Class B β-lactamases** are metallo-β-lactamases requiring metals such as zinc for catalysis. Their activities are inhibited by chelating agents. They are resistant to inactivation by carbapenems e.g., New Delhi metallo-β-lactamase, aztreonam, sulbactam and clavulanate (Rasmussen & Bush, 1997).

iii) **Class C β-lactamases** are also known as cephalosporinases. Except for *Klebsiella* and *Salmonella*, all Gram-negative bacteria produce the cephalosporinases. Class C β-lactamases hydrolysis the extended spectrum cephalosporins (Crichlow et al., 1999). Class C β-lactamases can bind to bulky extended spectrum penicillin because they have large cavities compared to class A β-lactamases which have small cavities. Class C β-lactamases include the Amp C β-lactamases. Except the carbapems, the Amp C β-lactamases are not inhibited by clavulanate (Lobkovsky et al., 1994).

iv) **Class D β-lactamases** are found predominantly in *P. aeruginosa* and *Enterobacteriaceae*, they hydrolysis oxacillin. These enzymes confer resistance to methicillin, oxacillin, cloxacillin and penicillin (Naas & Nordmann, 1999).

1.2.2.2.3 Aminoglycoside (AG) modifying enzymes (AMEs)

Aminoglycosides are neutralised by specific enzymes such as denylyl-transferases, nucleotidyl-transferases and phosphoryl-transferases. These aminoglycoside-modifying enzymes (AMEs) disrupts binding to the 30S ribosomal subunit thus reducing the affinity of antibiotics (Strateva & Yordanov, 2009). AMEs provides extended spectrum resistance to FQ and AG. AMEs have been identified in *S. pneumonia*, *E. faecalis* and *S. aureus* (Maurice et al., 2008).

A summary of the various classes of antibacterial drug, their site of action and their primary target sites is presented in Table 1.4 below. The mechanism of resistance against different classes of antibacterial drugs is presented in Table 1.5.

Table 1.4: Class of antibacterial drugs, site of action and their primary targets

Antibiotic Class	Antibiotic Name	Primary Target
Cell wall synthesis inhibitors		
β-lactams	Penicillins [Piperacillin, Carbenicillin, Ampicillin, Penicillin G, Cloxacillin] Cephalosporins [Cefadroxil, Cefaclor, Ceftazidime]	Penicillin-binding proteins

Lipopeptides	Polymyxin B	Disruption of inner and outer membranes through binding to lipopolysaccharide (LPS) in the outer membrane
Others	Alafosfalin	Peptidoglycan units terminal D-Ala-D-Ala dipeptide
	Bacitracin	50S-isoprenyl pyrophosphate
	D-cycloserine	D-alanine ligase and alanine racemase
	Fosfomycin	UDP-N-actetylglucosamine-3-enolpyruvyltransferase
DNA synthesis inhibitors		
Fluoroquinolones	Nalixidic acid, Ciprofloxacin, Levofloxacin, Sparfloxacin, Norfloxacin	Topoisomerase II (DNA gyrase), topoisomerase IV
Sulfonamides	Sulfamethazine, Sulfapyridine, Sulfamethoxazole, Sulfadiazine, Sulfamerazine	Competitive inhibitor for DHPS involved in folate synthesis
Others	Novobiocin	DNA gyrase
RNA synthesis inhibitors		
Rifamycins	Rifampicin, Rifabutin, Rifaximin	DNA-dependent RNA polymerase
Protein synthesis inhibitors		
Tetracyclines	Oxytetracycline, Doxycycline, Tetracycline, Demeclocycline, Minocycline	30S ribosome (inhibit aminoacyl tRNA binding to ribosome)
Aminoglycosides	Tobramycin, Gentamicin, Amikacin, Streptomycin, Spectinomycin	30S ribosome (mistranslation by tRNA mismatching)
Macrolides	Erythromycin, Clarithromycin, Midecamycin, Roxithromycin, Spiramycin, Azithromycin	50S ribosome (stimulating dissociation of the peptidyl-tRNA molecule from the ribosomes during elongation)
Amphenicols	Chloramphenicol, Thiamphenicol, Florfenicol	50S ribosome (inhibit elongation step)
Lincosamides	Clindamycin, Lincomycin	50S ribosome (stimulate dissociation of the peptidyl-tRNA molecule from the ribosomes during elongation)
Pleuromutilins	Tiamulin	50S ribosome (prevent correct positioning of the CCA ends of tRNA for peptide transferase)
DNA replication (intercalators)		
Anthracyclines	Doxorubicin, Epirubicin, Idarubicin	Intercalate DNA/RNA strand topoisomerase II
Others	Actinomycin D	Intercalates G-C base pairs and minor groove DNA at the transcription initiation complex
	Mithramycin	Intercalates GC-rich DNA strands
	Tetracenomycin	Intercalates DNA
Anaerobic DNA inhibitors		
Nitrofurans	Furazolidone, Nitrofurantoin	Highly reactive reduced form (by nitrofuran reductase)
Nitro-imidazole	Ornidazole	Damages bacterial DNA

(Hindler & Kelley, 1986; Peach et al., 2013).

Table 1.5: Mechanisms of Resistance against Different Antimicrobial Classes

ANTIMICRO-BIAL CLASS	MECHANISM OF RESISTANCE	SPECIFIC MEANS TO ACHIEVE RESISTANCE	EXAMPLES

Beta-lactams Examples: penicillin, ampicillin, mezlocillin, peperacillin, cefazolin, cefotaxime, ceftazidime, aztreonam, imipenem	Enzymatic destruction	Destruction of beta-lactam rings by beta-lactamase enzymes. With the beta-lactam ring destroyed, the antibiotic will no longer have the ability to bind to PBP (Penicillin-binding protein) and interfere with cell wall synthesis.	Resistance of *staphylococi* to penicillin;
	Altered target	Changes in penicillin binding proteins. Mutational changes in original PBPs or acquisition of different PBPs will lead to inability of the antibiotic to bind to the PBP and inhibit cell wall synthesis	Resistance of *staphylococci* to methicillin and oxacillin
	Decreased uptake	Porin channel formation is decreased. Since this is where beta-lactams cross the outer membrane to reach the PBP of Gram-negative bacteria, a change in the number or character of these channels can reduce beta-lactam uptake.	Resistance of *E. aerogenes, K. pneumoniae* and *P. aeruginosa* to imipenem
Glycopeptides Example: vancomycin	Altered target	Alteration in the molecular structure of cell wall precursor components decreases binding of vancomycin so that cell wall synthesis is able to continue.	Resistance of enterococci to vancomycin
Aminoglyosides Examples: gentamicin, tobramycin, amikacin, netilmicin, streptomycin, kanamycin	Enzymatic modification	Modifying enzymes alter various sites on the aminoglycoside molecule so that the ability of this drug to bind the ribosome and halt protein synthesis is greatly diminished or lost entirely.	Resistance of many Gram-positive and Gram-negative bacteria to aminoglycosides
	Decreased uptake	Change in number or character of porin channels (through which aminoglycosides cross the outer membrane to reach the ribosomes of gram-negative bacteria) so that aminoglycoside uptake is diminished.	Resistance of a variety of Gram-negative bacteria to aminoglycosides
	Altered target	Modification of ribosomal proteins or of 16s rRNA. This reduces the ability of aminoglycoside to successfully bind and inhibit protein synthesis	Resistance of *Mycobacterium* spp to streptomycin
Quinolones *Examples: ciprofloxacin, levofloxacin, norfloxacin, lomefloxacin*	Decreased uptake	Alterations in the outer membrane diminishes uptake of drug and/or activation of an “efflux” pump that removes quinolones before intracellular concentration is sufficient for inhibiting DNA metabolism.	Resistance of Gram-negative and staphylococci (efflux mechanism only) to various quinolones
	Altered target	Changes in DNA gyrase subunits decrease the ability of quinolones to bind this enzyme and interfere with DNA processes	Gram-negative and Gram-positive resistance to various quinolones

(Berger-Bachi, 2002; Forbes et al., 1998; Kapoor et al., 2017).

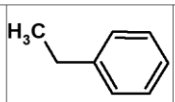
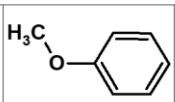
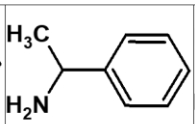
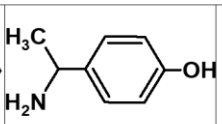
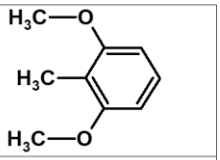
Modification of side chains of antibiotics could lead to an increase or decrease bioactivity or a complete loss of activity. In some instances, modification of a side-chain could retain the antibiotic characteristic of a drug as well as improve the solubility, bioavailable or safety. In penicillin, the spectrum of antibacterial activity is dependent on a number of factors. For e.g., different side-chain substitution on “R” will alter the spectrum of activity thus:

i) substituting R for NH_2 will reduce the polarity of the drug. This implies the drug can easily pass through porins leading to an increase bioactivity against Gram-negative and Gram-positive bacteria (Nooraldeen, 2016).

ii) Substituting R for α-COOH will increase the hydrophilicity of the drug and a decrease bioactivity against Gram-positive bacteria (e.g. carbenicillin) (Nooraldeen, 2016).

iii) Acid resistance of α-NH penicillins > α-OH (α-aminobenzylpenicillins > α-hydroxybenzylpenicillins) (Nooraldeen, 2016).

Table 1.6**:** Effect of alteration of the side chain of antibiotics on drug activity.

R-group					
Drug name	Penicillin G	Penicillin V	ampicillin	amoxicillin	methicillin
Spectrum of activity	Gram-positive and a few Gram-negative	Similar to penicillin G	Gram-positive and more Gram-negative than penicillin	Similar to ampicillin	Gram-positive only including β-lactamase producers
Route of administration	parenteral	oral	Parenteral and oral	Oral (better than ampicillin)	parenteral

Altering the side-chain of antibiotics could significantly alter its bioactivity, lead to an increase in the spectrum of activity and or stability of the antibiotics against enzymatic degradation and vice versa (Nooraldeen, 2016).

1.2.3 Fungal infection

Fungus (fungi – plural) were once viewed as crude members of the plant kingdom, just marginally more developed than bacterium. However, recent studies indicate that fungi are not primitive at all. Studies show that fungi and animals both belong to the group – Opisthokonta and they are more firmly identified with animals than they are to plants (Carris et al., 2012). Most fungi are heterotrophic in nature (obtain energy from other organisms), others are saprotrophs (obtain nutrients from dead matter), whereas some are necrotrophs – kill host cells to obtain their nutrients (Carris et al., 2012; Wilson, 2018). Thus, fungi are adaptive and can survive in many conditions.

In humans, mycosis (fungal infection) occur; the most common being dermatophytes-related mycosis (growths that colonize dead keratinized tissue including toenails, fingers and skin) (Carris et al., 2012). Infections such as ringworms are caused by dermatophytes and are difficult to treat, others are resident members of normal microflora in healthy

humans but become pathogenic in people with reduced immunity. An example is *Candida specie* that causes yeast infections in the mucosal tissues of many healthy people and is the cause of candidiasis (red, itchy rash on the skin) in immunocompromised individuals and babies (Wilson, 2018). Some fungi are inhaled as spores and initiates infections (such as valley fever) only when they get to the lungs, e.g. *Histoplasma capsulatum* and *Coccidioides immitis* (Carris et al., 2012). Another class of fungal pathogens (opportunistic fungi) are normally not associated with animals and humans. However, in immunocompromised individuals and in wounds of normal humans, opportunistic fungi can cause serious infection (Carris et al., 2012; Wilson, 2018).

The *C. albicans* fungus is a lifelong, harmless commensal member of the normal human microbiome. However, this fungus can cause infections that range from minor skin rashes to life-threatening blood / systemic infections under certain circumstances (Mayer et al., 2013). Several activities and factors have been identified which contributes to the pathogenic nature of this fungus. This include the yeast-to-hypha transition, biofilm formation, secretion of hydrolases, molecules which mediate invasion and adhesion to the host cells, phenotypic switching and thigmotropism (response of plants to direction, physical contact with solid object or touch) (Mayer et al., 2013). In humans, *C. albicans* is causes two main infections – superficial infections, such as vaginal or oral candidiasis and life-threatening systemic infections which could spread into vital organs (Wilson, 2018). *C. albicans* (up to 75%) is present in the oral cavity and remains benign in normal humans but could cause recalcitrant oral cavity infection in immunocompromised individuals and can affect the oropharynx and/or the oesophagus of humans with HIV/AIDS (and other immunocompromised individuals). Other risk factors include old age and wearing of dentures (Mayer et al., 2013; Wilson, 2018). About 75% of all women suffer from vulvovaginal candidiasis (VVC) at least once in their lifetime, with 40–50% experiencing at least one additional episode of infection (Sobel, 2007). 5-8% of women suffer from at least four recurrent VVC per year (Foxman et al., 1998). Hormone therapy, pregnancy, oral contraceptives, use of antibiotics and diabetes are some predisposing factors associated with VVC (Fidel, 2004). Despite the high morbidity associated with *C. albicans*, superficial *C. albicans* infections are non-lethal. Nonetheless, systemic candidiasis is responsible for high crude mortality rate, even with first line antifungal therapy (Pfaller & Diekema, 2010; Wilson, 2018).

1.2.3.1 Treatment of – Candidiasis (Fungal Infection)

Treatment of fungal infections such as candidiasis is challenging due to limited antifungal options in the clinics and with an increase in the incidence of drug resistance, interaction and toxicity. There is therefore a need to develop novel antifungal agents to increase treatment outcomes in patients with invasive fungal infections such as candidiasis, thus this research. The therapeutic strategies for treatment of candidiasis involves treatment in a timely manner with an antifungal agent that can overcome the limitation of resistance to antifungal agents reported by *C. albicans* and other fungi (Bassetti et al., 2016). Azoles (such as fluconazole, voriconazole, posaconazole, isavuconazole and itraconazole), echinocandins (micafungin, anidulafungin and caspofungin) and polyenes (amphotericin B) are current therapies in the treatment of candidiasis (Bassetti et al., 2016). Fluconazole and echinocandin are first-line treatment options depending on the patient's condition (Arendrup & Patterson, 2017). Antifungal resistance to candidiasis has been reported especially in patients with chronic mucocutaneous candidiasis (Nami et al., 2019). Among clinical isolates of *Candida*, *C. auris*, *C. glabrata* and *C. krusei* are least resistant to antifungal agents compared to *C. albicans* (Nami et al., 2019; Arendrup & Patterson, 2017). *C. albicans* and *C. krusei* are emerging global health threat that have caused intrinsic multidrug-resistant (MDR) relative to polyenes, triazoles and echinocandins (Nami et al., 2019; Vallabhaneni et al., 2017; Borman et al., 2016). The emergence of MDR *Candida* spp., issue of organ transplantation and the concomitant use of immunosuppressive drugs have caused problems in the effective treatment of invasive candidiasis. This limitation suggests the need for new therapeutic options thus this research – the isolation and synthesis of novel agents for the treatment of fungal infections associated with *C. albicans*. Table 1.7 represents some common antifungal drugs in clinical use.

Table 1.7: Common antifungal drugs

Class	**sub-class**	**Mechanism of action**	**Drug**	**Target groups**	**Prophylactic treatments**	**Ref(s)**
Azole	Tria-zole	Interact with the cytochrome demethylase system enzymes and inhibit the membrane oxidative enzymes, failure in the ergosterol synthesis, abnormal sterol retention, incomplete cell	Flucon-azole	First line treatment strategy for IC. Treatment of IC in (non)neutropenic patients	Organ transplan-tation	(Nucci et al., 2013)
			Vorico-nazole	Treatment of IC caused by fluconazole-resistant species, such as *C. krusei*. Treatment of non-neutropenic patients with candidemia.		(Pascual et al., 2008)
			Itracon-azole	Second line treatment strategy for IC.	Long-term application in children in	(Spellberg et al., 2006)

Class	sub-class	Mechanism of action	Drug	Target groups	Prophylactic treatments	Ref(s)
		wall formation, retention of intracellular phospholipids, and ultimately fungal cell death.			collaborated with FLC	
			Posaco-nazole	Post-transplantation treatment. As a treatment strategy in the failure of voriconazole-based treatment.	NM	(Kauffman, 2006; Ullmann et al., 2007)
Echino-candin		Inhibiting the activity of 1,3-beta-glucan synthase enzymes	Caspof-ungin	Primary treatment in patients with candidiasis caused by *C. krusei* or *C. glabrata*, which are previously received azole compounds, or patients who are unable tolerate the amphotericin B or azoles.	NM	(Mora-Duarte et al., 2002; Odio et al., 2004; Villanueva et al., 2001)
			Micafu-ngin	Infections by *C. albicans*, *C. glabrata*, and *C. krusei* Candidemia	NM	(Chandrasekar & Sobel, 2006; Pfaller et al., 2008)
			Anidula-fungin	Infection by *Candida* spp.	NM	(Reboli et al., 2007)
Polyene		Quickly attach to sterols penetrating the cell membrane leading ultimately to cell death.	Amphote-ricin B	Systemic fungal infection		(Nami et al., 2019)

NM: not mentioned.

1.3 Phytochemicals as Anticancer and Antimicrobial Agents

Phytochemicals are sources of important compounds for the treatment of infectious diseases and cancer and have become an important area of research for drug discovery in handling clinical challenges pose by resistant microorganisms and cancer cells, to drugs currently in use. In plants, isoprenoids serve as the backbone from which most phytochemicals are biosynthesised via the cytosolic mevalonate (MVA) pathway and the 2C-methyl-d-erythritol-4-phosphate (MEP) Pathway (Hemmerlin et al., 2012). Some phytochemicals (like the flavonoids) are synthesised via the phenylpropanoid pathway (Ferreyra et al., 2012). Phytochemicals obtained from natural products have exhibited antimicrobial inhibition against clinically resistant microbial strains such as *MRSA* (Sanchez & Doron, 2017), and cancerous growths (Seca et al., 2014), by interfering with the internal replication mechanism of the organism. Phytochemicals from natural products elicit their bioactivities by modulating various mechanisms in the bacteria or cancer such as angiogenesis, apoptosis, metastasis, differentiation and cell proliferation (van de Velde et al., 2017; Xuan et al., 2016) or by lysing the bacterial cell wall (Sanchez & Doron, 2017). The increase in research targeted at developing new chemotherapies for cancer and infectious diseases has gained momentum due to the cost effective and less toxic effect of phytochemicals.

1.3.1 The Mevalonate (MVA) and 2C-methyl-d-erythritol 4-phosphate (MEP) Pathway Provides Isoprenoid, the Building Block of Most Phytochemicals

In plants, isoprenoids (precursors for the synthesis of most phytochemicals) are biosynthesised via the MVA pathway in the cytosol (Hemmerlin et al., 2012). Isoprenoids are structurally and functionally the most diversified group of metabolites. Over 50,000 molecules have been identified (Thulasiram et al., 2007). Isoprenoids are dominant primary metabolites that functions in respiration, membrane fluidity and photosynthesis. As secondary metabolites, they are involved in the plant-pathogen and allelopathic interactions (Vranová et al., 2012). Many plant isoprenoids possess pharmacological, commercial and agricultural values as drugs, colourants, rubber, polymer and essential oils (Vranová et al., 2012). Isoprenoids are derived from isopentenyldiphosphate (IPP) which is synthesised by two different pathways in plants (Figure 1.11) – The cytoplasmic mevalonate (MVA) pathway and the plastidial 2C-methyl-d-erythritol 4-phosphate (MEP) pathway (Vranová et al., 2012; Rohmer et al., 1999). The (MVA) pathway begins with the condensation of three units of acetyl-CoA to 3-hydroxy-3-methylglutaryl-CoA (HMG-

CoA). This is reduced to mevalonate (MVA) and a subsequent phosphorylation steps at C-5 of mevalonate. Decarboxylation of mevalonate forms isopentenyl pyrophosphate (IPP) (Croteau et al., 2000). IPP is then used to synthesise mitochondrial and cytosolic isoprenoids such as sesquiterpene lactones, the side chain of ubiquinone and sterols (Vranová et al., 2012). The MEP pathway births plastid isoprenoids derived from IPP (Vranová et al., 2012). The first reaction in this pathway involves the condensation of C1 aldehyde group of D-glyceraldehyde 3-phosphate (GA-3P) with (hydroxyethyl)thiamin derived from pyruvate to produce deoxyxylulose-5-phosphate (DXP). Intracellular reduction and rearrangement of DXP by DXP reductoisomerase (CPZ) yields MEP which is subsequently converted to 2-C-methyl-D-erythritol 2,4-cyclodiphosphate (ME-2,4cPP).

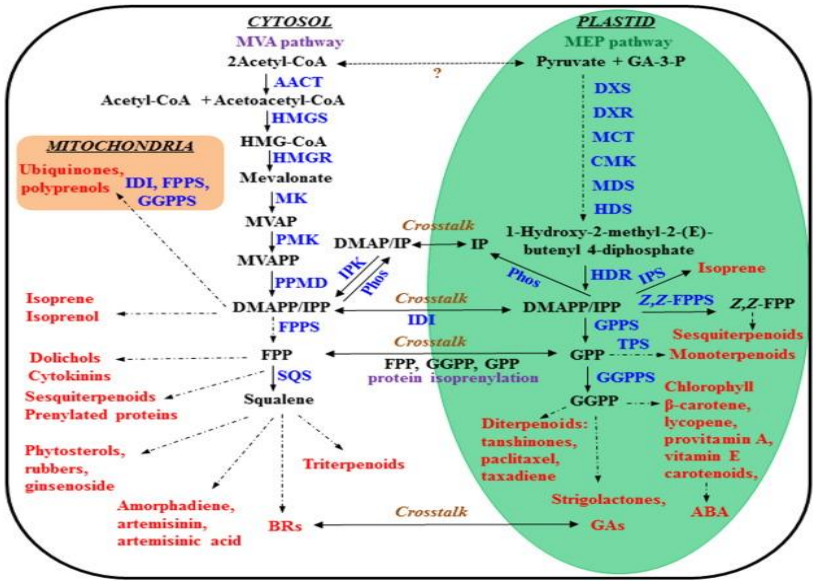


Figure 1.11: Isoprenoid biosynthesis pathway in plants.

"The MVA pathway occurs in the cytosol. It begins with the condensation of two units of acetyl-coA to acetoacetyl-CoA by the enzyme acetoacetyl-CoA thiolase. Condensation of acetyl-coA and acetoacetyl-coA to form S-3-hydroxy-3-methylglutaryl-coenzyme A. This step is catalysed by 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) synthase. 3-hydroxy-3-methylglutaryl-coenzyme A reductase converts 3-hydroxy-3-methylglutaryl-coenzyme A to MVA. MVA is then converted to mevalonate 5-phosphate via a phosphorylation reaction catalysed by mevalonate kinase. Mevalonate 5-phosphate is then converted to mevalonate 5-diphosphate in another phosphorylation reaction catalysed by phosphomevalonate kinase. Mevalonate 5-diphosphate is then converted to isopentenyl diphosphate. This step is catalysed by the enzyme diphosphomevalonate decarboxylase. Isopentenyl diphosphate synthesised from the MVA pathway is the universal precursor of isoprenoids including sesquiterpenes, triterpenes, isoprenes, artemisinin, dolichols, monoterpenes, cytokinins, dolichols, polyprenol, ubiquinone, prenylated proteins and diterpenes. The MEP pathway occurs in the plastid (green) with the enzymes in the MEP pathway as indicated. Enzymes are shown in blue. Substrates and intermediates are shown in black. Active components or end-products are shown in red. Arrows between plastid and cytosolic compartments indicate metabolic flow between them" (Liao et al., 2016).

This occurs in three steps (catalysed by enzymes). Reduction of ME-2,4cPP produces HMBPP in a reaction catalysed by 1-hydroxy-2-methyl-2-butenyl 4-diphosphate (HMBPP) synthase (HDS). HMBPP is converted to a mixture of dimethylallyl diphosphate (DMADP) and IPP by the enzyme HMBPP reductase (HDR) (Rodríguez-Concepción, 2006). IPP is

converted to DMAPP by IPP isomerase (IPPI) which reversibly converts IPP to DMAPP (Nakamura et al., 2001).

Longer chain isoprenoids are synthesised by condensation of IPP subunits (C5) to generate linear polymers such as C10, C15, C20 etc. These polymers have defined chain lengths. Different classes of isoprenoid compounds are then synthesis from these defined chain lengths (Figure 1.11). The synthesis of C10 (Emmerstorfer-Augustin, 2016; Tholl et al., 2004; Wang and Dixon, 2009), C20 (Sitthithaworn et al., 2001; Okada et al., 2000), C25 (Hsieh et al., 2011), C30 (Emmerstorfer-Augustin, 2016), C35 (Emmerstorfer-Augustin, 2016; Hsieh et al., 2011), C40 (Bartley et al., 1992; Emmerstorfer-Augustin, 2016), C45 (Hirooka et al., 2005; Emmerstorfer-Augustin, 2016) and polyprenyls in the range of C50–C130 (Cunillera et al., 2000; Oh et al., 2000; Sakaihara et al., 2000; Ibata et al., 1983) has been reported in plants. Isoprene, cytokinins and other hemiterpenoids are synthesised from DMAPP (C5). Monoterpenoids are synthesised from geranyl diphosphate – GPP (C10). **Sesquiterpenoids** – from farnesyldiphosphate (FPP; C15). Diterpenoids such as tocopherols, phylloquinone, the side-chain of chlorophyll and gibberellic acid are synthesised from geranylgeranyl diphosphate (GGPP; C20).

1.3.2 Phenylpropanoid Pathway for Synthesis of Flavonoids

In plants, two pathways exist for the biosynthesis of flavonoid-based compounds: the shikimic pathway and the acetate pathway (Nabavi et al., 2018). The shikimic pathway generates the phenyl propanoids (C6-C3) skeleton while the acetate pathway serves as the building block for polymeric 2-carbon units (Nabavi et al., 2018). The shikimate pathway is a seven-step metabolic route used by plants, algae, fungi, archaea, bacteria and some protozoans for the biosynthesis of folates and aromatic amino acids (tryptophan, tyrosine, and phenylalanine). Seven enzymes are involved in this pathway including DAHP synthase, 3-dehydroquinate synthase, 3-dehydroquinate dehydratase, shikimate dehydrogenase, shikimate kinase, EPSP synthase, and chorismate synthase (Herrmann & Weaver, 1999). The pathway starts with two substrates, erythrose-4-phosphate and phosphoenol pyruvate (PEP) and ends with chorismate, a substrate used for the synthesis of the aromatic amino acids required for the synthesis of flavonoids.

The fifth enzyme involved is the shikimate kinase, an enzyme that catalyses the ATP-dependent phosphorylation of shikimate to form shikimate 3-phosphate. The coupling of Shikimate 3-phosphate and phosphoenol pyruvate produces 5-enolpyruvylshikimate-3-phosphate. This step is catalysed by the enzyme 5-enolpyruvylshikimate-3-phosphate

(EPSP) synthase. Claisen rearrangement of chorismate by chorismate mutase leads to the synthesis of prephenic acid (not shown). Oxidative decarboxylation of prephenic acid gives p-hydroxyphenylpyruvate trans-aminated using glutamate to give α-ketoglutarate and tyrosine (Herrmann & Weaver, 1999). An overview of the biosynthetic pathway for flavonoids is presented in Figure 1.12. A detailed description of flavonoid chemistry and biological activity is presented in section 1.5. In this section, the biosynthesis and regulation of secondary metabolites involved in flavonoid synthesis shall be discussed. Flavonoids are the most diverse secondary metabolites in plants.

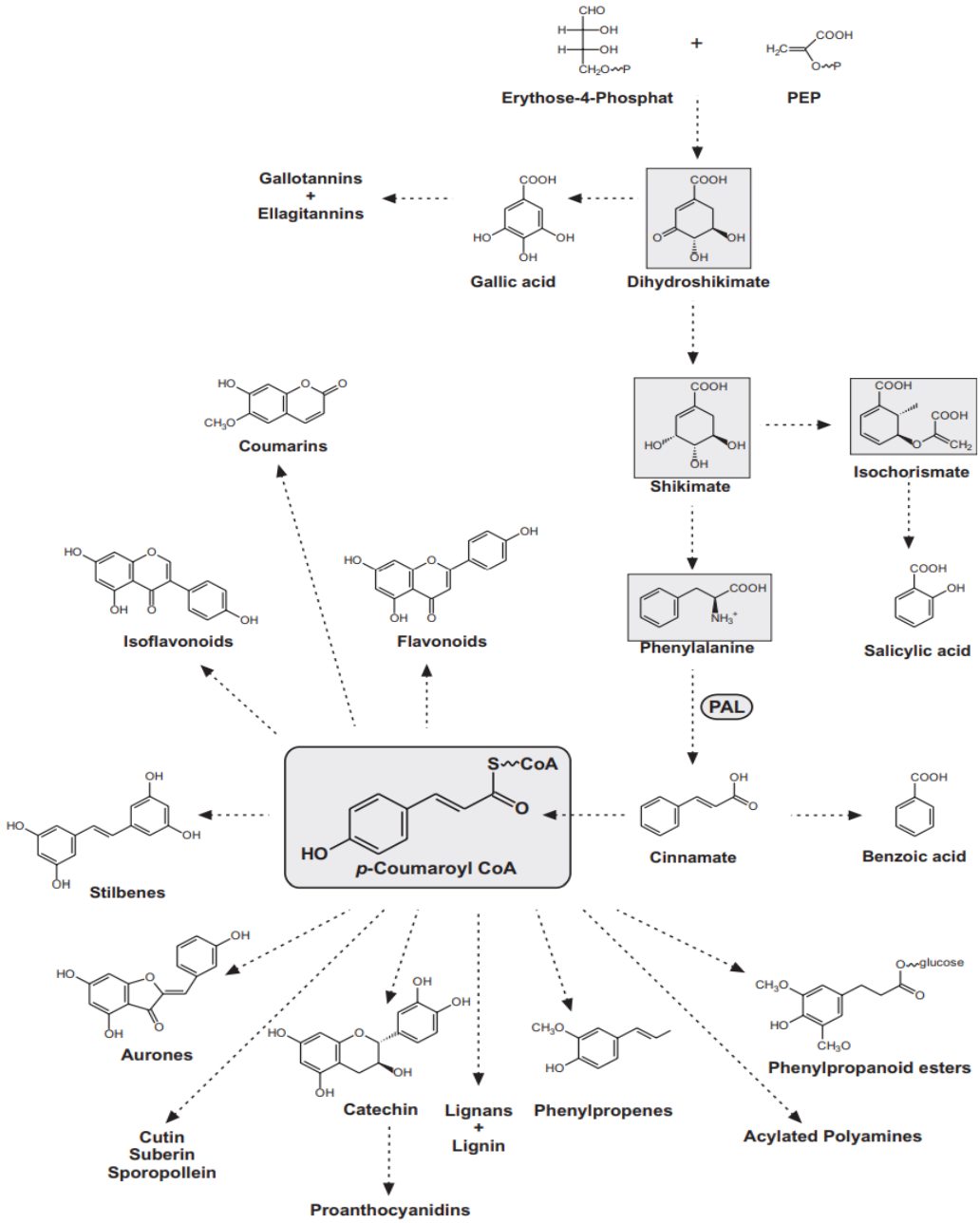


Figure 1.12: An overview of flavonoids biosynthetic pathway.

The shikimate pathway links metabolism of carbohydrates to biosynthesis of aromatic compounds. In a sequence of seven metabolic steps, phosphoenolpyruvate and erythrose 4-phosphate are converted to

chorismate, the precursor of the aromatic amino acids and many aromatic secondary metabolites. All pathway intermediates can also be considered branch point compounds that may serve as substrates for other metabolic pathways. The shikimate pathway is found only in microorganisms and plants, never in animals (Nabavi et al., 2018).

Flavonoids are a group of phenolic compounds that are constructed from a 15-carbon skeleton and composed of two benzene rings (A and B) (Figure 1.20, section 1.5, page 99), connected by 3-carbon linking chain; thus, they are called the C6-C3-C6 compounds (as seen in chalcone) (Ferreyra et al., 2012). In many flavonoids, the linker chain forms a further heterocyclic pyrone or pyran ring (C) (Corradini et al., 2011). Cyclisation of three malonyl-coA molecules (in acetate pathway) leads to the formation of ring-A. 4-coumaroyl-CoA are the substrates for the synthesis of ring-B from phenylalanine via the shikimate pathway (Nabivi et al., 2018). Chalcones are formed via the condensation of rings A and B. The chalcone scaffold serves as the building block from which all flavonoids are derived (Ferreyra et al., 2012). The central pathway for the synthesis of flavonoids is conserved in plants; however, a group of enzymes, such as $Fe^{2+/2-}$ oxoglutarate-dependent dioxygenases, hydroxylases, reductases and isomerases modify the basic flavonoid skeleton, leading to the different flavonoid subclasses (Martens et al., 2010). Isomerase-catalysed cyclisation of rings A and B leads to the formation of flavonone. Flavonones are then utilised for the synthesis of other flavonoids (Khoo et al., 2017). This biosynthetic pathway is common to all flavonoids with the flavan (2-phenylchroman) having the simplest structural units (Figure 1.12). The degree of oxidation and / or unsaturation of ring-C leads to structural diversity in flavonoids such as 2-phenylchromanes (flavan-3,4-diols, flavan-3-ols and flavans), 2-phenylochromones (di-OH-flavonols, flavanones, flavonols and flavones) and 2-phenylchromenyliums (anthocyanins / anthocyanidins) (Khoo et al., 2017).

1.4 *Inula* Species

Inula belongs to the compositae family of which over 100 species have been identified. It is widespread in Africa, Europe, and Asia and found predominantly in the Mediterranean area (Wang, Gao et al. 2018). *Inula helenium* is an herb, rigid in nature, and can grow to a height of 1.5 meters; with large and toothed leaves, the upper parts embracing the stem and the lower part stalked; its flowers are yellowish in colour, about 5cm wide, with many petals, each having at its extremity – three notches. The root is mucilaginous, thick and branching, bitter to taste, with an odour of camphor, and having sweet floral undertones (Amin et al., 2013; Nikolaev et al., 2006; Rong, 1979).

1.4.1 Traditional Uses of *Inula* Species

Inula has been used in ethnomedicine for decades especially in Chinese medicine. Due to their importance in traditional medicine, species such as *Inula britannica, Inula racemosa* and *Inula helenium* have been used in herbal preparation for commercial purposes (Han et al., 2010). *Inula helenium* has been used in Ventrofit, as an antiulcer drug (Nikolaev et al., 2006), and many of these medical applications of *Inula* have been documented in several European pharmacopeias (Trendafilova et al., 2010). *Inula helenium* is thought to be the herb used by Prophet Job who used it in the treatment of boils (Al-Gammal, 1998). Hippocrates also wrote that *Inula helenium* is an effective cure for treating skin infections and multiple itching, lung, stomach and head diseases (Al-Gammal, 1998). Jarić et al. (2011), in his review of medical manuscripts dating back to the 12th century reported the usefulness of this herb in the management of dandruff, freckles and wounds. The flowers and roots of *Inula helenium* were used in the treatment of bronchitis, emphysema and asthmatic conditions (Ram et al., 2011).

By using the root of *Inula helenium* in folklore medicine, the treatment of many ailments such as helminthic ailments, infectious diseases, tuberculosis, enterogastritis, indigestion, disorders of the lungs, bronchitis, cough and asthma including herpes, dermatitis and dark phlegm was achieved (Grimaud, 2009; Gyatso & Hakim, 2010; Huo et al., 2010; Huo et al., 2008). In China, *Inula* have been employed as antibacterial, antiemetics, diaphoretics, antitussives and expectorants, antidiabetic, antiulcer agents as well as in the cure of bronchitis (Qin et al., 2011). The roots and leaves of *Inula cappa* are utilized as a part of the treatment of jungle fever, hepatitis, dysentery, and arthritis in conventional medicine (Wang et al., 2012) as well as abdominal pain, laryngo-tracheitis and rheumatism (Zhao et al., 2013; Zou et al., 2008). Recent data suggests constituents

of *Inula helenium* are effective in treatment of atopic dermatitis-like skin infection by regulating the expression of cytokines (Wang et al., 2018).

Inula sasoloides, *Inula ammophila* and *Inula schugnanica* have been used in China in the treatment of diuresis and fever (Hu et al., 2011). Additionally, other *Inula* species such as *Inula asperrima, Inula nervosa, Inula venosa, Inula esquiroli* and *Inula nitida* are used in the treatment of rheumatism and stomach problems (Yan et al., 2010). *Inula helianthus* is used in treating cancers of the brain, colon, breast, gastric and oesophagus (Zeng et al., 2009).

Inula racemosa is employed in the treatment of dermal infections, tuberculosis, and used as bronchodilators and expectorants (Shishodia et al., 2008). *Inula racemosa* has been used in the management of diabetes (Krishnaraju et al., 2005), treatment of dyspnoea and angina, promoting the functioning of the circulatory system, and in the reduction of cholesterol (Mahmood et al., 2010; Mangethayaru et al., 2009; Millers, 1998). It is also used as an antibacterial agent and in the management of asthma (Vadnere et al., 2009; Xu & Shi, 2011).

Inula obtusifolia and *Inula royleana* has been used in the treatment of hypertension, used as diuretics, expectorants, antiseptics, anthelmintic, stimulants of the nervous system and hypotensive agents, as well as infections of the larynx and gastrointestinal system (Grimaud, 2009; Haq et al., 2011).

Additionally, the antiseptic, antipyretic, anti-inflammatory, anti-scabbies, antiphlogistic and basalmic activity of *Inula viscosa* have been documented (Fontana et al., 2007). *Inula viscosa* stem and roots are used in the treatment of catarrh and cough, and as expectorants in loosening phlegm from the mucus membrane and has been reported to heal over forty diseases in the holy land (Palevitch & Yaniv, 1991; Saad & Said, 2011), and in the treatment of infertility and muscle ache in Palestine (Kaileh et al., 2007).

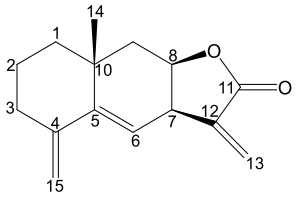
In Europe and China, *Inula britannica*, and *Inula japonica* finds application in the management of some tumours, hepatitis, viral infections, inflammation, bronchitis, digestive disorders, excessive sputum, hiccup, nausea, acute pleurisy, bronchitis and asthma (Khan et al., 2010; Liu et al., 2011; Nam et al., 2009; Zhao et al., 2006).

With these reports, *Inula* spp. can be said to be useful as: (ii) bactericidal and fungicidal agents in the treating dermatitis, (ii) in the treatment of biliary dyskinesia, (iii) in the

treatment of chronic cough, (iv) in the management of obesity and diabetes, (v) and as antipruritic agents.

1.4.2 Isolated Metabolites from *Inula*

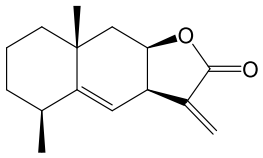
Most bioactive compounds isolated from *Inula* specie are the sesquiterpene lactone. In *Inula* japonica, sesquiterpene dimers – Inulanolide (Jin et al., 2006), bigelovin (Zeng et al., 2009), ergolide (Whan et al., 2001), acetylbritannilactone, (Liu et al., 2007), tomentosin, britanin (Lu et al., 2012), and inuviscolide (Rozenblat et al., 2008) have been isolated.



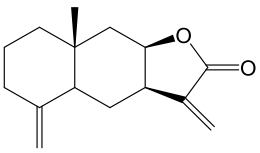
Structure 1.51: Numbering system for sesquiterpene lactones

In *Inula britannica*, five phytochemicals have been isolated, and they include the flavonoids, triterpenes, diterpenes, and sesquiterpene lactones. Sesquiterpene lactones are classified into the eudesmanolides, guaianolides, pseudoguaianolides, germacranolides, xanthanolides and the dimeric sesquiterpene lactones based on the chemical structures of these compounds (Fischedick et al., 2013; Seca et al., 2014).

Eudesmanolides (Structure 1.52 – 1.53) are the dominating group of metabolites present in *Inula* and the pharmacological activities of *Inula* are associated with the presence of these metabolites and their biological activities include: antibacterial, anticancer and anti-inflammatory actions (Picman 1986).



Structure 1.52: alantolactone



Structure 1.53: Isoalantolactone

Structure 1.52-1.53: basic structure of eudesmanolide (Jiang et al., 2011).

The following eudesmanolides have been extracted from *Inula racemosa* root: 11α,13-dihydroalantolactone, 12-hydroxyeudesmane-5(6)-7(11)-dien-12,8-olide, and other hydroxylated eudesmanolides (Zhang et al., 2012; Zhang et al., 2010).

From the aerial part of *Inula hupehensis*, 6α-hydroxy-4-epi-septuplinolide, 6α-hydroxyisoalloalantolactone, and 3-Oxo-6α-hydroxyeudesmane-4(5)11(13)-dien-12-8β-olide (Qin et al., 2012) have been isolated. Isolated from *Inula britannica* flowers are 1-

O-acetylbritanilactone, britanilactone, and 1,6-O,O-diacetyl-britannilactone (Qi et al., 2008). 5α-epoxyalantolactone, isoalantolactone, alantolactone and 4α,5α-epoxyalantolactone have been isolated from *Inula helenium* root (Jiang et al., 2011; Konishi et al., 2002).

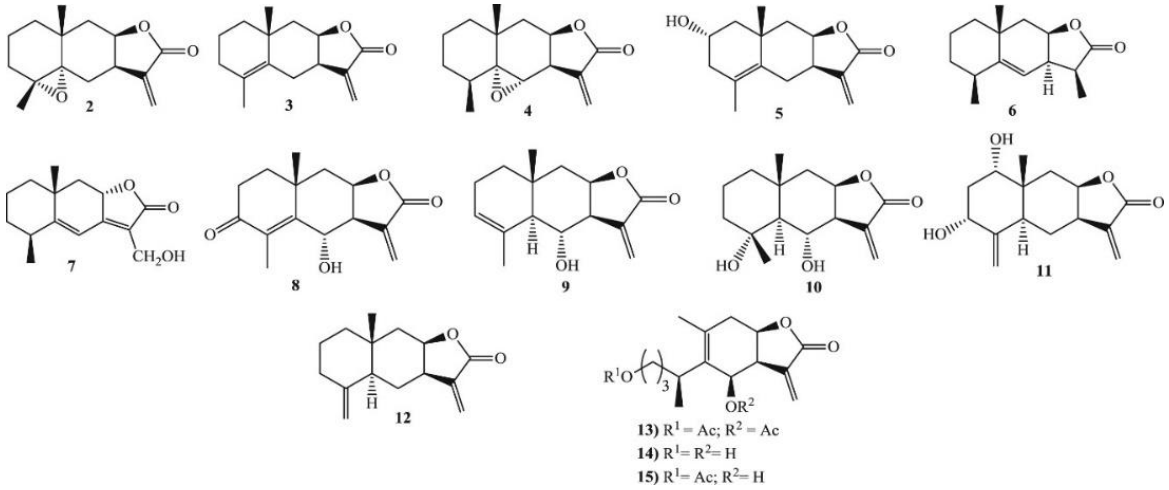
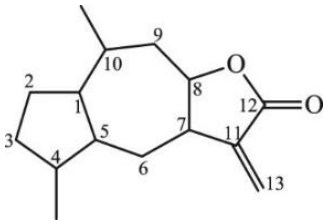


Figure 1.13: Structures of eudesmanolide derivatives isolated from *Inula* spp.
The numbers below the structures corresponds to the compound class and names presented in detail on Table 1.8 below. For instance (2) corresponds to 4α,5α-Epoxyalantolactone (Seca et al., 2014).

It therefore implies that the eudesmanolides are very useful sources of sesquiterpene lactones since they offer a range of pharmacologic activity which may be useful in clinical trials against pathologic conditions.

Guaianolides (Structure 1.54) have been isolated mainly from the whole parts of *Inula hookeri* and they include: inuchinenolide, 2α-acetoxy-inuviscolide, gaillardin, and 5α-6α-epoxy-2α-acetoxyl-4α-hydroxyl-1β,7α-guaiane-11-(13)-en-12,8α-olide (Cheng et al., 2012), and from the aerial part of *Inula hurpehensis*, and *Inula falconeri* and they include inuviscolide and its analogues, which are potent anti-inflammation agents (Cheng et al., 2011; Qin et al., 2011).



Structure 1.54: Guanolide – basic structure of guanolide - inuviscolide (Abraham et al., 2010; Seca et al., 2014).

Below is a diagramatic representation of bioactive guanolides isolated from *Inula spp.*

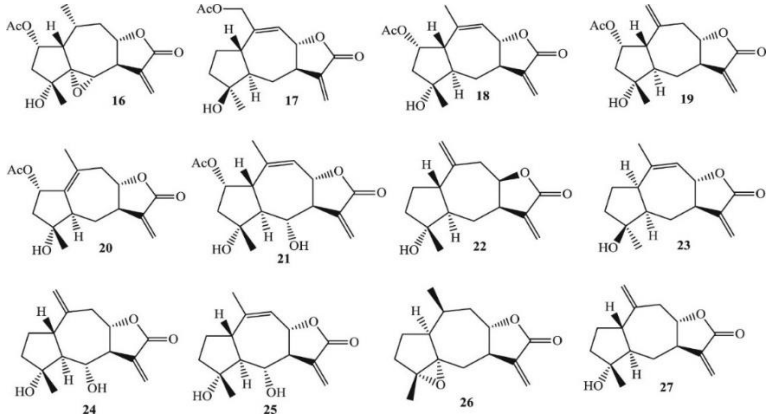
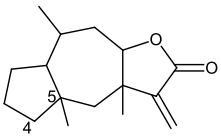
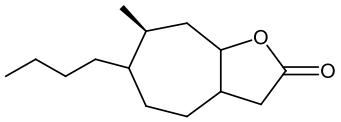


Figure 1.14: Structures of guaianolides isolated from *Inula* spp.
The numbers below the structures corresponds to the serial numbers on Table 1.8 below (Seca et al., 2014).

Pseudoguaianolides (Structure 1.55) have been extracted mainly from *Inula hupehensis* (aerial parts), and *Inula japonica*, and they include britanin (Lu et al., 2012), ergolide – from the leaves of *I. falconeri* (Cheng et al., 2011), and bigelovin – from the leaves and flowers of *Inula helianthus* (Zeng et al., 2009). Pseudoguaianolides inhibit the production of lipopolysaccharide instigated NO in RAW264.7 macrophage. Ethanol fractions of extracts from *Inula hupehensis* strongly inhibited the growth of U937 leukaemia tumour cells at very low concentration of 2μM which is supportive of the traditional application in the treatment of gastric cancer in China.



Structure 1.55: Pseudoguainolide



Structure 1.56**:** Germacranolide

(Seca et al., 2014).

Pseudoguanolides differs from guanolides in the arrangement of carbonyl groups at carbon 5, and not carbon 4 (in guanolides).

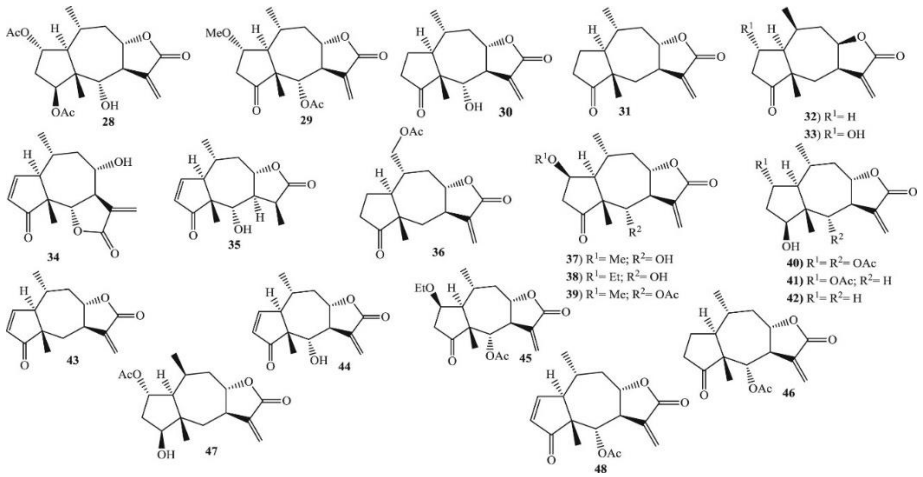
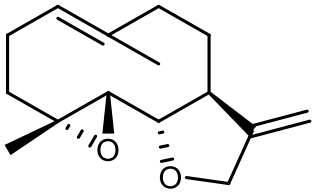


Figure 1.15: bioactive pseudoguainolides isolated from *Inula spp*.
The numbers below the structures corresponds to the serial numbers on Table 1.8 below

This is the reason *Inula* extracts acts against inflammation and diabetes (Qin et al., 2011; Zeng et al., 2009). Below is a diagrammatic representation of bioactive pseudoguanolides isolated from *Inula spp*

Germacranolide (Structure 1.56) is known to be an inducer of cytotoxicity to colorectal cancer cell line (HCT-116), and HeLa cells (Bai et al., 2006). A notable group of germacranolides are the parthenolide extracted from feverfew; it is currently undergoing clinical trials for anticancer activities (Ghantous et al., 2013; Jafari et al., 2018). Parthenolide (Structure 1.57) is a small molecule, the first of its kind, reported to exhibit selectivity against cancerous stem cells (Ghantous et al., 2013). This it does by targeting specific signal pathways and destroying tumour cells from its root (Ghantous et al., 2013). Other potent bioactive derivatives of parthenolide – such as (±)-7-epi-parthenolide (Li et al., 2016) and 9α-hydroxyparthenolide (Zaki et al. 2016) have been synthesised and they elicit interesting anticancer activities (Jafari et al., 2018; Seca et al., 2014). Derivatives or analogues of germacranolide can be used in treatment of tumours, which is supportive of their traditional uses (Hu et al., 2011).



Structure 1.57: parthenolide, a germacranolide.
(Kwok et al., 2001; Seca et al., 2014).

Other germacranolides isolated from the aerial parts of *Inula cappa, Inula verbasifolia*, and *Inula japonica* include ineupatolide, epoxygermacranolide, acetyl neobritannilactone B and eupatolide (Bai et al., 2006; Qin et al., 2010; Wang et al., 2012; Zhao et al., 2006). Below is a representation of the bioactive germacronolide isolated from *Inula spp*.

Epoxygermacranolide inhibited the growth of HCT116 cells (Harvala et al., 2002), Hela cells were susceptible to inhibition by neobritannilactone B (Bai et al., 2006), whereas acetylbritannilactone elicited apoptosis in AGS cells (Bai et al., 2006).

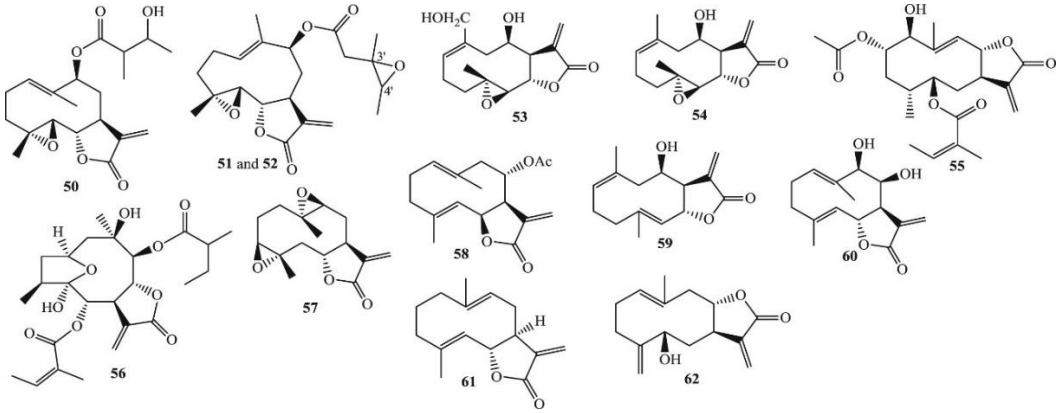
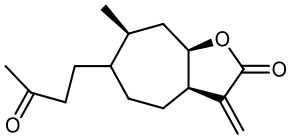


Figure 1.16: Structures of germacranolides isolated from *Inula* spp.
The numbers below the structures corresponds to the serial numbers on Table 1.8 below (Seca et al., 2014).

Xanthanolide isolated from *Inula hupehensis* (tomentosin), elicited interesting response on RAW264.7 macrophage. It inhibited RAW264.7 macrophage at (IC_{50} 9.9 μM) and its activity was comparable to the control agent aminoguanidine (IC_{50} 7.9 μM) (Qin et al., 2011).



Structure 1.58: Tomentosin – a xanthanolide
(Abraham et al., 2010; Seca et al., 2014).

Below are the structures of xanthanolide isolated from *Inula spp*.

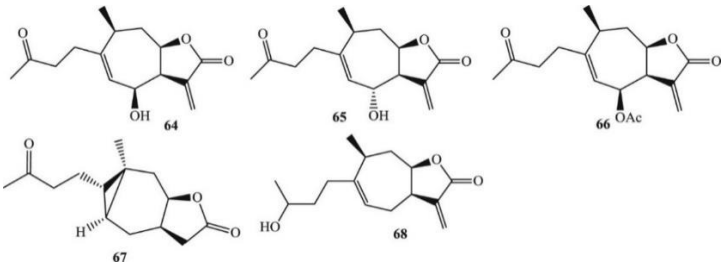
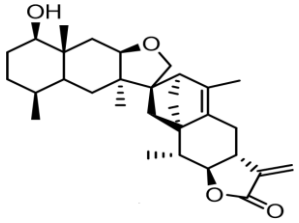


Figure 1.17: Xanthanolides isolated from *Inula spp*.
The numbers below the structures corresponds to the serial numbers on Table 1.8 below (Seca et al., 2014).

Xanthanolide are known to prevent ulcer by its cytoprotective activities on the gastric canal of the stomach by preventing sulfhydryl's nucleophilic attack on the gastric (Favier et al., 2005).

Dimeric sesquiterpenes have been isolated from *Inula Japonica* and they include: inulonide B and inulonide C (Jin et al., 2006; Xu et al., 2015), neojapanicone A, E, F, and J (Qin et al., 2010), and their anti-inflammatory potentials reported. Analogues from related compounds have shown promising results in their potential usefulness as anti-tumour and anti-inflammatory agents (Seca et al., 2014).



Structure 1.59: Japonicone - a dimeric sesquiterpene lactone
(Hu et al., 2012).

Recently, a potent sesquiterpene dimer Inulanolide E, and neojaponicone B have been isolated from *Inula japonica*, and tested against Jurkat cell and 6T-CEM cell lines.
Below are the structures of bioactive dimeric sesquiterpene lactones isolated from *Inula spp*.

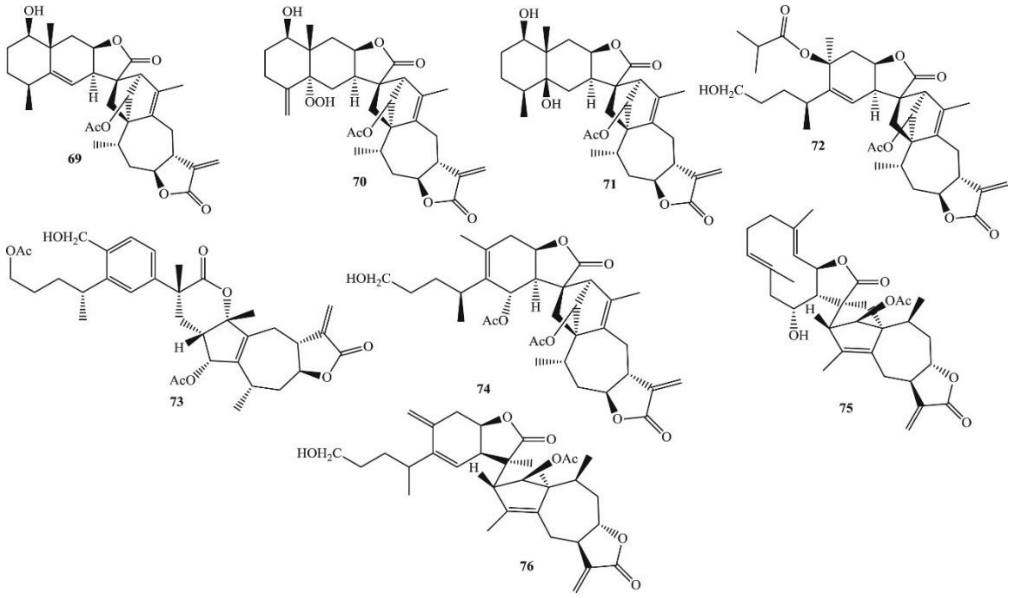
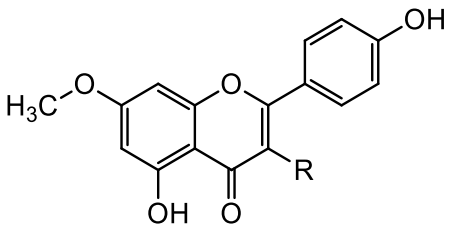


Figure 1.18: Dimeric sesquiterpene lactones isolated from *Inula spp.*
The numbers below the structures corresponds to the serial numbers on Table 1.8 below (Seca et al., 2014).

Both compounds elicited cytotoxicity against Jurkat and 6T-CEM cells (IC_{50} values 2.5 - 5.9 µM) (Xu et al., 2015). Japonicone F inhibited RAW 264.7 macrophage, preventing NO production induced by LPS (Qin et al., 2010).

Flavonoids are also present in *Inula viscosa* and are known for their antioxidant and radical scavenging properties and are known to be beneficial in ameliorating disease conditions where ROS plays a pivotal role (Nijveldt et al., 2001).



R = H Sukuranetin; R = OH 7-O-Methylaromadendrin

Structure 1.60: Flavonoids from *Inula viscosa*
(Hernández et al., 2007).

Flavonoids display important biological activities: antibacterial, antioxidants, anti-inflammatory and anticancer activities. Since flavonoids are known for their wide range of activities, it could therefore be stated that the traditional medicinal uses of *Inula* is because of their presence. Flavonoids such as 7-O-methylaromadendrin, 3-Acetylaromadendrin, sakuranetin have been isolated from *Inula britannica* and *Inula viscosa* (Seca et al., 2014).

Below are the structures of bioactive flavonoids isolated from *Inula spp*.

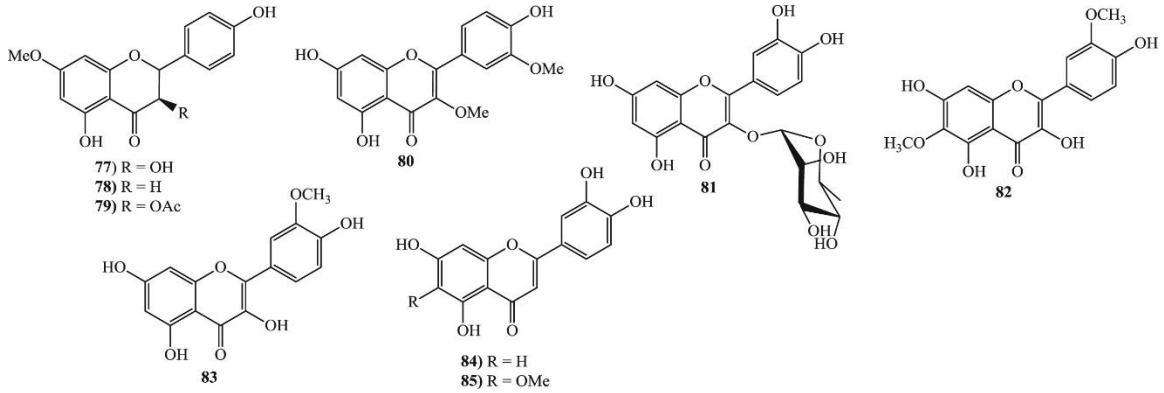


Figure 1.19: Bioactive flavonoids isolated from *Inula spp*.
The numbers below the structures corresponds to the serial numbers on Table 1.8 below (Seca et al., 2014).

Other secondary metabolites such as *Inula*vosin, quinic acid and its derivatives have been isolated from *Inula crithemoide*, *Inula nervosa* and the roots of *Inula hupehensis* (Seca et al., 2014).

Table 1.8: Bioactive compounds isolated from *Inula* specie

No.	Compound class and name	Species	Parts of plant	References
Eudesmanolides				
1	Alantolactone	*Inula helenium*	Roots	(Lawrence et al., 2001)
2	4α,5α-Epoxyalantolactone	*Inula helenium*	Roots	Jiang et al. (2011)
3	Diplophyllin	*Inula helenium*	Roots essential oil	Stojanović-Radić et al. (2012)
4	5α-Epoxyalantolactone	*Inula helenium*	Roots	Im et al. (2007)
5	2α-Hydroxyeudesma-4,11(13)-dien-12,8β-olide	*Inula racemose*	Roots	Zhang et al. (2012a)
6	11α,13-Dihydroalantolactone	*Inula racemose*	Roots	Zhang et al. (2010a)
7	12-Hydroxy-eudesma-5(6),7(11)-dien-12,8-olide	*Inula racemose*	Roots	Zhang et al. (2010a)
8	3-oxo-6α-Hydroxy-eudesma-4(5),11(13)-dien-12,8β-olide	*Inula hupehensis*	Aerial part	Qin et al. (2012b)
9	6α-Hydroxyisoalloalantolactone	*Inula hupehensis*	Aerial part	Qin et al. (2012b)
10	6α-Hydroxy-4-*epi*-septuplinolide	*Inula hupehensis*	Aerial part	Qin et al. (2012b)
11	Granilin	*Inula falconeri*	Aerial parts	Cheng et al. (2011)

12	5α,*H*-Eudesma-4(15),11(13)-dien-12,8β-olide (also named isoalantolactone)	*Inula japonica*	Aerial parts	Wang et al. (2007)
13	1,6-*O*,*O*-Diacetylbritannilactone	*Inula Britannica*	Flowers	Qi et al. (2008)
		Inula britannicavar	Flowers	Rafi et al. (2005)
14	Britannilactone	*Inula Britannica*	Flowers	Qi et al. (2008)
		Inula britannicava.	Flowers	Liu et al. (2009)
15	1-*O*-Acetylbritannilactone	*Inula Britannica*	Flowers	Qi et al. (2008)
		Inula britannicavar	Flowers	Rafi et al. (2005)
Guaianolides				
16	5α,6α-Epoxy-2α-acetoxy-4α-hydroxy-1β,7α-guaia-11(13)-en-12,8α-olide	*Inula hookeria*	Whole parts	Cheng et al. (2012)
17	14-Acetoxy-1β,5α,7αH-4β-hydroxy-guai-9(10),11(13)-dien-12,8α-olide	*Inula hookeri*	Whole parts	Cheng et al. (2012)
18	Gaillardin	*Inula hookeri*	Whole parts	Cheng et al. (2012)
19	2α-Acetoxy-inuviscolide	*Inula hookeri*	Whole parts	Cheng et al. (2012)
20	Inuchinenolide B	*Inula hookeri*	Whole parts	Cheng et al. (2012)
21	2α-Acetoxy-4α,6α-dihydroxy-1β,5α*H*-guai-9(10),11(13)-dien-12,8α-olide	*Inula hupehensis*	Aerial parts	Qin et al. (2011a)
22	8-*epi*-Inuviscolide	*Inula hupehensis*	Aerial parts	Qin et al. (2011a)
23	4-*epi*-Isoinuviscolide	*Inula hupehensis*	Aerial parts	Qin et al. (2011a)
24	6α-Hydroxyinuviscolide	*Inula falconeri*	Aerial parts	Cheng et al. (2011)
25	4α,6α-Dihydroxy-1β,5α,7α*H*-guaia-9(10),11(13)-dien-12,8α−olide	*Inula falconeri*	Aerial parts	Cheng et al. (2011)
26	4α,5α-Epoxy-10α,14*H*-1-*epi*-inuviscolide	*Inula falconeri*	Aerial parts	Cheng et al. (2011)
27	Inuviscolide	*Inula viscosab*	Leaves	Rozenblat et al. (2008)
Pseudoguaianolides				
28	Inuchinenolide C	*Inula hupehensis*	Aerial parts	Qin et al. (2011a)
29	(1*S*,2*S*,5*R*,6*S*,7*R*,8*S*,10*R*)-6-Acetoxy-2-methoxy-4-oxopseudoguai-11(13)-en-12,8-olide	*Inula hupehensis*	Aerial parts	Qin et al. (2011a)
30	Carpesiolin	*Inula hupehensis*	Aerial parts	Qin et al. (2011a)
31	Graveolide (2,3-dihydroaromaticin)	*Inula hupehensis*	Aerial parts	Qin et al. (2011a)
32	Confertin	*Inula hupehensis*	Aerial parts	Qin et al. (2011a)
33	Burrodin	*Inula hupehensis*	Aerial parts	Qin et al. (2011a)
34	(1*R*,5*R*,6*S*,7*R*,8*S*,10*R*)-8-Hydroxy-4-oxopseudoguai-2(3),11-(13)-dien-12,6-olide	*Inula hupehensis*	Aerial parts	Qin et al. (2011a)
35	(1*R*,5*R*,6*S*,7*S*,8*S*,10*R*,11*S*)-6-Hydroxy-4-oxopseudoguai-2(3)-en-12,8-olide	*Inula hupehensis*	Aerial parts	Qin et al. (2011a)
36	(1*S*,5*S*,7*R*,8*S*,10*R*)-14-Acetoxy-4-oxopseudoguai-11(13)-en-12,8-olide	*Inula hupehensis*	Aerial parts	Qin et al. (2011a)
37	(1*S*,2*R*,5*R*,6*S*,7*S*,8*S*,10*R*)-6-Hydroxy-2-methoxy-4-oxopseudoguai-11(13)-en-12,8-olide	*Inula hupehensis*	Aerial parts	Qin et al. (2011a)

38	(1*S*,2*R*,5*R*,6*S*,7*S*,8*S*,10*R*)-6-Hydroxy-2-ethoxy-4-oxopseudoguai-11(13)-en-12,8-olide	*Inula hupehensis*	Aerial parts	Qin et al. (2011a)
39	(1*S*,2*R*,5*R*,6*S*,7*R*,8*S*,10*R*)-6-Acetoxy-2-methoxy-4-oxopseudoguai-11(13)-en-12,8-olide	*Inula hupehensis*	Aerial parts	Qin et al. (2011a)
40	Britanin (also named britannin)	*Inula lineariifoliac*	Aerial parts	Nie et al. (2010),
		Inula japonica	Flowers	Lu et al. (2012)
		Inula aucherianad	Not identified	Moghadam et al. (2012)
41	2-*O*-Acetyl-4-*epi*-pulchellin	*Inula lineariifoliac*	Aerial parts	Nie et al. (2010)
42	2-Desoxy-4-*epi*-pulchellin	*Inula lineariifoliac*	Aerial parts	Nie et al. (2010)
43	Aromaticin	*Inula hookeri*	Whole parts	Cheng et al. (2012)
44	8-*epi*-Helenalin	*Inula hookeri*	Whole parts	Cheng et al. (2012)
45	6-Acetoxy-2-ethoxy-4-oxopseudoguai-11(13)-en-12,8-olide	*Inula hookeri*	Whole parts	Cheng et al. (2012)
46	Ergolide	*Inula falconeri*	Aerial parts	Cheng et al. (2011)
		Inula Britannica	Flowers	Park and Kim (1998)
47	2α-Acetoxy-4β-hydroxy-1α*H*,10α*H*-pseudoguai-11(13)-en-12,8α-olide	*Inula japonica*	Aerial parts	Qin et al. (2010b)
48	Bigelovin	*Inula helianthus-aquatica*	Leaves/flowers	Zeng et al. (2009)
Germacranolides				
50	Epoxygermacranolide	*Inula verbascifoliasubsp. Methaneae*	Aerial parts	Zhao et al. (2006), Chin et al. (2009)
51/52	Diastereomeric mixture of (3′*R*,4′*S*- and 3′*S*,4′*R*)-9β-(3′,4′-epoxy-3′-methylpentanoyloxy) parthenolide	*Inula montbretianaf*	Aerial parts (with flowers)	Gökbulut et al. (2012)
53	*Inula*salsolide	*Inula salsoloides*	Aerial parts	Hu et al (2011)
54	4α,5β-Epoxyeupatolide	*Inula salsoloides*	Aerial parts	Hu et al (2011)
55	*Inula*cappolide	*Inula cappa*	Whole plant	Xie et al. (2007)
56	Ineupatolide	*Inula cappa*	Aerial plant	Wang et al. (2012)
57	11(13)-Dehydroivaxillin	*Inula hupehensis*	Aerial plant	Qin et al. (2012b)
		Inula hookeri	Whole plant	Cheng et al. (2012)
58	Acetyl neobritannilactone B	*Inula britannica*	Flowers	Bai et al. (2006)
59	Eupatolide	*Inula japonica*	Aerial parts	Qin et al. (2010b)
		Inula britannicavar.	Aerial parts	Jin et al. (2006)
60	9β-Acetoxy-eupatolide	*Inula hupehensis*	Aerial parts	Qin et al. (2012b)
61	Isocostunolide	*Inula helenium*	Roots	Chen et al. (2007)
62	[1(10)*E*]-5β-Hydroxygermacra-1(10),4(15),11-trien-8,12-olide	*Inula racemose*	Roots	Zhang et al. (2012a)
Xanthanolides				
64	6β-Hydroxytomentosin	*Inula hupehensis*	Aerial parts	Qin et al. (2012b)
65	6α-Hydroxytomentosin	*Inula hupehensis*	Aerial parts	Qin et al. (2012b)
66	Inuchinenolide A	*Inula hupehensis*	Aerial parts	Qin et al. (2012b)

(Seca et al., 2014).

1.4.3 Pharmacological and Biological Activities

Some plants have been used as sources of medication in treating ailments and diseased conditions (Chinsembu 2016), because most plants (or metabolites isolated from these plants) are known to have little or no side effects; they are less toxic/ poisonous when compared to synthetic drugs (Ochwangi et al., 2018). Herbal extracts are also known for their efficacy in the treatment of several ailments and there are reports on their usefulness in the management of several disease conditions both in traditional medicine and in the laboratory (Mangethayaru et al., 2009; Greenwell & Rahman, 2015).

A review of literature of pharmacological activity of extracts and / or pure compounds from *Inula* specie reveals a striking finding; some authors in their report of antibacterial activity for instance, did not indicate the dosage and / or plant part used in their study. Others only mentioned the fraction (example hexane fraction) indicating the bioactivity without mentioning the dosage in which such bioactivity occurred. This noted, a summary of the plant part of *Inula* species, biological activity evaluated, solvent used for extraction and the activity of the extract are presented in Table 1.9 below.

Table 1.9: Summary of the plant part, biological activity evaluated, solvent used for extraction and the activity of the extracts from *Inula* spp.

Plant part	Biological activity evaluated	Tested material	Results	Reference
Inula viscosa				
Leaves	Abortifacient and anti-implantation (*in vivo*)	Petroleum ether, dichloromethane, methanol and aqueous extracts	The aqueous extract exhibited potent anti-implantation, mid-term abortion and luteolytic effects in pregnant rats but not acute toxicity	Al-Dissi et al., 2001
Leaves	Cytotoxicity (*in vitro*)	Metanolic extract	Growth inhibitory effect against SiHa and HeLa cell lines respectively harbouring HPV16 and HPV18 (IC_{50} 54 and 60 µg/mL, respectively)	Merghoub et al. (2009)
Leaves	Antibacterial (*in vitro*)	Ethanolic extract	The multi-drug resistant clinical isolates and reference bacteria were tested, and the most susceptible organism was the clinical bacteria *Pseudomonas aeruginosa* (MIC 8.0 mg/mL)	Oskay et al. (2009)
Leaves	Anti-hypertensive (*in vivo*)	Aqueous extract	This extract prevents significantly the development of hypertension induced by L-NAME and showed a dose-dependent negative inotropic effect in cardiac muscle	Kattouf et al. (2009)
Leaves	Cytotoxic (*in vitro*)	Dichloromethane/methanol (1:1)	The extract showed weak cytotoxicity against L929sA cell line (IC_{50}600±54 µg/mL)	Kaileh et al. (2007)

Plant part	Biological activity evaluated	Tested material	Results	Reference
Leaves	Cytotoxic and genotoxic (*in vitro*)	Aqueous extract	In *Allium cepa* test, extract (at 10 mg/mL) induced the cell death, ghost cells, cells with membrane damage, binucleated cells, chromosomal aberrations and micronuclei (MNC) formations	Çelik and Aslantürk (2010)
Aerial parts	Hypoglycaemic and hypolipidemic (*in vivo*)	Aqueous extract	The tested extract, at a dose of 20 mg/kg showed hypoglycaemic activity by reducing blood glucose levels, without effect on plasma insulin but not affect the total cholesterol and triglycerides	Zeggwagh et al. (2006)
Inula Britannica				
Whole plant	Acaricidal	Petroleum ether extract	The extract was active against *Tetranychus cinnabarinus* with a mortality of 92% at the concentration of 2 mg/mL	Duan et al. (2011)
Not stated	Anti-melanogenic (*in vitro*)	Ethanolic extract	The extract acts as moderate HSP70 inducer, with a safe induction index (SI) of 80 (SI-cell viability (%) at a concentration that doubles the expression of HSP70)	Yamashita et al. (2010)
Inula britannica*var.*chinensis				
Not stated	Antioxidant	Hexane, petroleum ether, chloroform, ethyl acetate and water fraction from 70% hydromethanolic extract	The water fraction showed the strongest antioxidant activity by DPPH (IC_{50} 20.7 µg/mL) and ABTS methods (IC_{50} 39.4 µg/mL) and it protects the cells from HO-induced damage (increasing cell survival by 35.0–77.0% at a concentration range of 62.5–250 µg/mL and decrease intracellular ROS of 34–39%)	Lee et al. (2009)
Inula japonica				
Aerial parts	Anti-inflammatory (*in vitro*)	60% Hydroethanolic extract	The extract exhibited a weak NO-production inhibitory activity but a histamine-release inhibition of 86% at 0.1 mg/mL was obtained	Wang et al. (2006)
Flower	Anti-asthmatic (*in vivo*)	Ethanolic extract	The extract inhibited the OVA-induced airway inflammation by reducing the leukocyte infiltration, AHR, Th2 cytokines, IgE level and mucus hypersecretation	Park et al. (2011)
Flower	Anti-allergic (*in vitro* and *in vivo*)	Ethanolic extract	The extract modulates eicosanoids generation and degranulation through the suppression of SCF-mediated signalling pathways	Lu et al. (2012)
Inula auriculata[b]				
Stem	Antifeedant	Methanolic extract	The *Leptinotarsa decemlineata* larvae was very sensitive to the extract (ED_{50}0.2 µg/cm^2) while *Spodoptera littoralis*had neglectable sensitivity (feeding deterrence index 6.3% at 500 µg/cm^2)	Pavela (2010)
Inula ensifolia				

Plant part	Biological activity evaluated	Tested material	Results	Reference
Flowers/fruits, leaves and roots	Cytotoxic (*in vitro*)	Hexane, chloroform, hydromethanolic and water extracts	The chloroform extract from flowers/fruits showed highest tumour cell-inhibitory activity against HeLa (IC_{50} 2.68 µg/mL) (cisplatin IC_{50}3.73 µg/mL; doxorubicin IC_{50}0.089 µg/mL)	Réthy et al. (2007)
Inula cuspidate				
Leaves	Anti-inflammatory (*in vivo*)	Petroleum ether, chloroform, acetone, methanol and water extracts	The maximum anti-inflammatory effect was observed in water extract followed by petroleum ether extract	Thapliyal et al. (2011)
Inula confertiflora				
Leaves	Antiviral (*in vitro*)	80% Hydromethanolic extract	The influenza A and HSV-1 virus were inhibited by the extract (IC_{50} 6.50 and 96.9 µg/mL, respectively) while the extract was well tolerated by the host cells of virus HeLa, MDCK and GMK (CC_{50}>150 µg/mL). The extract is inactive against CVB3	Gebre-Mariam et al. (2006)
Inula montbretiana				
Leaves flowers and stem	Antimicrobial (*in vitro*)	Ethanol, acetone and diethyl ether extracts of each plant parts	Weak but broad activity against a wide range of microorganisms, being the ethanol extracts of leaves and stem the most effective mainly against *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli* and *Candida albicans* (MIC value of 250–500 µg/mL to the extracts, 2.5–50 µg/mL to gentamicin and 1.25 µg/mL nystatin)	Kunduhoglu et al. (2011)
Inula falconeri				
Aerial parts	Allelopathic and antifungal	Hexane, chloroform, ethyl acetate, and water fractions of the methanolic extract	The hexane fraction presented 100% inhibitory effect to germination of lettuce seeds and hexane subfraction demonstrated 13.3 mm of inhibition zone at 5 mg/disc against *Alternaria alternata* and *Rhizoctonia*	Khan et al. (2010c)

(Seca et al., 2014)

The leaves extract of *Inula viscosa* have been reported to induce abortion in rats (*in vivo*) at doses above 626 mg/kg intra peritoneally, confirming its anti-fertility potential as previously reported in the traditional uses of aqueous extracts from this plant (Al-Dissi et al., 2001). The successful application of extracts from the flowers of *Inula viscosa* as antimicrobial agents in the laboratory has received further boost as its low toxicity on human cell lines (202.43 ± 3.70 µg/mL) has been confirmed (Talib & Mahasneh, 2010). Lu et al. (2012) stated the anti-inflammatory effect of extracts from *Inula Japonica* (ethanol

extract of the flower, no dosage mentioned), identifying tomentosin and britanin using HPLC as the active substances with anti-allergic effects. He stated further that extracts from this plant have been employed in treating inflammation, disorders of the gut and bronchitis in ethnomedicine. Antibacterial activities of extracts from *Inula helenium* have been reported against a clinical strain of *Staphylococcus aureus* (O'Shea et al., 2009). In his work, O'Shea et al., (2009) tested the antimicrobial activity of extracts of *Inula helenium* against 200 clinical strains of Irish *S. aureus* isolates consisting of the Methicillin-sensitive *Staphylococcus aureus* (MSSA) and the MRSA. By measuring the zones of inhibition, he reported a 100 % inhibition against all bacteria tested, with 93 % of these inhibitions falling between the +++ and ++ groups (+++ indicates good bacterial inhibition; ++ indicates moderate inhibition). The minimum bactericidal concentration ranged from 0.9 – 9.0 mg/mL against all bacteria including the MSSA and the MRSA (O'Shea et al., 2009).

Chloroform extracts from the flowers and fruit portion of *Inula ensifolia* elicited strong cytotoxic and anti-proliferative effects against HeLa cells and this activity was highly selective (36%) for dividing cells (Réthy et al., 2007).

Hexane extracts from the shoots and leaves of *Inula* specie elicited fungicidal activities against the *Candida albicans* fungus at inhibitory concentration of 10 - 30 µg/mL (Cohen, 1998; Cafarchia et al., 2003).

1.4.4 Cytotoxic / Antitumour Activity

Sesquiterpenes and other active components extracted from *Inula* such as Japonicone (extracted from *Inula japonica*) have been reported as possessing anti-tumour and cytotoxic activity. This dimeric sesquiterpene - Japanicone elicited potent *in-vivo* and *in-vitro* anticancer activities on Burkitt's lymphoma cells. Another interesting compound acetylbritannilactone (from *Inula britannica*) is known to suppress proliferation of abnormal cells of the vascular smooth muscle, inducing cell death (apoptosis) *in-vitro*. Anti-inflammatory activities of britanin – an active component extracted from *Inula britannica* have been reported (Seca et al., 2014; Wang et al., 2014). Sesquiterpenes from *Inula japonica* showed an IC_{50} value of 2.2 µM, and elicited cytotoxicity against Jurkat and 6T-CEM cell lines (Xu et al., 2015). These sesquiterpene lactones known as *Inula*lonide and neojapanicone B are dimers and elicit cytotoxicity at low concentrations (Xu et al., 2015). Sesquiterpenes containing α-methylene-γ-carbonyl groups isolated from *Inula wissmanniana* inhibited LPS-induced NO production at 1.04 µM IC_{50} value in RAW

264.7 macrophage (Cheng et al., 2014). The presence of α-methylene-γ-carbonyl groups mainly found in guaiane sesquiterpenes is the motif responsible for the anticancer efficacy of sesquiterpenes as seen in parthenolide, thapsiagargin and artemisinin of which some have been proposed for clinical trials because they tend to be selective against cancers and tumour stem cell (Ghantous et al., 2010; Lu, 2002). One important feature of sesquiterpene lactones which gives them advantage over some other compounds is their ability to elicit cytotoxicity against drug resistant and sensitive cell lines. Isolated sequiterpenes from *Inula britannica* elicited this cytotoxic effect (Fischedick et al., 2013).

Several isolates from *Inula* specie have been tested and found useful in the treatment of leukaemia cells (Cragg et al., 2006), of which sesquiterpenes have been reported as potent anticancer agents (Réthy et al., 2007; Zeng et al., 2009). Some products isolated from *Inula* species have shown anticancer activities against some cell lines such as MCF-7, HeLa, HL-60 and H549 (Seca et al., 2014) of which some have shown IC_{50} values lower than 10 μM which is comparable with those of conventional drug daunorubicin, and others have been shown to be effective against the leukaemia U-937 cell line (Seca et al., 2014). Japonicone A69 isolated from *Inula* is more potent than doxorubicin (DOX) against cancerous cells from lymphoblast leukaemia cells (CEM), colon LOVO and MDA-MB-435 (Table 1.10). Some studies have reported the cytotoxicity of extracts from *Inula britannica* (Qi et al., 2008) and inhibition of cancer growth in K-562, 293T, SGC-7901 and B16 cells (Zeng et al., 2009). However, one important point to note is that in these studies, neither the detailed mechanisms of action nor the controls used for validation were reported. Future research needs to be done to confirm their cytotoxicity and their mechanisms of action (Seca et al., 2014). A confirmation on some of the signalling pathways proposed by some research on the mechanism of action of these isolated agents; such as induction of apoptosis, JNK/MAPK signalling pathway activation, and activation of ROS (Pan et al., 2007), need to be verified. Some findings in the research of *Inula* species seems promising but some of these findings needs to be confirmed as some did not indicate the composition, purity and IC_{50} values of the extracted compounds (Seca et al., 2014).

Some secondary metabolites isolated from *Inula* species (Table 1.10) are more cytotoxic than conventional cancer therapeutics (Jordan et al., 1991a; Liu et al., 2005; Nathan et al., 2012). An isolated compound extracted from *Inula britannica*; acetylbritannilactone and its synthetic analogues exhibited stronger cytotoxicity against Bel-7402 and HL-60 cell lines (Liu et al., 2004). Despite the good cytotoxic activity of acetylbritannilactone and its synthetic analogues against cancer cell lines, the researcher did not mention any

positive control used for this experiment. The cytotoxicity of these compounds could be due to the activities of (HO)-1 group of heme oxygenase which is responsible for mopping oxidants, which are the agents responsible for the onset of many disease conditions. Sesquiterpene lactones have the structural unit called α-methylene-γ-butyrolactone (CH-BL), which can interact with these oxidants (Jeong et al., 2007). Therefore, the CH-BL backbone of sesquiterpenes is responsible for the cytotoxic activities elicited by them (Jeong et al., 2007). This is important, as the level of activities reported in sesquiterpene lactones is greatly dependent on the activities on the C-6 substitution (Seca et al., 2014). Cytotoxicity therefore depends on the substituted groups, their structures, and point of attachment (Seca et al., 2014). This finding seems to be different from sesquiterpene lactone group known as the pseudoguaianolides. Cytotoxicity elicited by pseudoguaianolides was stronger in HepG2, HeLa, MGC-803 and PC cell lines compared to cytotoxicity elicited by other groups of sesquiterpenes, implying that the α,-β-unsaturated cyclopentenones units but not the (CH-BL), is responsible for the cytotoxicity of the pseudoguaianolides (Seca et al., 2014).

Tomentosin and inuviscolide isolated from *Inula viscosa* leaves elicited anticancer activities against 1363-mel, 624-mel and SK-28 melanoma cell lines. These isolated compounds caused the cell cycle arrest at the G_2/M phase of the cell cycle, followed by a subsequent appearance of G0; an indication of apoptosis. This also altered the matrix metalloproteinases (MMP) which is caspase 3 dependent (Rozenblat et al., 2008). Inhibition of phosphorylation was observed at Tyr15 and Thr14 (of MMP), leading to a subsequent inhibition of the p53 anti-apoptosis protein of NF-kB (Rozenblat et al., 2008).

Bigelovin elicited strong inhibitory activities against the K-562, 293-T, B-16, and SGC-7901 cells (IC_{50} value 3 μM). An interesting result was seen which showed inhibitory activities at IC_{50} value of 0.47 μM against the histiocytic lymphoma (U937) cell line (Zeng et al., 2009). The mechanism of action of bigelovin includes arresting the cell cycles at G0/G1 stage, apoptosis induction (Gach & Janecka, 2014; Zeng et al., 2009), and inactivation of JAK2 via inhibition of STAT3 signalling (Zhang et al., 2015).

It appears that solvents play very important roles during extraction of phytochemicals from *Inula* as the hexane fraction elicited a significant activity against cell growth compared to the methanol extract. Hexane fraction was found to solubilise seven fractions from *Inula* species which elicited antiproliferative action against B16F10, Hela and MK-1 cell lines (Konishi, Kondo, & Uchiyama, 2008). Others have reported that cytotoxicity elicited by

petroleum ether extract, or methanol extracts at ratios ranging from 50-70% (solvent: water) ratio showed the most profound cytotoxicity (Attard & Cuschieri, 2009; Ojha et al., 2010) in *Inula crithmoides*. The ability of extracts from *Inula crithmoides* in counteracting oxidative stress in ochratoxin A, preventing kidney and liver damage, and thereby protecting these organs against mutagenesis has been reported (Abdel-Wahhab et al., 2008).

Some compounds extracted from *Inula* specie such as helenin – containing a mixture of 60% isoalantolactone and 40% alantolactone is more potent in the inhibition of the Raji lymphoblastoid cells when compared to conventional drugs such as fluorouracil and cyclophosphamide, an activity comparable to that of methotrexate (Spiridonov et al., 2005). 1-O-acetylbritannilactone at very low IC_{50} values of 2.91 to 6.78 µM elicited cytotoxicity against HeLa, HCT116 and HepG2 cells *in vitro*. This was comparable to cytotoxicity indicated by the standard anticancer drug – etoposide (IC_{50} 2.13 to 4.79 µM). Cytotoxicity of compounds isolated from plants are often selective against normal cells (Spiridonov et al., 2005). 1,6-O,O-diacetylbritannilactone (OABL), an analogue of acetylbritanilactone (ABL) isolated from *Inula britannica* showed cytotoxicity which was about ten times more potent than that elicited by ABL in MCF-7 and HL-60 cells (Bing-Nan et al., 1993; Liu et al., 2004). Semi-synthetic analogues of ABL elicited cytotoxicity against HeLa, HepG2 and HCT 116 cell lines. Insertion of a 12-carbon lauroyl group on the 6-OH increased the cytotoxicity of these compounds (Dong et al., 2014).

The proposed mechanism of action of OABL is apoptosis associated with activation of caspases 3, 8 and 9, elevation from the mitochondrial compartment of cytochrome C in HL-60 cell line (Pan et al., 2007). This enhanced activity of OABL is linked with the 6-hydroxy group (6-OH) at C-6 which makes this compound highly lipophilic (Dong et al., 2014; Liu et al., 2005). This increased activity is also potentiated by the availability of α-methylene-γ-lactone, an electrophilic moiety which has ability of binding to the thiol groups of proteins or can acts as an alkylating agent, and by inducing the fragmentation of DNA and apoptosis which is thought to occur due to depletion of glutathione in cells (Amslinger, 2010; Dong et al., 2014). Mechanisms of anticancer action of 5α-epoxyalantolactone and alantolactone isolated from *Inula* species include: induction of glutathione transferase (GST), quinone reductase and other detoxifying enzymes such as heme oxygenase, glutathione reductase and glutamylcysteine synthase which was reported in methanol extract of *Inula* species (Seo et al., 2008). This induction occurs via the potentiation of JNK and PI3K signal pathways, resulting into the efflux of Nrf2, and a

resultant encoding of ARE and Nrf2 (Seo et al., 2008), an activity regulated by (HO-1) which inhibits phosphorylation of IkBα and NF-Kb in RAW264.7 cells activated by LPS (Park et al., 2013). Anti-mutagenic effects are also responsible for cytotoxicity of compounds isolated from *Inula*.

Lawrence et al., (2001), reported the isolation of alantolactone and isoalantolactone from the roots of *Inula helenium*. Extraction was done using methanol and the brown root extracts were partitioned using chloroform, ethyl acetate and hexane. Alantolactone and isoalantolactone isolated from the hexane fraction indicated very good cytotoxic activity against K562 cell line with IC_{50} 0.7 and 1.2 μM. In a Michael type amine addition reaction of isoalantolactone and alantolactone, Lawrence et al. (2001) targeted the α-methylene lactone ring which they reported is responsible for the cytotoxic activity of these sesquiterpene lactones. Several derivatives of α-methylamino lactone were synthesised and screened against K562 cell lines. The diethylamine adduct of isoalantolactone possessed similar cytotoxicity IC_{50} 2.0 μM, and the ethylamine adduct of alantolactone indicated a similar cytotoxicity IC_{50} 0.7 μM, to the parent compound – alantolactone. These amines induced apoptosis by acting as alkylating agents. Due to the promising anticancer activities of alantolactone (AL) and isoalantolactone (IAL) isolated from *Inula*, *in vivo* studies on the metabolism of alantolactone and isoalantolactone has been investigated by (Zhou et al., 2018) who reported the conjugation of glutathione (GSH) to these compounds. Non conjugation with cysteine (Cys) was also observed giving rise to four metabolites: IAL-Cys, IAL-GSH, AL-Cys and AL-GSH; an indication that the thiol of Cys and GSH reacts with the α,β-unsaturated carbonyl (exomethylene carbon) of IAL and AL. When injected into rats, the bioavailability of these metabolites (IAL, IAL-GSH, IAL-Cys) and (AL, AL-GSH, AL-Cys) was 13.07 % and 8.39 %, to parent compounds IAL and AL are (1.88 %) and 2.32 % respectively. This finding indicates the bioavailability of AL and IAL when administered orally. AL and IAL metabolism *in-vivo* is mainly via the biotransformation of AL and IAL by addition of thiol groups at C11 and C13, confirming the bioavailability of these compounds *in vivo* (Yao et al., 2016).

Anti-proliferation and cytotoxicity of compounds isolated from *Inula* was via induction of apoptosis through various channels such as:

i) Blockage of Nf-kB signal pathway in Jurkat thymus cells as exemplified by ergolide and inuviscolide on SK-28 melanoma cells (Rozenblat et al., 2008).

ii) DNA damage and denaturation as elicited by alantolactone in HepG2, inuviscolide and tomentosin in SK-28 cells by alkylation of DNA which subsequently leads to activation of the kinases and a resultant cell cycle arrest at G2/M (Rozenblat et al., 2008; Lei et al., 2012).

iii) A decrease in survivin level; a member of a family known as inhibitors of apopotosis (Zhiqin et al, 2014) via Cyt-C discharge, splitting of ADP ribosyl-polymerase and caspase activation of 1,6-O-diacetylbritannilactone in U87 and HL-60 cell lines (Pan et al., 2007; Khan et al., 2012).

iv) Activation of JNK and MAPK, and release of ROS (Pan et al., 2007; Khan et al., 2010).

v) Alantolactone and isoalantolactone elicits cytotoxicity by acting as alkylating agents (Lawrence et al., 2001).

A summary of the anticancer activity of purified compounds isolated from *Inula* species are presented in Table 1.10 below:

Table 1.10: Summary of anticancer activity of purified compounds isolated from *Inula* species

Cell line	Compounds	IC_{50}values ($IC_{50}$to positive control)	References
B16F10	1 (Structure 1.52), 4 (Fig 1.14)	3.6–4.7 µM (5-Fluorouracil 1.1 µM)	Konishi et al. (2002)
MK-1	1 (Structure 1.52), 4 (Fig 1.14)	6.9 µM (5-Fluorouracil 19.2 µM)	Konishi et al. (2002)
T47D	13 (Figure 1.14)	7.50 µM	Rafi et al. (2005)
PA-I	13 (Figure 1.14)	8.00 µM	Rafi et al. (2005)
OVCAR	13 (Figure 1.14)	10.00 µM	Rafi et al. (2005)
DU-145	13 (Figure 1.14)	10.00 µM	Rafi et al. (2005)
DUPro-I	13 (Figure 1.14)	5.00 µM	Rafi et al. (2005)
DUPro-I	15 (Figure 1.14)	10.00 µM	Rafi et al. (2005)
MCF-7	13 (Figure 1.14)	5.00 µM	Rafi et al. (2005)
MCF-7	15 (Figure 1.14)	10.00 µM	Rafi et al. (2005)
MCF-7	44 (Figure 1.16)	9.1 µM	Park and Kim (1998)
MCF-7	85 (Figure 1.20)	5.87 µg/mL (Vincristine sulfate 10.03 µg/mL, SI >17)	Talib et al. (2012)
Malme3	44 (Figure 1.16)	8.3 µM	Park and Kim (1998)
HeLa	1 (Structure 1.52), 4 (Fig 1.14)	6.5–6.9 µM (5-Fluorouracil 12.3 µM)	Konishi et al. (2002)
HeLa	48 (Figure 1.16)	5.5 µM (DOX 0.4 µM)	Cheng et al. (2012)
HeLa	55 (Figure 1.17)	1.2 µM	Xie et al. (2007)
K-562	55 (Figure 1.17)	3.8 µM	Xie et al. (2007)
KB	55 (Figure 1.17)	5.3 µM	Xie et al. (2007)

Cell line	Compounds	IC_{50}values ($IC_{50}$to positive control)	References
Jurkat T	46 (Figure 1.16)	3.56 µM	Song et al. (2005)
AGS	58 (Figure 1.17)	5.4 µM	Bai et al. (2006)
HL-60	56 (Figure 1.17)	1.50 µM (DOX 0.059 µM)	Wang et al. (2012)
HL-60	46, 48 (Figure 1.16)	2.7–5.6 µM (DOX 0.15 µM)	Park and Kim (1998)
HCT-116	50 (Figure 1.17)	0.39 µg/mL (Daunorubicin 0.28 µg/mL)	Chin et al. (2009)
HCT-15	44 (Figure 1.16)	8.7 µM	Park and Kim (1998)
HepG-2	40 (Figure 1.16)	2.2 µg/mL	Moghadam et al. (2012)
HepG-2	61 (Figure 1.17)	2.0 µg/mL	Chen et al. (2007)
HepG-2	43, 44, 45, 48, (Figure 1.16); 57 (Figure 1.17)	2.5–4.1 µM (DOX 0.2 µM)	Cheng et al. (2012)
PC-3	16 (Figure 1.15), 43, 44, 45, 48 (Figure 1.16)	1.3–2.9 µM (DOX 0.3 µM)	Cheng et al. (2012)
PC-3	30 (Figure 1.16)	8.7 µM (DOX 0.3 µM)	Cheng et al. (2012)
MGC803	43, 44, 48, (Figure 1.16)	1.0–1.4 µM (DOX 0.3 µM)	Cheng et al. (2012)
MGC803	16, 17, 18, 19, 20 (Figure 1.15), 30, 31, 45, 46 (Figure 1.16), 68 (Figure 1.18)	6.5–10.1 µM (DOX 0.3 µM)	Cheng et al. (2012)
U-937	48 (Figure 1.16)	0.47 µM	Zeng et al. (2009)
A-549	40 (Figure 1.16)	3.5 µg/mL	Moghadam et al. (2012)

1.4.5 Antimicrobial Activity of *Inula*

Ethanol extracts from *Inula crithemoides* and *Inula helenium* have shown inhibitory activities against a range of bacterial and fungal species. Extracts of *Inula* species showed potent activities against all the tested bacteria - *Staphylococcus aureus, Escherichia coli, Enterococcus faecalis, Bacillus cereus,* and *Bacillus subtilis* (Diguţă et al., 2014; Jallali et al., 2014), and also on anaerobic bacteria such as *Porphyromonas gingivalis, Parvimonas micra, Fusobacterium nucleatum* and *Prevotella intermedia* at a MIC (minimum inhibitory concentration) range of 0.06 -10.00 mg/mL, and also inhibited oral bacteria (Karygianni et al., 2014). This is an indication that *Inula* species may be employed as anti-bacterial agents considering the wide range of activities reported by these researchers. The thymol derivatives from *Inula hupehensis* elicited strong anti-fungal activities against pathogenic fungi of plants in *Rhizoctonia solani, Peronophythora litchi* and *Phytophthora melonis* at very low concentrations (EC_{50} 157, 141, and 180 µg/ mL) (Zhao et al., 2010).

Studies on alantolactone (ALA), and isoalantolactone (IAL) reveals its antibacterial activities are comparable to that of streptomycin, against *B. cereus, P. aeruginosa* and *Shigella dysenteriae* (Lokhande et al., 2007). Lokhande et al., (2007) reported a zone of inhibition of 21 mm (ALA), 17 mm (IAL) and 27 mm for streptomycin against *B. cereus*; 21 mm (ALA), 18 mm (IAL) and 28 mm for streptomycin against *P. auruginosa* and 16 mm (ALA), 12 mm (IAL) and 24 mm for streptomycin against *S. Shigella dysenteriae*. The mechanism of eliciting this effect is not known (Lokhande et al., 2007), and isoalantolactone inhibited the expression (at very low concentration of 8 µg/mL) of α-toxin in *S. aureus* (Qiu et al., 2011). Alantolactone can elicit a more potent antibacterial effect if combined with another agent. For instance, a combination of alantolactone, isoalantolactone and diplophylin elicited a potent inhibition against *S. aureus* at MIC 20 µg/ml (Stojanović-Radić et al., 2012). Another isolate from *Inula,* 4α,5α-epoxyalantolactone elicited a more potent inhibitory activities (MIC 15.5 µg/mL) when compared to ampicillin (MIC, 25.0 µg/ml) against *Bacillus aureus* (Jiang et al., 2011), an activity attributed to the presence of the methylene ring on the alantolactone (Jiang et al., 2011).

Some isolates such as epoxyalantolactone were more potent than ampicillin against a strain of *Bacillus cereus* (Jiang et al., 2011). Alantolactone, isoalantolactone and diplophyllin have been identified as the agents eliciting activity against *Staphylococcus* in essential oils extracted from the roots of *Inula helenium* (Stojanović-Radić et al., 2012).

Quercetin and its derivatives were found to elicit antimicrobial activity against *Mycobacterium tuberculosis*, *Salmonella typhimorium* and *Bacillus cereus* by inhibiting isocitrate lyase, and damaging the cell walls of bacteria and the cytosolic membrane in *Salmonella typhimorium* and *Bacillus cereus* (Shukla et al., 2015; Talib et al., 2012).

Anti-fungal activity of tomentosin has been reported against *Trichophyton mentagrophytes, Microsporum gypseum,* and *Microsporum canis* at concentration of 1 mg/ml (Cafarchia et al., 2001), whereas isoalantolactone has been used against the fungi *Candida albicans, Geotrichum candidum, Aspergillus niger, Candida tropicalis* and *Aspergillus flavu*s at doses of 25, 25, 50, 25 and 50 µg/mL (Tan et al., 1998), as well as *Plasmopara viticola*, a fungus which causes mildew (Cohen et al., 2006).

Most of the antibacterial activities on isolates from *Inula* were carried out on mixtures or impure compounds (Seca et al., 2014). Purification of isolates before application on

bacterial strains is therefore necessary to further confirm the pharmacological activities of extracts from *Inula.*

Even though over 100 species of plants belong to the genus *Inula*, only very few of these species have been characterised chemically. The species characterised mostly include *Inula japonica, Inula racemosa, Inula britannica, Inula hupehensis and Inula viscosa.* There is therefore a need to study the anticancer – against cancer cell lines such as Caco-2 etc., and physiological conditions such as hypoxia (which has not been previously investigated) and antimicrobial activities of *Inula helenium*. Investigation of the anticancer activity of sesquiterpene lactones isolated from *Inula helenium* against the leukaemia cell line (K562) during physiological conditions such as hypoxia and hypoglycaemia, could offer insight into the subject matter of the potential use of these sesquiterpene lactones against cancers in low oxygen concentrations and in hyperglycaemic conditions such as cancers associated with diabetes. This area has not been investigated despite the significant cytotoxic inhibition of the leukaemia cell line (K562) reported by Lawrence et al., (2001). There also reports of isolation of bioactive flavonoids with significant anticancer activities in some *Inula* species but not in *Inula helenium*. A review of the literature suggests there are flaws in the extraction and purification techniques used in extraction of bioactive compounds from *Inula helenium* as no author reported the use of the centrifugation technique (as a pre-purification technique) prior to the use of high-performance liquid chromatography (HPLC). This could aid in the isolation of compounds which may be present in traces or small quantities.

1.5 Flavonoids

Flavonoids are a group of substances, naturally occurring, with various phenolic ring structures, usually found in wine, tea, flowers, stems, roots, barks, grains, vegetables and fruits and have beneficial health effects. Due to their beneficial health effects, isolation of phyto-active compounds from flavonoids has been made, and flavonoids are indispensable components of cosmetics, medicines, pharmaceuticals and nutraceuticals due to their anti-carcinogenic, anti-mutagenic, anti-inflammatory and anti-oxidative properties, and their ability to modulate key enzymes involved in cellular functions (Panche et al., 2016). Flavonoids are also inhibitors of many enzymes such as phosphoinositide 3-kinase, lipoxygenase, cyclo-oxygenase and xanthine oxidase and are classified into several subgroups such as isoflavones, flavonols, flavones and chalcones, depending on the C ring carbon on which the B ring attaches to, the oxidation of the C-ring and the degree of unsaturation (Panche et al., 2016).

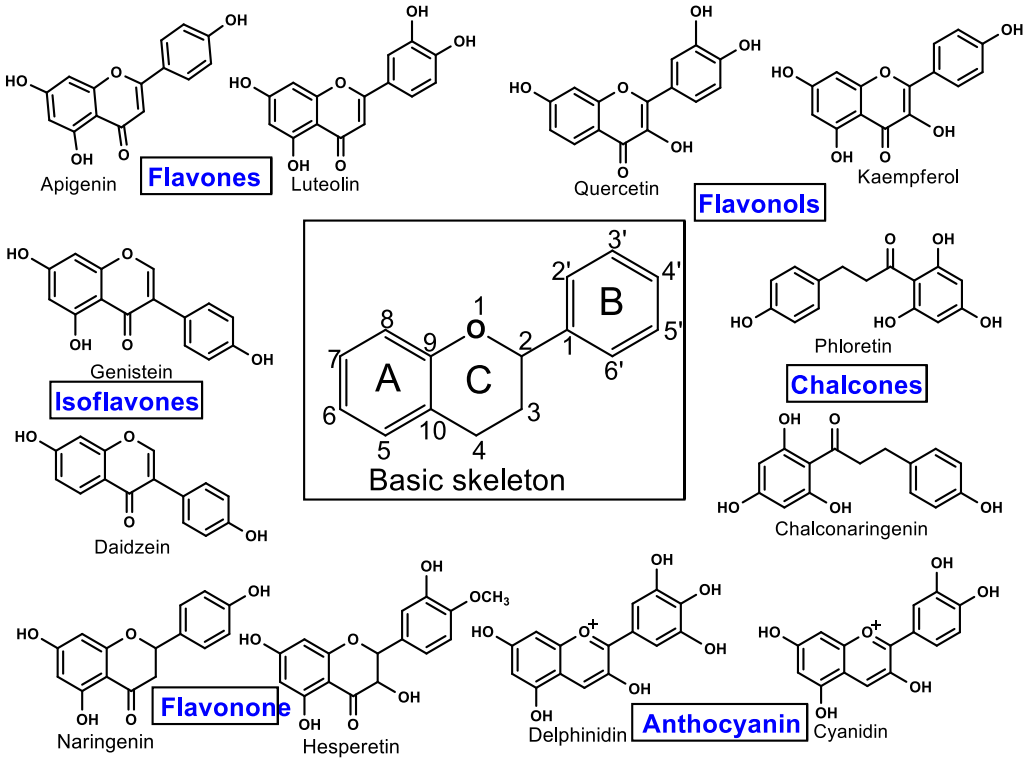


Figure 1.20: Flavonoids – classes of flavonoids and their skeletal structure.
Flavonoids are classified into flavones, flavonols, isoflavones, flavonols, chalcones and anthocyanins (Panche et al., 2016).

Isoflavones are flavonoids where the B ring is linked to the C ring in position 3. Neoflavones occur when the B ring is linked to the C ring in position 4. When the B ring is linked to the C ring in position 2, several subgroups of flavonoids results, and they are grouped base on the structural features on the C ring, giving rise to chalcones,

anthocyanins, catechins or flavanols, flavanonols, flavanones and flavonols (Figure 1.20) (Panche et al., 2016).

1.5.1 Classification

1.5.1.1 Flavanones

Flavanones also known as dihydroflavones, are present mostly in citrus fruits – grapes, lemons and oranges, giving the peel and juices from citrus fruit a bitter taste. Eriodictyol, naringenin and hesperitin are examples. Theses flavanones exert cholesterol-lowering, blood lipid-lowering, anti-inflammatory and antioxidant effects. Their C ring is saturated, and the double bond between position 2 and 3 is saturated unlike in flavones where they are unsaturated (Iwashina, 2013).

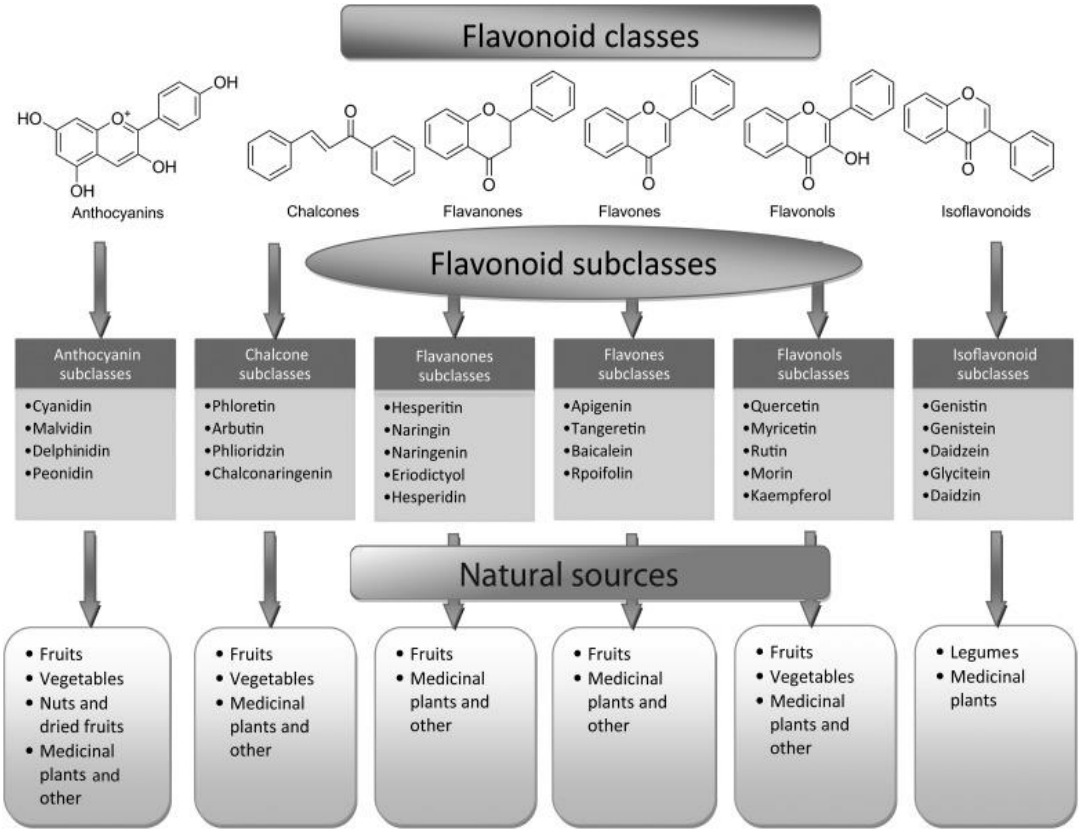


Figure 1.21: Flavonoid natural sources, classes and subclasses (Panche et al., 2016).

1.5.1.2 Flavones

Flavones are found mainly in the fruits, flowers and leaves of glucosides. Ginkgo biloba, mint, chamomile, red peppers, parsley and celery are rich in flavones. Tangeritin, apigenin and luteolin are examples of flavones (Figure 1.21). Flavones have a ketone in position 4 of the C ring and a double bond between position 2 and 3. Sinensetin, nobiletin, tageretin, chrysin and polymethoxylated flavones are also found in the peels of citrus.

Other flavones have a hydroxyl group in position 7 of the ring A, others in position 5 of the ring A, or the 3’ and 4’ position of ring B (Panche et al., 2016).

1.5.1.3 Flavonols

Flavonols contain a ketone group and provides the building block for proanthocyanins and are abundant in some vegetables and fruits such as berries, grapes, apples, tomatoes, lettuce, kale and onions.

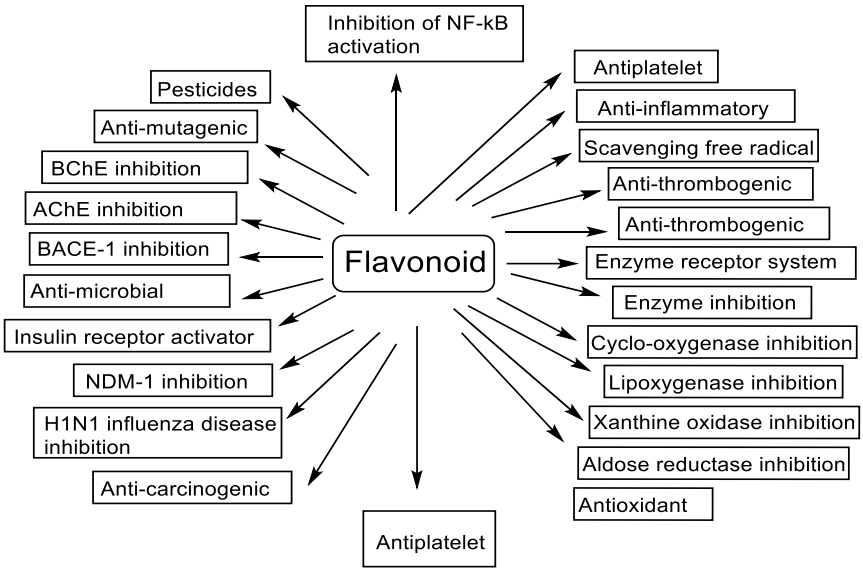


Figure 1.22: Bioactive roles of flavonoids.

Bioactive roles of flavonoids include; inhibition of NF-kB activation, anti-inflammatory, antimicrobial, antimutagenic, anticarcinogenic etc. (Panche et al., 2016).

Flavonols include fisetin, myricetin, quercetin and kaempferol (Figure 1.21) which are potent antioxidant agents. Unlike flavones, hydroxyl group is attached to position 3 of ring C. Glycosylation may also occur at this position 3. Due to the numerous glycosylation pattern observed in flavonols, they are the largest group of flavonoids known (Iwashina, 2013; Panche et al., 2016).

1.5.1.4 Chalcones

Chalcones lack ring C of flavonoid skeleton (Figure 1.21) and a sub-class of flavonoids. Chalcones include chalconaringenin, phloretin, arbutin and phloridzin (Figure 1.21). Chalcones are present in wheat bearberries, strawberries, pears and tomatoes. Chalcones are known to be potent anticancer, antioxidant, antimicrobial agents, and also elicit numerous biological activities as highlighted in Figure 1.22 (Panche et al., 2016). Chalcones have a wide range of biological activities including inhibition of cyclooxygenase, lipoxygenase and the NF-κB activation pathways.

1.5.1.5 Isoflavonoids

Isoflavonoids are mainly present in legumes especially soybeans, and in microbes. They induce metabolic and hormonal changes which activate the disease fighting potential of isoflavonoids. They act on oestrogen, inducing hormonal changes, which activate the machinery necessary for fighting infection. Daidzein and genistein are example of isoflavonoids (Szkudelska & Nogowski, 2007). Isoflavonoids are therefore angiogenesis inhibitors.

1.5.1.6 Neoflavonoids

Neoflavonoids differ from other flavonoids because they have a 4-phenylchromen backbone, whereas other flavonoids possess the 2-phenylchromen-4-one backbone. Hydroxyl group substitution is also lacking at position 2 of neoflavonoids. They contain 15 carbons. They were first isolated in the seed of *Calophyllum inophyllum* in 1951 (Nishimura et al, 2000).

1.5.1.7 Catechins, flavan-3-ols or flavanols

Catechins are also known as dihydroflavanols or flavanonols. They are a divers and multi-substituted subgroup also referred to as flavan-3-ol because the position 3 of the C ring is where the hydroxyl group binds. It is distinct from other flavonoids because no double bond exists between position 2 and 3. Pears, peaches, blueberries, apples and bananas are rich in catechins (Panche et al., 2016).

1.5.1.8 Anthocyanins

Antacynins are pigments which are responsible for the diverse colours in fruits, flowers and plants. Peonidin, pelargonidin, malvidin, delphinidin and cyanidin are the most studied anthocyanins. They are predominantly found in the outer layers of fruits. The intensity of the colour of anthocyanins depends on factors such as acylation, methylation and pH on the A and B ring (Iwashina, 2013).

1.5.2 Pharmacological Activities of Flavonoids

The following activities have been reported for flavonoids: combating neurodegenerative diseases, anti-inflammation, xanthine oxidase inhibition, radical scavenging, disease-combating activity, countering antibiotic resistance, xanthine oxidase modulators, steroid-

genesis modulators, anti-inflammatory and anti-cholinesterase activity (Panche et al., 2016).

1.5.2.1 Anti-inflammatory activity

The anti-inflammatory activity of flavonoids involves inhibition of activities and synthesis of several proinflammatory mediators such as C-reactive protein, adhesion molecules, cytokines and eicosanoids (Serafini et al., 2010; Ribeiro et al., 2015). Flavonoids molecular activities includes activation of nuclear factor-erythroid 2-related factor 2 (Nrf2) and inhibition of transcription factors such as NF-kappaB (Serafini et al., 2010). Flavones and flavonols which contain 2, 3-double bond are preferential inhibitors of COX-2, an enzyme expressed during inflammatory responses. COX-2 synthesises prostaglandins which induces inflammation and pain. Flavonoids such as celecoxib, naringenin, taxifolin, esculatin, daidzein, genistein, hesperitin, scopoletin, galangin and silbinin are effective inhibitors of COX-2 (Madeswaran et al., 2012; Panche et al., 2016).

1.5.2.2 Anticancer Activities of Flavonoids

1.5.2.2.1 Flavonoid containing 2-phenyl-4H-chromen-4-one system

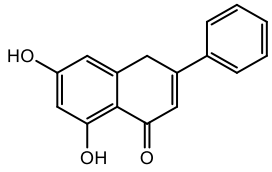
Anticancer activity of flavonoids has been reported against a variety of cell lines and in a certain study, the seed extracts of *Abrus precatorius* elicited anticancer effect against nineteen cancer cell lines (Patil et al., 2015), with HepG2, HeLa, NCI-H460 and MCF 7 susceptible to these flavonoids. Flavonoids from *Arrabidaea chica* extracts inhibited the growth of Ehrlich solid tumour in rats and vicenin. 4′,7-dimethylapigenin and kaempferol were the phyto-active constituents eliciting these activities (Barbosa et al., 2008).

Importantly, flavonoids kill cancer cells without affecting normal cells as seen in isolinariin A, isolinarin B, linariin and pectolinarin, isolated from the genus *Linaria*. These compounds indicated cytotoxicity on C32, Caco-2, COR-L23 and MCF-7 cell lines without killing normal cell lines – MRC-5. Isolates from *Heterotheca inuloides* such as eriodictol, luteolin, kaempferol, catechin and quercetin were cytotoxic against MCF7, HCT-15, and K562 cell lines with IC_{50} as low as 1.45 µM (Rodríguez-Chávez et al., 2017; Thamere et al., 2005).

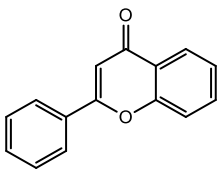
The anticancer activities of chrysin, quercetin, hesperetin, rutin, flavone and 3-Hydroxyflavone were studied using 5-fluorouracil as standard drug against Caco-2 cell line. Interestingly, Chrysin, flavone and 3-Hydroxyflavone were more potent against

Caco-2 IC_{50} (5.5, 21.01 and 42.1 μM) compared to an IC_{50} of 60 μM for 5-Fluorouracil, which was comparable to the anticancer activity indicated by hesperetin (IC_{50} = 66.67 μM) whereas quercetin was less active against Caco-2. rutin and hesperedin were inactive. These results are a pointer that the chemical group substitution plays a vital role in the biological responses observed. The OH group in A ring of chrysin increased its cytotoxicity. It implies that the position of the OH group in flavonoids is vital to their cytotoxicity; 3-Hydroxyflavone with an OH group at C ring indicated a decreased activity compared to chrysin and flavone. Additional OH groups at ring B – as seen in hesperetin (Structure 1.66), further decreased their cytotoxicity (Ibrahim et al., 2014) an indication that a free B ring is an important feature of an anticancer flavonoid against colon cancers. Flavonoids such as genistein, formononetin, 2′-Hydroxyflavonone, apigenin and quercetin elicit their anticancer activity by inducing apoptosis and overexpressing NAG-1 protein. NAG-1 belong to the TGF-β family and is being regulated by transcription factors PPARγ, Sp1, ATF-3, p53, EGR-1. These transcription factors are also flavonoid targets such as 2′-hydroxyflavonone which inactivates AKT/STAT3, changing Bcl-2 gene expression, resulting in growth inhibition and apoptosis (Wu et al., 2014; Yang et al., 2014).

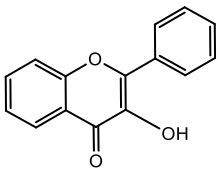
Unlike some groups of anticancer agents, flavonoids can interfere with tumour cells growth in all phases by modulating different receptors and enzymes which partake in reverse multidrug resistance, metastasis, angiogenesis, apoptosis, differentiation and cell proliferation. Flavonoids acts mainly by inhibiting protein kinases and topoisomerases: serine, threonine kinases / protein tyrosine kinase and phosphatidylinositol 3-kinase (Kaleem & Ahmad, 2018).



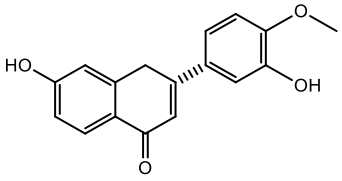
Structures 1.61: Chrysin



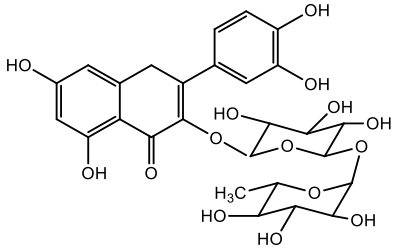
1.62: Flavone



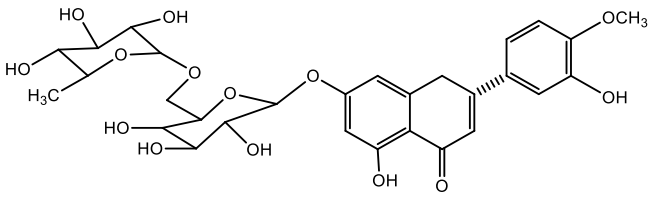
1.63: Hydroxyflavone



1.64: Hesperetin



Structure 1.65: Rutin



Structure 1.66: Hesperidin

Structures 1.61 – 1.66: bioactive flavonoids isolated from plants (Abdallah et al., 2015; Frutos et al., 2019; Kosari-Nasab et al., 2018; Rha et al., 2019)

Flavonoids also act as anti-angiogenic agents by regulating the expression of EGFR, MMPs and VEGF and inhibition of ERK ½, PI3-K/AkT and NFkB signal pathways. Flavonoids also elicit antioxidant activities via alteration of processes involved in P-glycoprotein expression, they mitigate nitric oxide induced oxidative stress, chelate trace metals and inhibit oxidases involved in production of superoxide anion and scavenging of ROS etc. (Abdallah et al., 2015; Ravishankar et al., 2013). Flavonoids are therefore important sources of anticancer agents.

1.5.2.2.2 Pro-apoptotic flavonoids

As described in section 1.1.1.1 (page 1), apoptosis or programmed cell death involves a plethora of processes such as apoptosis induction, pro-apoptotic proteins activation, caspase cascade (cysteine proteases), reorganization / degradation of organelles in cells, cell fragmentation into apoptotic bodies, and phagocytosis of fragmented cells. This process is sophisticated and complex, requiring a cascade of energy dependent cellular events via inhibition of signal pathways and / or expression of several biomolecules involving caspases, MAPS, p53, FAS, TNF, TRAIL and Bcl-2 family (Raffa et al., 2017).

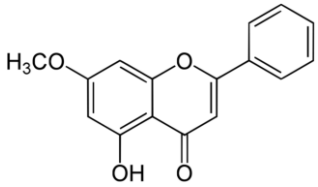
Casticin, Jaceosidin and other flavonoids interfere with various mechanisms of apoptosis such as inhibition of growth factors, by activating or transducing transcription and by ROS / mitochondrial mediated apoptotic pathways (Millimouno et al., 2014; Raffa et al., 2017).

The PI3K/Akt/FoxO1, PI3K/MEK/ERK, JNK/ caspase/mitochondria, transcription factors B of NF-kB1 family and the p53 tumour suppressor family are the main targets of flavonoids (Raffa et al., 2017).

Clinical trials involving quercetin are in the late phase, an indication of promising antitumour activity (Akhlaghi et al., 2018). The mechanism of action of quercetin involves proliferation of antiapoptotic proteins, downregulation of cell survival, angiogenesis and induction of apoptosis via inhibition of Akt and NF-κB signalling pathways. Up-regulation of Bcl2, down-regulation of p53, bax and caspase 3 is responsible for quercetin pro-apoptotic activity (Primikyri et al., 2014; Akhlaghi et al., 2018). Quercetin and kaempferol indicated an IC_{50} of 1.54 and 12.05 ng/ml against A375 cell line (Gopal et al., 2015), and quercetin was bound to Bcl-xL protein and the BH3 domain of Bcl2, resulting in a BH3-mimetic property which led to apoptosis (Primikyri et al., 2014). For instance, kaempferol 3-O-α-l-rhamnopyranoside inhibited K562 cancerous growth at IC_{50} of 4.1 µM and acted

via inhibition of NF-kB. No toxicity was recorded on non-tumour cell at IC_{50} of 50 µM (Okoye et al., 2015).

Targeting TRAIL (TNF-Related Apoptosis Inducing Ligand) receptors as a means of inducing apoptosis has offered promising results as TRAILS do not kill normal cells but inhibit cancerous growths. Chrysin was shown to inhibit STAT3, downregulating Mcl-1 anti-apoptosis protein, enabling apoptotic induction of TRAILS. Similarly, tectochrysin (Structure 1.67) increases the expression of Fas and DR3 and inhibits phosphorylation of STAT3, resulting in apoptosis (Lirdprapamongkol et al., 2013).



Structure 1.67: Tectochrysin
(Lirdprapamongkol et al., 2013).

Other flavonoids such as icariin, wogonin, dihydromyricetin, sylibin, fisetin, flavone B, flavone A, calycopterin, vitexin, fisetin, hesperetin, myricetin, luteolin and apigenin also induce apoptosis via multiple and different signal transduction pathways. These flavonoids are naturally occurring (Raffa et al., 2017) and are readily available.

1.5.2.2.3 Flavonoids affecting caspase cascade

Caspases are enzymes responsible for cleavage of proteins at specific aspartate residues during apoptosis. When caspase activation malfunctions, apoptosis occurs. By targeting caspases, unwanted cells can be killed. Myricetin, luteolin, apigenin, HLBT-100, isoquercitrin, kaempferol and quercetin are naturally occurring flavonoids which target caspases.

The pro-apoptotic flavonoid – luteolin inhibited the growth of MCF-7 cancer cells via inactivation of PARP, activation of caspase cascades and expression of DR5 death receptors. Furthermore, luteolin induces the release of cytochrome C, collapse of mitochondrial membrane potential, inhibits Bcl-2 expression and an increase in Bax expression (Park et al., 2014). Apigenin inhibited the growth of cancers of the prostate, colon, leukaemia, thyroid, skin and breast by apoptotic cell death, inhibition of invasion and migration of T24 cancer cells of the bladder, leading to apoptosis via caspase-3 and PARP activation, regulation of Bcl-2 family and Akt/ PI3K pathways (Zhu & Zhu, 2013).

Isoquercitrin, isolated from extract of *Bidens pilosa* inhibited cancer cells of the pancreas in *in vitro* and *in vivo* studies, inducing apoptosis and G1 cell cycle arrest via activation of caspases-3, 8 and 9; this reduces the potential of the mitochondrial membrane. Isoquercitrin also inhibited δ opioid receptors, promoting the MAPK signalling pathways (Chen et al., 2015), thus caspases play important roles in cancer therapy and by targeting caspases; apoptosis can be achieved.

1.5.2.2.4 Flavonoids acting on DNA topoisomerases

Topoisomerases unwinds and reseals broken DNA strands. Two types exist: topoisomerase I responsible for cleavage of one DNA strand, and topoisomerase II responsible for double strand cleavage, and generates a staggered double-strand break. Chemotherapeutics with potentials to interfere with topoisomerase can therefore kill cancers. Pinostrobin, isolated from *Kaempferia pandurate* acts on both DNA and topoisomerase I, forming a stable complex which makes them candidate for clinical trials. Cudraflavanone A, morin, fisetin, rutin, quercetin, silybin, wogonin, epigallocatechin-3-gallate, luteolin, kaempferol, apigenin, genistenin and **chrysin** are natural flavonoids with the potential of targeting DNA isomerases in humans (Raffa et al., 2017).

1.5.2.2.5 Flavonoids endowed with anti-angiogenic activity

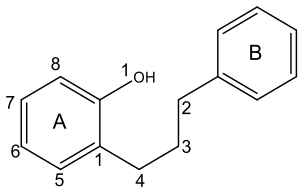
Angiogenesis describes the formation of new blood vessels in disease and good health. Cancer cells have an increase demand for nutrients and oxygen, and for this to be sustained, mediators of pro-angiogenesis such as matrix-degrading enzymes, bioactive lipids and cytokines come to prominence. New blood vessels can therefore be prevented if angiogenesis factors are targeted as these blood vessels serves as invasion routes for the spread of cancers (Fernando et al., 2015). The anti-angiogenic activity of luteolin against T47-D and BT-474 hormone-responsive breast cancer cells has been described to include blockage of secretion of the progestin-dependent VEGF in these hormone-responsive cancer cell lines and this effect was superior for luteolin compared to apigenin, which elicited a similar response (Cook et al., 2015).

1.5.2.2.6 Activities of other flavonoids-like structures

1.5.2.2.6.1 Chalcones

Chalcones or 1,3-diphenyl-2-propen-1-ones differs from conventional flavonoids due to the presence of an open ring (Structure 1.69) and the chalcone – naringin is the precursor for flavonoids (Evranos et al., 2011). Naringenin chalcone inhibited the production of pro-

inflammatory mediators (nitric oxide, MCP-1 and TNF-α) in RAW 264 LPS stimulated macrophages, promotes PPARγ transcription by enhancing secretion and expression of adiponectin from adipocyte, and enhances AdipoR2 expression (Hirai et al., 2007). In HepG2 cells, naringenin chalcone inhibits the synthesis of triglyceride by inhibiting the expression of DGAT-1 and activity of MTP (Casaschi et al., 2004), inhibits the activation of NF-κB and pro-inflammatory gene expression in hepatocytes and HSC (Fernando et al., 2015).



Structure 1.68: Basic structure of Chalcone and Flavonoid backbone (Raffa et al., 2017).

Xanthohumol exerted anti-proliferative, pro-apoptotic and anti-migratory effect against HepG2 and HCC cell lines. Anti-inflammatory effect was elicited via inhibition of interleukin-8 and TNF-α induced NF-κB activity (Dorn et al., 2010).

Butein isolated from *Toxicodendron vernicifluum* induced apoptosis by activating the apoptotic pathway, and inhibition of proliferation of hepatoma cancers at G2/M phase of the cell cycle which is dependent on activation of JNK and generation of ROS. *In vivo,* butein inhibited the activity of uPA and MMP-9 by down-regulation of ATP synthesis, suppression of cancer metastasis and suppression of NF-κB and signalling pathways involving Akt/mTOR/p70S6K (Liu et al., 2014). Other chalcone such as licochalcone A and phloretin have been studied in rat models and in HepG2 cells. Their anticancer activities involve uPA inhibition by downregulation of JNK/MKK4 pathways, activation of NF-κB, and inhibition of Bcl-2, Akt and GLUT2 signalling pathways. These compounds stimulate both extrinsic and intrinsic apoptotic pathways, exerting anti-tumour activities both *in vitro* and *in vivo* (Tsai et al., 2014; Wu et al., 2009). These activities were also reported for MCF-7 cancer cell lines (Kang et al., 2017).

Since flavonoids are present in natural food sources, their cytoprotective, anti-inflammatory and antioxidant effects may be exploited in daily dietary intake.

1.5.3 Chrysin – a Flavonoid

1.5.3.1 Source of Chrysin

Chrysin (Structure 1.61) is a flavonoid that is present predominantly in honey, blue passion flower, and propolis and it possess great medicinal and economic impact such as anticancer, antibacterial, anti-inflammatory activities (Mani & Natesan, 2018).

1.5.3.2 Pharmacological Activities of Chrysin

Chrysin possesses numerous health benefits which are still nascent and poorly exploited due to poor absorption and bioavailability of chrysin. It is a potent inhibitor of HIV virus, and aromatase (Li et al., 2014). Chrysin also indicates anticancer, antioxidant, and anti-inflammatory activities via induction of apoptosis in a range of cancer cell lines, thus the following activities of chrysin have also been reported: antiarthritic, antidepressant, antidiabetic, antiasthmatic, antibacterial, antienteroviral, and neuroprotective (Mani & Natesan, 2018).

1.5.3.2.1 Anticancer Activity of Chrysin

Anticancer activities of chrysin on several cancer cell lines including cancers of the prostate, pancreas, thyroid, glioblastoma, liver, cervical, nasopharyngeal, breast, lungs, and haematological cancers such as MCF-7, DU145, HCT 116, HepG2, Hep3B, A549, H1975, H157, H460, TNBC, 4T1, JB6 P+, HTh7; KAT18 have been reported with IC_{50} ranging from 1.0-50.0 μM, with A549 indicating the lowest IC_{50} reported (Mani & Natesan, 2018). Chrysin elicits its action via apoptosis, inhibition of cell proliferation and removal of inflammatory responses (Xue et al., 2016), inhibition of β-arrestin-2 expression, suppression of NF-κB and angiogenesis. It also stabilises the expression of p53 thereby activation of ERK1/2 which allows phosphorylation of p53 in HepG2 (Li et al., 2015). Due to the promising role of chrysin as an anticancer agent, and the role of organotin(iv)compounds as anticancer agents, chrysin-Sn complex was synthesised and tested against MCF-7, HeLa and A549 cancers *in vitro*. Interestingly, the anti-proliferative effect of chrysin-Sn complex was much stronger compared to chrysin and this effect was time and dose dependent (Xuan et al., 2016). Inhibition of cell proliferation was by apoptosis due to activation of caspase 3, increase in the level of LC3-II/LC3-1 ratio, suggesting that autophagy and apoptosis were responsible for the anti-proliferative effect of chrysin-Sn complex (Xuan et al., 2016).

Chrysin also preferentially kill cancer cells but not normal cells such that even cancer cells with acquired or primary drug resistance can be killed by chrysin. Wang et al. (2007), reported the suppression of interleukin-6 dehydrogenase expression in NSCLC cells via AKR1C1/1C2 superfamily responsible for aldo-keto reaction, and overcame drug resistance to Adriamycin and cisplatin. Chrysin also increase the sensitivity of BEL-7402 to doxorubicin (BEL-7402 is resistant to doxorubicin) by inhibition of nrf2 expression and its genes downstream - MRP5, AKR1B10 and Homooxygenase-1 gene by suppressing PI3K-Akt and ERK pathways (Gao et al., 2013).

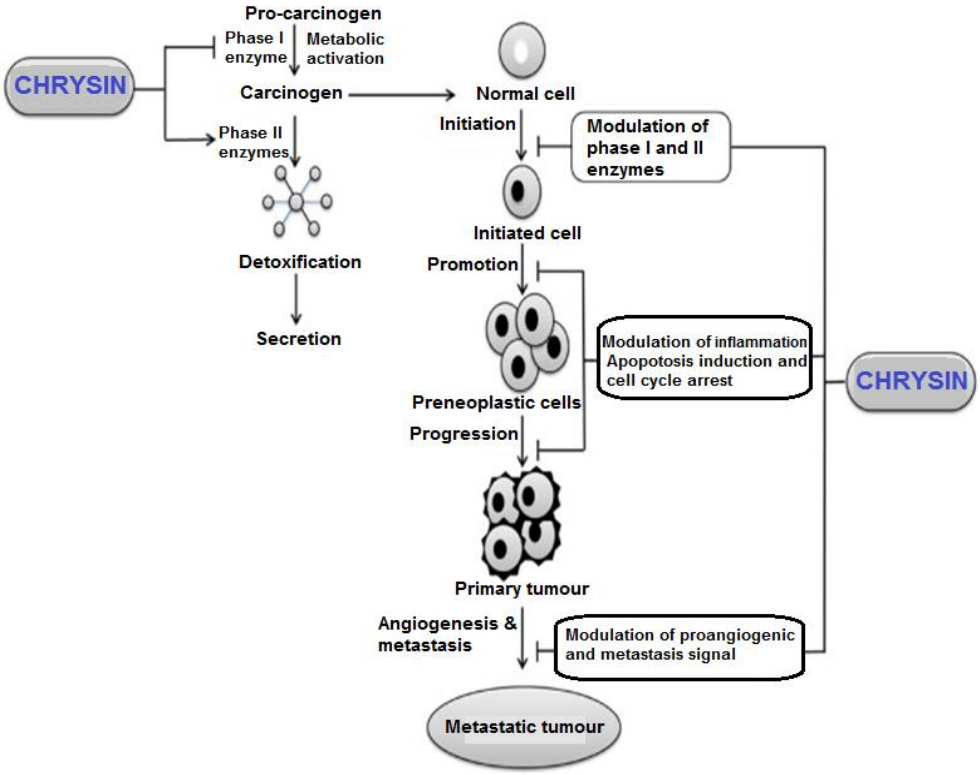


Figure 1.23: the role of detoxifying enzymes in prevention of carcinogenesis.

Diagram representing multi-step carcinogenesis and how chrysin intervenes in inhibiting cancer cell progression. Carcinogenesis begins with the transformation of healthy cells into cancer cells. These cells (cancer cells) progresses into preneoplastic cells and then into neoplastic cells. Carcinogenesis is inhibited by chrysin via inhibition of procarcinogen activation, or via stimulation of the detoxification of carcinogens, inhibition and suppression of growth via apoptosis, inhibition of cell proliferation by inhibition of cell inflammation, metastasis, invasion and angiogenesis (Kasala et al., 2015).

BCRP-mediated multi drug resistance (MDR) was also reversed by chrysin as it inhibited the efflux of BCRP, allowing BCRP to accumulate in cells, restoring the MDR cells sensitivity to anticancer agents (Zhang et al., 2004). Hursting et al., (1999), proposed the mechanism of action of chrysin to include inhibition of cancer processes such as initiation, proliferation and progression. Carcinogen biotransformation is modulated, allowing the mopping up of free radicals and alteration of gene expression in genes which take part in signalling pathways. Initiation of carcinogenesis can be prevented by detoxifying

enzymes such as NAD(P)H quinone oxidoreductase or glutathione S-transferase (Figure 1.23).

Chrysin can also prevent the effect of chemically induced cancers, including models of xenograft tumours by inducing detoxification and antioxidant enzymes, inhibition of cell proliferation, induction of apoptosis and inhibition of cyt-p450 monooxygenases. Carcinogenesis from 7,12-dimethylbenz(a)anthracene – induced carcinoma in hamster buccal pouch was inhibited by chrysin via several mechanisms such as metastasis, angiogenesis, inhibition of tumour cell proliferation, induction of apoptosis, invasion, glutathione reductase, glutathione peroxidase, upregulation of antioxidant and pro-carcinogen inhibition (Karthikeyan et al., 2013). In breast cancer cells, chrysin induces the BCRP (breast cancer resistance protein) by modulating enzymes of phase I and II, through the AhR (aryl hydrocarbon receptor) - Figure 1.23 and Figure 1.24 (Karthikeyan et al., 2013). Chrysin has been reported to also inhibit carcinogenesis resulting from sulphation mediated by phenolsulphotransferases in HepG2 (Eaton et al., 1996; Kasala et al., 2015).

Chrysin also exert anticancer activities *in vivo*. Using animal models, chrysin at doses of 20 and 40 mg/kg corrected ferric nitrilotriacetate and N-nitrosodiethylamine (DEN) induced renal carcinogenesis by ameliorating inflammation and oxidative stress via NF-κB pathway (Burkard et al., 2017) and also suppressed the growth of colorectal cancer induced by 1,2-dimethylhydrazine in Wistar rats by reducing cell proliferation, nitrosative stress, and the recovery of the antioxidant mineral in intestinal mucosa (Rehman et al., 2013; Sequetto et al., 2013). Chrysin also ameliorates lung carcinogenesis induced by benzo(a)pyrene in Wistar rats by decreasing the levels of both non-enzymatic (vitamin C, vitamin E and glutathione) and enzymatic antioxidants such as glutathione reductase, glutathione peroxidase, catalase and superoxide dismutase. Immunoblotting and histopathology analysis indicated downregulation of the expression of NF-κB, COX-2 and PCNA (Kasala et al., 2015).

Dimethylchrysin, a chrysin derivative also attenuated the signalling of NF-κB pathways by upregulating the expression of apoptotic genes in DEN induced hepoto-carcinogenesis in Wistar rats and inhibited colon carcinogenesis induced by azoxymethane in rats (Khan et al., 2011).

Xenograft model studies indicate chrysin elicited antitumour effects against 4T1, HTh7, MDA-MB-231 and Ehrlich ascites carcinoma cells via induction of apoptosis and

angiogenesis. In leukaemia xenograft model of mouse induced by implantation of WEHI-3 cells in Balb/c mouse, chrysin inhibited tumour growth by promoting phagocytosis of macrophage, and enhancing B and T-cells, and inhibition of cytotoxicity induced by natural killer cells (Lin et al., 2012). Chrysin therefore exhibit anticancer, anti-angiogenic, anti-metastatic, anti-invasive, apoptosis and antiproliferative properties both *in vivo* and *in vitro*.

Mechanism of action of chrysin therefore include apoptosis, inhibition of metastasis, angiogenesis, cell proliferation, NF-κB, Wnt signalling, EGFR, PPARγ, Ubiquitin-proteasome and the Nrf2 pathways (Figure 1.24) (Fonseca et al., 2017; Gao et al., 2013; Kasala et al., 2015).

Despite the cytotoxic activities of chrysin, poor bioavailability of chrysin has been observed because it is poorly absorbed, rapidly metabolised and eliminated rapidly from the system. It has been reported that some athletes including body-builders, ingest up to 3g of chrysin daily without any side effect. Another study indicates that after oral intake of 400 mg chrysin, below 0.1 μM mean plasma concentration was found. This was traced to systematic hepatic and intestinal sulphation, glucuronidation, hydrolysis and efflux of systemic metabolite into the intestine and elimination via the faeces. Administration of 5mg/kg chrysin orally (single dose), chrysin was found in faeces and only tiny amounts of glucoronides of chrysin were found in the urine. After intraperitoneal and intravenous administration of chrysin (1-5 mg/kg), a 10-fold increase in glucoronides of chrysin were found in the bile, 10-times higher than chrysin sulphates (Walle et al., 2001). Doses of chrysin – up to 625 mg were administered orally to humans without toxicity, an indication of efficacy and safety of chrysin.

Due to poor bioavailability of chrysin, efforts have been made to improve its bioavailability such as the use of nanoparticles of chrysin (Sabzichi et al., 2017), the use of liposomal chrysin (Kasala et al., 2015), and by use of synthetic chrysin analogues (Kasala et al., 2015; Mani & Natesan, 2018; Patel et al., 2016). By conjugation of Sn to chrysin (Xuan et al., 2016), chrysin-piperazine conjugates (Patel et al., 2016) and other chrysin conjugates (Mani & Natesan, 2018), an increased bioavailability and absorption of chrysin has been observed. There are also indications that the use of chrysin and other anticancer compounds potentiates the anticancer activities of these compounds with little systemic toxicities (Mani & Natesan, 2018). There is therefore a need to synthesize analogues of chrysin that can overcome these limitations thus this research project.

1.5.3.2.2 Mechanism of Anticancer Activity of Chrysin

Chrysin exerts its anticancer activity by acting thus: i) as an apoptotic gene, ii) as proliferation markers, iii) triggering the release of protein kinases, iv) alteration of activities of key metabolic enzymes, v) inhibition of angiogenesis, vi) inhibition of growth-factor releasing hormone etc (Figure 1.25) (Khoo et al., 2010).

Nuclear factor-E2-related factor 2 (Nrf2) is an important cytoprotective transcription factor which plays important roles in detoxification and antioxidant processes (Gao et al., 2013) and development of chemoresistance is associated with the constitutive activation of the Nrf2-mediated signalling pathway in many types of cancer cells (Gao et al., 2013). Investigation of the drug reversal effect of chrysin on Nrf2-associated drug resistance BEL-7402/ADM (doxorubicin resistant BEL-7402) cells indicated a significantly higher level of Nrf2 and its target proteins in BEL-7402/ADM cells compared to BEL-7402 cell only (Gao et al., 2013). Significantly lower intracellular Nrf2 protein level were also reported (Gao et al., 2013). ADM resistance was reversed by Nrf2 siRNA in BEL-7402/ADM cells (Gao et al., 2013), an indication that chrysin is a potent inhibitor of Nrf2 which sensitizes BEL-7402/ADM cells to ADM and increases intracellular concentration of ADM (Gao et al., 2013).

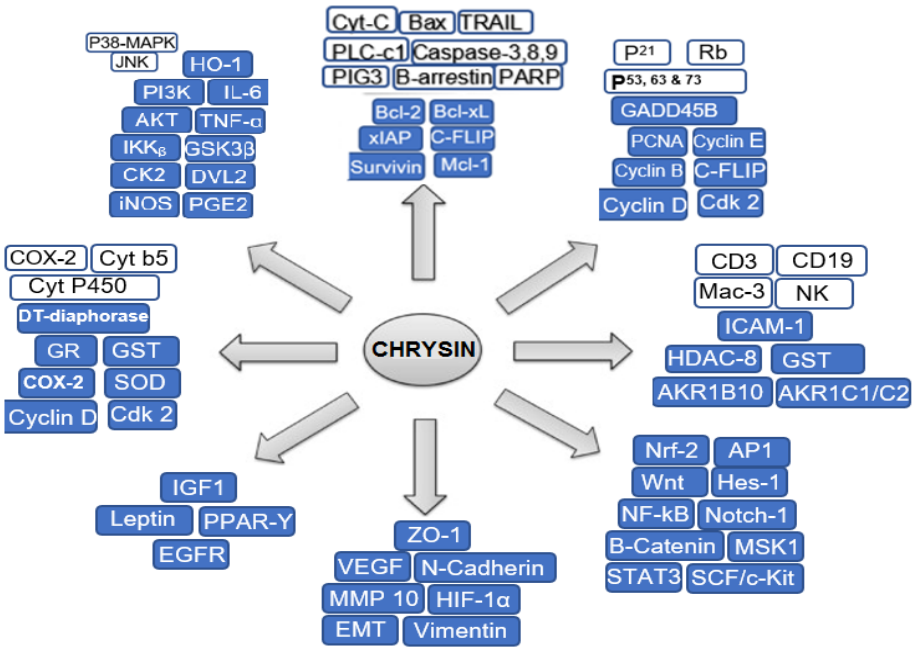


Figure 1.24: Molecular target of chrysin.
Dark and light-coloured rectangular boxes denote the targets inhibited and promoted by chrysin respectively (Kasala et al, 2015).

Chrysin therefore reduces the expression of Nrf2 at both the mRNA and protein levels down-regulating ERK and PI3K-Akt pathways (Figure 1.24). Consequently, expression of

MRP5, AKR1B10 and HO-1 (Nrf2-downstream genes) were reduced and the Nrf2-dependent chemoresistance was suppressed (Gao et al., 2013). Chrysin therefore acts as an effective sensitizer to reduce anticancer drug resistance by down-regulating Nrf2 signalling pathway.

Chrysin also acts by upregulating the apoptotic genes Cyt c, Bax, TRAIL, PLC-c1, caspases 3, 8 and 9, PIG3, B-arrestin and PARP (Gao et al., 2013), and down-regulation of Bcl2, Bcl-xL, C-Flip, survivin and Mcl-1 (Gao et al., 2013).

In a study by Zhang et al., (2004), he demonstrated that phosphorylated derivatives of chrysin at the 5-OH and 7-OH positions indicated better cytotoxicity in human cervical carcinoma (HeLa) cell via induction of apoptosis and down-regulation of the proliferating cell nuclear antigen (PCNA) in cells. Chrysin also induce apoptosis via induction of the p38 and activation of NFkappaB/p65 in the HeLa cells (von Brandenstein et al., 2008). Chrysin also sensitises the TNFalpha-induced apoptosis in nasopharyngeal carcinoma cell line (CNE-1), human liver cancer cell line (HepG2) and in human colorectal cancer cell line (HCT-116). This sensitization is associated with inhibitory effect on NFkappaB activation in Hela cells (Li et al., 2010). Although both chrysin and its phosphorylated adduct inhibited proliferation of cancer (HeLa) cells and induced apoptosis, the phosphorylated adducts (IC_{50} 9.8 μM) indicated better cytotoxic inhibition against HeLa cells than chrysin (IC_{50} 14.2 μM) (Zhang et al., 2004). Against the human leukaemia cell U937, apoptotic induction is associated with caspase-3 activation involving inactivation of Protein Kinases B (PKB) or Akt signalling and down-regulation of X-linked inhibitor of apoptosis protein (XIAP) in the U937 cells (Woo et al., 2004). This finding unlocked a more detailed molecular mechanism of induction of apoptosis via Akt dephosphorylation of the phosphoinositide-3-kinase (PI3K) signalling pathway in leukaemia cells.

The signalling pathway (requiring Akt) from PI3K to phosphoinositide-dependent kinase-1 (PDK1) and from PDK1 to Akt is responsible for apoptotic induction in cancer cells (Figure 1.25). Phosphorylation of Akt prevents apoptosis whereas dephosphorylation initiates apoptosis (Hou et al., 2018). Phosphorylation of Akt activates caspase-9 and phosphorylates Bcl-2-associated death protein (BAD). Phosphorylated BAD binds to the 14-3-3 proteins in the cytosol, preventing the heterodimerization with Bcl-2 at the mitochondrial membrane (Bui et al., 2018). BAD is released from cytosolic 14-3-3 proteins via dephosphorylation which subsequently form heterodimers with Bcl-2 family proteins and migrate into the mitochondrial membrane.

This migration signals the release of cytoplasmic cytochrome c (Inamdar et al., 2018; Pelengaris et al., 2002). Cytoplasmic cytochrome C then combines with caspase-9 and apaf-1 to form an apoptosome (a complex). Caspase-9 activation via phosphorylation (by ATP) is required for the formation of apoptosomes (Attaran-Bandarabadi et al., 2017; Pelengaris et al., 2002). Activation of caspase-9 leads to activation of caspase-3 (downstream executor caspase), leading to apoptosis (Debatin, 2004; Zamaraev et al., 2017). Consequently, activation of caspase-9 by phosphorylated Akt prevents formation of the apoptosome complex, and therefore inhibition of the downstream event of apoptosis (Debatin, 2004).

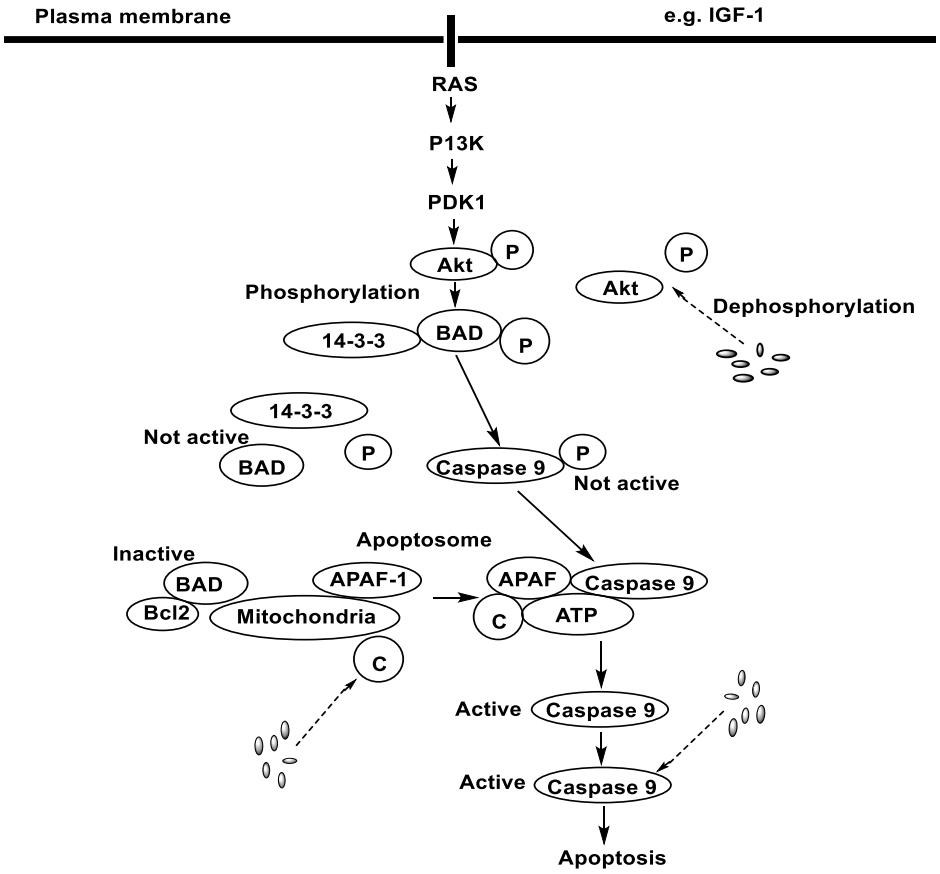


Figure 1.25: The PI3K/Akt signalling pathway.

Chrysin acts via activation of caspases and inactivation of Akt signalling in leukaemia cells. (•) depicts chrysin (Ediriweera et al., 2019; George et al., 2018; Khoo et al., 2010).

A summary of bioactive flavonoids, their mechanism and site of action is presented in Table 1.11 below.

Table 1.11: Mechanisms of action and target sites of bioactive flavonoids

Flavonoid	Target	References
Apigenin	Direct DNA damage, ATM mediated cell cycle checkpointsignallingactivation, p53 accumulation, down-regulation of anti-apoptotic Bcl-2, up-regulation of apoptotic Apaf-1 and p21, inhibition of PI3K/Atk and NF-kBsignallingpathway.	(Meng et al., 2017; Erdogana et al., 2017)

Flavonoid	Target	References
Luteolin	Activation of MEK/ERKsignallingpathway, inhibition of gene expression, production and secretion of MUC5AC mucin via regulation of NF-kBsignallingpathway.	(Meng et al., 2016; Lee et al., 2015)
Myricetin	RSK2 binding, increased expression of Mad1.	(Feng et al., 2015)
Hesperetin	Alteration of JAK/STAT pathway, KIT receptorsignallingand growth hormone receptor signaling.	(Adan & Baran 2016)
Fisetin	Alteration of JAK/STAT pathway, KIT receptorsignallingand growth hormone receptor signaling.	(Adan & Baran 2016)
Vitexin	Activation of MEK/ERKsignallingpathway.	(Yang et al., 2013)
Flavone A	Decrease unphosphorylated ERK and S6, inactivation of the survival proteins Bcl-xL or Bcl-2.	(LeJeune et al., 2015)
Flavone B	Upregulation of the activated forms of ERK and c-JUN.	(LeJeune et al., 2015)
Dyhydromyricetin	AMPK and $p38^{MAPK}$ activation, downregulation of Sox2.	(Sengupta et al., 2016)
Calycopterin	ROS production, Bcl-2 family protein expression, mitochondria depolarization and MMP disruption.	(Esmaeili et al., 2014; Kim et al., 2013)
Wogonin	Inhibition in E6/E7 expression, induction and increase in p53 mRNA expression.	(Chen et al., 2013; Kim et al., 2013)
Icariin	Increases in the EC109 and TE1 cell apoptotic index, Caspase 9 activity, reactive oxygen species (ROS), and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity. Upregulated the levels of ERS-related molecules (p-PERK, GRP78, ATF4, *p*-eIF2α, CHOP), and a pro-apoptotic protein (PUMA). Downregulation of Bcl-2.	(Fan et al., 2016)
Sylibin **53**	Modulation of receptor tirosin kinases, androgen receptor, STATs, NF-kB,signallingpathways, downregulation of MicroRNA-21 and MicroRNA-155, upregulation of CASP-9 and BID.	(Gandara et al., 2014; Zadeh et al., 2016)

(Raffa et al., 2017)

1.5.3.2.3 Antibacterial Activity of Chrysin Derivatives

The antibacterial activities of propolis has previously been reported against Gram-positive and Gram-negative bacteria. Ruffato et al., (2018) reported a MIC of 125 µg/ml for the crude ethanol extracts of Brazillian propolis against *Staphylococcus aureus* with the hexane fraction indicating an MIC of 62.5 µg/ml. Chrysin has been identified as the main constituent of propolis (Surech et al., 2006). Antimicrobial activities of propolis with MIC ranging from 62.5 – 500 µg/ml against a range of bacteria has been documented (Braca et al., 2008, Cabral et al., 2012, Castro et al & Daltro 2009, Fabri et al., 2011, Gonçalves et al., 2011).

Suresh et al., (2006) reported the antibacterial activity of chrysin against three Gram-positive bacteria – *Bacillus subtilis Bacillus spaericus* and *S. aureus* at MIC values 50, 50 and 25 µg/ml, and three Gram-negative bacteria – *Chromobacterium violaceum* and *Klebsiella aerogenes* at MIC values 25 and 50 µg/ml but no inhibition against *Pseudomonas aeruginosa*. Surech et al., (2006) further synthesised derivatives of chrysin, grouping them into three series: series 1 (3-carbon spacer in between chrysin and heterocyclic moiety), series 2 (separated by 4-carbon spacer) and series 3 compounds (separated by 6-carbon spacer in between chrysin and the substituent). All chrysin derivatives synthesised showed antibacterial activities ranging from 6.25 – 25 µg/ml against all bacteria tested except *P. aeruginosa*. There was no peculiar difference between the pattern of inhibition between the Gram-negative and Gram-positive bacteria (except *P. aeruginosa*). These activities were better than those indicated by chrysin and comparable to those indicated by Streptomycin and Penicillin (MIC 1.56 to 12.5 respectively). The work of Surech et al., (2006) is a clear indicator that the modification of a compounds could significantly alter its biological activities.

1.5.4 7-O-Chrysin Derivatives Previously Synthesised

1.5.4.1 7-O-bromochrysin adducts

7-O-Bromochrysin adducts: 7-O-2-bromoethylchrysin, 7-O-3-bromopropylchrysin, 7-O-4-bromobutylchrysin, 7-O-5-bromopentylchrysin, 7-O-6-bromohexylchrysin have been previously made and their anthelminthic activities on nematodes investigated (Valdez-Calderón et al., 2016). These chrysin-adduct inhibited the growth of nematode at the larval stage. The method of synthesis of these compounds used involved dissolving the reactants in acetone and allowing the reacting mixture to run for 24 hours.

1.5.4.2 7-O-Alkylchrysin Adducts

7-O-alkylchrysin adducts: 7-O-butylchrysin and 7-O-hexyl chrysin have been synthesised previously and their antidiabetic activity investigated (Cheng et al., 2014). These compounds inhibited α-glucosidase, and a subsequent reduction in sugar levels *in vivo*. 7-O-Isopropylchrysin has been made previously and reported to modulate cancer cell resistance to cancer therapy and to enhance binding affinity towards P-glycoprotein (Comte et al., 2001). Longer chain 7-O-alkylchrysin adducts have not been synthesised.

1.5.5 Justification / Rational behind the Synthesis of 7-O-Chrysin Derivatives

Even though chrysin has indicated significant biological activities in *in-vitro* and *in-vivo* studies, the limitation of this molecule has made it difficult to transform the use of this molecule with promising biological activities into clinical trials. This study therefore aims to synthesise 7-O-Chrysin derivatives with different functional groups in an attempt to make molecules that are more cytotoxic to bacteria, fungi and cancer cells, yet indicating less toxicity to normal cells. The synthesis of 7-O-chrysin derivatives with different functional groups and chain length could also overcome the limitations of chrysin (poor absorption and bioavailability) in living systems. Although some 7-O-bromochrysin and 7-O-alkylchrysin derivatives have been previously made (Comte et al., 2001; Valdez-Calderón et al., 2016), no bio-availability data has been provided for these compounds. Zhou et al., (2012) has postulated that the 5-OH position of chrysin is the active site, whereas Babu et al., (2006) has postulated that the aromatic ring is the active site of this molecule. Synthetic chrysin derivatives at the 5-OH position or at the aromatic ring position might be a waste of resources as some authors have postulated that these regions are the active sites of the chrysin molecule. 5-O-chrysin derivatives are relatively inactive biological molecules due to the hydrogen-bonding effect at the 5-OH position (Zheng et al., 2016) and also because the 5-OH position is the active site of this molecule (Zhou et al., 2012). Blocking them could imply total loss of bioactivity.

One of the aims of this project (section 1.6) is to synthesise derivatives of chrysin (Structure 1.61) with increased cytotoxicity compared to the parent compound. The –OH groups at C-5 and C-7 are important for the cytotoxicity indicated by chrysin. Dao et al. (2003) and Shin et al., (1999) synthesised chrysin derivatives by targeting the –OH groups at both C-5 and C-7 of the chrysin molecule and reported weak anticancer activities at the 5-OH position and also reported an increase in cytotoxic activity via the introduction of a bromide ion in chrysin molecule. Comte et al. (2001), Valdez-Calderón et al. (2016) and Cheng et al. (2014) also synthesised chrysin derivatives by targeting other positions (position 6 and 8) of the chrysin molecule and reported the biological activities of these compounds. Based on these previous works, there is a need to synthesise chrysin derivatives with focus on C-7 and not C-5 of chrysin.

1.5.6 Flavonoids in Clinical Use

Despite the promising antibacterial and anticancer activities reported in the literature, no flavonoids are in clinical use for treatment of bacterial infection or as a cancer chemotherapeutics. However, quercetin was successfully used in phase I clinical trials as an inhibitor of tyrosine kinase (anticancer activity) up to a dose of 1400 mg/m2 without an indication of adverse side effects and has been recommended for phase II clinical trials (Ferry et al., 1996). Randomized, double-blind, placebo-controlled, crossover trials also indicated significant reduction of systolic blood pressure with no significant adverse effects on inflammatory biomarkers or cardiovascular (CVD) risk factors (Zahedi et al., 2013). Administration of quercetin-3-glycoside had no effect in CVD risk factors, insulin resistance or flow-mediated dilation (Dower et al., 2015a), and contributed to cardioprotection by reduction of inflammation and improvement of endothelial functions (Dower et al., 2015b).

1.6 Project Aims

This work is divided into two distinct sections: a) focussing on extraction isolation and characterisation of novel drug candidates from a plant (*Inula helenium*) with well-known use in herbal focussing on using chemical synthesis to improve the potency and safety index of a well-known cytotoxic agent. This work is also subdivided into two sections: i) antimicrobial studies using compounds isolated from a) and compounds synthesised from b) above and ii) anticancer studies using compounds isolated from a) and compounds synthesised from b).

The choice of bacteria and fungus – *Candida albicans* selected for this work is based on the clinical importance associated with them as described in section 1.2.1 above with emphasis on extended spectrum beta lactamase (ESBL) bacteria.

The choice of cancer cell lines selected for these studies is based on their diversification (origin): leukaemia – K562 and Molt-4 cell lines, colorectal cancers – Caco-2 (for compounds obtained as described in objective *a)* and *b)* above) and HCT 116 cell lines), breast cancer (MCF-7 and MDA-MB 468 cell lines), liver cancer – HepG2 cell line, lung cancer – (A549 cell line), skin cancer (HacaT cell line) and mesothelioma (Mero 14 cell line). This is meant to track the cytotoxicity of synthetic adducts on a range of cancer cell lines of different origins. The cytotoxicity of compounds isolated from *Inula helenium* (but not compounds synthesised from chrysin) on leukaemia cell line (K562) during hypoxia and normoxia, hyperglycaemia and normoglycaemia, shall also be investigated as there is no published data on the inhibition of leukaemia cancer cell (K562) growth by compounds from *Inula helenium* during hypoxia and hyperglycaemia despite their potent cytotoxicity – alantolactone and isoalantolactone (IC_{50} 0.7 and 1.2 µM) against the K562 cell line (Lawrence et al., 2001). The specific objectives of this work are highlighted below:

1.6.1 Isolation Work

The search for novel anticancer agents from plant – *Inula helenium*:

i) To obtain extracts from *Inula helenium* using different extraction methods: Microwave-assisted-extraction (MAE), ultrasound-assisted extraction (UAE) and Soxhlet extraction (SE), determine the extraction yields, and test their anticancer and antibacterial (see definition of antibacterial drugs on section 1.2.1) activities.

ii) To fractionate the extracts obtained from MAE, UAE and SE using different solvents: methanol, ethyl acetate and hexane.

iii) To purify and characterise bioactive components using different purification and characterisation techniques such as HPLC, NMR, MS and infrared spectroscopy.

iv) To test purified compounds isolated from *Inula helenium* on bacteria (see section 1.2.1.1) and study the cytotoxicity of bioactive compounds on K562 cell line in hypoxia, normoxia, hypoglycaemia and hyperglycaemia. This is because the cytotoxicity of compounds isolated from *Inula helenium* on cancer cell line has been well documented but no report on the cytotoxicity of these compounds in hypoxia, hypoglycaemia and hyperglycaemia has been documented.

1.6.2 Synthetic Work

The synthetic work is based on chrysin (Structure 1.61), a natural occurring flavonoid found in honey. This study is based broadly on the chemistry of the 7-OH (hydroxyl group). The 7-OH of the chrysin molecule interacts with other molecules, strengthening the hydrogen bond at position 5-OH (Cai et al. 2017), thus making the chrysin molecule insoluble in most organic solvents (except DMSO) (Zhou et al. 2014), which is also the reason for the poor bioavailability of chrysin in living systems (Walle et al., 2001). By targeting the 7-OH position of chrysin, its derivatives with better bioactivity, safety indices and solubilities could be synthesised thus overcoming the challenges of poor solubility and bioavailability. The aims are listed below.

i) To synthesise 7-O-Chrysin derivatives with increased cytotoxicity compared to the parent compound.

ii) Focussing on the 7–OH position of chrysin, to synthesise chrysin-aryl derivatives with different functional groups attached to the aryl ring and to investigate their anticancer and antimicrobial activities.

iii) Focussing on the 7–OH position of chrysin, to synthesise 7-O-haloalkylchrysin adducts and to investigate the role of halogenation of 7-O-Haloalkylchrysin-adduct in cancer and microbial growth inhibitions.

iv) Focussing on the 7–OH position of chrysin, to synthesize 7-O-alkylchrysin adducts lacking halogen groups and to compare the cytotoxic and microbial activities of these adducts.

v) Focussing on the 7–OH position, to synthesis aliphatic 7-O-alkylchrysin adducts containing branched chains and to investigate their anticancer and antimicrobial activities compared to aliphatic chrysin adducts without branch chains.

vi) To synthesise other 7-O-chrysin derivatives and to investigate their anticancer and antimicrobial activities.

Chapter 2 Methodology

2.1 Isolation and Purification of Natural Products Experimental

2.1.1 Extraction of compounds from *Inula helenium*

Three extraction techniques were used: Soxhlet extraction (SE), ultrasound-assisted extraction (UAE) and microwave-assisted extraction (MAE). Three different solvents were used to fractionate compounds extracted from *Inula helenium*: methanol, ethyl acetate and hexane. 1100 g of dry root sample of *Inula helenium* (crushed to fine powder) was purchased from Bristol Botannicals, UK. 1000g of this sample was divided into three equal parts: 333.3 g and extracted in 70 % methanol using SE, MAE and UAE.

The Soxhlet apparatus for this experiment consists of an extraction flask (1000 mL), a Soxhlet chamber and a condenser. Dried root (33.3 g x 10) were placed in an extraction thimble with 400 mL of methanol in an extraction flask, and the extraction allowed occurring in three cycles of extractions (such that the liquid in the extraction chamber becomes colourless). Analytical grade methanol (99.9%) was used for extraction (Fischer Scientific, UK).

The Soxhlet extractor operates via heat reflux. Heat is applied to the base of flask, causing the vapour to travel into the extraction chamber housing the extraction thimble and the solute to be extracted. The warm distilled solvent causes the solute in the extraction chamber to dissolve, dissolving some desired compounds. As the solvent fills the extraction chamber, the solvent is siphoned back to the flask. The extraction thimble prevent insoluble compounds from passing into the extraction flask. As each cycle of siphoning occurs, substances are constantly being dissolved. After several cycles of siphoning, the desired compounds become concentrated in the distillation flask. The apparatus is disassembled, and the concentrated extracts concentrated further by means of a rotary evaporator.

MAE was done using Sonicor Instrument Corporation, Copiague, New York. Dried roots of *Inula helenium* were extracted such that the temperature remained steady. This was done by using fluctuating radiation, and extraction conditions chosen as stated by Nkhili et al. (2009). Power was 400 W and temperature 60 °C for 40 minutes. The solvent used for extraction was 99.9 % analytical grade methanol (Fischer Scientific, UK).

MAE has the advantage over conventional extraction methods in that it requires less quantity of polluting non-environmentally friendly solvent and is less time-consuming,

leading to increased yield and quality of compound extracted from natural products (Xiao et al., 2013). This is achievable because the sample and solvents are enclosed in a sealed extraction vessel under controlled conditions of pressure and temperature. This allows for a quick rise in temperature of the solvents which allows efficient bond breaking of the compounds to be extracted in a very short period, leading to an eventual shortening of extraction time and an increased yield (Simić et al., 2016). MAE is therefore known as a "green" extraction procedure because it requires lower extraction time and relatively inexpensive (Karabegović et al., 2013).

The energy transfers and heating mechanism in MAE occurs via two mechanisms: ionic conduction and dipole rotation through displacement and reversal of charged ions present in the solvent and the solute. Unlike other heating methods which utilise radiation, conduction and convection of heat energy through to material to be extracted, MAE involves direct delivery of energy to the solute / solvent through interaction of molecular species with the electromagnetic field, thereby converting electromagnetic energy into heat (Thostenson & Chou, 1999).

UAE was performed using the Cole Parmer Sonicor instruments, Illinois, USA; ultrasonic model (power 130 W, 2.8 L, 28H) was used in carrying out the extraction. Dried roots (33.3 g) of *Inula helenium* were placed in a beaker with 300 mL of solvent with operational conditions of 25 °C, frequency of 50 kHz for 60 minutes. It consists of an amplitude control which allowed the ultrasound vibration probe to be set / adjusted to any limit within the 10 – 100 % range of the nominal power.

On completion of sonication, extracts were centrifuged for 8 minutes at 3000 rpm to sediment the extracts of interest.

2.1.2 Determination of extraction yield

Extracts of various components were evaporated in a rotary evaporator to a total volume of 5 – 20% and stored at -30ºC freezer and were then freeze-dried in a bench-top freeze-dryer - SJIA-10N-50 (Shanghai Beiyi Bioequip Information Co., Ltd.) to obtain dried samples. The extraction yield of various procedures was expressed as percent of mass of the extract obtained in relation to the dry matter of *Inula helenium* that was used in the extraction, as stated thus:

$$\frac{\text{Mass of extract (g)}}{\text{Mass of dry matter (g)}} \times 100$$

2.1.3 Statistical analysis

The extraction process was divided into two sets based on the experimental design. The experiment sets were compared in terms of extraction yield. One-way analysis of variance (ANOVA) was applied to detect differences among all the groups whereas the student t-test was applied in detecting differences within groups. Analysis were performed using the Origin 9.1 software. A significant level of $\alpha = 0.05$ was selected and significant difference was considered at $p < 0.05$. The factors examined are the extraction methods (three levels; SE, MAE and UAE) and the extraction solvent (three types methanol, n-hexane and ethyl acetate).

2.1.4 High-performance liquid chromatography (HPLC)

2.1.4.1 Apparatus and chromatographic conditions

High performance liquid chromatography (HPLC) analysis was performed using Agilent 1200 LC system (Agilent Technologies Deutschland, Germany) equipped with a G1313A auto-sampler, a G1316A column thermostat, a G1315B UV–vis photodiode array detector, G1354A solvent delivery unit, a fraction collector, an Ace 5 C18-Amide column (Hichrom Limited, Reading, UK), (250 mm × 21.2 mm, 6 µM). Methanol-water was used as the mobile phase starting from a gradient of 30:70, 40:60, 50:50, 60:40, 75:25 (v/v) and the column temperature was set at 40 °C, the flow rate was set at 5 ml / min, and detection wavelength 250 nm. 0.1 % formic acid was also introduced to the water-methanol used as the mobile phase to enhance good resolution of peaks. Freeze-dried extracts from *Inula helenium* were analysed by HPLC under optimum analytical conditions and the various fractions obtained collected by an automated fraction collector. The same parameters were used for qualitative HPLC analysis except that a column size (250 mm × 4.6 mm, 5 µM) and a flow rate of 1 ml / min were used.

2.1.4.2 Pre-HPLC sample preparation

Prior to HPLC analysis, only precipitates obtained from the hexane fractions were re-dissolved in 80 % methanol. A total of 14.57 g of precipitates from hexane fraction (dissolved in 80 % methanol at 10 mg / mL) was injected into the HPLC compartment and pure compounds collected in the auto-sample collector.

2.2 Synthetic Chemistry of Chrysin derivatives Methodology

The numbering system used for analysis follows the numbering system in chrysin (Structure 1.61, page 104). All NMR spectra data were recorded on a Bruker AC400 spectrometer using CDCl3 as an internal standard unless stated otherwise (7.28 ppm for 1H NMR and 77.0 ppm for 13C NMR). All solvents used for NMR analysis were purchased from Sigma Aldrich, UK.

Mass spectrometry data were recorded on an Agilent Technology 6120 Quadrupole LC/MS. High Resolution Mass Spectrometry was recorded using the positive electrospray ionisation time-of-flight mass spectrometry (TOF MS ES) on Waters i-class UPLC at Cambridge Analytical Services, University of Cambridge, UK.

Infrared spectrometry data were recorded on Thermo-scientific Nicolet iS10.

Melting point analysis was recorded on a Stuart Melting Point SMP 20.

Silica gel chromatography was conducted using silica gel 60 Å with a pore size of 40-63 µM (Fluorochem Limited). Silica thin layer chromatography was performed on pre-coated aluminium sheets with 0.2mm thickness obtained from Thermo-Fisher Scientific, UK. Two methods: microwave irradiation and the method previously used by Cheng et al., 2014) was used in synthesis of these chrysin adducts with modifications in solvent type used, reaction time and purification procedures.

Novel analytical data can be found in the appendix.

2.2.1 Synthesis of 7-O-bromoalkylchrysin derivatives (C13-C17)

Potassium carbonate (4.00 eq, 0.52 g, 8.4 mmol) was suspended in acetonitrile (10.0 ml) and stirred in a microwave tube for 30 minutes. 5,7-Dihydroxyflavone (0.12 g, 0.47 mmol) and the corresponding dibromoalkane was added and the resulting suspension stirred for 24 hours at 80 °C in the presence of bright light to produce a pale-yellow suspension. The resulting suspension was allowed to cool to room temperature and poured into crushed ice, leading to the formation of pale-yellow precipitate which was left at 4 °C for 24 hours. After 24 hours, the precipitate was filtered and washed with ice cold water, followed by petroleum ether and eluted in silica gel using 2:1 ethyl acetate: petroleum ether, v/v. The solvent was evaporated, and the precipitate dried in an oven for 24 hours at 50 °C to yield the pure compound.

2.2.2 Synthesis of 7-O-alkylchrysin Derivatives (C4-C12)

2.2.2.1 Method A for synthesis of 7-O-Alkyl derivatives of Chrysin

The aliphatic (7-O-alkyl) derivatives of chrysin were made by suspending potassium carbonate (2.00 eq, 0.26 g, 4.2 mmol) in acetonitrile (10.0 ml). The corresponding bromoalkane (2.00 eq) was added to a thick-walled microwave tube and the resulting suspension irradiated vigorously in a microwave reactor at 120 °C for 8 minutes at 900 W. The reaction was cooled rapidly by means of an auto-installed cooler system to a temperature of about 30 °C and poured unto crushed ice. The precipitate formed was left at 4 °C for 8 – 12 hours after which the precipitate was washed with ice cold water, followed by petroleum ether and dried in an oven at 50 °C for 12 hours to yield the pure compounds.

2.2.2.2 Method B for synthesis of 7-O-alkylchrysin derivatives

To chrysin (0.12 g, 0.47 mmol), potassium carbonate (2.00 eq, 0.26 g, 4.2 mmol) and tetrabutylammonium bromide (13.0 mg, 0.040 mmol) in dimethylformamide (DMF) was added the bromoalkane (2.00 eq, 4.72 mmol) in a microwave reaction bottle. This was irradiated in a microwave reactor in 8-successive 3.0 minutes microwave irradiations at 900 W (with a pause time to return the reaction to 35 °C). Ice cold water was added to the mixture which was subsequently acidified with 6 N HCl and extracted with ethyl acetate. The organic layer was concentrated to dryness and the brown residue obtained chromatographed on silica gel using ethyl acetate: petroleum ether (1:3 to 4:1) mobile phase to yield between 62 – 84 % yields of pure compounds.

2.2.2.3 Method C for synthesis of 7-O-alkylchrysin derivatives

7-O-alkyl derivatives of chrysin were made by dissolving potassium carbonate (2.0 eq, 0.26 g, 9.44 mmol) in acetone (10 mL). 5,7-Dihydroxyflavone (0.12 g, 0.47 mmol) was added and stirred for 15 minutes. The bromoalkane (2.00 eq) was added and the resulting suspension stirred for 72 hours at 70 °C The resulting suspension was allowed to cool to room temperature and poured into crushed ice, leading to the formation of pale-yellow precipitate which was left at 4 °C for 24 hours. After 24 hours, the precipitate was filtered and washed with ice cold water, followed by petroleum ether and eluted in silica gel using 2:1 ethyl acetate: petroleum ether, v/v. The solvent was evaporated, and the precipitate dried in an oven for 24 hours at 50 °C to yield the pure compounds with yields ranging from 54 – 68 %. The percentage yields for method A and C are reported in Table 4.1.

2.2.3 Synthesis of Methylated 7-O-alkylchrysin derivatives

One of the aims of this Project is to synthesise methylated 7-O-alkylchrysin derivatives and to study the antimicrobial effect of alkylation of these derivatives. The first compound synthesised in this category is 5-hydroxy-7-isopropoxy-3-phenyl-4H-chromen-4-one.

2.2.3.1 Synthesis of 7-O-Isopropylchrysin 5-Hydroxy-7-isopropoxy-3-phenyl-4H-chromen-4-one (C4)

2.2.3.1.1 Method A: Synthesis using the microwave reactor

Tetrabutylammonium bromide (0.08 mmol; 0.2 eq) was suspended in acetonitrile (10.0 ml) and stirred for 1 minute at room temperature. 5,7-Dihydroxyflavone (1 e.q., 0.12 g, 0.47 mmol), potassium carbonate (4.00 eq, 0.522 g, 8.4 mmol) and 2-bromopropane (4.0 eq, 152 µl, 12.4 mmol) were added and the resulting suspension irradiated in a microwave reactor for 10 minutes at 120 °C, 900 W. The resulting suspension was cooled rapidly in dry ice to a temperature of about 4 °C and left at this temperature for 24 hours. After 24 hours, the pale brown precipitate was filtered and washed with ice cold water, followed by petroleum ether and eluted in silica gel using 2:1 ethyl acetate: petroleum ether, v/v. The solvent was evaporated, and the precipitate dried in an oven for 24 hours at 50 °C to yield pure 5-hydroxy-7-isopropoxy-3-phenyl-4H-chromen-4-one.

2.2.3.1.2 Method B: Stirring the Reaction Mixture

To a solution of chrysin (100 mg of chrysin (0.40 mmol), 0.120 g K_2CO_3 (0.79 mmol; 2.0 eq) and 0.025 g of tetrabutylammonium bromide (0.08 mmol; 0.2 eq) in dimethylformamide (10 mL) was added 2-bromopropane (88 µL, 0.94 mmol, 2.0 eq). The reaction was allowed to stir for 72 hours at 50 °C. After 72 hours, the mixture was diluted with ice cold water, acidified with HCl (6 N) and extracted with ethyl acetate. The organic layer was concentrated to yield a yellowish-brown precipitate which was chromatographed on silica gel using ethyl acetate: petroleum ether (4:1) as mobile phase to yield pure 5-hydroxy-7-isopropoxy-3-phenyl-4H-chromen-4-one.

2.2.3.2 5-Hydroxy-7-isobutoxy-3-phenyl-4H-chromen-4-one or 7-O-Chrysin-2-Methylpropane (C5) – novel

To a solution of chrysin (100 mg, 0.40 mmol), 0.120 g K2CO3 (0.79 mmol; 2.0 eq) and 0.025 g of tetrabutylammonium bromide (0.08 mmol; 0.2 eq) in dimethylformamide (10 mL) was added 1-bromo-2-methylpropane (84 µL, 0.99 mmol, 2.0 eq). The reaction was

allowed to stir for 72 hours at 50 °C. After 72 hours, the mixture was diluted with ice cold water, acidified with HCl (6 N) and extracted with ethyl acetate. The organic layer was concentrated to yield a yellowish-brown precipitate which was chromatographed on silica gel using ethyl acetate: petroleum ether (20:80 to 100:0) as mobile phase to yield pure 5-hydroxy-7-isobutoxy-3-phenyl-4H-chromen-4-one.

2.2.4 Synthesis of other Chrysin Derivatives (C1-C3)

2.2.4.1 Synthesis of 7-O-Chrysinbutyl acetate [4-((5-Hydroxy-4-oxo-2-phenyl-4H-chromen-7-yl)oxy)butyl acetate] – (C1)

2.2.4.1.1 Method A: Irradiated in a microwave reactor

Potassium carbonate (4.0 eq, 0.52 g, 8.4 mmol) was suspended in acetone (10.0 ml). Chrysin (5,7-Dihydroxyflavone) (0.12 g, 4.7 mmol) and 4-bromobutyl acetate (2.0 eq, 184 µl, 9.4 mmol) were added and the resulting suspension irradiated in a microwave reactor for 10 minutes at 120 °C, 900 W. This was rapidly cooled by means of a vapour coolant to room temperature. This was then poured into crushed ice, leading to the formation of a pale brown precipitate which was left at 4 °C for 24 hours. After 24 hours, the precipitate was filtered and washed with ice cold water, followed by petroleum ether and eluted in silica gel using 2:1 ethyl acetate: petroleum ether, v/v. The solvent was evaporated and the precipitate dried in an oven for 24 hours at 50 °C to yield pure 4-((5-Hydroxy-4-oxo-2-phenyl-4H-chromen-7-yl)oxy)butyl acetate: (yield 0.196 g, 80.20 %); m.p. 122 – 124 °C; R_f = 0.74 (Silica 60 Å, 2:1 ethyl acetate: petroleum ether, v/v).

2.2.4.1.2 Method B: Stirring the reaction mixture

The method for synthesis reported previously by Cheng et al., (2014) was used for synthesis of 7-O-Chrysinbutyl acetate with some modifications.

Potassium carbonate (4.0 eq, 0.52 g, 8.4 mmol) was suspended in acetone (10.0 ml) and stirred vigorously in a thick-walled microwave tube (not microwave reactor) for 10 minutes. 5,7-Dihydroxyflavone (0.12 g, 4.7 mmol) was added and the resulting suspension stirred for 1 hour. 4-Bromobutyl acetate (2.0 eq, 184 µl, 9.4 mmol) was added and the resulting suspension allowed stirring for 19 hours. The resulting suspension was allowed to cool to room temperature and poured into crushed ice, leading to the formation of ivory white precipitate which was left at 4 °C for 24 hours. After 24 hours, the precipitate was filtered and washed with ice cold water, followed by petroleum ether. This was dried

in an oven for 24 hours at 50 °C to yield pure 4-((5-Hydroxy-4-oxo-2-phenyl-4H-chromen-7-yl)oxy)butyl acetate

2.2.4.2 Synthesis of 7-O-Chrysin-3,5-dimethylbenzene [7-((3,5-Dimethylbenzyl)oxy)-5-hydroxy-2-phenyl-4H-chromen-4-one] – (C2)

2.2.4.2.1 Method A: Irradiation in a microwave reactor

7-O-Chrysin-3,5-dimethylbenzene was prepared by alkylation of chrysin at 7-OH with 1-(Bromomethyl)-3,5-dimethylbenzene (2.00 eq, 0.085 g, 6.4 mmol). Potassium carbonate (4.0 eq, 0.52 g, 8.4 mmol) was suspended in acetonitrile (10.0 ml). Chrysin (5,7-Dihydroxyflavone) (0.12 g, 4.7 mmol) was added and the resulting suspension irradiated in a microwave reactor for 5.0 minutes at 80 °C, 900 W. This was rapidly cooled by means of a vapour coolant to room temperature. This was then poured into crushed ice, leading to the formation of a floral-white precipitate which was left at 4 °C for 24 hours. After 24 hours, the precipitate was filtered and washed with ice cold water, followed by petroleum ether and eluted in silica gel using 2:1 ethyl acetate: petroleum ether, v/v. The solvent was evaporated and the precipitate dried in an oven for 24 hours at 50 °C to yield pure 4-((5-Hydroxy-4-oxo-2-phenyl-4H-chromen-7-yl)oxy)butyl acetate.

2.2.4.2.2 Method B: Stirring the reaction mixture

7-((3,5-dimethylbenzyl)oxy)-5-hydroxy-2-phenyl-4H-chromen-4-one (C2), was made by dissolving potassium carbonate (2.0 eq, 0.26 g, 9.44 mmol) in acetonitrile (10 mL). 5,7-Dihydroxyflavone (0.12 g, 0.47 mmol) was added and stirred for 15 minutes. 1-(Bromomethyl)-3,5-dimethylbenzene (2.00 eq, 0.08 g, 6.4 mmol) was added dropwise and the resulting suspension stirred for 4.0 hours at room temperature to form a floral-white suspension.

The resulting suspension was poured into crushed ice, leading to the formation of floral white precipitate which was left at 4 °C for 4 hours. After 4 hours, the precipitate was filtered and washed with ice cold water, followed by petroleum ether. This was dried in an oven for 24 hours at 50 °C to yield pure 7-(3,5-dimethylphenoxy)-5-hydroxy-3-phenyl-4H-chromen-4-one.

2.2.4.3 Synthesis of 7-O-Chrysin-3,5-dimethylbenzene [7-(2,4-Dinitrophenoxy)-5-hydroxy-3-phenyl-4H-chromen-4-one] – (C3)

2.2.4.3.1 Method A: Irradiation in a microwave reactor

Potassium carbonate (4.00 eq, 0.522 g, 8.4 mmol) was suspended in methanol (10.0 ml) with vigorous stirring in a thick- walled microwave tube. 5,7-Dihydroxyflavone (0.12 g, 0.42 mmol) and 1-bromo-2,4-dinitrobenzene (116.6 g, 4.7 mmol) was added and the resulting suspension irradiated in a microwave reactor for 10 minutes at 120 °C, 900 W. The resulting suspension was poured into crushed ice, leading to the formation of a pale brown precipitate which was left at 4 °C for 30 minutes. This is because the precipitate formed was very distinct in appearance. After 30 minutes, the precipitate was filtered and washed with ice cold water, followed by petroleum ether and eluted in silica gel using 2:1 ethyl acetate: petroleum ether, v/v. The solvent was evaporated, and the precipitate dried in an oven for 24 hours at 50 °C to yield pure 7-(2,4-dinitrophenoxy)-5-hydroxy-3-phenyl-4H-chromen-4-one.

2.2.4.3.2 Method B: Stirring the reaction mixture

Potassium carbonate (4.00 eq, 0.52 g, 8.4 mmol) was suspended in acetonitrile (10.0 ml) and stirred for 30 minutes. 5,7-Dihydroxyflavone (0.12 g, 0.47 mmol), and 1-bromo-2,4-dinitrobenzene (116.6 g, 4.7 mmol) was added and the resulting suspension stirred for 36 hours. The resulting suspension was cooled rapidly in dry ice to a temperature of about 4 °C. At 4 °C, the suspension was transferred to crushed ice at about 4 °C and left for 24 hours. After 24 hours, the pale brown precipitate was filtered and washed with ice cold water, followed by petroleum ether and eluted in silica gel using 1:3 ethyl acetate: petroleum ether, v/v. The solvent was evaporated, and the precipitate dried in an oven for 24 hours at 50 °C to yield pure 7-(2,4-Dinitrophenoxy)-5-hydroxy-3-phenyl-4H-chromen-4-one. This was dried in an oven for 48 hours at 50 °C to yield pure 7-(2,4-Dinitrophenoxy)-5-hydroxy-3-phenyl-4H-chromen-4-one.

2.2.5 Determination of Hydrophilicity of Chrysin Derivatives

Hydrophilicity of chrysin derivatives were established using the function HC = R_fX / R_fC after TLC on C18 reverse-phase silica gel 20- × 20-cm plates in solvent : water (8:2). Methanol, dimethylformamide (DMF) and tetrahydrofuran (THF) where HC is the hydrophilicity index of chrysin derivatives (ability to dissolve in polar solvents). R_fX = R_f

value (ratio of the distance moved by the solute to the distance moved by the solvent) of chrysin derivatives. R_fC = R_f value of chrysin.

2.3 Antimicrobial Studies Experimental

2.3.1 Culture Media and Microbial Strain

Nutrient agar (Sigma-Aldrich, UK) was used to culture the bacteria to be tested whereas the Mueller Hinton Broth (Sigma-Aldrich, UK) was used for minimum inhibitory concentration (MIC) assay (Kuete et al., 2011). The Sabouraud dextrose was used to culture the *Candida albicans* fungus (MTCC 227). The following bacterial strains were used for antimicrobial assay studies:

Gram positive bacteria:

i) *Enterococcus faecalis* NCIMB 13280 (Kuch, et al., 2012).
ii) *Staphylococcus aureus* 25923 (Taleb-Contini et al., 2003).
iii) *Bacillus cereus* ATCC 10876 (Fakruddin et al., 2012).
iv) *(MRSA252* obtained from Public Health England) (Holden et al., 2004).

Gram negative bacteria:

i) *Escherichia coli* NCTC 13353 – strain EO 487. CTX-M-15 ESBL producer (Woodford et al., 2004).
ii) *Klebsiella pneumoniae* NCTC 13439 – VIM-1 metallo-carbapenemase (outbreak strain (Woodford et al., 2006).
iii) *Pseudomonas fluorescens* ATCC 13525 (Yadav et al., 2014).
iv) *Pseudomonas aeruginosa* NCTC 13437 – VIM-10 metallo-carbapenemase; VEB-1 ESBL (Woodford et al., 2008).

2.3.2 Bacterial Preparation

Strains of bacteria were cultured overnight (22 - 25 h) at 37 °C on nutrient broth for the preparation of cell suspensions. Suspensions of bacterial cells (dissolved in PBS) were homogenised such that the spectrophotometry standard of 5 × 105 CFU/mL (0.5 McFarland standards) was obtained.

2.3.3 Bacterial positive control

Gentamycin (Sigma-Aldrich, UK) was the reference antibiotic (RA) used. Isolates from *Inula helenium* were used in ascertaining microbial growth inhibition.

2.3.4 Agar well diffusion assay

Agar well diffusion assay was done as described before (Balouiri et al., 2016). The test compounds were assayed to determine antimicrobial activity via the disc diffusion method against MTCC microbial strains. The antibacterial activity of each extracts was determined in duplicate and compared to standard antibiotic disc of Gentamycin 10 µg/mL. Mueller Hinton agar plates were pre-warmed and inoculated with 106 CFU/mL of bacterial strain. Test compound were dissolved in DMSO (10 µg/mL); 40 µL of each test compound (100 µg/mL) was pipetted unto 10 mm sterile paper disc which was placed on the surface of inoculated agar plate and incubated for 23-25 hours at 37 °C and the inhibition band of each extracts recorded. Antibacterial activity was expressed as the diameter of zone of inhibition recorded by these extracts. DMSO served as the negative control. Only fractions from extraction methods which indicated good inhibitory activities via the disc diffusion method were tested for MIC.

2.3.5 Colorimetric assay for Minimum Inhibitory Concentration (MIC)

A rapid p-iodonitrotetrazolium chloride (INT) colorimetric assay was carried out as previously described (Kuete et al., 2009). The assay measures the production of NADH by coupling it to the reduction of 2-(p-iodophenyl)-3(p-nitrophenyl)-5-phenyl tetrazolium chloride (INT). INT accepts an electron via an intermediate electron carrier, either lipoamide dehydrogenase or phenazine methosulfate and is reduced to a red-coloured formazan, which can be quantified by the spectrophotometer. All samples to be tested were dissolved in Mueller Hinton Broth (MHB) / DMSO such that the DMSO final concentration was below 5%. This was necessary to prevent interference of microbial growth by DMSO. The resulting solution was added to MHB. This was diluted serially in the 96 well plates. Inoculum prepared in appropriate broth – MHB (100 µL) was then added (Kuete et al., 2009; Kuete et al., 2008). These plates were sealed and agitated using a plate shaker (in order to properly mix the contents of the well), incubated for 18 h at 37 °C. This assay was repeated at least thrice. A negative control (wells containing broth, DMSO and 100 µL of inoculum to a final concentration of 2.5 %) was also prepared. In order to determine MIC, 40µl of 0.2 mg / mL of INT was added after incubation for 18 h at 37 °C. The concentration of sample which prevented colour change of the media and inhibited bacterial growth completely is known as the MIC (Eloff, 1998). INT was added to aid the reading of the MIC / MBC result (colour change from violet to red). MBC was done by adding 60 µl aliquot from the preparations which did not indicate growth activity

during the MIC assay to 140 µl of 7H9 broth, and was incubated for 48 h at 37 °C. The concentration of fraction which did not indicate a colour change after INT was added as stated above is known as MBC.

2.3.6 Screening for antifungal activity

Antifungal activity against the *Candida albicans* fungus was done using the broth microdilution with each well containing about 103 fungus cells-inoculum and the different test compounds at 400 µg / mL in RPMI 1640 (L-glutamine containing medium without sodium bicarbonate) (Sigma-Aldrich, UK). This was buffered with 0.165 M MOPs (3-(N-morpholino)-propanesulfonic acid) at pH 7.0. The plates were incubated for 48 h at 37 °C. Minimum inhibitory concentration (MIC) was done in triplicate and the mean MIC taken (Riesselman et al., 2000). Fluconazole (Sigma-Aldrich, UK) was used as an antifungal standard drug (Riesselman et al., 2000).

2.4 Anticancer Studies Experimental

2.4.1 Cell lines

Ten cancer cell lines – breast cancer (MCF-7 and MDA-MB 468) (Seca et al., 2014), colorectal cancer (HCT-116 and Caco-2) (Seca et al., 2014), Leukaemia cancer (K562 and Molt-4) (Seca et al., 2014), hepatocellular cancer (HepG2) (Seca et al., 2014), lung cancer (A549) (Seca et al., 2014), keratinocytes (HacaT) (Nzengue et al., 2008) and one normal cell line: BEAS-2 (Park et al., 2015), were used for anticancer inhibition studies.

MCF-7 was isolated from a 69-year-old woman in 1970 (Soule, Vazquez, Long, Albert, & Brennan, 1973). It is a breast cancer cell line known as an oestrogen receptor (ER) positive cell line because of its ability to process oestrogen to oestradiol. When treated with “anti-oestrogen, modulation and secretion of insulin-like growth factor binding protein” ensues. MCF-7 is inhibited by “tumour necrosis factor alpha (TNF alpha)” (Lacroix, Toillon, & Leclercq, 2006). It is an adherent cell. Dulbecco's Modified Eagle Medium (DMEM), Sigma Aldrich UK, was used as culture media.

K562 cells are “immortalised myelogenous leukaemia cell lines of the erythroleukaemia type, and the line is derived from a 53-year-old female chronic myelogenous leukaemia patient in blast crisis” (Lozzio & Lozzio, 1975). They are suspension cells and were cultured using Roswell Park Memorial Institute (RPMI) media. Sigma Aldrich, UK.

HacaT cells are immortal keratinocytes cells which are spontaneously transformed aneuploidy cells with ability to proliferate and differentiate *in vitro*. Widely used to study vitamin D3 metabolism (Lehmann 1997), cancers of the skin (Chorachoo et al., 2016). It is an adherent cell. DMEM was used as culture media.

Caco-2 cell line is a colorectal adenocarcinoma cell of epithelial origin. In pharmaceutical industries, Caco-2 is used in studying the absorption of drugs administered orally across the small intestine mucosa (Fogh & Trempe, 1975). It is an adherent cell. RPMI was used as culture media. DMEM was used as culture media supplemented with 20 % Fetal Bovine Serum (FBS, Sigma Aldrich, UK) as these cells grow slowly.

HepG2 is a perpetual epithelial cell with a well differentiated hepatocellular carcinoma. It is derived from the liver tissues and they secret transferrin, albumin, fibrinogen and plasminogen and is usually used in the study of liver disease. It is implicated in hepatocellular carcinoma (Ma et al., 2016). It is an adherent cell. RPMI media was used as culture media.

MDA-MB 468 was isolated from pleural effusion of breast tissue / mammary gland is used in the study of invasion of breast cancer, migration and metastasis. It is an oestrogen (ER) alpha negative human breast cancer cell line (Wang et al., 1997). It is an adherent cell. RPMI was used as culture media.

A549 cell line is an adenocarcinoma human alveolar basal epithelial cell line which is responsible for transport of substances including electrolytes and water across the alveoli of lungs. They contain high lipids (Foster, Oster, Mayer, Avery, & Audus, 1998) (unsaturated fatty acids) and can synthesise lecithin. They are squamous adherent cells. A549 cell line was cultured in RPMI media

HCT-116 is a colon adenocarcinoma cancer cell line. It is an adherent colon cancer cell from human. It was cultured on McCoy 5a media, Sigma Aldrich, UK.

Molt-4 is “human T lymphoblast; acute lymphoblastic leukaemia cell line”. It was grown on RPMI media. It is a suspension cell.

Mero 14 is a mesothelioma cell line of human origin, derived from malignant mesothelioma due to exposure to asbestos. It was derived from a 60-year-old man. Mero 14 cell line was cultured in DMEM media.

Beas-2 is an adherent, epithelial-like, normal human bronchial epithelial cell. These cells release cytokines in the lungs which fights organic pollutants inhaled into the lungs

thereby preventing respiratory and cardiovascular diseases (Fuentes-Mattei et al., 2010). BEAS-2B cell line was cultured in Bronchial Epithelial Basal Medium (BEBM) from Lonza.

2.4.2 Maintenance of cells

Cell culture personal protective equipment comprising of blue sterile nitrile gloves, and blue laboratory coat was used. RPMI, DMEM, BEBM, Mc-Coy media and PBS were used in the cell feeding procedure (as stated in section 2.4.1) above. Unless stated otherwise, all media used cytotoxicity studies were purchased from Sigma Aldrich, UK. BEBM was purchased from Lonza, UK. Temperature and CO_2 conditions of 37 °C and 5% CO_2 were not exceeded in all the cell work. Cell handling was done in a sterilised manner by using Virkon followed by 70% v/v EtOH, in cleaning the cell culture Hood. Every two to three days, old media was replaced by fresh media and the flask returned to the incubator. Everything was then decontaminated in Virkon (Sigma-Aldrich, UK).

2.4.3 Splitting Matured Confluent Cells

Once the cells confluence reaches 70% or above, the cells were either split into three flasks, or passaged and split into 1/3. PBS was used to wash metabolic wastes off the cells; cells were trypsinised (5 mL) and detached from the flask which was incubated for 2-5 minutes at 37 °C. After incubation, detached cells were split into 3 new flasks and returned to the incubator. Nevertheless, Caco-2 cells were passaged once the confluence was about 40-60%. This is because once confluent, they are difficult to disaggregate as they adhere very tightly unto the plate and do not plate well.

2.4.4 Dosing procedure

The cell lines used in this study were dosed (unless stated otherwise) as described in Table 2.1 below – see the plate map below.

Table 2.1: Dosing design of cancer cell lines in 96 well plate in MTT assay

	Compound 1 (µM)			Compound 2 (µM)			Positive control (µM)			(DMSO + CELL) (µM)		
	1	2	3	4	5	6	7	8	9	10	11	12
A	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
B	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0
C	25.0	25.0	25.0	25.0	25.0	25.0	25.0	25.0	25.0	25.0	25.0	25.0
D	12.5	12.5	12.5	12.5	12.5	12.5	12.5	12.5	12.5	12.5	12.5	12.5
E	6.3	6.3	6.3	6.3	6.3	6.3	6.3	6.3	6.3	6.3	6.3	6.3
F	3.1	3.1	3.1	3.1	3.1	3.1	3.1	3.1	3.1	3.1	3.1	3.1
G	1.6	1.6	1.6	1.6	1.6	1.6	1.6	1.6	1.6	1.6	1.6	1.6
H	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8

The dosage used in this study ranged from 0.8 – 100 µM (unless stated otherwise) for cancer cells assays. The dose was increased to concentrations up to 400 (µM) in order to investigate the interaction between the normal cell line in comparison to the cancer cell lines.

2.4.5 Cell viability assay using MTT

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was done as described previously by Holst and Oredsson (2005). 100 µl aliquots of cell suspension containing 3000 cells per well (30,000 cell/mL) were seeded in wells of 96 well plates. After 24 hours of seeding, compounds were added. The concentration range used in this assay was between 2.0 and 125 µg/mL in addition to appropriate DMSO which was used as negative control in the same proportion as the compound. MTT solution (5 mg/ml MTT in PBS) was added to the 96 wells and the plate returned to the incubator and left for 4 hour. The medium containing MTT was removed. Live cells were reduced by the formation of purple formazan. 200 µl DMSO was added to dissolve the formazan precipitate. Absorbance was monitored Thermo Labsystem Multiskan Ascent plate reader (Labsystems Oy, Helsinki, Finland) using the Ascent software at 540 nm. Dose response curves were drawn based on % of control in Origin 9.1 Software. The inhibitory concentration 50 (IC_{50}) was determined from the curves (Mossman, 1983).

2.4.6 MTS Assay

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTS) assay was done as described before (Mosmann 1983). MTS assay (cell proliferation) is a sensitive colorimetric assay that quantifies viable cells in cytotoxic and proliferative assay. Its principle is based on the ability of viable cells to reduce MTS tetrazolium compound, generating a coloured formazan product which dissolves in cell culture medium. The enzyme – NAD(P)H-dependent dehydrogenase is thought to be responsible for this conversion in active cells. Absorbance of 540 nm was used to measure the colour intensity of the formazan formed (Mossman, 1983).

Unlike MTT assay, MTS reagent is added directly into the cell culture media. DMSO detergent is not needed to solubilise the cells as in MTT assay. MTS assay finds application in cytotoxic analysis of anticancer compounds, cell proliferation in response to nutrients, mitogens, cytokines and growth factors.

Chlorpromazine hydrochloride (CPZ) was used as the positive control anticancer agent because of its broad-spectrum cytotoxicity against cancer cells *in-vitro* (Shenoy et al., 1982; Jessica et al., 2013). Many anticancer drugs in clinical use are specific for certain cancer cells and inactive against others. To compare the cytotoxicity of compounds tested in this study especially against ten cancer cell lines, there is need to maintain consistency in the criteria (anticancer agent) used.

2.4.7 Cell Culture and dosing of samples used for Hypoxia, Normoxia, Hyperglycaemia and Normoglycaemia Studies

The technique reported for cell culture as stated in section 2.4.1 – 2.4.6 was used with some modifications. Two media – one containing high glucose (0.20 mg/mL) and the other containing low glucose levels (0.02 mg/mL) were used. Lactose (0.20 mg/mL) was added to the high glucose media only and this media was marked as “high glucose media”. The media without glucose was marked “low glucose media”. Maintenance, splitting and dosing of cell lines was done as stated in section 2.4.1 – 2.4.6. Cells were incubated under normal oxygen conditions (normoxia), and under reduced oxygen i.e. < 30 mmHg (hypoxia – in Baker Ruskinn *in vivo* tube). RPMI media was used. Cells were incubated for 72 hours. Results are expressed as the mean ± standard error of mean (SEM).

Chapter 3 Extraction and Purification of Bioactive Agents from *Inula helenium*

The quantity of bioactive constituents of natural products are fairly low. Extraction and isolation of bioactive constituents in the laboratory is intensive and time consuming. This has been the bottle-neck in drug discovery from natural product, as bioactive products (especially those present in minute quantities) could be missed if proper extraction and isolation techniques are not used. There was therefore a need to carefully select appropriate / optimised methods that will allow for isolation of novel bioactive compounds (with good yield). After extraction, there is a need to fractionate the extracted compounds using solvents with different polarities. By fractionating extracts, the biological activities of fractionated compounds could be tested. Fractions indicating poor or no bioactivity could be discarded while fractions with good bioactivity were subjected to further purification to obtain pure compounds. The results obtained from these extraction methods are presented below.

3.1 Extracts from *Inula helenium*

3.1.1 Extraction of compounds from *Inula helenium*

Three extraction techniques: Soxhlet extraction (SE), ultrasound-assisted extraction (UAE) and microwave-assisted extraction (MAE), and three solvents – with different polarities (methanol, ethyl acetate and hexane) were used in the extraction of compounds from *Inula helenium*.

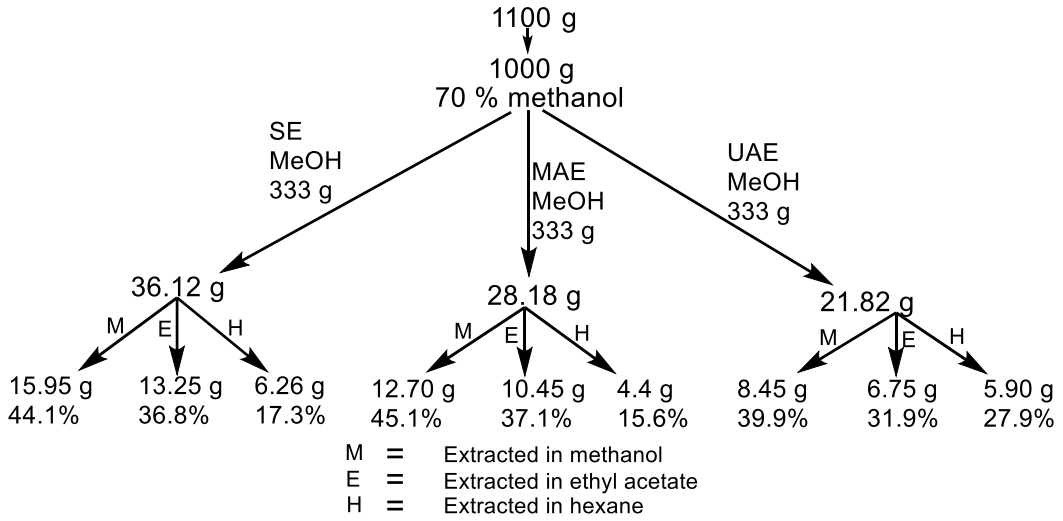
1000 g (out of the 1100 g) of dry root sample of *Inula helenium* purchased from Bristol botannicals, UK, was divided into three approximate parts: 333.3 g and extracted in 70 % methanol using SE, MAE and UAE to yield 36.12 g, 28.18 g and 21.82 g respectively. These were further partitioned in hexane, ethyl acetate and methanol – Scheme 3.1.

For **SE**, 15.95 g (44.1 %) was extracted using methanol, 13.25 g (36.8 %) using ethyl acetate and 6.26 g (17.3 %) using hexane (Table 3.1). For **MAE**, 12.70 g (45.1 %) was extracted using methanol, 10.45 g (37.1 %) using ethyl acetate and 4.4 g (15.6 %) using hexane (Table 3.1). For **UAE**, 8.45 g (39.9 %) was extracted using methanol, 6.75 g (31.9 %) using ethyl acetate and 5.9 g (27.9 %) using hexane (Table 3.1).

Results obtained from the extraction yield of extracts from *Inula helenium* (Table 3.1 – Table 3.2) indicate that SE and methanol were the best extraction method, and the solvent of choice for the extraction of compounds from *Inula helenium* in terms of product

yield. The highest yields of extracts were obtained from the SE (34.45 g) whereas the lowest yield was obtained from UAE (21.1 g).

From the solvents used, the highest extract was recorded using methanol as the extraction solvent (Table 3.2) and the least reported for hexane extracts.



Scheme 3.1: Percentage yield of compounds extracted from dry roots of *Inula helenium*

Although the total yield obtained using hexane was significantly low compared to the yields recorded for methanol and ethyl acetate, the percentage yield recorded for UAE using hexane was significantly higher than those recorded for SE and MAE (p-value, 0.000007), and this yield was comparable to those of UAE using methanol and ethyl acetate – Scheme 3.1.

With **SE**, the student t-test indicated that the yield is significantly affected. Although the total yield obtained using hexane was significantly low compared to the yields recorded for methanol and ethyl acetate, the percentage yield recorded for UAE using hexane was significantly higher than those recorded for SE (p-value, 0.03) and MAE (p-value, 5.6 x 10^9). The yield was comparable to those of UAE using methanol and ethyl acetate (Table 3.1– 3.2).

Table 3.1: The yield of compounds extracted from *Inula helenium* using different extraction methods and different solvents

Dry 100 g *Inula helenium powder*				
Method of extraction	solvent used	Dry matter obtained from 100 g *Inula helenium* powder		Total yield (g)
		Yield (g)	Yield (%)	
Soxhlet extraction (SE)	Methanol	15.95	44.1	34.45
	Ethyl acetate	13.25	36.8	
	Hexane	6.26	17.3	
Microwave-assisted	Methanol	12.70	45.1	27.55

extraction (MAE)	Ethyl acetate	10.45	37.1	
	Hexane	4.40	15.6	
Ultrasound-assisted extraction (UAE)	Methanol	8.45	39.9	21.1
	Ethyl acetate	6.75	31.9	
	Hexane	5.90	27.9	

The yields recorded for the various extraction methods (SE, MAE and UAE) base on the solvents used is presented in Table 3.2.

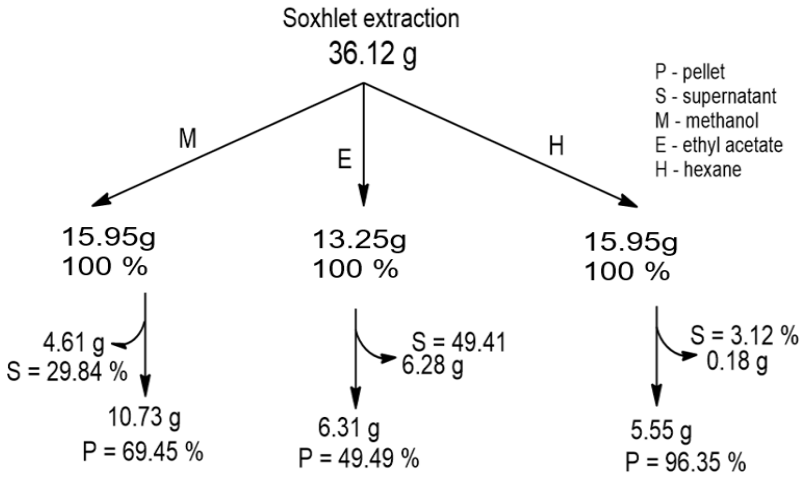
Table 3.2: Yield recorded from SE, MAE and UAE base on the solvent used.

Solvent used	Soxhlet extraction (g)	Microwave-assisted extraction (g)	Ultrasound-assisted extraction (g)	Total yield (g)
Methanol	15.95	12.7	8.45	37.1
Ethyl acetate	13.25	10.45	6.75	30.45
Hexane	6.25	4.4	5.9	16.55

The yield also for polar solvent – methanol was higher than the yields recorded for less-polar solvents (ethyl acetate and hexane). The most effective solvents in terms of output (yield) for SE were methanol – 15.95 % (100% v/v), whereas the less-polar solvents – ethyl acetate and hexane showed the least yields (13.25 %, and 6.25 % respectively per gram of dry plant matter) (Table 3.2).

3.1.2 Centrifugation of compounds extracted from *Inula helenium*

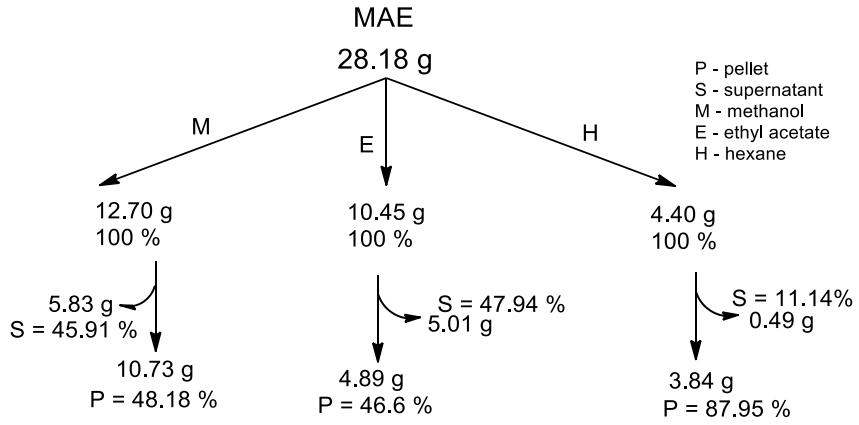
Dried samples extracted from *Inula helenium* (Table 3.1) were re-suspended in methanol and centrifuged in an Allegra X-14R Benchtop Centrifuge at 500 r.p.m. for 1 minute to obtain the pellet and supernatants - Scheme 3.2.



Scheme 3.2: Schematic representation of extraction yields from Soxhlet extraction using different solvents

For SE, 10.73 9 (69.45 %) yellowish-brown precipitate was obtained from the methanol fraction and 4.61 g (29.84 %) yellow precipitate was obtained from the supernatant. 6.31 g (49.49 %) and 6.28 g (49.41 %) was obtained from the pellet and supernatants obtained from the ethyl acetate fraction. There was however a colour change in the colour of the precipitate obtained from the hexane fraction. 5.55 g (96.35 %) white precipitate from the pellets, and 0.18 g (yellow) precipitate from the supernatant were obtained (Scheme 3.2). The white precipitate was isolated and characterised as sesquiterpene lactones as will be discussed in the next section.

For MAE, 10.73 g (48.18 %) yellowish-brown precipitate, and 5.83 g (45.91 %) yellow precipitate, were obtained from the pellets and supernatant respectively for the methanol fraction - Scheme 3.3. 4.89 g (46.6 %) and 5.01 g (47.94 %) were obtained from the pellets and supernatants of the ethyl acetate fraction respectively.



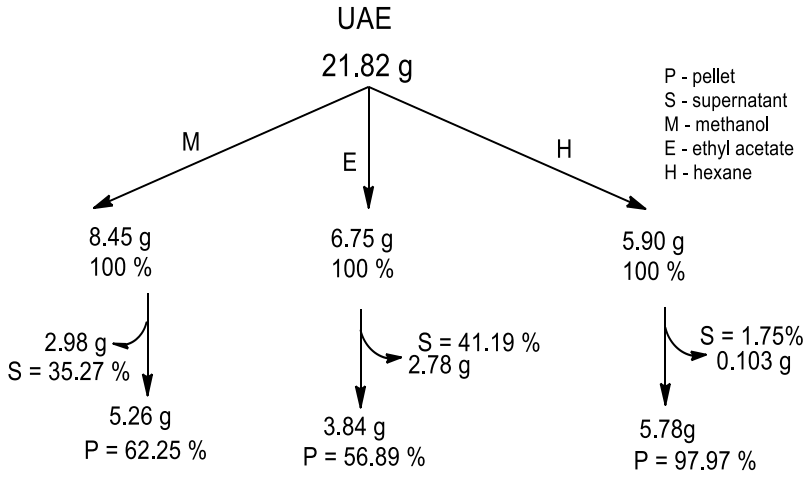
Scheme 3.3: Schematic representation of extraction yields from MAE using different extraction solvents

As observed for the precipitates obtained from the SE, 3.84 g (87.95 %) and 0.49 g (11.14 %) white precipitate was obtained from the pellets from the hexane fraction - Scheme 3.3. The precipitate obtained from the supernatant was yellowish in colour. The white precipitate was also characterised as a sesquiterpene lactone. For UAE, a yellow-brown precipitate - 5.26 g (62.25 %), and a yellow precipitate – 2.98 g (35.27 %), were obtained from the pellets and supernatant obtained from the methanol fraction. Similarly, 3.84 g (56.89 %) and 2.78 g (41.19 %) yellow-brown precipitate and yellow precipitate, were obtained from the pellets and supernatants obtained from the ethyl acetate fraction - Scheme 3.4.

Like the pellets obtained from SE and MAE, the pellets obtained from the hexane fraction were white in colour and were characterised to be sesquiterpene lactones. 5.78 g (97.97 %) white precipitates were obtained from the pellets, and 0.103 g (1.75 %) pale yellow

precipitates from the supernatant (Scheme 3.4). The precipitates obtained from the supernatants and the pellets from each of the extraction methods – SE, MAE and UAE, were tested against cancer cell lines, and microbial strains as shall be discussed in the next section. Precipitates which indicated promising biological activities were further purified via HPLC, isolated, tested on bacterial strains and cancer cell lines, and characterised thereafter using NMR, MS and infrared spectroscopy and their melting points determined.

Prior to HPLC analysis, only precipitates obtained from the hexane fraction indicated good bioactivities against cancer cell lines and bacterial strains. Since one of the aims of this project was to isolate, purify and characterise bioactive components of *Inula helenium*, compounds which did not meet this criterion were dropped and only compounds obtained from the hexane fraction were further purified and characterised.



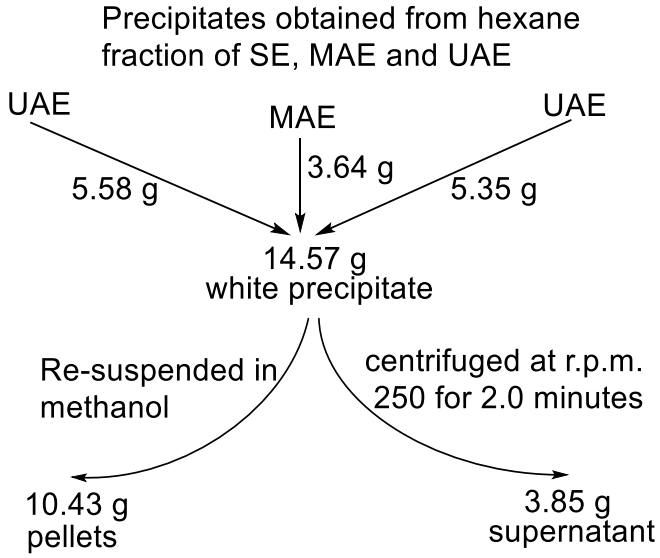
Scheme 3.4: Schematic representation of extraction yields from UAE using different extraction solvents

To do this, the white precipitates obtained from SE, MAE and UAE in Scheme 3.2 - Scheme 3.4 were combined because they indicated similar spots on a thin layer chromatography (TLC plate). 0.2 g of these precipitates was used for biological analysis on cancer cell lines and bacterial strains for each of SE, MAE and UAE, thus 5.58 g (from SE), 3.64 g (from MAE) and 5.35 g (from UAE) were summed together; a total yield of 14.57 g of precipitates from hexane fraction.

It was observed that the particle sizes were uneven as some were larger and folded into small white balls, while others were powdery in nature. The white precipitate was re-dissolved in methanol and centrifuged at 250 r.p.m for 2 minutes to obtain a white supernatant (3.85 g) and a milky colour precipitate (10.43 g) - Scheme 3.5.

The bioactivity of the supernatant and the pellets were assayed on some cancer cell lines and bacteria. The bioactive components were present in the supernatant. The precipitate

obtained from the supernatant (3.85 g) was chromatographed (HPLC) as described in section 3.1.3 below, to yield three sesquiterpene lactones – alantolactone, isoalantolactone and costunolide.



Scheme 3.5: Precipitate obtained from Soxhlet extractor.

The precipitates obtained were centrifuged at r.p.m 250 for 2 minutes to obtain 10.43 g pellets and 3.85 g supernatant. The pellets were re-suspended in methanol, evaporated and purified further via the HPLC.

Interestingly, this is the first time costunolide has been reported (isolated) from *Inula helenium*. Purification and characterisation of bioactive compounds isolated from *Inula helenium* is described in detail in section 3.1.3.1 (below).

3.1.3 High-performance liquid chromatography (HPLC)

3.1.3.1 Purification of bioactive compounds from *Inula helenium*

An auto-sample collector, a component of the HPLC system as described in section 2.1.4 above was used to collect the pure samples. Vials 9 and 18 (Figure 3.1) were eluted at 35.62 minutes and were characterised via nuclear magnetic resonance (NMR), mass spectroscopy (MS) and infrared (IR) spectroscopy to be alantolactone, vial 17 was eluted at 37.04 minutes and was characterised to be isoalantolactone. Vial 16 was eluted at 39.97 minutes and was characterised to be costunolide – a novel compound isolated from *Inula helenium*. These vials (9, 18, 17 and 16 were characterised becaused they indicated good bioactivity). The boiling point (b.p), nuclear magnetic resonance (NMR) and masss spectroscopy (MS) data of alantolactone, isoalantolactone and costunolide isolated from *Inula helenium* via HPLC is reported below. Figure 3.1 and Figure 3.2 represents the HPLC spectrum of pure sesquiterpene lactones isolated from *Inula helenium*.

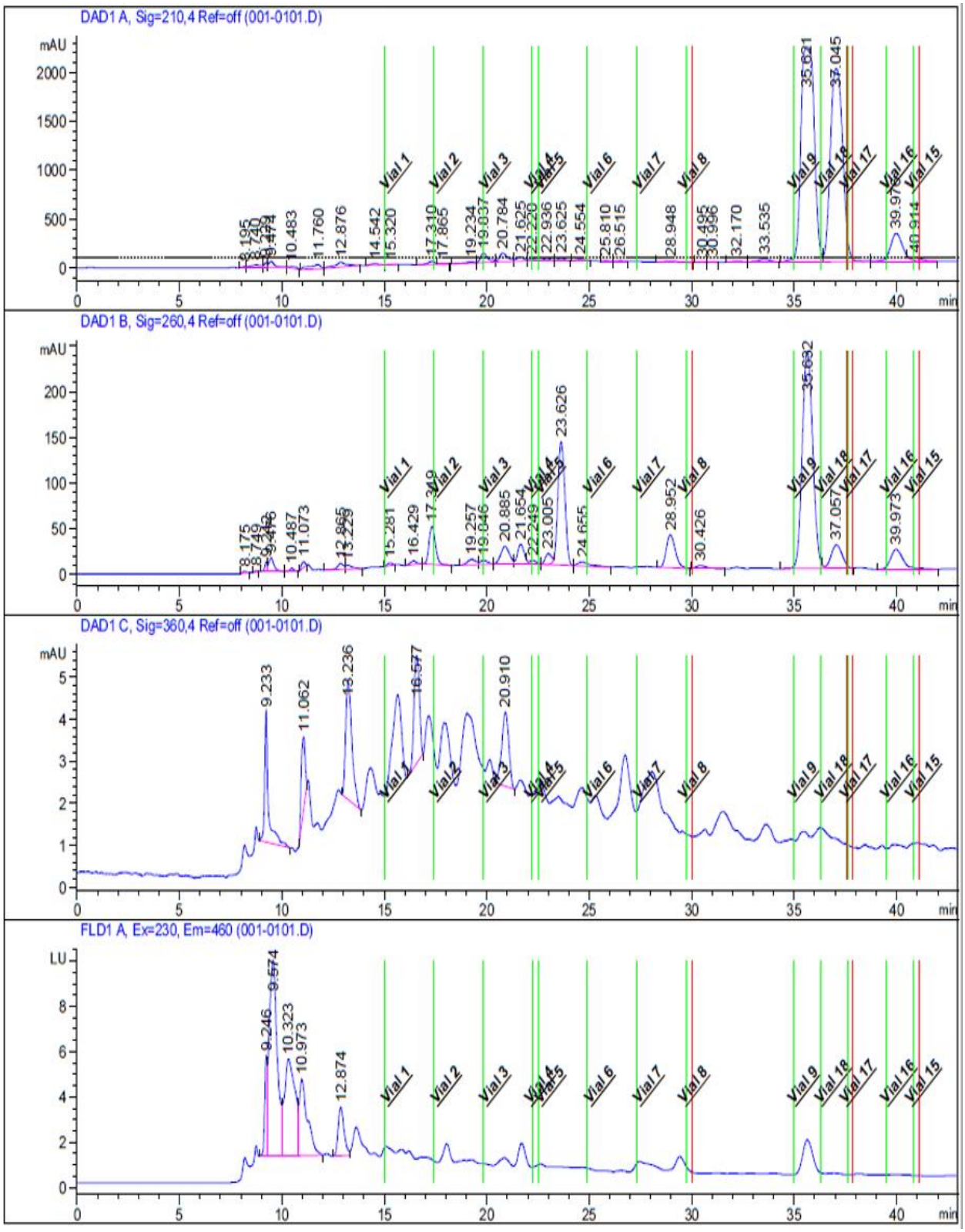


Figure 3.1: chromatograms showing sample collection at the auto-sample collection

DAD indicates the ultraviolet (UV) absorbance readings measures at different wavelengths: DAD1A at 210 nm, DAD1B at 260 nm, DAD 1C at 360 nm. FLD1 indicates the infrared (IR) reading measured at 230 nm. The measurement was performed using different UV wavelengths and an HPLC-FT-IR in order to ascertain the purity of the purified compounds. Purity obtained via HPLC was ≥ 98 %. Vials 9, 16 and 18 represents pure alantolactone, costunolide and isoalantolactone respectively. The green lines indicate the start and end point of sample collection. The red lines indicate sample collection but also an indication that less sample is collected at that vial position.

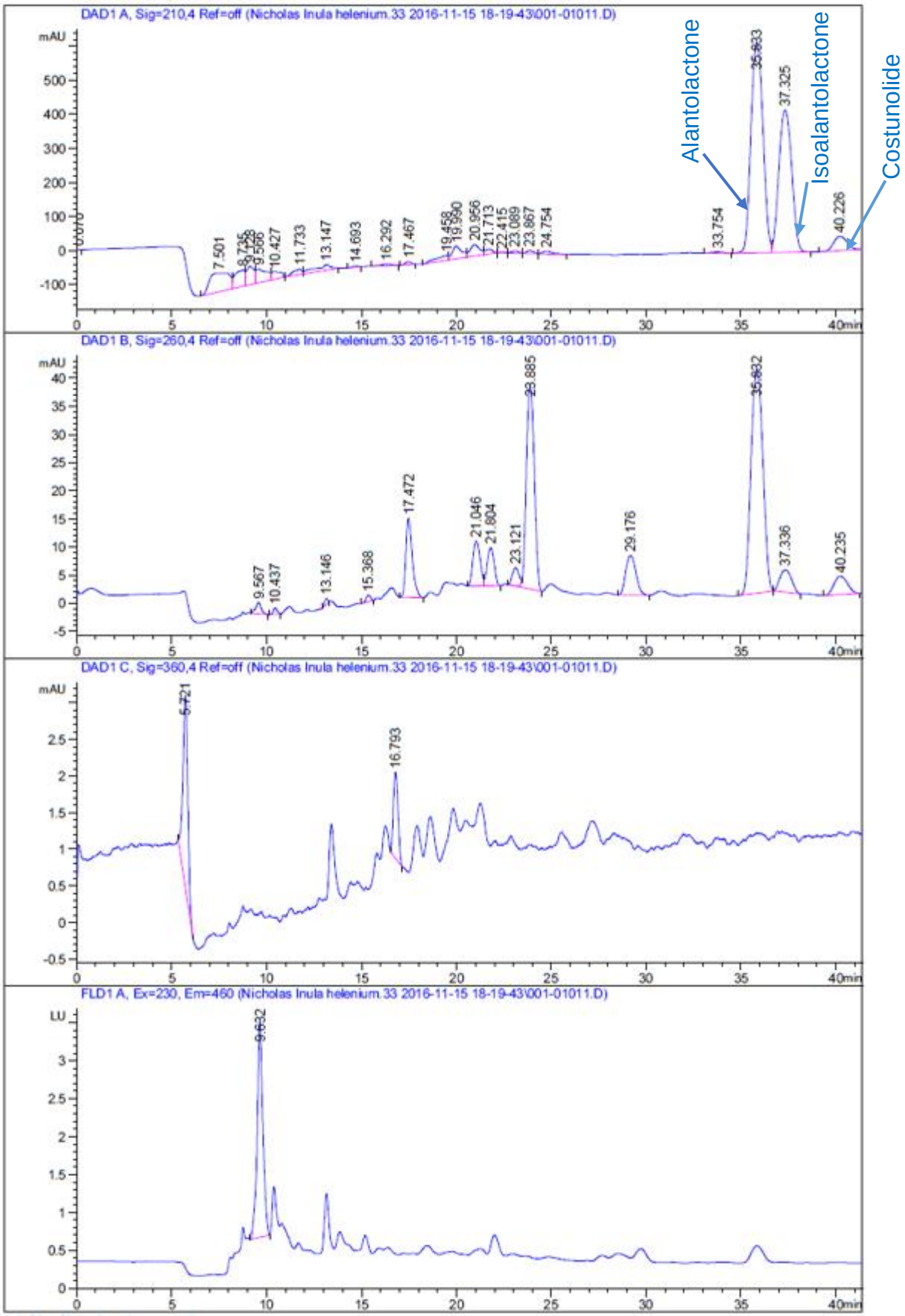


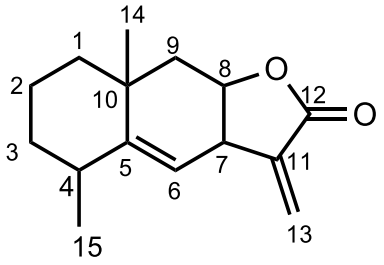
Figure 3.2: chromatograms obtained from the UV and HPLC-FT-IR detectors.

This represents the chromatogragram obtained at the HPLC detectors. Different wavelengths were used to ensure pure compounds with purity of over 98 % were obtained by HPLC. The clustering effect of the sesquiterpene lactones was overcome by introduction of 0.1% formic acid into the solvent medium.

Although costunolide has been isolated from some other plants, this is the first time costunolide has been isolated and characterised from *Inula helenium*. Figure 3.2 indicate the HPLC chromatogram of pure bioactive compounds isolated from *Inula helenium* – alantolactone, isoalantolactone and costunolide. It should be noted that to ensure these compounds were pure (above 98 %), different UV wavelengths: 210 nm, 260 nm and 360 nm and an HPLC-FT-IR wavelength 230 nm were used to detect the compounds in the chromatogram. From the chromatogram in Figure 3.2, pure compounds were isolated. The sesquiterpene lactones eluted late because the concentration gradient of methanol to water was increased from 50 % to over 60 % after 30 minutes of elution. Sesquiterpene lactones are poorly soluble in water and non-soluble in methanol. This is the first time costunolide, a known sesquiterpene lactone has been isolated from *Inula helenium* (by HPLC).

3.1.3.1.1 Alantolactone (D1)

Alantolactone (D1), was obtained as a white powdery precipitate by HPLC with spectroscopic data given below:



D 1

Structure 1.52: Alantolactone

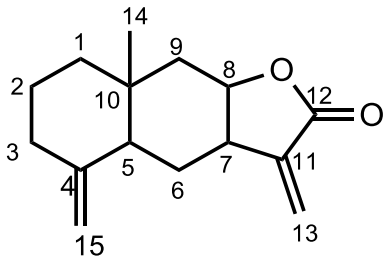
Elution time: 35.83 minutes (methanol : water (65 : 35 % v/v)); (1.3978 g, 36.3 %); m.p. 75 – 77 °C, R_f = 0.73 (Silica 60 Å, 1 : 1 hexane : ethyl acetate, v/v ^{1}H NMR (400 MHz, $CDCl_3$, δ ppm) 1.09 – 1.20 (4H, d, J= 6.7 Hz), 1.21 (3H, s), 1.42 – 1.50 (1H, dddd, J = 13.3, 10.2, 4.5, 3.1 Hz), 1.54 – 1.67 (4H, dtt, J = 13.4, 10.2, 3.2 Hz), 1.78 – 1.91 (1H, m, J = 13.4, 10.2, 3.2, 2.7 Hz), 2.12 – 2.17 (1H, tt, J = 13.4), 3.60 (1H, t, J = 6.7 Hz), 4.85 (1H, ddd, J = 10.3, 10.1, 3.5), 5.19 (1H, d, J = 6.9 Hz), 5.64 (1H, s), 6.22 (1H, s); ^{13}C NMR (100 MHz, $CDCl_3$, δ ppm) 16.73 (CH_3), 22.53 (CH_2), 28.56 (CH_3), 32.64 (CH_2), 32.70 (C), 37.58 (CH), 39.48 (CH), 41.71 (CH_2), 42.64 (CH_2), 76.42 (C-O), 118.75 (C), 121.64 (CH_2), 139.82 (C), 149.05 (C), 170.43 (C=O); MS (ESI) m/z: calculated for $C_{15}H_{20}O_2$ [M+H]+ = 232.1463, found 232.32 g/mol.

The first approach aimed at isolating pure compound (D1, D2 and D3) was by using varying concentrations of the methanol and acetonitrile. This was unsuccessful as these compounds (D1, D2 and D3) clustered together. Acetonitrile was replaced with water and a gradient of methanol : water (v/v) was allowed to run. It was still difficult to isolate pure compounds. In order to overcome this challenge, 0.1 % formic acid was introduced to the HPLC solvent system. Pure compounds were then isolated (Figure 3.2).

Alantolactone (D1) was isolated as a powdery white precipitate and its melting point was consistent with that reported in the literature (Naemura & Nakazaki, 1969). In the ^{1}H NMR spectrum of alantolactone (D1), the 3 protons (3 H, d, J = 6.9 Hz) having chemical shift δ 1.08 ppm, corresponds to the protons attached to position 15 (methyl group) of alantolactone. The 3 singlet protons at chemical shift 2.00 in D1 correspond to the methyl group at position 14 of D1. The 3 protons stated above appear as part of the multiplet of five protons (5 H) with a chemical shift range of δ 1.63 – 1.79 ppm. The other two protons are those at position 8 of D1. The 1 H multiplet, 1H triplet-triplet in the aliphatic region along with two deshielded 1H (triplet) and 1H (doublet-doublet-doublet) at δ 3.60 and 4.85 gave evidence for the lactone ring. The 13C NMR spectrum of D1 provided evidence for the presence of a carbonyl group through the signals at 170.43. It also provides evidence for the existence of a methelene bond at C-6 position of D1 in the ^{13}C NMR spectrum (δ 118.75 ppm). The mass of 232.32 g/mol is also consistent with the proposed structure.

3.1.3.1.2 Isoalantolactone (D2)

Isoalantolactone (D2), was isolated as a white needle-like precipitate by HPLC with spectroscopic data given below:



D 2

Structure: 1.53 Isoalantolactone

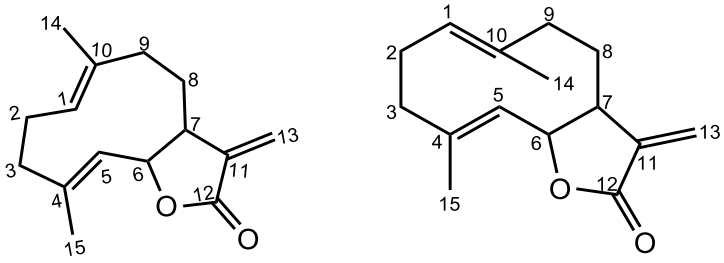
Elution time: 37.34 minutes (methanol : water (65 : 35 % v/v)); (1.2374 g, 32.14 %); m.p. 105 - 107 °C, R_f = 0.72 (Silica 60 Å, 1 : 1 hexane : ethyl acetate, v/v; ^{1}H NMR (400 MHz, $CDCl_3$, δ ppm) 0.83 (3H, s), 1.21 – 1.30 (1H, m, J = 13.3, 10.3, 10.2, 3.5, 2.6 Hz), 1.34 - 1.47 (1H, ddd, J = 13.2, 13.0, 2.6 Hz) 1.50 – 1.66 (4H, m, J = 14.2, 10.3, 10.2 Hz), 1.73

– 1.80 (1H, m, J = 14.2, 10.3, 10.2 Hz), 1.86 (1H, d, J = 3.2 Hz), 2.03 (1H, m, J = 14.4, 10.2, 3.5 Hz), 2.21 (1H, dd, J = 3.5, 3.2 Hz), 2.35 (1H, tt, J = 10.2, 10.0 Hz), 3.0 (1H, m, 10.2, 10.0, 2.4 Hz), 4.46 (1H, s), 4.51 (1H, t, J = 10.20 Hz), 4.79 (1H, s), 5.60 (1H, s), 6.14 (1H, s); ^{13}C NMR (100 MHz, $CDCl_3$, δ ppm) 17.63 (CH_2), 22.64 (CH_2), 27.42 (CH_3), 34.24 (CH_2), 36.77 (C), 40.48 (CH_2), 41.32 (CH), 42.14 (CH_2), 46.16 (CH), 77.32 (C-O), 106.57 (CH_2), 120.00 (CH_2), 142.17 (C), 148.92 (C), 170.59 (C=O); MS (ESI) m/z: calculated for $C_{15}H_{20}O_2$ $[M+H]^+$ = 232.1463, found 232.32 g/mol

Isoalantolactone (D2) was isolated as a white needle-like precipitate and its melting point was consistent with that reported in the literature (Naemura & Nakazaki, 1969). In the ^{1}H NMR spectrum of isoalantolactone (D2), the methyl group at position 15 of alantolactone is replaced by a methylene group ($C=CH_2$). In place of 3 protons in alantolactone, only 2 protons are present in isoalantolactone. This difference is observed as doublets at δ 4.65 ppm and 4.75 ppm with coupling constants 1.4 Hz respectively. The 3 singlet protons at δ 0.83 corresponds to the methyl protons attached to position 14 of D2. The ^{13}C NMR spectrum reveals the appearance of a methelene carbon at δ 120 ppm corresponding to C15. The ^{13}C NMR spectrum of D2 also reveals the absence of a peak at δ 118.75 ppm (as seen with D1). This observation is an indication that the methelene bond at position 6 of D1 is absent in D2. The ^{13}C NMR spectrum of D2 at position 6 gives a signal at δ 22.64 corresponding to a CH_2 group. The mass of 232.32 g/mol is also consistent with the proposed structure.

3.1.3.1.3 Costunolide (D3)

Costunolide (D3) was isolated as a white semi-crystalline needle-like precipitate by HPLC with spectroscopic data given below:



D 3

Structure 3.1: Costunolide

Elution time: 40.24 minutes (methanol : water (65 : 35 % v/v)); (0.0568 g, 1.48 %); m.p. 102 – 105 °C; R_f = 0.78 (Silica 60 Å, 1:1 hexane : ethyl acetate, v/v; ^{1}H NMR (400 MHz, $CDCl_3$, δ ppm) 1.44 (3H, s), 1.65 – 1.76 (4H, dddd, J = 13.6, 9.6, 5.6, 1.6 Hz), 2.01 – 2.38

(7H, m, J = 13.6, 9.6, 5.6, 1.6 Hz), 2.46 (1H, m, J = 13.6, 9.6, 5.6, 1.6 Hz), 2.60 (1H, ddd, J = 7.8, 5.6, 2.5 Hz), 4.60 (1H, t, 9.6 Hz), 4.75 (1H, dd, J = 9.3, 6.0 Hz), 4.88 (1H, dd, J = 9.3, 6.0 Hz), 5.64 (1H, d, J = 3.2 Hz), 6.25 (1H, d, J = 3.2 Hz); ^{13}C NMR (100 MHz, $CDCl_3$, δ ppm) 16.12 (CH_3), 17.35 (CH_3), 26.20 (CH_2), 28.04 (CH_2), 39.46 (CH_2), 40.99 (CH), 50.41 (CH_2), 81.92 (C-O), 119.68 (CH_2), 127.05 (CH), 127.27 (CH), 136.97 (C), 140.09 (C), 141.50 (C), 170.50 (C-O)**;** calculated for $C_{15}H_{20}O_2$ $[M+H]^+$ = 232.1463, found 232.32 g/mol.

Costunolide (D3) was isolated as a white semi-crystalline needle-like precipitate and its melting point was consistent with that reported in the literature (Naemura & Nakazaki, 1969). Costunolide (D3) has a chemical shift δ 1.43 ppm and appears as a singlet (3 protons) at position 15 of D3. In D3 there is ring opening at position 5. This opening gives a chemical shift of δ 1.73 ppm, s, corresponding to the 3 protons (methyl group) at position 14 of costunolide. The chemical shift δ 1.65 - 1.76 appearing as a 4-protons is reality are 3-protons. Evidence from ^{13}C NMR and ^{13}C-APT, gives an indication that the signal is actually a methyl group. The appearance of the methyl groups at chemical shifts δ 1.44 (singlet) and 1.65 – 1.76 (doublet-doublet) gives an indication that these methyl groups may be oriented asymetrically. To confirm this, ^{13}C NMR and ^{13}C-APT and two-dimensional ^{1}H NMR were done. In the ^{13}C NMR spectrum, chemical shifts were observed at very close range to each other in pairs at the aliphatic region – δ 16.12 and 17.35; 26.20 and 28.04; and 39.46 and 40.99 respectively. These signals give an indication that the carbon atoms represented by these signals may be asymmetrical in orientation. To verify this, ^{13}C APT (associated proton test) was conducted. From the APT signals (see appendix), the asymmetric nature of these carbons was verified. Signals at 16.12 and 17.35 ppm corresponds to the methyl carbon atoms at positions 14 and 15; signals at 26.20 and 28.04 ppm corresponds to CH_2. Signals at δ 39.46 and 40.99 also corresponds to CH_2 carbon atoms. The signal at δ 50.41 and 81.92 ppm gives an indication that a lactone ring is present in D3. The signal at δ 170.50 corresponds to a carbonyl carbon. The actual mass found in the mass spectrum (232.32 g/mol) was also in agreement with the proposed structure.

In the ^{13}C NMR spectrum, D1 has a chemical shift δ at 16.73 ppm (CH_3), D2 at 106.57 ppm (CH_2) and D3 at 17.35 ppm, corresponding to carbon atom 15 of these compounds respectively. The significant difference observed in the chemical shifts of D2 (106.57 ppm) compared to D1 (16.73 ppm) and D3 (17.35 ppm) is the presence of the methylene bond at C15 of D2.

In this study, the bioactive compounds were extracted by solid-liquid phase extraction using 70 % methanol (v/v) as solvent, and the extraction yield obtained was 8.61 %. This result is similar (10.50 %) to the one obtained by Chahmi et al. (2015) studying *I. viscosa* leaves from Morocco. Chahmi et al. (2015) also reported yields of 13.35 %, 20.8 % and 23.90 (percentage of dry matter w/w) using ethanol. These extracts were obtained from different regions of Morocco (Imouzzer, Sefrou and Taounate respectively). The extract obtained from this work is lower than values obtained by Bssaibis et al. (2009) in the same part of plant of the same origin. The variability of physicochemical properties influencing the extraction of bioactive compounds emanates from this structural diversity (Koffi et al., 2010). The variation observed can be explained by differences in the geographical origin of the plant, climatic factors, the harvest period, and the methodological used for extraction procedure.

Although a conventional method of extraction of natural products, SE offers some advantages over other methods, as fresh solvent is constantly coming into contact with the sample, enhancing the extraction of compounds from these samples (Luque de Castro & Priego-Capote, 2010), this constant contact between the sample and the solvent tends to override its draw-back of high solvent requirements, energy and time consumption (Luque de Castro & Priego-Capote, 2010). SE also produces an increased yield of compounds compared to other methods of extraction. Soxhlet extraction also has the ability of extracting both high molecular weight compounds and volatile compounds (Zhao & Zhang, 2014).

The increased yield obtained from methanol extract may be as a result of the polarity (dissolving power) of the hydroxyl group which combines with hydrogen to form water and water has a high dielectric constant (78.5 at room temperature) and this dielectric constant can be decreased by simply increasing its temperature (Hartonen et al., 2007). This enhances the solute-solvent interaction of water, thus the increased yield. On the other hand, less-polar solvents such as ethyl acetate and hexane are considered appropriate for extraction of non-polar compounds including lipids, terpenes and carotene, whereas water, methanol and ethanol are suitable for extraction of phenolic compounds (Barreira et al., 2014; Vázquez et al., 2013), anthocyanins and flavonoids (Pereira et al., 2016).

For **SE**, the results indicate that solvent polarity plays a role in determining the yield of compounds as previous studies have confirmed (Kaneria & Chanda, 2012; Pereira et al.,

2016). The low extraction yields recorded for ethyl acetate and hexane suggest these solvents are not very suitable for extraction of compounds from *Inula helenium* (in terms of yield). It can also be said that the polarity of the solvent, coupled with an increased temperature, potentiates the solubilizing ability of solvents on the analyte and subsequently decreasing the viscosity of solvents, and allowing a better penetration of solvents into matrices of dry matter (Bae et al., 2012; Pereira et al., 2016). The hydroxyl group in methanol which has the higher polarity than ethyl acetate therefore showed the highest yield, followed by ethyl acetate. It could be seen also that the yield of methanol was higher than the yield for hexane. The increased yield could also be attributed to the rehydration of the dried plant matter by water as rehydration entails: imbibing of vapour into dried organic matter, swelling and leaching of soluble substances (Krokida & Maroulis, 2001), especially if the dielectric constant of water is reduced by increasing its temperature (Hartonen et al., 2007). Water therefore plays a major role in the yield of extracts from *Inula helenium*, allowing cellular contents to be leached out of the cells. The result for SE indicates the important role played by solvents in the extraction yields of compounds isolated from *Inula helenium*; a trend that has been confirmed by various studies (Kaneria & Chanda, 2012; Yang et al., 2007).

For **MAE** (Table 3.2), the role of hydroxyl group in increasing the yield was also noticed. The polar solvent - methanol indicated the highest yields – 45.1 % (Scheme 3.3), compared to the non-polar or less polar solvents: hexane and ethyl acetate (15.6 % and 37.1 %). Polarity also plays an important role here as water appears to penetrate into the matrices of the dry matter, rehydrating the dry matter, and enabling the interaction between solute and solvent, leading to the leaching of soluble substances from the plants (Bae et al., 2012; Krokida & Maroulis, 2001), and the efficiency of extraction is potentiated by the effect of increased temperature and pressure within the microwave (Zhao & Zhang, 2014). However, the yield indicated for MAE was similar (Table 3.2) to the corresponding yields for SE and UAE using less polar solvents – hexane and ethyl acetate. In this situation, temperature and pressure (but not polarities) are the major parameters influencing the extraction efficiency. As the MAE begins, the pressure and temperature of the microwave increases, and these conditions favour a rise in the fluid density and a corresponding increase in solubility of the solute especially as the pressure begins to increase (Zhao & Zhang, 2014). Thus, microwave enables the rapid penetration of solvents through dry tissues, breaking them, and releasing substances in the tissues.

Because MAE radiation is non-ionised, extraction of materials depends on the heating effect and substances which can absorb the energy generated by the microwave can be heated thus solvent free extraction can occur in a microwave for extraction of especially volatile products. Another factor responsible for the increased yield recorded for the MAE is the choice of solvent used as compounds from *Inula helenium* are relatively soluble in the solvents used. As the compounds tend to dissolve in the extraction solvent, a solvent film is established and a sort of an equilibrium which allows solvents influx into the cells, and solute efflux out of the cells, leading to swelling of the cell. As the cell swells, the increased pressure and the high temperature in the cellular compartment cause the cell to burst, releasing its contents into the solution.

UAE employing polar solvents – methanol indicated the highest yields 39.9 % compared to less polar solvents – ethyl acetate (31.9 %) and hexane (27.9 %). Among the different extraction techniques used in this study, UAE indicated the lowest yields of compounds compared to SE and MAE. This is an indication that an increase in temperature and pressure (as in SE and MAE) potentiates the yield of extracts from *Inula helenium*. Polarity of the solvent also potentiates the yields of extracts from *Inula helenium*. However, an increased yield of extracts from hexane fraction using UAE was recorded compared to the percentage yield using other extraction techniques.

Interestingly, there was a significant difference ($p < 0.0004$) between SE and UAE using non-polar solvent - hexane (Table 3.2), with a higher yield recorded for methanol. This is an indication that the amplitude of sonication (in UAE) is mainly responsible for disruption of the cell walls of plant by the phenomena of cavitation, increasing surface area of contact between the solute and solvent, allowing influx of solvents into the plant material and a subsequent rupture of cell walls and release of solutes into the solvent media (Jerman et al., 2010; Kazemi et al, 2016) but not the effect of temperature and pressure as seen in the extraction yields recorded for SE. The increase in extraction yield using polar solvents, compared to ethyl acetate and hexane is an indication that the combination of sonication and polar solvents, sends down shock-waves into the cell wall, and by imbibing the water (polar solvents) into the cells, disruption of the cell membrane occurs because cavitation produces microscopic bubbles. This releases the cell contents into the solvent. Thus polarity (in SE) potentiates the efficiency of extraction of compounds due to the interaction of the -OH group in methanol which interacts with hydrogen atom to form water, which is imbibed by the dry plant matter, causing it to swell, rupture and burst; releasing its contents into the extraction system. The increased

extraction yield using MAE is also an indication that the increased pressure and temperature accelerates the swelling and softening of compounds, and violently releasing them thus the increased yield for MAE compared to UAE (Tao & Sun, 2015; Yao, 2016). The effect of a combination of increased temperature (≥ 50 ºC) and polarity in increasing the ability of solvent to solubilize the analyte, with a subsequent increased yield of extracted compounds has been reported (Bae et al., 2012; Gardeli et al., 2008), which confirms the findings in this work. Increasing the temperature also decreases the viscosity of the extraction solvent (especially the polar solvents), allowing a better penetration of solvent into the matrix (Bae et al., 2012; Gardeli et al, 2008).

This finding indicates that UAE using hexane, resulted in an increase yield of compounds from *Inula helenium* compared to other extraction techniques and UAE is an important extraction technique when increase in yield is a factor to consider and hexane is important in the extraction of sesquiterpene lactones from natural products as we found out in the course of this project. Although the percentage yield recorded using methanol as an extraction solvent was higher than those obtained using hexane, it was found that the hexane fraction indicated a high yield of sesquiterpene lactones (which are the bioactive components isolated from *Inula helenium* – chapter 3) whereas the methanol fraction contained biologically inactive constituents extracted from *Inula helenium*. Of interest is the fact that UAE using hexane produced the highest yield (%) (Scheme 3.4) compared to other extraction solvents and the biologically active components were isolated from the hexane fractions.

It has been documented that UAE offers a better extraction yield of up to 50 % at room temperature compared to other extraction methods and the extraction time was reduced to 10 hours instead of 28 days for conventional extraction methods and an increased yield is often obtained when the temperature is increased from room temperature to a temperature over 60 °C (Vinatoru 2001) and UAE is a technique of choice used in the isolation of compounds of choice compared to other methods as reported in this project. A similar finding was also reported by Sun et al., (2011) and Sun (2011) while comparing Soxhlet extraction, maceration and other extraction techniques. They reported low solvent consumption and an increased extraction yield for extraction of isoflavones from *Inula tectorum* using UAE and in addition to the increased extraction yield of the targeted compounds, a significant shorter process and extraction time was recorded compared to other extraction techniques. This findings by Sun et al. (2011) and Sun (2011) agrees with our findings where we report an increased extraction yield of targeted compounds

(sesquiterpene lactones) from *Inula helenium*) using hexane and a shorter extraction time compared to other extraction methods.

UAE using hexane also provided a good selectivity for extraction of sesquiterpene lactones which were the major components of the hexane fraction of compounds isolated from *Inula helenium*. Ultrasound like any sound wave is propagated through rarefaction and compression waves which is induced in the medium and molecules through which it passes. When the rarefaction and compression power become so great, it exceeds the forces of attraction of the molecules, forming cavitation bubbles in the liquid. Rectified diffusion therefore results, allowing the bubbles to grow. This implies that gasses or vapour enters the bubble from the medium and this bubble is trapped inside the molecule and is not expelled during the compression and expansion phase. As successive cycles of compression occur, energy is generated, and this great intensity and frequency of compression allows cellular contents to be emptied into the extraction medium (Sun et al., 2010; Sun et al., 2011).

Cavitation is responsible for UAE and operates via the passage of ultrasonic waves across the solvent; cavitation exerts some hydrodynamic forces creating small localised vacuums in the solvent which implodes rapidly, creating shock waves on the sample matrix as the solvents pass through the sample; this allows greater penetration of solvents through the biological organisation, increasing the area of interaction between the liquid and solid phase (Yolmeh et al., 2014). UAE can drastically decrease particle size thereby increasing reactive surfaces. The solvents used were methanol, ethyl acetate and hexane.

The result from this finding indicates that UAE provided a better yield when compounds from *Inula helenium* were extracted using hexane. This improved yield could be attributed to the collapse of acoustic cavity within the molecule as a result of improved mass transfer which disrupts the cell, milling it, with a consequent improved penetration of the solvent into the matrices of the molecule (Dezhkunov & Leighton, 2004; Luo et al., 2013). In addition, there is a possibility that the application of an ultrasound generates an ultrasonic capillary effect. This effect is related to anomalous rise of a liquid in a capillary tube. This effect was discovered in 1950 (Luo et al., 2013). Logically, it is supposed that plant material has capillaries which could exert the capillary effect. This effect has been ignored since its discovery. It has been linked to sonoluminescence (Dezhkunov & Leighton, 2004). There is usually a movement of materials contained in pores of plant materials.

Luo et al., (2013) proved that they exist alternating electrical field at both ends of a capillary tube containing an electrolyte solution when an ultrasound is applied. There are certainly ionic / polar compounds in plants which can occupy the capillaries and crevices within the plant material which could be affected by the capillary effect.

0.5 g of compounds extracted from the respective extraction techniques were tested against several cancer cell lines and bacterial strains and compounds extracted from the hexane fraction indicated good cytotoxic activities against the cancer cell lines and bacterial strains tested; these compounds were further isolated and purified by other purification techniques including High-performance liquid chromatography (HPLC). After preparation of samples as stated in Scheme 3.5, dry-freeze precipitates obtained from the supernatant (3.85 g) were diluted in 70 % methanol (v/v) and eluted via the HPLC system. Bioactive compounds isolated from *Inula helenium* were isolated / purified via the high-performance liquid chromatography (HPLC) to obtain three-known bioactive sesquiterpene lactones – alantolactone (ALA), isoalantolactone (ISL) and costunolide CST). ALA and ISL has been previously isolated from *Inula helenium* (Jiang et al., 2011; Lawrence et al., 2001; Qiu et al., 2011; Seo et al., 2008; Trendafilova et al., 2010). However, this is the first time costunolide has been isolated from the roots of *Inula helenium*. CST has been isolated previously from the root extracts of *Saussurea costus* (Eliza et al., 2009). ALA and ISL were previously isolated from the petroleum ether fraction of *Inula helenium* using the reverse phase C18 column chromatography (Jiang et al., 2011; Lawrence et al., 2001). This finding agrees with the findings in this work as petroleum ether is a non-polar solvent having the same polarity as hexane (0.1). Most authors isolated ALA and ISL using column chromatography which involves multiple steps of purification via collection of fractions, comparing similar fractions and oftentimes fractions obtained are relatively impure. HPLC (which was used in purification of ALA, ISL and CST) offers a better advantage over column chromatography as compounds can be monitored and purified via an auto-detector. ALA and ISL were isolated using a gradient of 55 % methanol and 45 % water. ALA was isolated first at a retention time of 35.62 minutes, followed by ISL at a retention time of 37.04 minutes. This observation differed from those of Xiao & Hu, (2007) and Huo et al., (2010) who isolated ISL first, followed by ALA using Agilent Zorbax XDB-C18 column and Phenomenex Kromasil C18 columns respectively. In this study, Agilent ACE C18-Amide coloum was used in purifying the compounds via HPLC. This may be attributed to the increased polar retention and alternative selectivity of ACE C18-Amide column with ability to efficiently separate polar,

phenolic, acidic and basic compounds. ACE C18-Amide column can also separate products efficiently at pH ranging from 2 – 11 (Schellinger et al., 2005; Schellinger et al., 2008a). Agilent ZORBAX Eclipse XDB columns – C18 is efficient at a pH range of 2-3, and 6-8. Tailing interactions with compounds to be separated usually occur in the Agilent ZORBAX Eclipse XDB columns – C18 columns at pH range outside the one stated above (Schellinger et al., 2008a; Schellinger et al., 2008b). This tailing effect is due to the silanols constituents of this column. This tailing interaction is also common with the Phenomenex Kromasil C18 columns (Schellinger et al., 2008a). The tailing effect, and lack of increased polar retention and alternative selectivity of the later columns may be responsible for the appearance of ISL before ALA in the HPLC fractions.

ALA and ISL were isolated as white powders. The LC-MS spectrum displayed a molecular ion peak at m/z 232.2 $[M+Na]^+$ consistent with a molecular formula of $C_{15}H_{20}O_2$. This finding is consistent with those reported by other authors (Jiang et al., 2011; Lawrence et al., 2001; Huo et al., 2010). CST was isolated as a crystalline solid. The HR-ESI-MS spectrum displayed a molecular ion peak at m/z 232.1538 $[M+Na]^+$ consistent with a molecular formula of $C_{15}H_{20}O_2$. This finding is also consistent with those previously reported by Eliza et al., (2009) who isolated costunolide from the roots of *Costus speciosus*.

It was not surprising isolating costunolide from the root extracts of *Inula helenium* using HPLC. This is because previous studies focussed mainly on the use of column chromatography (Huo et al., 2010; Lawrence et al., 2001) or the use of acetonitrile / water as the mobile phase (Yang et al., 2017) instead of methanol (used in this study). A higher concentration of acetonitrile (≥55%) in the mobile phase is associated with poor resolution and separation of ALA and ISL (Yang et al., 2017). It therefore suggests that the type of solvent used in the mobile phase in the separation of sesquiterpene lactones could affect the resolution or isolation of bioactive compounds. Other authors have not reported the use of centrifugation technique in the isolation of compounds from *Inula helenium*. By centrifuging the hexane fraction, it was possible to force the tiny amount of costunolide which was in the suspension into the pellets. Considering the tiny amount of costunolide isolated from hexane fraction (1.48 %), compared to the yield of alantolactone (35 %) and isoalantolactone (32.14 %), other authors may have missed the fractionation of costunolide via the use of column chromatography and / or acetonitrile (associated with poor resolution and separation of ALA and ISL).

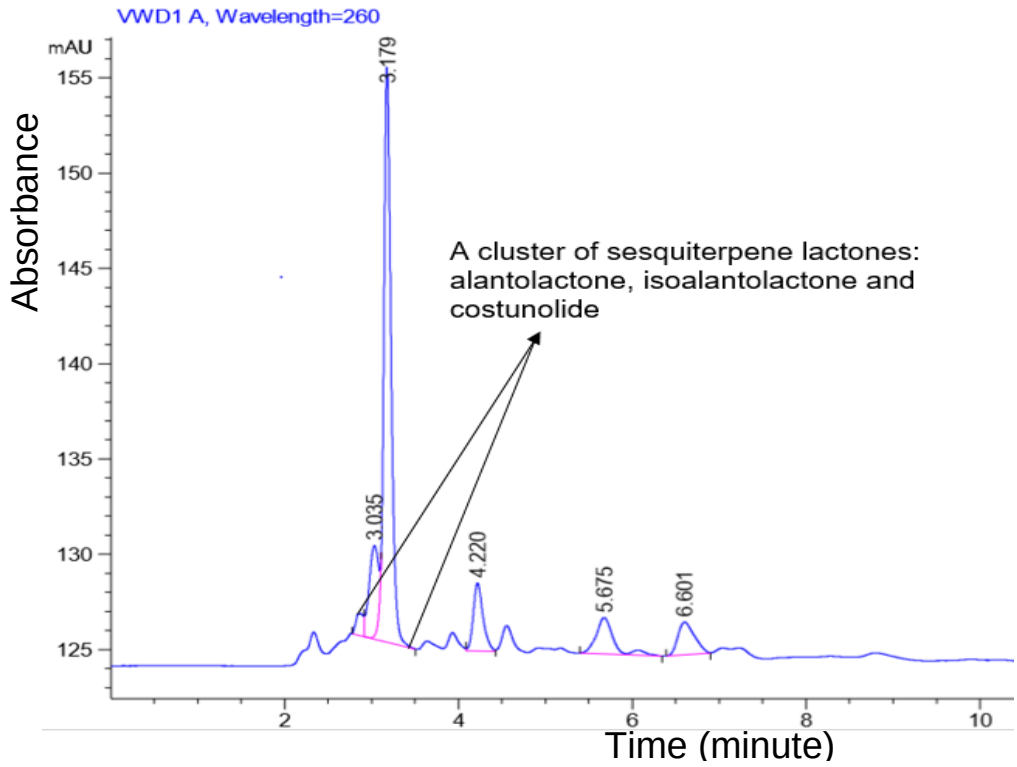


Figure 3.3: Peaks were poorly resolved.
Good peak resolution was achieved via the introduction of 0.1 % formic acid (Figure 3.2).

It was possible to isolate CST from the roots of *Inula helenium* because 0.1 % formic acid was used to enhance good peak resolution. The effect of use of formic acid or metallic ion (such as silver ion) in enhancing good peak resolution has previously been reported (Dobson et al., 1995; Yang et al., 2017). It is possible that costunolide or other compounds may be appearing on the chromatogram as impurities or clustered to alantolactone or isoalantolactone due to the tiny amount of this compound in *Inula helenium* (1.48 % - isolated from hexane fraction). The effect of peak clustering was observed (Figure 3.3) when 0.1 % formic acid was not introduced thus preventing proper peak resolution. Huo et al., (2010) and Lawrence et al., (2001) did not indicate the use of formic acid for good resolution of peaks. This may account for the inability to isolate costunolide from *Inula* specie. It is also possible that the geographical location, climate and season where the plant's (*Inula helenium*) extract was collected influenced the quantity of phytochemicals present in the plant extract (Chahmi et al., 2015).

Chapter 4 Chemical Synthesis of Modified Chrysin Derivatives to

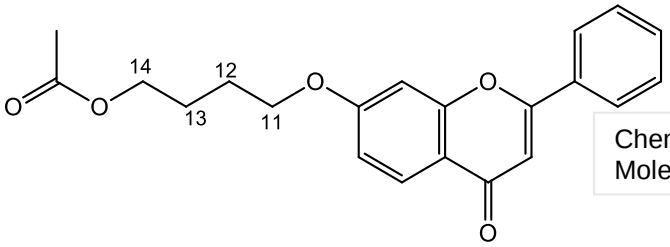
This chapter is a presentation of data for chemical synthesis of modified chrysin-derivatives using different synthetic methods such as microwave irradiation, stirring at regulated temperatures and / or the use of catalysts. Reaction conditions were modified many times during the experiments to synthesise compounds with high yield and purity.

It should be noted that the mass spectroscopy data for novel compounds are presented to 4-decimal places (accurate mass spectroscopy data) whereas data for known data is reported to 2-decimal places (measured using the LC-MS).

The proton NMR data as well as the mass spectrometry data for novel compounds are presented in the appendix.

4.1 4-((5-Hydroxy-4-oxo-2-phenyl-4H-chromen-7-yl)oxy)butyl acetate (C1) or 7-O-Chrysinbutyl acetate – novel compound

C1 was synthesised as described in section 2.2.4.1 (page 125). C1 is a novel compound and the yield / spectral data can be found below:



Chemical formula: $C_{21}H_{20}O_6$
Molecular Weight: 368.13 g/mol

Structure 4.1 – Compound C1

Method A: Yield (0.196 g, 80.2 %); m.p. 122 – 124 °C; R_f = 0.74 (Silica 60 Å, 2:1 ethyl acetate: petroleum ether, v/v).

Method B: Yield 0.166 g, 68.0 %); m.p. 122 – 124 °C; R_f = 0.75 (Silica 60 Å, 2:1 ethyl acetate: petroleum ether, v/v).

Spectral data:

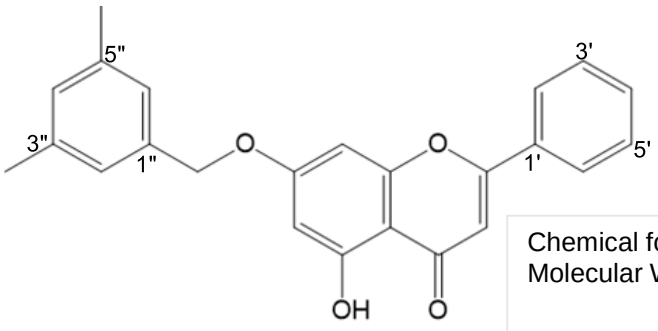
^{1}H NMR (400 MHz, $CDCl_3$, δ ppm) 1.83-1.94 (4H, 1.87 (quint, *J* = 7.5 Hz), 1.90 (tt, *J* = 7.5, 7.1 Hz)), 2.09 (3H, s), 4.06-4.24 (4H, 4.09 (t, *J* = 7.1 Hz), 4.18 (t, *J* = 7.5 Hz)), 6.38 (1H, d, *J* = 2.2 Hz), 6.51 (1H, d, *J* = 2.2 Hz), 6.69 (1H, s), 7.50-7.60 (3H, 7.53 (dddd, *J* = 7.8, 7.3, 1.5, 0.4 Hz), 7.57 (tt, *J* = 7.3, 1.5 Hz)), 7.92 (2H, dtd, *J* = 7.8, 1.4, 0.4 Hz, 12.71 (1H, s, 5-OH); ^{13}C NMR (100 MHz, $CDCl_3$, δ ppm) 20.99 (CH_3), 25.29 (CH), 25.63 (CH), 63.92 (OCH_2), 67.96 (OCH_2), 93.12 (CH), 98.53 (CH), 105.71 (CH), 105.90 (C), 126.29

(2', CH), 126.29 (6', CH) 129.10 (3' CH), 129.10 (5', CH), 131.34 (4', CH) 131.84, (1', C) 157.80 (C-O), 162.17 (C-O), 163.98 (C-OH), 164.91 (C-O), 171.15 (COO), 182.46 (C=O); IR 1165.42 cm^{-1} (C-O-C, large ring C-O stretch), 1031.25 cm^{-1} and 1242.32 cm^{-1} (C-O, phenolic), 1588.12 cm^{-1} (C=C aromatic), 1605.21 cm^{-1} (C=O, ketone), 1751.27 cm^{-1} (O-C=O); MS (ESI) m/z: calculated for $C_{21}H_{20}O_6$ $[M+H]^+$ = 368.1338, found 368.1330 g/mol.

The initial reaction conditions used require stirring the reaction mixture at 30 °C for 12.0 hours. 2.0 eq potassium carbonate and 1.0 eq of 4-bromobutyl acetate were used. This produced 22 % yield. It was found that the reaction conditions could be optimised to give a maximum yield of 68 % when the mixture was stirred at 70 °C for 20 hours and the concentration of potassium carbonate increased to 4 eq and 4-bromobutyl acetate increased to 2.0 eq. In the ^1H NMR spectrum of the novel compound – C1, the two methylene protons at positions 12 and 13 provides evidence for the proposed structure and they appear as 4-protons at δ 1.83 – 1.94 ppm. The two methylene protons at positions 11 and 14 appears at δ 4.06-4.24 ppm because they are deshielded compared to the former protons. It should be noted that the protons at position 14 would appear first before those at position 11 because the protons at position 14 are attached to an oxygen that is oriented towards the aliphatic region whereas the protons at position 11 are attached to the oxygen that is oriented toward the aromatic region. This provides further evidence for the propose structure. The proton δ 12.80 ppm corresponds to the hydroxyl group at the 5-OH position of the molecule. In the ^{13}C NMR spectrum, the signals δ 63.92 (C-14) and 67.96 (C-11) provides further evidence for the synthesis of C1. The mass spectroscopy data (ESI) m/z of 368.1330 g/mol is also consistent with the proposed structure.

4.2 7-((3,5-Dimethylbenzyl)oxy)-5-hydroxy-2-phenyl-4H-chromen-4-one (C2) or 7-O-Chrysin-3,5-dimethylbenzene – novel compound

C2 was synthesised as described in section 2.2.4.2 (page 126). C2 is a novel compound and the yield / spectral data is presented below:



Chemical formula: $C_{24}H_{22}O_4$
Molecular Weight: 372.14 g/mol

Structure 4.2 – Compound C2

Spectral data:

^{1}H NMR (400 MHz, $CDCl_3$, δ ppm) 2.29 (6H, s), 4.49 (2H, s), 6.36 (1H, d, *J* = 1.9 Hz), 6.50 (1H, d, *J* = 1.9 Hz), 6.58 (1H, s), 6.91 (1H, t, *J* = 1.9 Hz), 6.98 (2H, dd, *J* = 1.9, 1.4 Hz), 7.40-7.50 (3H, 7.46 (dddd, *J* = 7.8, 7.3, 1.5, 0.4 Hz), 7.80 (2H, dd, *J* = 7.8, 1.4, 0.4 Hz), 12.80 (1H, s, 5-OH); ^{13}C NMR (100 MHz, $CDCl_3$, δ ppm) 21.31 (CH_3), 21.31 (CH_3), 70.63 (OCH_2), 93.54 (CH), 98.94 (CH), 105.85 (CH), 105.91 (C), 125.39 (CH), 125.39 (CH) 126.31 (2', CH), 126.31 (6', CH), 129.10 (3' CH), 129.10 (5' CH), 130.07 (4', CH), 131.35 (1', C), 131.84 (CH), 135.56 (C), 138.42 (C), 138.42 (C), 157.77 (C-O), 162.20 (C-O), 164.01 (C-OH), 164.81 (C-O), 182.50 (C=O); IR 1158.49 cm^{-1} (C-O-C, large ring C-O stretch), 1029.80 cm^{-1} and 1246.45 cm^{-1} (C-O, phenolic), 1583.85 cm^{-1} (C=C aromatic), 1602.10 cm^{-1} (C=O, ketone), 2854.31 (C-H, alkane), 3001.46 cm^{-1} (C-H, aromatic); MS (ESI) m/z: calculated for $C_{24}H_{22}O_4$ $[M+H]^+$ = 372.1439, found 372.1433 g/mol.

It was difficult to make compound (C2) with the initial conditions used involving the use of acetone and methanol, and temperature ranging from 100 - 130 °C in a microwave reactor for a time period ranging from 10 – 15 minutes. Precipitates – a mixture of brown, brick-red and orange colours were obtained. Attempts were made to purify these precipitates without success as the targeted product was not synthesised. TLC analysis indicated the presence of lots of impurities and debris.

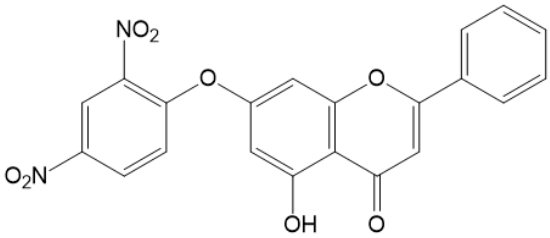
The same conditions stated above were used in an attempt to synthesise (C2) except that the reaction was stirred at 70 °C for 24 hours; a microwave reactor was not used. It was still difficult to make (C 2). The solvents were changed, and acetonitrile used. The reaction was stirred at 70 °C for 24 hour and monitored using a TLC after it was purified via the chromatographic column. This time, 26 % yield was obtained. In order to optimise the yield, the reaction conditions were optimised by stirring the reaction at room temperature for 4 hours in acetonitrile. A yield of 81.3 % was recorded.

In the ^{1}H NMR spectrum of chrysin adduct (C2), the singlet proton (2H, s, OCH_2 aromatic) and having chemical shift δ 4.49 ppm, corresponds to the point of attachment of 1-(bromomethyl)-3,5-dimethylbenzene. The proton 6H corresponding to two methyl groups on 1-(bromomethyl)-3,5-dimethylbenzene having chemical shift of 2.25 (s) ppm, which is absent in the chrysin molecule. The proton δ 12.80 ppm corresponds to the hydroxyl group at the 5-OH position of the molecule. In the ^{13}C NMR spectrum, the signals at δ 138.42 ppm corresponds to the C-1" and C-5" whereas the signals for C-1' and C-4"

(which exist in a similar chemical environment is seen at δ 131.35 (C-1') and 131.84 (C-4"). This is an evidence for the synthesis of the proposed compound. The mass spectroscopy data (ESI) m/z of 372.1433 g/mol is also consistent with the proposed structure.

4.3 7-(2,4-Dinitrophenoxy)-5-hydroxy-3-phenyl-4H-chromen-4-one (C3) or 7-O-Chrysin-2,4-dinitrobenzene – novel compound

C3 was synthesised as described in section 2.2.4.3, page 123. C3 is a novel compound and the yield / spectral data is sated below.



Chemical formula: $C_{22}H_{16}O_8$
Molecular Weight: 420.32 g/mol

Structure 4.3 – Compound C3

Method A: Yield (0.109 g, 46.2 %); m.p. 292 - 294 °C; R_f = 0.49 (Silica 60 Å, 2:1 ethyl acetate: petroleum ether, v/v.

Method B: Yield (0.211 g, 89.6 %); m.p. 292 - 294 °C; R_f = 0.47 (Silica 60 Å, 2:1 ethyl acetate: petroleum ether, v/v).

Spectral data:

^{1}H NMR (400 MHz, DMSO-d_6, δ ppm) 6.73 (1H, d, *J* = 2.1 Hz), 7.12 (1H, d, *J* = 2.1 Hz), 7.20 (1H, s), 7.57-7.69 (4H, 7.60 (dd, *J* = 7.9, 0.5 Hz), 7.63 (dddd, *J* = 7.8, 7.3, 1.5, 0.4 Hz), 7.66 (tt, *J* = 7.3, 1.5 Hz)), 8.16 (2H, dtd, *J* = 7.8, 1.4, 0.4 Hz), 8.60 (1H, dd, *J* = 7.9, 1.9 Hz), 8.90 (1H, dd, *J* = 1.9, 0.5 Hz), 13.00 (1H, s, 5-OH); ^{13}C NMR (100 MHz, DMSO-d_6, δ ppm) 98.02 (CH), 102.02 (CH), 105.72 (CH), 107.72 (C) 122.04 (CH), 122.95 (CH), 126.60 (2', CH), 126.60 (4', CH), 126.60 (6', CH), 129.16 (3', CH), 129.16 (5', CH) 130.23 (1', C), 132.45 (CH), 140.65 (C), 143.18 (C), 152.43 (C-O), 157.14 (C-O), 160.43 (C-O), 161.76 (C-OH), 163.02 (C-O), 182.43 (C=O); IR 1149.55 cm^{-1} (C-O-C, large ring C-O stretch), 1025.24 cm^{-1} and 1248.32 cm^{-1} (C-O, phenolic), 1358 cm^{-1} and 1535 cm^{-1} (N=O, aromatic) 1593.47 cm^{-1} (C=C aromatic), 1623.80 cm^{-1} (C=O, ketone); MS (ESI) m/z: calculated for $C_{22}H_{16}N_2O_8$ $[M+H]^+$ = 420.0671, found 420.0661.

Synthesis of (C3) via method B offered an increased yield compared to method A. However, method A offered a quick synthesis of C3.

The ^{1}H NMR spectrum shows a doublet at δ 8.98 ppm and a doublet at 8.57 ppm corresponding to the protons at the point of attachment of 1-bromo-2,4-dinitrobenzene. It also has a multiplet (4 H) at δ 7.51 – 7.67 ppm, corresponding to the protons at 3', 4' and 5' positions of chrysin, and C-6" of (C 3). For chrysin, the chemical shift of the protons at 3', 4' and 5' positions is a multiplet (3 H) at δ 7.56 – 7.69 ppm. The proton corresponding to 5-OH is a singlet at δ 12.95 ppm for all Series 1 compounds. In the ^{13}C NMR spectrum, aliphatic carbon atoms were absent as seen in C1 (δ 20.00 – 50 ppm). The signals appeared beginning from δ 98.02 ppm (corresponding to a C-O signal). The absence of aliphatic carbon signals is consistent with the proposed structure. Narrow stretch vibrations were observed at 1358 and 1535 cm^{-1} corresponding to NO_2 in the IR spectrum. The mass spectroscopy data (ESI) m/z of 372.1433 g/mol is also consistent with the proposed structure.

Series 1 compounds – C1 and C2, are soluble in chloroform and ethyl acetate (R_f values 0.75 and 0.71). C3 is partially soluble in chloroform and ethyl acetate (R_f = 0.49) and soluble in dimethyl sulfoxide (DMSO).

A higher yield was obtained from method A (synthesis using microwave reactor) for C1 80.2 % compared to the yield from method B (stirring for 24 h) – 68.0 %. This was different for C2 and C3 which recorded poor yields with method A (53.34 and 46.2 %) compared to the increase yields recorded for C2 and C3 using method B (83.1 and 89.6 %).
In order to investigate the degrading effect of the benzene ring by the microwave, the reaction time for synthesis of these compounds – C1 and C2 were reduced from 10 minutes to 5 minutes (other conditions remaining constant), the impurities recorded on the TLC plate for C2 and C3 faded. To investigate further this observation, the reaction time was increased from 10 minutes to 14 minutes; more impurities were observed on the TLC plate compared to those observed at 10 minutes irradiation. This finding suggests that when 7-O-chrysin adducts containing benzene rings are irradiated in the microwave reactor for longer periods (≥ 10 minutes), degradation of these compounds may occur.

4.4 Synthesis of Methylated 7-O-alkylchrysin derivatives

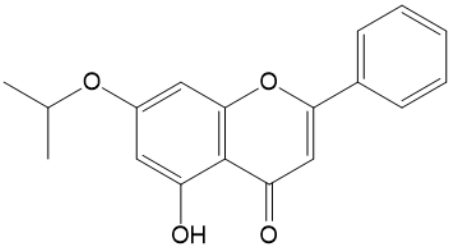
One of the aims of this project is to synthesise methylated 7-O-alkylchrysin derivatives and to study the antimicrobial effect of alkylation of these derivatives. The first compound synthesised in this category is 5-hydroxy-7-isopropoxy-3-phenyl-4H-chromen-4-one.

4.4.1 5-Hydroxy-7-isopropoxy-3-phenyl-4H-chromen-4-one (C4) or 7-O-Isopropylchrysin

C4 was synthesised as described in section 2.2.3.1, page 124. The yield / spectral data is presented below.

Method A: Yield (0.209 g, 95.0 %); m.p. 145 – 146 °C; R_f = 0.58 (Silica 60 Å, 2:1 ethyl acetate: petroleum ether, v/v).

Method B: Yield (0.138 g, 62.7 %) m.p 145 – 146 °C; R_f = 0.58 (Silica 60 Å, 2:1 ethyl acetate: petroleum ether, v/v).



Chemical formula: $C_{18}H_{16}O_4$
Molecular Weight: 296.32 g/mol

Structure 4.4 – Compound C4

Spectral data:

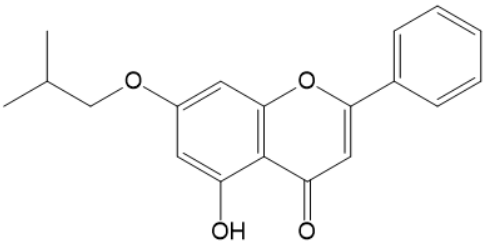
^{1}H NMR (400 MHz, $CDCl_3$, δ ppm) 1.42 (6H, d, *J* = 6.6 Hz), 4.67 (1H, sept, *J* = 6.6 Hz), 6.38 (1H, d, *J* = 2.2 Hz), 6.50 (1H, d, *J* = 2.2 Hz), 6.68 (1H, s), 7.58 (3H, dd, *J* = 1.5, 0.4 Hz), 7.9 (2H, dtd, *J* = 7.8, 1.4, 0.4 Hz, 12.74 (1H, s, 5-OH); ^{13}C NMR (100 MHz, $CDCl_3$, δ ppm) 21.92 (CH_3), 21.92 (CH_3), 70.78 (C-O), 93.94 (CH), 99.28 (CH), 105.42 (CH), 105.81 (C), 126.27 (2', CH), 126.27 (6', CH), 129.07 (3', CH), 129.07 (5', CH), 131.38 (4', CH), 131.79 (1', C), 157.86 (C-O), 162.17 (C-O), 163.89 (C-OH), 164.16 (C-O), 182.40 (C=O); IR 1143.62 cm^{-1} (C-O-C, large ring C-O stretch), 1041.05 cm^{-1} and 124.87 cm^{-1} (C-O, phenolic), 1589.68 cm^{-1} (C=C aromatic), 1622.29 cm^{-1} (C=O, ketone), 2897.36 (C-H, alkane), 2956.87 (CH_3, alkane), 3076.84 (C-H, aromatic); MS (ESI) m/z: calculated for $C_{18}H_{16}O_4$ $[M+H]^+$ = 296.1048, found 296.10.

4.4.2 5-Hydroxy-7-isobutoxy-3-phenyl-4H-chromen-4-one (C5) or 7-O-Chrysin-2-methylpropane - novel

C5 was synthesised as described in section 2.2.3.2, page 124. The yield and spectral data are presented below.

Method A: Yield (0.205 g, 74.7 %); m.p. 143 – 145 °C; R_f = 0.69 (Silica 60 Å, 2:1 ethyl acetate: petroleum ether, v/v.

Method B: Yield (0.138 g, 62.7 %) m.p 145 – 146 °C; R_f = 0.58 (Silica 60 Å, 2:1 ethyl acetate: petroleum ether, v/v).



Chemical formula: $C_{19}H_{18}O_4$
Molecular Weight: 310.34 g/mol

Structure 4.5 – Compound C5

^{1}H NMR (400 MHz, $CDCl_3$, δ ppm) 1.05 (6H, d, *J* = 6.6 Hz), 2.15 (1H, sept, *J* = 6.7, 6.6 Hz), 3.81 (2H, d, *J* = 6.7 Hz), 6.40 (1H, d, *J* = 2.2 Hz), 6.52 (1H, d, *J* = 2.2 Hz), 6.69 (1H, s), 7.55 (3H, dd, *J* = 1.5, 0.4 Hz), 7.90 (2H, dd, *J* = 7.8, 1.4 Hz), 12.78 (1H, s, 5-OH); ^{13}C NMR (100 MHz, $CDCl_3$, δ ppm) 19.15 (CH_3), 19.15 (CH_3), 28.11 (CH), 74.95 (OCH_2), 93.12 (CH), 98.68 (CH), 104.3 (CH), 105.84 (C), 126.28 (2', CH), 126.28 (6', CH), 129.09 (3', CH), 129.09 (5', CH), 131.40 (4', CH), 131.80 (1', C), 157.81 (C-O), 162.13 (C-OH), 163.91 (C-O), 165.32 (C-O), 182.48 (C=O); IR 1149.55 cm^{-1} (C-O-C, large ring C-O stretch), 1035.01 cm^{-1} and 123.99 cm^{-1} (C-O, phenolic), 1587.00 cm^{-1} (C=C aromatic), 1614.79 cm^{-1} (C=O, ketone), 2859.02 (C-H, alkane), 2953.21 (CH_3, alkane), 3076.84 (C-H, aromatic); MS (ESI) m/z: calculated for $C_{19}H_{18}O_4$ $[M+H]^+$ = 310.1283, found 310.1278

In the ^{1}NMR spectrum of C4, the 6-protons appearing as a doublet at δ 1.42 ppm are signals for the two methyl protons. The proton appearing as a septuplet at δ 4.67 ppm corresponds to the aliphatic proton in C4 which is the point of attachment of the reactants. The hydroxyl proton at position 5 of the chrysin molecule is seen as a signal δ 12.72 ppm. The dimethyl carbon atoms are observed in δ 21.92 ppm in the ^{13}C NMR. The ^{1}H NMR spectrum of C5, a novel chrysin derivative shows 6-protons appearing as doublet at δ 1.05 ppm. At the point of attachment of the reactants, the signals appear as 2-protons (doublet) at δ 3.81 ppm. In the ^{13}C NMR spectrum, the dimethyl carbon protons appear at δ 19.15 ppm. The mass spectroscopy data (ESI) m/z of 310.1278 g/mol is also consistent with the proposed structure.

4.5 7-O-alkylchrysin derivatives

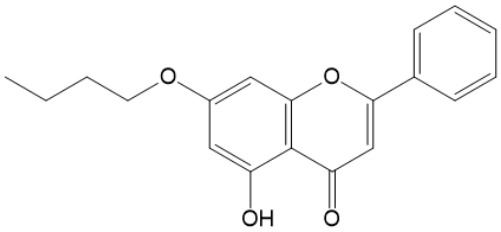
One of the aims of this project is to synthesise chrysin derivatives with aliphatic chain, attached to 7-OH with increasing carbon chain where C ≥ 3 with the aim of investigating their biological activities against cancer cell lines and bacterial strains. The first compound that was synthesised was 7-butoxy-5-hydroxy-3-phenyl-4H-chromen-4-one.

7-O-alkylchrysin derivatives were synthesised as described in section 2.2.2, page 123. The yields recorded for method A ranged from 46 – 58 %. The yields recorded for method

B ranged from 54 – 68 %. The yields recorded for method C ranged from 46.0 – 68.4 %. Only the yields recorded for method B is reported below. The percentage yields for method A and C are reported in Table 4.1.

4.5.1 7-butoxy-5-hydroxy-3-phenyl-4H-chromen-4-one (C6) or 7-O-Butylchrysin

5,7-dihydroxyflavone (0.12 g, 0.47 mmol), potassium carbonate (2.00 eq, 0.26 g, 4.2 mmol) and 1-bromobutane (2.00 eq, 0.130 µl, 4.72 mmol) were suspended in acetonitrile / DMF / acetone (10.0 mL) and stirred / irradiated in a microwave reactor as described in section 2.2.2, page 123 (methods A, B and C respectively) to yield pure 7-butoxy-5-hydroxy-3-phenyl-4H-chromen-4-one.



Chemical formula: $C_{19}H_{18}O_4$
Molecular Weight: 310.34 g/mol

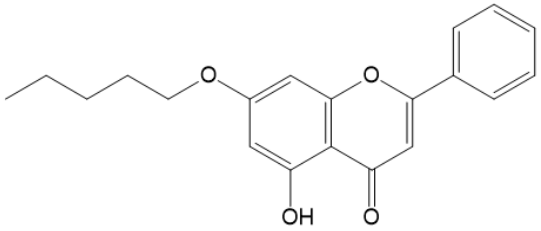
Structure 4.6 – Compound C6

Yield (0.138 g, 78.9 %); m.p. 138 – 139 °C; R_f = 0.68 (Silica 60 Å, 2:1 ethyl acetate: petroleum ether, v/v; ^{1}H NMR (400 MHz, $CDCl_3$, δ ppm) 1.02 (3H, t, *J* = 7.1 Hz), 1.52 (2H, quint, *J* = 7.4, 7.1 Hz), 1.88 (2H, quint, *J* = 7.4 Hz), 4.10 (2H, t, *J* = 7.4 Hz), 6.40 (1H, d, *J* = 2.2 Hz), 6.58 (1H, d, *J* = 2.2 Hz), 6.70 (1H, s), 7.50-7.60 (3H, 7.47 (dddd, *J* = 7.8, 7.3, 1.5, 0.4 Hz), 7.94 (tt, *J* = 7.3, 1.5 Hz)), 7.92 (2H, dd, *J* = 7.8, 1.4, 0.4 Hz), 12.78 (1H, s, 5-OH); ^{13}C NMR (100 MHz, $CDCl_3$, δ ppm) 27.62 (CH_3), 29.28 (CH_2), 33.21 (CH_2), 67.54 (OCH_2), 93.07 (CH), 98.56 (CH), 105.73 (CH), 105.87 (C), 126.28 (2', CH), 126.28 (5', CH), 129.09 (3', CH), 129.09 (5', CH), 131.31 (4', CH), 131.85 (1', C), 157.77 (C-O), 162.19 (C-O), 163.97 (C-OH) 164.84 (C-O), 182.46 (C=O); IR 1167.10 cm^{-1} (C-O-C, large ring C-O stretch), 1023.65 cm^{-1} and 1276.54 cm^{-1} (C-O, phenolic), 1589.89 cm^{-1} (C=C aromatic), 1625.97 cm^{-1} (C=O, ketone), 2852.93 cm^{-1} (C-H, alkane), 2957.69 cm^{-1} (C-H, alkane), 3010.01 cm^{-1} (C-H, aromatic); MS (ESI) m/z: calculated for $C_{19}H_{18}O_4$ $[M+H]^+$ = 310.1283, found 310.12

4.5.2 5-hydroxy-7-(pentyloxy)-3-phenyl-4H-chromen-4-one (C7) or 7-O-Pentylchrysin – novel compound

Using the method described in section 2.2.2, page 123, 5-hydroxy-7-(pentyloxy)-3-phenyl-4H-chromen-4-one (C7) was made using potassium carbonate (2.00 eq, 0.26 g,

4.2 mmol), 5,7-dihydroxyflavone (0.12 g, 0.47 mmol) and 1-bromopentane (2 eq, 142.6 µl, 9.4 mmol). 5-hydroxy-7-(pentyloxy)-3-phenyl-4H-chromen-4-one was obtained as a pale-yellow powder:



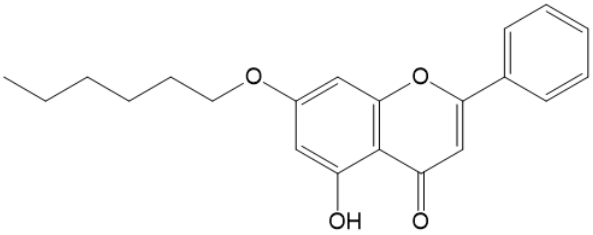
Chemical formula: $C_{20}H_{20}O_4$
Molecular Weight: 324.37 g/mol

Structure 4.7 – Compound C7

Yield (0.259 g, 88.1 %); m.p. 138 - 140 °C; R_f = 0.69 (Silica 60 Å, 2:1 ethyl acetate: petroleum ether, v/v; ^{1}H NMR (400 MHz, $CDCl_3$, δ ppm) 0.99 (3H, t, *J* = 7.0 Hz), 1.49 (4H, quint, *J* = 7.5, 7.0 Hz), 1.89 (2H, quint, *J* = 7.5 Hz), 4.08 (2H, t, *J* = 7.5 Hz), 6.40 (1H, d, *J* = 2.2 Hz), 6.52 (1H, d, *J* = 2.2 Hz), 6.68 (1H, s), 7.55 (3H, ddd, *J* = 7.3, 1.5, 0.4 Hz), 7.90 (2H, dd, *J* = 7.8, 1.4, 0.4 Hz), 12.78 (1H, s, 5-OH); ^{13}C NMR (100 MHz, $CDCl_3$, δ ppm) 14.02 (CH_3), 22.41 (CH_2), 28.09 (CH_2), 28.66 (CH_2), 68.70 (OCH_2), 93.07 (CH), 98.62 (CH), 105.56 (CH), 105.80 (C), 126.26 (2', CH), 126.26 (6', CH), 129.07 (3', CH), 129.07 (5', CH), 131.34 (4', CH), 131.79 (1', C), 157.78 (C-O), 162.10 (C-O), 163.88 (C-OH), 165.22 (C-O), 182.44 (C=O); IR 1168.90 cm^{-1} (C-O-C, large ring C-O stretch), 903.80 cm^{-1} and 1272.38 cm^{-1} (C-O, phenolic), 1587.06 cm^{-1} (C=C aromatic), 1615.56 cm^{-1} (C=O, ketone), 2945.43 cm^{-1} (C-H, alkane), 2952.33 cm^{-1} (C-H, alkane), 3064.25 cm^{-1} (C-H, aromatic); MS (ESI) m/z: calculated for $C_{20}H_{20}O_4$ $[M+H]^+$ =324.1361, found 324.14

4.5.3 7-(Hexyloxy)-5-hydroxy-3-phenyl-4H-chromen-4-one (C8) or 7-O-Hexylchrysin

Using the method described in section 2.2.2, page 123, 7-(hexyloxy)-5-hydroxy-3-phenyl-4H-chromen-4-one (C8) was made using potassium carbonate (2.00 eq, 0.26 g, 4.2 mmol), 5,7-dihydroxyflavone (0.12 g, 0.47 mmol) and 1-Bromohextane (2 eq, 142.6 µl, 9.4 mmol). 7-(hexyloxy)-5-hydroxy-3-phenyl-4H-chromen-4-one was obtained as a pale-yellow powder:



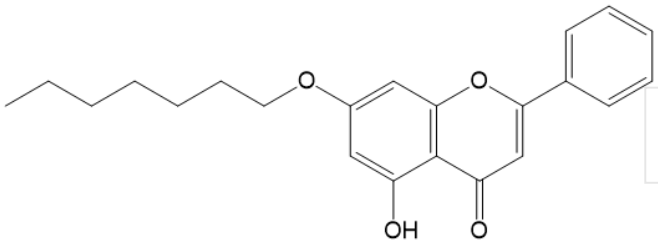
Chemical formula: $C_{21}H_{22}O_4$
Molecular Weight: 338.40 g/mol

Structure 4.8 – Compound C8

Yield (0.246 g, 81.2 %); m.p. 136 – 137 °C; R_f = 0.60 (Silica 60 Å, 2:1 ethyl acetate: petroleum ether, v/v; ^{1}H NMR (400 MHz, $CDCl_3$, δ ppm) 0.96 (3H, t, *J* = 7.0 Hz), 1.36 (4H, quint, *J* = 7.0 Hz, *J* = 7.0 Hz, 1.50 (2H, tt, *J* = 7.5, 7.0 Hz), 1.86 (2H, quint, *J* = 7.5 Hz), 4.05 (2H, t, *J* = 7.5 Hz), 6.40 (1H, d, *J* = 2.2 Hz), 6.50 (1H, d, *J* = 2.2 Hz), 6.70 (1H, s), 7.55 (3H, ddd, *J* = 7.3, 1.5, 0.4 Hz), 7.90 (2H, dd, *J* = 7.8, 1.4, 0.4 Hz), 12.78 (1H, s, 5-OH); ^{13}C NMR (100 MHz, $CDCl_3$, δ ppm) 14.04 (CH_3), 22.59 (CH_2), 25.62 (CH_2), 28.92 (CH_2), 31.51 (CH_2), 68.72 (OCH_2), 93.12 (CH), 98.62 (CH), 105.59 (CH), 105.87 (C), 126.29 (2', CH), 126.29 (6', CH), 129.09 (3', CH), 129.09 (5', CH), 131.40 (4', CH), 131.80 (1', C), 157.81 (C-O), 162.14 (C-O), 163.92 (C-OH), 165.24 (C-O), 182.48 (C=O); IR 1169.58 cm^{-1} (C-O-C, large ring C-O stretch), 905.02 cm^{-1} and 1272.92 cm^{-1} (C-O, phenolic), 1505.98 cm^{-1} (C=C aromatic), 1662.90 cm^{-1} (C=O, ketone), 2643.55 cm^{-1} (C-H, alkane), 2944.69 cm^{-1} (C-H, alkane), 3012.13 cm^{-1} (C-H, aromatic); MS (ESI) m/z: calculated for $C_{21}H_{22}O_4$ $[M+H]^+$ = 338.1518, found 338.15

4.5.4 7-(Heptyloxy)-5-hydroxy-3-phenyl-4H-chromen-4-one (C9) or 7-O-Heptylchrysin – novel compound

Using the method described in section 2.2.2, page 123, 7-(heptyloxy)-5-hydroxy-3-phenyl-4H-chromen-4-one (C9) was made using potassium carbonate (2.00 eq, 0.26 g, 4.2 mmol), 5,7-dihydroxyflavone (0.12 g, 0.47 mmol) and 1-bromoheptane (2 eq, 142.6 µl, 9.4 mmol). 7-(heptyloxy)-5-hydroxy-3-phenyl-4H-chromen-4-one was obtained as a pale-yellow powder:



Chemical formula: $C_{22}H_{24}O_4$
Molecular Weight: 352.42 g/mol

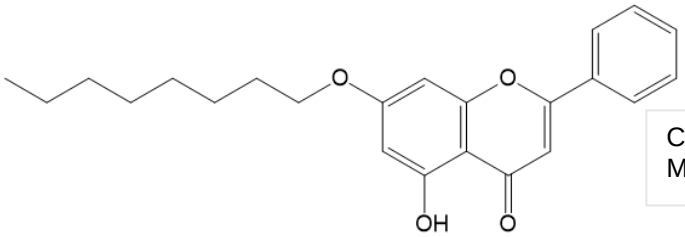
Structure 4.9 – Compound C9

Yield (0. 235 g, 81.4 %); m.p. 118 – 120 °C; R_f = 0.69 (Silica 60 Å, 21 ethyl acetate: petroleum ether, v/v; ^{1}H NMR (400 MHz, $CDCl_3$, δ ppm) 0.89 (3H, t, *J* = 7.0 Hz), 1.30-1.42 (6H, quint, *J* = 7.1, 7.0 Hz), 1.47 (2H, quint, *J* = 7.5, 7.1 Hz), 1.85 (2H, quint, *J* = 7.5 Hz), 4.03 (2H, t, *J* = 7.5 Hz), 6.37 (1H, d, *J* = 2.2 Hz), 6.50 (1H, d, *J* = 2.2 Hz), 6.68 (1H, s), 7.58 (3H, dddd, *J* = 7.8, 7.3, 1.5, 0.4 Hz), 7.90 (2H, dtd, *J* = 7.8, 1.4, 0.4 Hz), 12.78 (1H, s, 5-OH); ^{13}C NMR (100 MHz, $CDCl_3$, δ ppm) 14.09 (CH_3), 22.61 (CH_2), 25.90 (CH_2), 28.96 (CH_2), 28.99 (CH_2), 31.76 (CH_2), 68.72 (OCH_2), 93.11 (CH), 98.62 (CH), 105.59 (CH), 105.87 (C), 126.28 (2', CH), 126.28 (6', CH), 129.08 (3' CH), 129.28 (5', CH), 131.40

(4', CH), 131.79 (1', CH), 157.81 (C-O), 162.14 (C-O), 163.91 (C-OH), 165.24 (C-O), 182.47 (C=O); IR 1161.22 cm^{-1} (C-O-C, large ring C-O stretch), 998.27 cm^{-1} and 1281.39 cm^{-1} (C-O, phenolic), 1512.26 cm^{-1} (C=C aromatic), 1666.37 cm^{-1} (C=O, ketone), 2651.27 cm^{-1} (C-H, alkane), 2937.24 cm^{-1} (C-H, alkane), 2946.41 cm^{-1} (C-H, alkane), 3021.03 cm^{-1} (C-H, aromatic); MS (ESI) m/z: calculated for $C_{22}H_{24}O_4$ $[M+H]^+$ = 352.1674, found 352.17

4.5.5 5-Hydroxy-7-(octyloxy)-3-phenyl-4H-chromen-4-one (C10) or 7-O-Octyloxychrysin

Using the method described in section 2.2.2, page 123, 5-hydroxy-7-(octyloxy)-3-phenyl-4H-chromen-4-one (C10) was made using potassium carbonate (2.00 eq, 0.26 g, 4.2 mmol), 5,7-dihydroxyflavone (0.12 g, 0.47 mmol) and 1-bromoocane (2 eq, 142.6 µl, 9.4 mmol). 5-hydroxy-7-(octyloxy)-3-phenyl-4H-chromen-4-one was obtained as a pale-yellow powder with the following spectroscopic data:



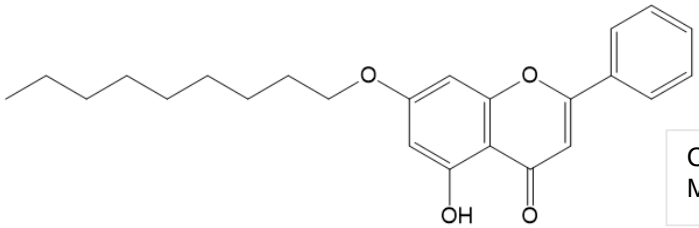
Chemical formula: $C_{23}H_{26}O_4$
Molecular Weight: 366.45 g/mol

Structure 4.10 – Compound C10

Yield (0.228 g, 75.0 %); m.p. 77 - 80 °C; R_f = 0.67 (Silica 60 Å, 2:1 ethyl acetate: petroleum ether, v/v; ^{1}H NMR (400 MHz, $CDCl_3$, δ ppm) 0.92 (3H, t, *J* = 7.0 Hz), 1.29-1.43 (8H, quint, *J* = 7.1, 7.0 Hz), 1.50 (2H, quint, *J* = 7.5, 7.1 Hz), 1.82 (2H, quint, *J* = 7.8, 7.3, 1.5, 0.4 Hz), 4.05 (2H, t, *J* = 7.5 Hz), 6.40 (1H, d, *J* = 2.2 Hz), 6.52 (1H, d, *J* = 2.2 Hz), 6.70 (1H, s), 7.58 (3H, dddd, *J* = 7.8, 7.3, 1.5, 0.4 Hz), 7.92 (2H, dtd, *J* = 7.8, 1.4, 0.4 Hz), 12.78 (1H, s, 5-OH); ^{13}C NMR (100 MHz, $CDCl_3$, δ ppm) 14.11 (CH_3), 22.64 (CH_2), 25.94 (CH_2), 28.96 (CH_2), 29.22 (CH_2), 29.22 (CH_2), 31.81 (CH_2), 68.73 (OCH_2), 93.10 (CH), 98.63 (CH), 105.57 (CH), 105.83 (C), 126.27 (2', CH), 126.27 (6', CH), 129.08 (3', CH), 129.08 (5', CH), 131.37 (4', CH), 131.80 (1', C), 157.80 (C-O), 162.13 (C-O), 163.91 (C-OH), 165.24 (C-O), 182.47 (C=O); m/z [M+H]+ =; IR 1169.51 cm^{-1} (C-O-C, large ring C-O stretch), 1033.91 cm^{-1} and 1272.96 cm^{-1} (C-O, phenolic), 1587.89 cm^{-1} (C=C aromatic), 1607.79 cm^{-1} (C=O, ketone), 2643.39 cm^{-1} (C-H, alkane), 2918.76 cm^{-1} (C-H, alkane), 3023.26 (C-H, aromatic); MS (ESI) m/z: calculated for $C_{23}H_{26}O_4$ $[M+H]^+$ = 366.1909, found 366.1899

4.5.6 5-Hydroxy-7-(nonyloxy)-3-phenyl-4H-chromen-4-one (C11) or 7-O-Nonylchrysin – novel

Using the method described in section 2.2.2, page 123, with some modifications, 5-hydroxy-7-(nonyloxy)-3-phenyl-4H-chromen-4-one (C 11) was made using potassium carbonate (2.00 eq, 0.26 g, 4.2 mmol), 5,7-dihydroxyflavone (0.12 g, 0.47 mmol) and 1-Bromononane (2 eq 195 µl, 9.45 mmol). This was stirred for 48 hours at room temperature. On completion of the reaction, the contents were transferred immediately into -80 °C refrigerator and allowed for 5 minutes. This was transferred into a -25 ºC refrigerator and stored for 48 h. 5-Hydroxy-7-(nonyloxy)-3-phenyl-4H-chromen-4-one was obtained as a bright yellow semi-powder:



Chemical formula: $C_{24}H_{28}O_4$
Molecular Weight: 380.47 g/mol

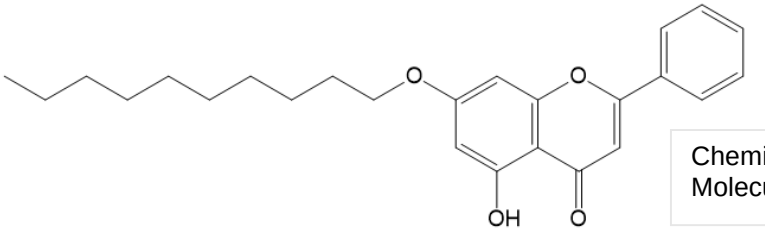
Structure 4.11 – Compound C11

Yield (0.221 g, 66.5 %); m.p. 73 - 76 °C; R_f = 0.63 (Silica 60 Å, 2:1 ethyl acetate: petroleum ether, v/v; ^{1}H NMR (400 MHz, $CDCl_3$, δ ppm) 0.92 (3H, t, *J* = 7.0 Hz), 1.25-1.43 (10H, quint, *J* = 7.0 Hz), 1.49 (2H, quint, *J* = 7.5, 7.0 Hz), 1.83 (2H, quint, *J* = 7.5 Hz), 4.05 (2H, t, *J* = 7.5 Hz), 6.40 (1H, d, *J* = 2.2 Hz), 6.52 (1H, d, *J* = 2.2 Hz), 6.70 (1H, s), 7.58 (3H, 7.47 m, *J* = 7.8, 7.3, 1.5, 0.4 Hz), 7.92 (2H, dtd, *J* = 7.8, 1.4, 0.4 Hz), 12.78 (1H, s, 5-OH); ^{13}C NMR (100 MHz, $CDCl_3$, δ ppm) 14.12 (CH_3), 22.67 (CH_2), 25.93 (CH_2), 28.95 (CH_2), 29.25 (CH_2), 29.32 (CH_2), 29.51 (CH_2), 31.87 (CH_2), 68.72 (OCH_2), 93.12 (CH), 98.61 (CH), 105.58 (CH), 105.87 (C), 126.28 (2', CH), 126.28 (6', CH) 129.08 (3' CH), 129.08 (5' CH), 131.40 (41, CH), 131.79 (1', C), 157.81 (C-O), 162.12 (C-O), 163.91 (C-OH), 165.23 (C-O), 182.47 (C=O); IR 1169.51 cm^{-1} (C-O-C, large ring C-O stretch), 1033.91 cm^{-1} and 1272.96 cm^{-1} (C-O, phenolic), 1505.20 cm^{-1} (C=C aromatic), 1660.79 cm^{-1} (C=O, ketone), 2323.48 cm^{-1} (C-H, alkane), 2643.39 cm^{-1} (C-H, alkane), 2918.76 cm^{-1} (C-H, alkane), 3018.56 cm^{-1} (C-H, aromatic); MS (ESI) m/z: calculated for $C_{24}H_{28}O_4$ $[M+H]^+$ = 380.2065, found 380.2057

4.5.7 7-(Decyloxy)-5-hydroxy-3-phenyl-4H-chromen-4-one (C12) - or 7-O-Decylchrysin – novel compound

Using the method described in section 2.2.2, page 123, 7-(decyloxy)-5-hydroxy-3-phenyl-4H-chromen-4-one (C12), was made using potassium carbonate (2.00 eq, 0.26 g, 4.2

mmol), 5,7-dihydroxyflavone (0.12 g, 0.47 mmol) and 1-bromodecane (2 eq 235.3 µl, 9.8 mmol). 7-(Decyloxy)-5-hydroxy-3-phenyl-4H-chromen-4-one was obtained as a bright yellow semi-solid compound:



Chemical formula: $C_{25}H_{30}O_4$
Molecular Weight: 394.50 g/mol

Structure 4.12 – Compound C12

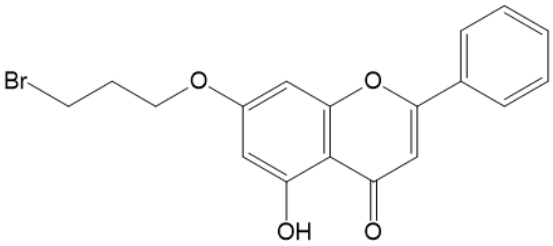
Yield (0.231 g, 65.2 %); m.p. 74 - 76 °C; R_f = 0.54 (Silica 60 Å, 2:1 ethyl acetate: petroleum ether, v/v; ^{1}H NMR (400 MHz, $CDCl_3$, δ ppm) 0.92 (3H, t, *J* = 7.0 Hz), 1.24-1.42 (12H, 1.28 (m, *J* = 7.0, 6.6 Hz), 1.50 (2H, m, *J* = 7.5, 7.0 Hz), 1.83 (2H, quint, *J* = 7.5 Hz), 4.05 (2H, t, *J* = 7.5 Hz), 6.40 (1H, d, *J* = 2.2 Hz), 6.52 (1H, d, *J* = 2.2 Hz), 6.70 (1H, s), 7.58 (3H, m, *J* = 7.8, 7.3, 1.5, 0.4 Hz), 7.92 (2H, dtd, *J* = 7.8, 1.4, 0.4 Hz), 12.78 (1H, s, 5-OH); ^{13}C NMR (100 MHz, $CDCl_3$, δ ppm) 14.13 (CH_3), 22.69 (CH_2), 25.94 (CH_2), 28.96 (CH_2), 29.33 (CH_2), 29.33 (CH_2), 29.56 (CH_2), 29.56 (CH_2), 31.90 (CH_2), 68.72 (OCH_2), 93.10 (CH), 98.62 (CH), 105.58 (CH), 105.85 (C), 126.27 (2', CH), 126.27 (6', CH), 129.08 (3', CH), 129.08 (5', CH), 131.38 (4', CH), 131.79 (1', C), 157.80 (C-O), 162.13 (C-O), 163.88 (C-OH), 165.23 (C-O), 182.46 (C=O); m/z $[M+H]^+$ = 394.2208; IR 1169.51 cm^{-1} (C-O-C, large ring C-O stretch), 1026.75 cm^{-1} and 1283.27 cm^{-1} (C-O, phenolic), 1512.15 cm^{-1} (C=C aromatic), 1663.49 cm^{-1} (C=O, ketone), 2286.36 cm^{-1} (C-H, alkane), 2323.33 cm^{-1} (C-H, alkane), 2645.76 cm^{-1} (C-H, alkane), 3014.51 cm^{-1} (C-H, aromatic); MS (ESI) m/z: calculated for $C_{25}H_{30}O_4$ $[M+H]^+$ = 394.2222, found 394.2208

4.6 7-O-Bromoalkane-derivatives of Chrysin

One of the aims of this project was to make 7-O-bromoalkylchrysin derivatives. The first compound made was 7-(3-bromopropoxy)-5-hydroxy-3-phenyl-4H-chromen-4-one. The 7-O-bromoalkane-derivatives of chrysin were made as described in section 2.2.1, page 123.

4.6.1 7-(3-Bromopropoxy)-5-hydroxy-3-phenyl-4H-chromen-4-one (C13)

The dibromoalkane used for synthesis of C13 is 1,3-dibromopropane (93 µl, 4.72 mmol). The yield and spectral data obtained is presented below.

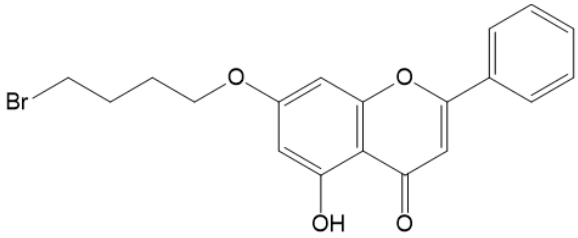


Chemical formula: $C_{18}H_{15}BrO_4$
Molecular Weight: 375.21 g/mol

Structure 4.13 – Compound C13

Yield (0.245 g, 80.2 %); m.p. 142 - 143 °C; R_f = 0.47 (Silica 60 Å, 1:2 ethyl acetate: petroleum ether, v/v; ^{1}H NMR (400 MHz, $CDCl_3$, δ ppm) 2.39 (2H, tt, *J* = 7.5, 6.5 Hz), 3.65 (2H, t, *J* = 6.5 Hz), 4.24 (2H, t, *J* = 7.5 Hz), 6.42 (1H, d, *J* = 2.2 Hz), 6.54 (1H, d, *J* = 2.2 Hz), 6.70 (1H, s), 7.55 (3H, m *J* = 7.8, 7.3, 1.5, 0.4 Hz), 7.92 (2H, dtd, *J* = 7.8, 1.4, 0.4 Hz), 12.78 (1H, s, 5-OH); ^{13}C NMR (100 MHz, $CDCl_3$, δ ppm) 30.94 (CH_2), 31.48 (CH_2-Br), 66.29 (OCH_2), 93.24 (CH), 98.54 (CH), 105.03 (CH), 105.34 (C), 126.45 (2', CH), 126.45 (6', CH), 129.13 (3', CH), 129.13 (5', CH), 130.56 (4', CH), 132.16 (1', C), 157.36 (C-O), 161.15 (C-O), 163.49 (C-OH), 164.37 (C-O), 182.08 (C=O); IR 692.00 cm^{-1} (C-Br), 1100.16 cm^{-1} (C-O-C, large ring C-O stretch), 1032 cm^{-1} and 1244.19 cm^{-1} (C-O, phenolic), 1607.29 cm^{-1} (C=C aromatic), 1651.28 cm^{-1} (C=O, ketone), 285.08 cm^{-1} (C-H, alkane), 2917.41 cm^{-1} (C-H, aromatic); MS (ESI) m/z: calculated for $C_{18}H_{15}BrO_4$ $[M+H]^+$ = 374.0153, found 374.02

4.6.2 7-(4-Bromobutoxy)-5-hydroxy-3-phenyl-4H-chromen-4-one (C14)



Chemical formula: $C_{19}H_{17}BrO_4$
Molecular Weight: 389.24 g/mol

Structure 4.14 – Compound C14

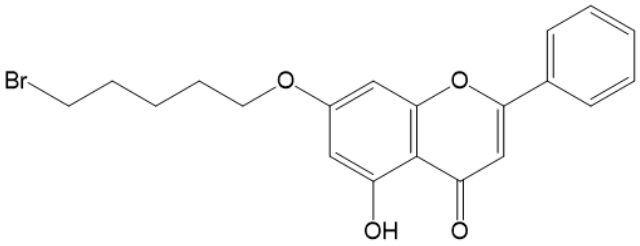
The dibromoalkane used for synthesis of C14 is 1,4-dibromobutane (1 eq 102 µl, 4.72 mmol). The yield and spectral data obtained is presented below.

Yield (0.284 g, 93.3 %); m.p. 143 – 144 °C; R_f = 0.62 (Silica 60 Å, 2:1 ethyl acetate: petroleum ether, v/v; ^{1}H NMR (400 MHz, $CDCl_3$, δ ppm) 1.98-2.18 (4H, 2.05 (quint, *J* = 7.5 Hz), 2.10 (tt, *J* = 7.5, 6.9 Hz)), 3.54 (2H, t, *J* = 6.9 Hz), 4.12 (2H, t, *J* = 7.5 Hz), 6.42 (1H, d, *J* = 2.2 Hz), 6.54 (1H, d, *J* = 2.2 Hz), 6.70 (1H, s), 7.57 (3H, m, *J* = 7.8, 7.3, 1.5, 0.4 Hz), 7.92 (2H, dtd, *J* = 7.8, 1.4, 0.4 Hz), 12.78 (1H, s, 5-OH); ^{13}C NMR (100 MHz, $CDCl_3$, δ ppm) 27.62 (CH_2), 29.82 (CH_2), 33.20 (CH_2-Br), 67.54 (OCH_2), 93.09 (CH), 98.56 (CH), 105.75 (CH), 105.90 (C), 126.29 (1', CH), 126.29 (6', CH), 129.10 (3', CH),

129.10 (3', CH), 131.33 (4', CH), 131.86 (C), 157.80 (C-O), 162.21 (C-O), 164.00 (C-OH), 164.84 (C-O), 182.47 (C=O); IR 685.38 cm^{-1} (C-Br), 1160.46 cm^{-1} (C-O-C, large ring C-O stretch), 1038 cm^{-1} and 1252.36 cm^{-1} (C-O, phenolic), 1609.38 cm^{-1} (C=C aromatic), 1643.54 cm^{-1} (C=O, ketone), 2894.36 cm^{-1} (C-H, alkane), 3089.23 cm^{-1} (C-H, aromatic); MS (ESI) m/z: calculated for $C_{19}H_{17}BrO_4$ $[M+H]^+$ = 388.0310, found 388.03

4.6.3 7-((5-Bromopentyl)oxy)-5-hydroxy-3-phenyl-4H-chromen-4-one (C15)

The dibromoalkane used for synthesis of C15 is 1,5-dibromopentane (1 eq 108 µl, 4.72 mmol). The yield and spectral data obtained is presented below.



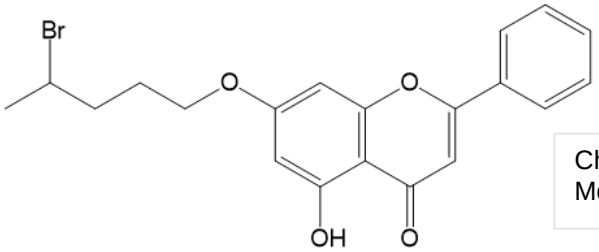
Chemical formula: $C_{20}H_{19}BrO_4$
Molecular Weight: 403.27 g/mol

Structure 4.15 – Compound C15

Yield (0.286 g, 94.0 %); m.p. 120 – 122 °C; Rf = 0.72 (Silica 60 Å, 2:1 ethyl acetate): petroleum ether, v/v; ^{1}H NMR (400 MHz, $CDCl_3$, δ ppm) 1.68 (2H, quint, *J* = 7.5 Hz), 1.85-2.05 (4H, 1.82 (quint, *J* = 7.5, 6.9 Hz), 3.65 (2H, t, *J* = 6.9 Hz), 4.24 (2H, t, *J* = 7.5 Hz), 6.42 (1H, d, *J* = 2.2 Hz), 6.54 (1H, d, *J* = 2.2 Hz), 6.70 (1H, s), 7.58 (dddd, *J* = 7.8, 7.3, 1.5, 0.4 Hz), 7.92 (2H, dtd, *J* = 7.8, 1.4, 0.4 Hz), 12.78 (1H, s, 5-OH); ^{13}C NMR (100 MHz, $CDCl_3$, δ ppm) 24.73 (CH_2), 28.15 (CH_2), 32.37 (CH_2), 33.48 (CH_2-Br), 68.26 (OCH_2), 93.10 (CH), 98.57 (CH), 105.67 (CH), 105.87 (C), 126.28 (1', CH), 126.28 (6', CH), 129.09 (3' CH), 129.09 (5' CH), 131.34 (4', CH), 131.83 (1', C), 157.79 (C-O), 162.17 (C-O), 163.95 (C-OH), 164.99 (C-O), 182.47 (C=O); IR 673.50 cm^{-1} (C-Br), 1160.48 cm^{-1} (C-O-C, large ring C-O stretch), 1035.84 cm^{-1} and 1251.54 cm^{-1} (C-O, phenolic), 1585.17 cm^{-1} (C=C aromatic), 1605.89 cm^{-1} (C=O, ketone), 2851.36 cm^{-1} (C-H, alkane), 2925.09 cm^{-1} (C-H, alkane), 2949.90 cm^{-1} (C-H, alkane), 3058.38 cm^{-1} (C-H, aromatic); MS (ESI) m/z: calculated for $C_{20}H_{19}BrO_4$ $[M+H]^+$ = 402.0466, found 402.05

4.6.4 7-((4-Bromopentyl)oxy)-5-hydroxy-3-phenyl-4H-chromen-4-one or 7-O-2-Bromopentylchrysin (C16) - novel

The dibromoalkane used for synthesis of C16 is 1,4-dibromopentane (1 eq 108 µl, 4.72 mmol). The yield and spectral data obtained is presented below.



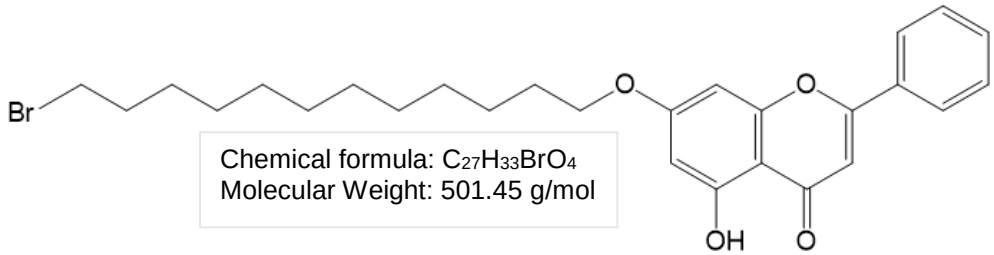
Chemical formula: $C_{20}H_{19}BrO_4$
Molecular Weight: 404.27 g/mol

Structure 4.16 – Compound C16

Yield (0.286 g, 94.0 %); m.p. 123 – 124 °C; R_f = 0.74 (Silica 60 Å, 2:1 ethyl acetate): petroleum ether, v/v; ^{1}H NMR (400 MHz, $CDCl_3$, δ ppm) 1.78 (3H, d, *J* = 6.8 Hz), 1.88-2.16 (4H, 1.99 (td, *J* = 7.4, 6.8 Hz), 2.04 (td, *J* = 7.4, 6.8 Hz), 2.07 (tt, *J* = 7.5, 7.4 Hz), 2.11 (tt, *J* = 7.5, 7.4 Hz)), 4.10 (2H, t, *J* = 7.5 Hz), 4.25 (1H, h, *J* = 6.8 Hz), 6.38 (1H, d, *J* = 1.9 Hz), 6.50 (1H, d, *J* = 1.9 Hz), 6.70 (1H, s), 7.56 (3H, dd, *J* = 7.8, 7.3 Hz), 7.93 (2H, dtd, *J* = 7.8, 1.4, 0.4 Hz) 12.78 (1H, s, 5-OH); ^{13}C NMR (100 MHz, $CDCl_3$, δ ppm) 26.58 (CH_3), 27.41 (CH_2), 37.51 (CH_2), 50.94 (CH_2-Br), 67.76 (OCH_2), 93.07 (CH), 98.57 (CH), 105.73 (CH), 105.89 (C), 126.29 (2', CH), 126.29 (2', CH), 129.10 (3', CH), 129.10 (3', CH), 131.33 (4', CH), 131.85 (1', C), 157.79 (C-O), 162.19 (C-O), 163.07 (C-OH), 164.89 (C-O), 182.47 (C=O); IR 765.53 cm^{-1} (C-Br), 1172.08 cm^{-1} (C-O-C, large ring C-O stretch), 1101.61 cm^{-1} and 1270.95 cm^{-1} (C-O, phenolic), 1603.28 cm^{-1} (C=C aromatic), 1621.23 cm^{-1} (C=O, ketone), 2854.31 cm^{-1} (C-H, alkane), 2943.23 cm^{-1} (C-H, alkane), 2965.54 cm^{-1} (C-H, alkane), 3068.12 cm^{-1} (C-H, aromatic); MS (ESI) m/z: calculated for $C_{20}H_{19}BrO_4$ $[M+H]^+$ = 402.0466, found 404.0521

4.6.5 7-((12-Bromododecyl)oxy)-5-hydroxy-3-phenyl-4H-chromen-4-one (C17)

The dibromoalkane used for synthesis of C17 is 1,4-dibromopentane (0.17 g, 4.75 mmol). The yield and spectral data obtained is presented below.



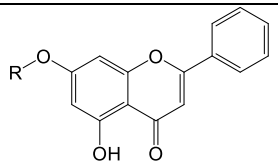
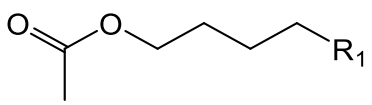
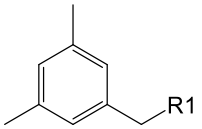
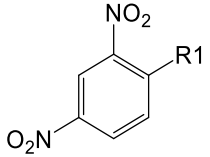
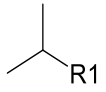
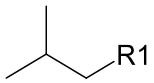
Chemical formula: $C_{27}H_{33}BrO_4$
Molecular Weight: 501.45 g/mol

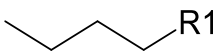
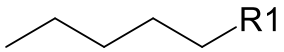
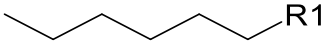
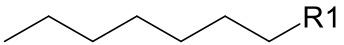
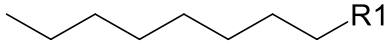
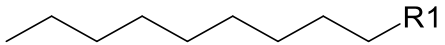
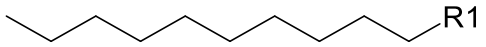
Structure 4.17 – Compound C17

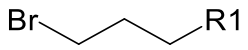
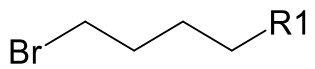
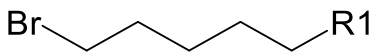
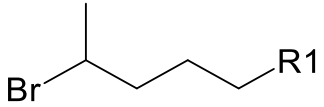
Yield (0.258 g, 89.0 %); m.p. 91 – 92 °C; R_f = 0.65 (Silica 60 Å, 2:1 ethyl acetate: petroleum ether, v/v); ^{1}H NMR (400 MHz, DMSO-d_6, δ ppm) 1.28-1.53 (16H, 1.35 (tt, *J* = 7.0, 6.6 Hz), 1.42 (tt, *J* = 7.5, 7.0 Hz), 1.44 (tt, *J* = 7.0, 6.6 Hz), 1.46 (quint, *J* = 7.0 Hz), 1.48 (tt, *J* = 7.0, 6.6 Hz), 1.76-1.86 (4H, 1.80 (tt, *J* = 7.5, 6.9 Hz), 1.82 (quint, *J* = 7.5 Hz)),

4.10 (2H, t, *J* = 6.9 Hz), 4.25 (2H, t, *J* = 7.5 Hz), 6.38 (1H, d, *J* = 2.2 Hz), 6.50 (1H, d, *J* = 2.2 Hz), 6.70 (1H, s), 7.55 (3H, dddd, *J* = 7.8, 7.3, 1.5, 0.4 Hz), 7.92 (2H, dtd, *J* = 7.8, 1.4, 0.4 Hz), 12.78 (1H, s, 5-OH); ^{13}C NMR (100 MHz, $CDCl_3$, δ ppm) 14.11 (CH_2), 26.15 (CH_3), 28.18 (CH_2), 28.77 (CH_2), 28.95 (CH_2), 29.31 (CH_2), 29.43 (CH_2), 29.52 (CH_2), 29.67 (CH_2), 32.83 (CH_2), 34.08 (CH_2-Br), 68.71 (OCH_2), 93.10 (CH), 98.62 (CH), 105.58 (CH), 105.85 (C), 126.28 (1', CH), 126.28 (6', CH), 129.08 (3', CH), 129.08 (5', CH), 131.38 (4', CH), 131.80 (1', C), 157.80 (CH), 162.13 (C-O), 163.90 (C-OH), 165.22 (C), 182.82 (C=O); IR 684.90 cm^{-1} (C-Br), 1118.35 cm^{-1} (C-O-C, large ring C-O stretch), 1166.82 cm^{-1} and 1300.67 cm^{-1} (C-O, phenolic), 1584.66 cm^{-1} (C=C aromatic), 1611.43 cm^{-1} (C=O, ketone), 2851.08 cm^{-1} (C-H, alkane), 2917.41 cm^{-1}, 3071.03 cm^{-1} (C-H, aromatic); MS (ESI) m/z: calculated for $C_{20}H_{19}BrO_4$ $[M+H]^+$ = 500.1562, found 500.16

Table 4.1: Structures and properties of synthetic chrysin derivatives made in this study. Eight novel chrysin derivatives were synthesised.

Compound	Structure	Name / Properties	Yield (%)	m.p (°C)	Novel?
Chrysin		N/A	N/A	284 – 286 (Comte et al., 2001)	
C1	R = 	4-((5-hydroxy-4-oxo-2-phenyl-4H-chromen-7-yl)oxy)butyl acetate or 7-O-chrysinbutylacetate Chemical formula: $C_{21}H_{20}O_6$ Exact Mass: 368.1330 Molecular Weight: 368.13 g/mol	68.0	122 – 124	✓
C2	R = 	7-((3,5-dimethylbenzyl)oxy)-5-hydroxy-2-phenylchroman-4-one or 7-O-chrysin-3,5-dimethylbenzene Chemical formula: $C_{24}H_{22}O_4$ Exact Mass: 372.1433 Molecular Weight: 372.14 g/mol	83.1	189 – 190	✓
C3	R = 	7-((2,4-dinitrobenzyl)oxy)-5-hydroxy-2-phenylchroman-4-one or 7-O-chrysin-2,4-dinitrobenzene Chemical formula: $C_{22}H_{16}N_2O_8$ Exact Mass: 420.0661 Molecular Weight: 420.32 g/mol	89.6	292 – 294	✓
C4	R = 	5-hydroxy-7-isopropoxy-2-phenyl-4H-chromen-4-one or 7-O-isopropylchrysin Chemical formula: $C_{18}H_{16}O_4$ Exact Mass: 296.10 Molecular Weight: 296.32 g/mol	62.7	145 – 146	-
C5	R = 	5-hydroxy-7-isobutoxy-2-phenyl-4H-chromen-4-one or 7-O-2-methylpropylchrysin Chemical formula: $C_{19}H_{18}O_4$ Exact Mass: 310.1278 Molecular Weight: 310.34 g/mol	74.7	138 – 139	✓

C6	R = 	7-butoxy-5-hydroxy-2-phenyl-4H-chromen-4-one or 7-O-butylchrysin Chemical formula: $C_{19}H_{18}O_4$ Exact Mass: 310.12 Molecular Weight: 310.34 g/mol	A– 51.4 B– 78.8 C– 57.0	138 – 139	-
C7	R = 	5-hydroxy-7-(pentyloxy)-2-phenyl-4H-chromen-4-one or 7-O-pentylchrysin Chemical formula: $C_{20}H_{20}O_4$ Exact Mass: 324.13 Molecular Weight: 324.37 g/mol	A – 46.4 B – 88.1 C – 66.0	138 – 140	
C8	R = 	7-(hexyloxy)-5-hydroxy-2-phenyl-4H-chromen-4-one or 7-O-hexylchrysin Chemical formula: $C_{21}H_{22}O_4$ Exact Mass: 338.15 Molecular Weight: 338.40 g/mol	A – 58.2 B – 81.2 C – 68.4	136 – 137	-
C9	R = 	7-(heptyloxy)-5-hydroxy-2-phenyl-4H-chromen-4-one or 7-O-heptylchrysin Chemical formula: $C_{22}H_{24}O_4$ Exact Mass: 352.17 Molecular Weight: 352.42 g/mol	A – 46.1 B – 81.4 C – 4.64	118 – 120	
C10	R = 	5-hydroxy-7-(octyloxy)-2-phenyl-4H-chromen-4-one or 7-O-octylchrysin Chemical formula: $C_{23}H_{26}O_4$ Exact Mass: 366.1899 Molecular Weight: 366.45 g/mol	A – 49.3 B – 75.0 C –57.6	77 – 80	✓
C11	R = 	5-hydroxy-7-(nonyloxy)-2-phenyl-4H-chromen-4-one or 7-O-nonylchrysin Chemical formula: $C_{24}H_{28}O_4$ Exact Mass: 380.2057 Molecular Weight: 380.47 g/mol	A – 47.0 B – 66.5 C – 54.7	73 – 76	✓
C12	R = 	7-(decyloxy)-5-hydroxy-2-phenyl-4H-chromen-4-one or 7-O-decylchrysin Chemical formula: $C_{25}H_{30}O_4$ Exact Mass: 394.2208 Molecular Weight: 394.50 g/mol	A – 56.2 B – 65.2 C – 66.7	84 – 86	✓

C13	R = 	7-(3-bromopropoxy)-5-hydroxy-2-phenyl-4H-chromen-4-one or 7-O-bromopropylchrysin Chemical formula: $C_{18}H_{15}BrO_4$ Exact Mass: 374.02 Molecular Weight: 375.21 g/mol	80.2	142 – 143	
C14	R = 	7-(4-bromobutoxy)-5-hydroxy-2-phenyl-4H-chromen-4-one or 4-bromobutylchrysin Chemical formula: $C_{19}H_{17}BrO_4$ Exact Mass: 388.03 Molecular Weight: 389.24 g/mol	93.3	143 – 144	
C15	R = 	7-((5-bromopentyl)oxy)-5-hydroxy-2-phenyl-4H-chromen-4-one, or 7-O-bromopentylchrysin Chemical formula: $C_{20}H_{19}BrO_4$ Exact Mass: 402.05 Molecular Weight: 403.27 g/mol	94.0	120 – 122	
C16	R = 	7-((4-bromopentyl)oxy)-5-hydroxy-2-phenyl-4H-chromen-4-one or 4-bromopentylchrysin Chemical formula: $C_{20}H_{19}BrO_4$ Exact Mass: 404.0521 Molecular Weight: 404.27 g/mol	94.0	123 – 124	✓
C17	R = 	7-((12-bromododecyl)oxy)-5-hydroxy-2-phenyl-4H-chromen-4-one, or 12-bromododecylchrysin Chemical formula: $C_{27}H_{33}BrO_4$ Exact Mass: 500.16 Molecular Weight: 501.45 g/mol	89.0	91 – 92	

A - Yield reported for method A used in synthesis of the compound. Method A is synthesis via the microwave reactor.
B - Yield reported for method B used in synthesis of the compound. Method B involves stirring the reaction mixture under controlled conditions of temperature and other reaction conditions.
C - Yield reported for method C.
N/A - Not applicable
R1 - Chrysin molecule without hydrogen proton at 7-OH

The synthesis of these compounds was approached from different perspectives in order to provide the highest yield and atom economy with the fewest possible steps. This study has been able to achieve this via the use of a microwave reactor which offered a shorter period (≤ 10 minutes) for the synthesis of these compounds compared to a period of 24 – 48 hours using the conventional method of stirring the reaction mixture. The use of a microwave reactor in driving the quick synthesis of these compounds implies that a large-scale production of these compounds within a short time period is possible and feasible via the methods developed in this study. Although some 7-O-bromoalkyl, 7-O-alkyl and 7-O-chrysin derivatives have been previously made (Cheng et al., 2014; Valdez-Calderón et al., 2016), this is the first time a microwave reactor has been used in the synthesis of these compounds. This is also the first time the antimicrobial and anticancer activities of these compounds have been reported. It is also the first time 7-O-bromoalkylchrysin derivatives with carbon atoms ≥ 7, and aliphatic 7-O-chrysin derivatives (7-O-bromododecylchrysin and 7-O-4-bromopentylchrysin) have been reported.

The 7-O-chrysin derivatives synthesised are divided into three series for this study:

Series 1: 7-O-chrysin derivatives (C1 – C3).
Series 2: 7-O-alkylchrysin derivatives comprising of C4 – C12.
Series 3: 7-O-bromoalkylchrysin derivatives comprising of C13 – C17.

The names of these compounds are listed in Table 4.2 below.

Table 4.2: name and series of compounds synthesised in this study.

Compound	**Name of Compound**	**Series name**	
C1	7-O-chrysinbutyl acetate	Series 1	
C2	7-O-chrysin-3,5-dimethylbenzene		
C3	7-O-chysin-2,4-dinitrobenzene		
C4	7-O-isopropylchrysin	Methylated	Series 2
C5	7-O-chrysin-2-methylpropane		
C6	7-O-butylchrysin	Non-methylated	
C7	7-O-pentylchrysin		
C8	7-O-hexylchrysin		
C9	7-O-heptylchrysin		
C10	7-O-octylchrysin		
C11	7-O-nonylchrysin		
C12	7-O-decylchrysin		

C13	7-O-3-bromopropylchrysin	Series 3
C14	7-O-4-bromobutylchrysin	
C15	7-O-5-bromopentylchrysin	
C16	7-O-4-bromopentylchrysin	
C17	7-O-12-bromododecylchrysin	
Chrysin	5,7-dihydroxyflavone	Parent compound
CPZ	Chlorpromazine hydrochloride	Positive control drug

Series 1 compounds made in this study are 7-O-chrysinbutyl acetate (C1), 7-O-chrysin-3,5-dimethylbenzene (C2) and 7-O-chrysin-2,4-dinitrobenzene (C3). These are novel compounds.

Series 2 compounds synthesised in this study are classified into 2:

Group A: methylated series-2 compounds – 7-O-Isopropylchrysin (C4) and 7-O-2-Methylpropylchrysin (C5) – novel compound.

Group B: Non-methylated series-2 compounds – 7-O-butylchrysin (C6), 7-O-pentylchrysin (C7), 7-O-hexylchrysin (C8), 7-O-heptylchrysin (C9), 7-O-octylchrysin (C10), 7-O-nonylchrysin (C11), 7-O-decylchrysin (C12). Compounds synthesised using method B were used for biological studies.

Series 2 compounds were made via three methods: method A, involving irradiation of the reactants in a microwave reactor using acetonitrile as the reaction solvent. Method B involves irradiation of the reactants in a microwave reactor using dimethylformamide (DMF). Unlike method A, tetrabutylammonium bromide (0.04 mmol) was added to the reactants. Method C involves stirring of the reactants for 24 – 48 hours.

The yields recorded for these compounds was higher with method B (yields > 75 %, except for C12) compared to method A (< 60 %) and method C (< 70 %). This increase in yield may be attributed to the quaternary ammonium salt (tetrabutylammonium bromide) added to the reactants, whose bromide counter-ion may have acted as a phase transfer catalyst, thus the increase yield recorded with method B.

In the ^{1}H NMR, the singlet proton with δ 12.61, corresponds to the 5-OH of chrysin, and a triplet with δ 4.05, corresponding to 2-protons (OCH_2, J = 7.1), at the point of attachment of the various reactants. Unusual differences in melting point was observed for these compounds: the methylated series 2 adducts (C4 and C5) recorded higher melting points (m.p.) of 145 – 146 °C and 143 – 145 °C. The m.p. of C4 was slightly higher than C5 which had an increased chain length ($-CH_3$). Similarly, the m.p. pattern observed for the non-methylated series 2 compounds showed a decreasing m.p. as the number of carbon chain

increases. C6 with 4-carbon chain indicated m.p. of 138 – 139 °C, C7 with 5-carbon chain (m.p. 138 – 140 °C), C8 with 6-carbon chain (136 – 137 °C), C9 with 7-carbon chain (118 – 120 °C), C10 with 8-carbon chain (77 – 80 °C), C11 with 9-carbon chain (73 – 76 °C) and C10 (74 – 76 °C).

This suggests that increasing the chain length (methylation) of chrysin at 7-OH, reduces the melting point of chrysin. It also suggests that the attachment of a branched aliphatic chain (as in C4) near the 7-OH of chrysin, increases the melting point, and if further apart (as in C5), the melting point tends to decrease. The m.p. of series 2 compounds decreased drastically for C11 and C12 (having 9 and 10 carbon atoms).

It was difficult to precipitate out C11 and C12 using the conventional methods developed in this study as these compounds were semi-liquids. After some unsuccessful attempts to precipitate C11 and C12, the reaction conditions were modified such that the reaction was made to progress at room temperature for 48 h and not at 80 °C. After 48 hours, the reaction was transferred immediately into a -80 °C refrigerator for 5 minutes, and then into a -25 °C refrigerator where it was stored for 48 h. The solution was not frozen as acetonitrile freezes at -45 °C. This enabled the precipitation of C11 and C12 respectively. C11 was partly oily. C12 was partly oily and translucent on the filter paper used. To precipitate C12, very cold water was used to filter C12.

C4 (Comte et al., 2001), C6, C7 and C8 have been previously made (Cheng et al., 2014). In this study, in addition to C4, C6, C7 and C8 (which have been previously made), we synthesised novel 7-O-chrysin adducts: 7-O-octylchrysin (C9), 7-O-nonylchrysin (C10) and 7-O-decylchrysin (C12). Although the spectral data of C7 and C9 have been reported on PubMed, no biological investigation has been reported by any author.

Series 3 compounds includes the bromoalkane-derivatives of chrysin. Five (5) compounds which include: 7-O-bromopropylchrysin, 7-O-bromobutylchrysin, 7-O-5-bromopentylchrysin, 7-O-4-bromopentylchrysin (novel compound) were synthesised in this group. Compounds synthesised using method B were used for biological studies. It is believed that the methyl, halogen, acetate and nitrite groups attached at some points in the molecules of chrysin derivatives could influence the biological activities of the newly synthesised compounds, and the biological activities of these new molecules could then be investigated. Compounds synthesised in this study were made using two or three different methods; by radiating the reactants in a microwave reactor, by stirring the reactants for a certain period or. The spectral data of chrysin is presented in the appendix. The first derivatives of chrysin that was synthesised were the series 1 compounds (C1, C2 and C3).

All series 1 compounds made in this study are novel 7-O-chrysin derivatives. Compounds synthesised using method B were used for biological studies.

The first chrysin adduct that was made is 7-O-chrysinbutyl acetate (C1) – page 154. In the ^{1}H NMR spectrum of the chrysin adduct (C 1), proton 12-H and 15-H, have chemical shift of 3.98 – 4.15 ppm (O-CH_2). This corresponds to the attachment of the 4-bromobutyl acetate to the 7-OH position of chrysin. Attachment of 4-bromobutyl acetate at the 5-OH is difficult without a strong base like NaOH due to the development of hydrogen bond between the hydrogen atom at C-5 and the carbonyl oxygen at C-3 of chrysin. This hydrogen bond therefore tends to prevent the interaction of chemical groups with the –OH group at C-5 of chrysin, allowing interaction with the –OH group at C-7 of chrysin. This assumption was confirmed in a research by Zheng et al., (2016) and Lapkin et al. (2014), who exploited the effect of hydrogen bonding in chrysin and galangin. They concluded that a strong hydrogen bond and interaction exist in chrysin molecule, which prevents the interaction of the hydrogen atom at position C-5 of chrysin. The stronger this hydrogen bond, interaction with other molecules becomes difficult giving rise to a strong covalent and electrostatic interaction at the hydroxyl group at the C-5 position of chrysin. Spectroscopic data: 1D (1H, 13C, C-APT) and 2D (COSY, gHMBC) and IR support the findings that the 7-OH position is where the reaction occurred. Additionally, in the ^{1}H NMR spectrum of the chrysin adduct (C1), the chemical shift at 12.61 (1 H, s, 5-OH) corresponds to the 5-OH hydroxyl atom.

In order to investigate the effect of the hydrogen bond at the 5-OH position of the chrysin molecule, the concentration of 4-bromobutyl acetate and potassium carbonate were increased to 10 eq respectively and the product obtained monitored on a TLC plate. There was no evidence of formation of chrysin adduct at 5-OH of chrysin as confirmed by spectroscopic data. This is a confirmation of previous observation of the existence of hydrogen bond at 5-OH of chrysin (Lapkin et al., 2014; Zheng et al., 2016) which limits the reactivity of chrysin at the 5-OH position. This noted, other chrysin adducts made showed a similar pattern with a 1H NMR spectrum of the chrysin adducts corresponding to –OH group at C-5 seen around 12.61 – 13.01 ppm.

Although a colour change (brown precipitate) was observed between the C1 obtained using method A (synthesis using the microwave reactor) and method B (ivory white precipitate), the NMR spectrum of both compounds was identical. The yield obtained using the microwave extractor (method A) – 80.20 % was significantly higher than the yield obtained using method B (68.0 %). The reason for these colour changes is not certain but may have resulted from the heating effect of the reaction mixture in the microwave reactor at a higher

temperature (120 °C) and power of 900 W. To investigate the effect of increased temperature on the colour change observed, the temperature of the microwave reactor was reduced to 100 °C (other variables remaining constant). This time, an ivory white precipitate was obtained but the yield declined from 80.2 % obtained previously to 72.85 % (result obtained after taking an average of 3 reactions). This suggests that the colour change observed was due to the increased temperature and the environment at the microwave reactor. This finding also suggests that at high reaction temperatures, the yield of C1 increases using the microwave reactor (Jensen et al., 2017).

The second chrysin derivative synthesised was 7-O-chrysin-3,5-dimethylbenzene (C2). The ease with which this reaction occurred with acetonitrile but not acetone or methanol may be attributed to the nucleophilic addition of the nitrile giving rise to intermediates which undergo further reactions with the –OH to form the product. The structure of the solvent can influence the outcome of the reaction especially when ionic liquids such as acetonitrile are used due to the entropy changes occurring in the reaction medium. In a study by Keaveney et al., (2016) and Keaveney et al., (2017), the role of ionic solvents was investigated, and it was concluded that ionic liquids increase the effective concentration of reactants, allowing interaction between reactants especially those containing short chain alkyl groups as non-polar ionic domains are created in these molecules, allowing an increase in the rate constants for ionic liquids. The positioning of the methyl groups at the meta positions (3, 5) in the benzene ring may have allowed the rapid progression of the reaction, allowing for the electrophilic substitution of the bromine atom, and the formation of (C 2). It was difficult to increase the yield of C2 when reaction temperature was ≥ 70 ºC. C2 was made at room temperature while C1 and C3 were made at temperatures ≥ 60 ºC.

These findings suggest that the microwave reactor degrades the benzene ring due to the polarization effect of microwave which oxidizes the benzene rings in C2 and C3 (Zhang et al., 2012). This was visible in the impurities spotted on the TLC plate when C2 and C3 (from the microwave reactor) were eluted on TLC plates. These impurities were absent on C1. These impurities were also absent in C2 and C3 synthesised via method B (stirring).

Series 2 compounds include the 7-O-alkylchrysin derivatives (C4 -12). In the 1H NMR spectrum, these compounds indicated similar spectra at δ (ppm) ranging from 7.47 – 7.50 (2 H, H-3’ and H-5’); 7.91 - 7.92 corresponding (2H, H-2’ and H-6’); and 12.64 – 13.01 corresponding to (1 H, s, 5-OH). These corresponds to 1H NMR spectra reported by Cheng et al., (2014) and Kingkaew et al., (2018). In this study, series 2 compounds were synthesised using acetonitrile as reaction medium and not acetone as previously

synthesised (Cheng et al., 2014; Kingkaew et al., 2018). Acetonitrile (as a reaction medium) offered better yield of 7-O-butylchrysin (C6) – 78.86 % compared to previous study using acetone (74.5 %) (Cheng et al., 2014), and a better yield of 7-O-hexylchrysin (C8) (88.12 %), compared to the yield reported by Comte et al., (2001) using acetone (59.1%). Nevertheless, the yield reported by Cheng et al., (2014) for C8 using acetone (89.7 %) is comparable to that reported in this study.

Similarly, a higher yield of 7-O-isopropylchrysin (C4) synthesised via the microwave reactor, was recorded in this study using acetonitrile as a reaction medium, 62.7 % yield was recorded in this study. This yield is comparable to that reported by Comte et al., (2001) and Pan et al., 2015 (65.8 %). The increased yield of series 2 chrysin derivatives recorded in this study could be attributed to the use of dry ice / ice-cold water in rapidly cooling and washing the products. This process is hypothesised to favour the equilibrium towards the yield of products, converting reactants to products. Other reports did not cool the reaction medium rapidly, and the researchers did not wash / store their products in ice-cold water. To confirm this hypothesis, the same reaction conditions were employed, and the products allowed to cool to room temperature and then washed with water at 25 ºC. 50 – 82 % yields (compared to the yields recorded when the products were rapidly cooled in dry-ice / ice-cold water) was recorded. The increased yield recorded in this study using acetonitrile, compared to those reported by other researchers using acetone could be attributed to the fact that that acetonitrile is an ionic liquid (Keaveney et al., 2016). Ionic liquids increase the effective concentration of reactants, allowing interaction between reactants especially those containing short chain alkyl groups as non-polar ionic domains are created in these molecules, allowing an increase in the rate constants for ionic liquids (Keaveney et al., 2016; Keaveney et al., 2017). To compare the role of acetonitrile and acetone in the yield of chrysin derivatives, acetone was used as a reaction medium (with other variables remaining constant); it was observed that the yields recorded for acetonitrile was higher than the yields recorded for acetone (as reaction solvents).

Although some series-3 chrysin adducts made in this study have been previously made, this is the first time the antibacterial and anticancer activities of these compounds have been reported. Experimental data obtained in this study also compares to data previously reported Valdez-Calderón et al. (2016) has previously made three series 3 compounds synthesised in this study – 7-O-bromopropylchrysin, 7-O-bromobutylchrysin and 7-O-5-bromopentylchrysin and studied the anthelminthic activities of these compounds but not their antibacterial and anticancer activities. Cheng et al. (2014), has previously made some series 2 compounds (7-O-butylchrysin and 7-O-hexylchrysin), studying their inhibitory

activity against α-glucosidase, an enzyme implicated in diabetes. The 7-O-bromoalkane-derivatives of chrysin offered a better yield compared to other chrysin adducts.

One of the aims of this study was to make 7-O-chrysin adducts such that chrysin adducts synthesised were attached to the 7-OH of chrysin. This noted, an attempt was made to synthesise 7-O-chloro-chrysin adducts like the 7-O-bromoalkylchrysin adducts of series 1 using 1-chlorobutane and 1-chlorohexane (using the methods described for the synthesis of 7-O-bromoalkylchrysin adducts). Unlike the chrysin adducts reported above, little precipitates were formed for these compounds. The microwave reactor was used for the synthesis of these chloro-chrysin adducts as well but little no precipitates were formed. One reason ice cold water was used in precipitating the Chrysin derivativesmade was to ensure the products were stabilised before extraction as the bromoalkane and alkanes used for the synthesis of these chrysin adducts had relatively low melting points (−112.5 °C for 1-bromobutane). Ice cold water was used to stabilise the chloro-adducts of chrysin, little precipitate was formed. Precipitates formed were poured unto crushed ice and left for 24 hours at –80 °C; little precipitates were formed. Precipitates formed were filtered as described for the synthesis of 7-O-bromoalkylchrysin adducts using ice cold water. The precipitate formed dissolved in water. This is because chlorine is water soluble in most instances. The reason water was used in washing the precipitate is because unreacted chrysin was easily washed off the precipitate during filtration using the vacuum pump. Another reason water was used is because potassium carbonate dissolves easily in water but not in other solvents. This implies a pure compound of interest can be obtained even without subjecting the product to further purification using column chromatography. We attempted to purify the chloro-chrysin adducts using different purification techniques (except HPLC) but we obtained very poor yield (≤ 10 %). The yield obtained was impure when checked using the TLC plate as minor impurities were present. Nevertheless, on TLC plate, the impurities were not chrysin as the retardation factor (Rf) values for these impurities were different from the Rf value of chrysin. It was reasoned that these impurities could be 5-O-chrysin adducts and both 5-O- and 7-O-chrysin adducts in combination with the 7-O-chrysin adducts which were of interest to us in this study. Because no literature has been published for aliphatic chloro-chrysin adducts, this finding is worthy of note for future researchers who should seek to synthesize and purify aliphatic chloro-chrysin adducts with the aim of studying their biological activities. This is important because the magnitude of anticancer activities observed against MCF 7 and HCT 116 cell lines was interesting compared to those indicated by chrysin, and the Rf values suggests these impurities were not chrysin.

The ^{13}C NMR spectra for series 1 compound indicated fifteen signals of chrysin and their chemical shifts do not differ from each compound's chemical shift by more than 1.8 ppm technically. A characteristic similar to all compounds in series were δC 105.8 ppm (with minor differences ≤ 0.1 ppm). Oxygen bearing vinyl or aromatic carbon atoms was observed at δC 159 – 162 ppm (C7), 105.72 ppm (C2), 162 – 164 ppm (C5), 157.77 – 158.35 ppm (C9). For ring B carbon atoms, signals were detected at δC 131.33 – 131.39 ppm (1'), 129.60 – 129.99 ppm (C4'), 129.09 – 130.44 ppm (3', 5'), 126.27 – 126.38 ppm (C2' and C6'). For C3 and C10 aromatic carbons, overlapping signals between δC 182.45 – 182.53 ppm (C3) and 67.76 – 67.91 ppm (C10). Oxygen bearing aliphatic carbon (O-CH2) was detected at δC 66.20 – 68.21 ppm. Signals for bromine bearing atoms (Br-CH2) were seen at δC 29.35 – 32.63 ppm. All other aliphatic carbon atoms were observed between δC 25.35 – 33.87 ppm. Proton and carbon atom assignments were corroborated by gHMBC and gHSQC experiments and HMBC correlation supported the O-alkylation of Chrysin derivatives at C7. These corresponds to signals previously reported (Comte et al., 2001; Cheng et al., 2014; Valdez-Caldron et al., 2016). NMR data matched the mass spectroscopy (MS) data for all synthesised chrysin derivatives. MS data for novel compounds are presented in appendix I.

Previously, some chrysin adducts have been made and their biological activities evaluated. Among the chrysin adducts made include the geranylated, prenylated, O-benzylated, C-benzylated, isopropylated and methylated chrysin derivatives by targeting positions 6, 7 and 8 of the chrysin molecule (Comte et al, 2001). Comte et al., (2001) reported a lower KD value (a quantitative measurement of antibody affinity), which was 500 folds lower than that of chrysin and concluded that alkylation of the chrysin molecule strengthens the interaction of chrysin at the modulatory site which binds hydrophobic compounds, and this methylation also inhibited the efflux of daunomycin from the cell domain, thus an increased activity of these methylated chrysin adducts. In this study, we synthesised novel methylated chrysin adducts (C5 and C16), which are structural isomers of C4 and C15. The boiling points of methylated chrysin adducts was slightly higher than those of the isomers without a methyl group with an increase ≥ 2 ˚ C. The methylated chrysin adducts also had a flaky powdery appearance (fine powders) compared to chrysin and other chrysin adducts. Chrysin adducts were soluble in chloroform unlike chrysin which was partially soluble in chloroform but very soluble in DMSO. The only chrysin adduct which was partially soluble in chloroform is C3 (lacks an alkyl group). Interestingly, like C3, C2 also had a phenyl substitution at position 7 (phenyl-7-O-chrysin) of the chrysin molecule but unlike the NO2 groups in C3, C2 has dimethyl groups attached to positions 3 and 5 of the phenyl ring, and

a methyl group separating the benzyl ring from the chrysin molecule at the 7-OH position. Thus, C2 was soluble in chloroform, C3 was partially soluble. This suggests that methylation of the chrysin molecule increases its solubility in chloroform and this finding agrees with those of Comte et al., (2001)

4.6.6 Hydrophilicity of Chrysin Derivatives

The solubility of chrysin derivatives synthesised in this work in polar solvents was determined as stated in section 2.25, page 128 and the results presented below.

Table 4.3: Hydrophilic index of synthesised chrysin derivatives in comparison to chrysin

Hydrophilicity in 75 % (v/v) solvent in water							
Abbre-viation	R_f Methanol	R_fX Methanol	R_f DMF	R_fX DMF	R_f THF	R_fX THF	Compound
C1	0.75	**1.07**	0.75	**1.15**	0.75	**1.25**	7-O-chrysinbutyl acetate
C2	0.70	**1.00**	0.70	**1.08**	0.70	**1.17**	7-O-chrysin-3,5-dimethylbenzene
C3	0.25	0.36	0.45	0.69	0.55	0.92	7-O-chysin-2,4-dinitrobenzene
C4	0.25	0.36	0.30	0.46	0.30	0.50	7-O-isopropylchrysin
C5	0.30	0.43	0.30	0.46	0.35	0.58	7-O-chrysin-2-methylpropane
C6	0.15	0.21	0.25	0.38	0.25	0.42	7-O-butylchrysin
C7	0.15	0.21	0.20	0.31	0.30	0.50	7-O-pentylchrysin
C8	0.15	0.21	0.20	0.31	0.35	0.58	7-O-hexylchrysin
C9	0.15	0.21	0.25	0.38	0.20	0.33	7-O-heptylchrysin
C10	0.10	0.14	0.20	0.31	0.30	0.50	7-O-octylchrysin
C11	0.10	0.14	0.20	0.31	0.30	0.50	7-O-nonylchrysin
C12	0.15	0.21	0.30	0.46	0.35	0.58	7-O-decylchrysin
C13	0.70	**1.00**	0.65	**1.00**	0.70	**1.17**	7-O-3-bromopropylchrysin
C14	0.50	0.71	0.55	**0.85**	0.50	**0.83**	7-O-4-bomobutylchrysin
C15	0.45	0.64	0.50	0.77	0.40	0.67	7-O-5-bromopentylchrysin
C16	0.35	0.50	0.45	0.69	0.45	0.75	7-O-4-bromopentylchrysin
C17	0.20	0.29	0.20	0.31	0.20	0.33	7-O-12-bromododecylchrysin
Chrysin	0.70	1.00	0.65	1.00	0.60	1.00	5,7-dihydroxyflavone

R_f = R_f value (ratio of the distance moved by the solute to the distance moved by the solvent); R_fX = hydrophilic index; DMF = dimethylformamide; THF = tetrahydrofuran; HC= hydrophilic index. The hydrophilic index is a measure of the potential ability of a molecule to dissolve in polar solvents. Hydrophilic compounds tend to be better candidates for pharmaceutical agents. C1, C2 and C13 indicated better hydrophilicity to chrysin.

From the R_f values of chrysin derivatives recorded using the thin-layer chromatography (TLC) plates, hydrophilicity indices – ability of chrysin derivatives to dissolve in polar

solvents (75 % v/v polar solvent in water) of these compounds (chrysin derivatives) was calculated using the equation: $HC = \frac{R_fX}{R_fC}$

The R_f values reported per chrysin derivative is an average of at least two independent experiments. The HC data indicates that the solubility of chrysin derivatives in the mixture of methanol, DMF or THF and water increases as from methanol to THF. This implies that chrysin derivatives dissolves better in 75 % v/v THF than DMF and methanol. From results obtained from Table 4.3, the solubility values of chrysin derivatives are at least 1.1 to 2.2 times more soluble in 75 % v/v DMF than in 75 % v/v methanol and 1.1 to 3.2 times more soluble in in 75 % v/v THF than in 75 % v/v methanol. Comparing the solubility of chrysin derivatives between THF and DMF (75 % v/v), it is observed that these compounds are about 1.0 to 2.0 times more soluble in THF than in DMF. It could therefore be inferred that the solubility of chrysin derivatives in the solvents investigated follows the order: THF > DMF > methanol. The HC of these compounds was calculated such that chrysin is a constant with numerical value 1.0. It could be hypothesised that chrysin derivatives with HC values greater than 1.0 are more lipophilic (soluble in polar solvents) than chrysin. Chrysin derivatives with HC 0.8 to 1.0 could be hypothesised to indicate similar lipophilicity to chrysin and those with HC ≤ 0.8 could be said to be less hydrophilic, compared to chrysin. Results presented on table 4.3 indicate that 7-O-chrysinbutyl acetate (C1) is more lipophilic than chrysin in all solvents used. 7-O-3-bromopropylchrysin (C13) is also more lipophilic than chrysin in 75 % v/v THF. C13 also indicated similar lipophilicity in 75 % v/v methanol and DMF respectively. 7-O-chrysin-3,5-dimethylbenzene (C3) also showed similar lipophilicity in THF to chrysin. Although the 7-O-bromoalkyl chrysin derivatives were more hydrophobic (less lipophilic) than chrysin, an interesting finding is observed. 7-O-bromoalkyl chrysin derivatives are more lipophilic than their 7-O-alkylchrysin derivatives (Table 4.3). This behaviour could be due to the similarity of chrysin polarity to these solvents. The aromatic ring present in the chrysin molecule makes chrysin less polar. Unfortunately, no work on the solubility of chrysin derivatives synthesised in this work in aqueous mixtures of THF, DMF and methanol (75 % v/v) has been reported in the literature for comparison. However, Zhou et al., (2014) has previously reported the mole fraction solubility values of chrysin in pure water at 250 °C to be 1.86 × 10− 5 moldm^{-3}. The better lipophilicity indicated by 7-O-bromoalkyl derivatives of chrysin compared to their 7-O-alkylchrysin derivatives could be because of the influence of the bromide attached to these compounds.

The role of the bromine atom in improving solubility of compounds in organic solvents have been previously reported (Christov, 2012; Królikowska & Hofman, 2019).

Chapter 5 Results / Discussion: Biological Activities and Selectivity of Sesquiterpene Lactone Extracts and Synthesised Chrysin Derivatives

Biological activities investigated in this project are divided into two: antibacterial activities and anticancer activities. This project has been divided into two distinct sections (see the project aims). The results for the antibacterial and anticancer activities of sesquiterpene lactones isolated in this study, and the results for the antibacterial and anticancer activities of synthetic chrysin derivatives are presented below.

5.1 Results / Discussion for Antibacterial Activities of Isolates from *Inula helenium*

Dried *Inula helenium* extracts obtained from Soxhlet extraction (SE), microwave-assisted extraction (MAE) and ultrasound-assisted extraction (UAE), fractionated using different solvents (methanol, ethyl acetate and hexane) were tested against the microbial species used in this study and the respective zone of inhibition is reported in Table 5.1 below.

Table 5.1: Zone of inhibition (mm) of extracts of *Inula helenium* against standard microbial strains

Microorganisms	Soxhlet extraction (SE) (100 µg / mL)			Microwave-assisted extraction (MAE) (100 µg / mL)			Ultrasonic assisted extraction (UAE) (100 µg / mL)			Standard Gentamycin / Fluconazole (10 µg /mL)
	ZOI (mm)			ZOI (mm)			ZOI (mm)			ZOI (mm)
	M	E	H	M	E	H	M	E	H	DMSO
Escherichia coli NCTC 13353	2	4	15	2	3	13	2	3	16	27
Pseudomonas aeruginosa NCTC 13437	-	4	16	2	-	13	3	2	18	27
Bacillus cereus ATCC 10876	6	3	21	-	3	15	2	3	21	25
Staphylococcus aureus ATCC 25923	5	5	19	4	4	15	2	4	21	24
MRSA ATCC 252 NCIMB 13280	-	-	5	-	-	5	-	3	7	20
Enterococcus faecalis NCIMB 13280	3	4	10	-	-	13	-	2	12	24
Pseudomonas flores-cens ATCC 13525	2	4	11	3	-	7	2	2	14	25
Klebsiella pneumoniae NCTC 13439	-	4	5	-	2	6	-	2	5	26
Candida albicans MTCC 227	2	2	4	2	3	4	2	2	5	-

M – Methanol; E – Ethyl acetate; H – Hexane; – means no activity observed.

NCTC - National Collection of Type Culture
NCIMB - National Collection of Industrial Food and Marine Bacteria
ATCC - American Type Culture Collection
MTCC - Microbial Type Culture Collection
ESBL - Extended Spectrum β-Lactamase

Extracts indicating inhibition zones ≥ 8 mm were isolated (because one of the aims of this work are to isolate bioactive compounds) and purified via high-performance liquid chromatography (HPLC) to obtain three pure bioactive sesquiterpene lactones – alantolactone (D1), isoalantolactone (D2) and costunolide (D3). These pure compounds were tested to determine the minimum inhibitory concentration (MIC) against these microbial strains. Disc diffusion assay was not done for these compounds (D1, D2 and D3) because they were the predominant compounds present in the hexane fraction isolated from *Inula helenium* (accounting for over 89 % of total compounds isolated from the hexane fraction). The result for the bactericidal inhibition indicated by these sesquiterpene lactones is presented in Table 5.2 below.

Table 5.2: MIC / MBC of sesquiterpene lactones isolated from *Inula helenium*

Microorganisms	AL (µg /mL)	IAL (µg /mL)	CST (µg /mL)	Gentamycin/ Fluconazole (µg /mL)
Escherichia coli NCTC 13353	500 / ND	500 / ND	- / ND	4 / ND
Pseudomonas aeruginosa NCTC 13437	- / ND	- / ND	- / ND	8 / ND
Bacillus cereus ATCC 10876	50 / 100	50 / 100	125 / 250	8 / ND
Staphylococcus aureus ATCC 25923	65 / 130	65 / 130	125 / 125	4 / ND
MRSA ATCC 252 NCIMB 13280	- / ND	- / ND	- / ND	16 / ND
Enterococcus faecalis NCIMB 13280	250 / ND	250 / ND	500	8 / ND
Pseudomonas florescens ATCC 13525	250 / ND	500 / ND	- / ND	8 / ND
Klebsiella pneumoniae NCTC 13439	- / ND	- / ND	- ND	16 / ND
Candida albicans MTCC 227	> 500	> 500 / ND	250 / ND	150 / ND

AL - Alantolactone
IAL - Isoalantolactone
CST - Costunolide
\- - No activity observed at MIC ≥ 500 µg /mL.
ND - Not done

The result presented for (Agar diffusion assay) – Table 5.1, indicates that the isolates from *Inula helenium* elicited antibacterial activities. The best antibacterial activity was recorded with hexane fraction obtained by ultrasound-assisted extraction (UAE) followed by hexane fraction from the Soxhlet extractor. Compounds isolated from ethyl acetate and methanol fraction indicated weak antimicrobial activity for all the extraction methods used. From the results presented in Table 5.1, only the hexane fractions (from SE, MAE and UAE) were

further purified and three bioactive sesquiterpene lactones were purified by HPLC from the hexane fractions of *Inula helenium* – alantolactone (D1), isoalantolactone (D2) and costunolide (D3). These fractions were combined because the analysis on TLC plate suggests they contain similar spots and an assumption was made that they contained similar compounds.

Compounds isolated from UAE (hexane fraction) showed inhibition zones of 27 mm on ESBL producing *E. coli* NCTC 13353 and *P. aeruginosa* NCTC 13437*,* 26 mm on *K*. *pneumonia* NCTC 13439, 25 mm on *B. cereus* ATCC 10876 and *P. florescence* ATCC 13525, 24 mm on *S. aureus* ATCC 25923 and *E*. *faecalis* NCIMB 1328 and 20 mm on *MRSA* ATCC 252. Activities indicated by hexane fractions from MAE and UAE were comparable with those from the UAE. Poor inhibition (inhibition > 125 µg/ml) was recorded for fractions from methanol and ethyl acetate in all extraction methods used Table 5.1.

The compounds isolated and purified from the hexane fraction of *Inula helenium* – alantolactone, isoalantolactone and costunolide were tested against bacteria and fungus species using the micro broth dilution method.

The sesquiterpene lactones isolated inhibited the bacterial species tested except *P. aeruginosa* and *K. pneumonia*. The compound alantolactone elicited good inhibitory action against *B. cereus* (MIC 50 µg/ml) and *S. aureus* (MIC 65 µg/ml). Alantolactone also indicated moderate inhibition of *E. faecalis* and *P*. *florescens* (MIC 250 µg/ml) and poor inhibitory action against *E. coli* (500 µg/ml). *MRSA*, *K. pneumonia* and *P. aeruginosa* were not sensitive to inhibition by alantolactone at doses ≤ 1000 µg/ml. The compound isoalantolactone elicited a good antibacterial action, similar to that indicated by alantolactone on *B. cereus* (MIC 50 µg/ml), *S. aureus* (MIC 65 µg/ml); moderate inhibition (MIC 250 µg/ml) against *E*. *faecalis* and poor inhibitory actions (500 µg/ml) against *P. florescence* and *E. coli*, and like alantolactone, *MRSA, K. pneumonia* and *P. aeruginosa* were not sensitive to inhibition by isoalantolactone. Costunolide indicated moderate inhibition against *B. cereus* and *S. aureus* (MIC 125 µg/ml), poor inhibitory action against E. *faecalis* and no inhibition against *E. coli, P. aeruginosa, MRSA* and *P. pneumonia*. Alantolactone and isoalantolactone indicated similar antibacterial activity while costunolide indicated moderate to weak antibacterial activities against all bacterial strains tested.

The bioactive compounds isolated from *Inula helenium* belongs to a group of compounds known as sesquiterpene lactones and do have diverse chemical structures and a wide spectrum of biological activities such as cytotoxic, antibacterial, antifungal, constituent of

plant growth regulating hormone, insect anti-feedant, antitussive, anti-tumourigenic and anti-protozoal activities (Seca et al., 2014). This study reports the isolation and antibacterial activities of alantolactone, isoalantolactone and costunolide isolated from the roots of *Inula helenium*. Disc diffusion assay with hexane fraction from SE, UAE and MAE all indicated good zone of inhibition (ZOI) against all bacteria tested (MIC 15 – 27 mm) except *MRSA* (ZOI 5 – 6 mm). Kunduhoglu et al., (2011) reported that ethanol extracts contained the bioactive components of *Inula helenium*. Conversely, Stanojević et al., (2010) reported activity for aqueous extracts. Talib & Mahasneh, (2010) reported a good inhibitory action for methanol extracts against *MRSA* but in our findings, methanol and ethanol fractions did not indicate antibacterial activity against *MRSA*; only the hexane fraction did. Methanol and ethyl acetate extracts indicated poor activities with ZOI (2 – 6 mm) on all bacteria tested. In this study, water extracts initially tested for antimicrobial activity indicated no ZOI against bacterial species and for this reason, it was discontinued (data not shown). The sesquiterpene lactones were responsible for the antibacterial activity reported in this study. Sesquiterpene lactones were fractionated from the hexane fraction; an indication that they are soluble in non-polar solvents (hexane) and poorly soluble in polar solvents (such as water and methanol). Since water is more polar than methanol, this could account for the lack of ZOI recorded for the water fraction.

Diguţă et al. (2014) has previously reported the antimicrobial activity of *Inula* specie against some bacterial strains using agar disk diffusion method. Comparison of antimicrobial activities of ethanolic extracts of powdered roots of *Inula helenium* using 30%, 50% and 70% ethanol. The 70% ethanolic extract showed high inhibitory activity against *E. coli,* the 50% ethanolic extract showed moderate inhibitory activity against *E. coli*, whereas low inhibitory activity was recorded for the 30% extract. Although the researcher did not indicate the concentrations used, these findings agrees with our findings, and indicates that hydrophobicity of extraction solvents alone is not enough in the extraction of compounds from *Inula helenium*. Our report also indicates that as polarity of solvents increased, the antimicrobial activities of the plants extracts also increased. For instance, compounds fractionated using hexane indicated better inhibitory activities against bacterial strains. These activities were better than those indicated by methanol – a polar solvent, and ethyl acetate, a less polar solvent, indicated minimal inhibition against bacterial strains. It could therefore be suggested that polarity of solvents is responsible for extraction of active compounds from *Inula helenium*.

Diguţă et al. (2014) also reported similar trend for *S. aureus* and *E. faecalis*. No inhibitory activity was reported in the case of 30% ethanolic extract against *S. aureus* and *E. faecalis*

whereas the 70% ethanolic extract indicated moderate inhibitory activities against *S. aureus* and *E. faecalis*. These findings are similar to the findings in this study. For instance, good and significant activities were recorded against *E. coli, S. aureus* and *E. faecalis* using the hexane fraction. These activities were poor in the extracts obtained from polar solvents – methanol and ethyl-acetate.

Qiu et al., (2011) and Zhao et al., (2010) reported antibacterial activities of alantolactone and isoalantolactone against Gram-negative bacteria with isoalantolactone also acting as an inhibitor of α-toxin, (which confers virulence to bacteria), preventing its expression. The differences in inhibitory activities of these isomeric sesquiterpene lactones could be attributed to their conformational orientations at the carbonyl moiety and the stearic hindrances at the axial methyl groups of these compounds (Blagojevic & Radulovic, 2012).

Compounds isolated from *Inula helenium* – alantolactone, isoalantolactone and costunolide indicated poor inhibitory activities against Gram-negative bacteria tested – *E. coli, P. aeruginosa, K. pneumonia* and *P*. *florescens* with MIC ≥ 250 µg /mL. These results were comparable to those reported by Jiang et al., (2011), except for *E. coli.* Jiang et al., (2011) reported an MIC of 125 µg /mL for alantolactone against *E. coli*. The poor antibacterial activity of these compounds could be attributed to the outer membrane in Gram-negative bacteria which prevents influx of these compounds into the bacterial cell. Most bacteria also develop enzymes such as β-lactamase, which inactivates drugs (Kapoor et al., 2017; Mandal & Das, 2015).

Antibacterial activity of ethanolic extracts of *Saussurea lappa* (MIC 0.5 µg /mL) against *Streptococcus mutans* was reported by Yu et al., (2007). There are sketchy data on the antibacterial activity of costunolide. In this work, costunolide indicated moderate bacterial inhibition against *B. cereus* and *S. aureus* at MIC 125 µg /mL) but no inhibition against all bacterial strains tested at MIC < 500 µg /mL. The poor bacterial inhibition shown by costunolide (compared to alantolactone and isoalantolactone) may be because of the orientation (annulation) of the γ-lactone ring (Ludwiczuk et al., 2017). Alantolactone and isoalantolactone are classified as eudesmanolide sesquiterpene lactones (Seca et al., 2014) and are orientated in the 8,12-olides annulation (Ludwiczuk et al., 2017) whereas costunolide, a germacronolide sesquiterpene lactone (Seca et al., 2014) is oriented in the 6,12 annulation (Ludwiczuk et al., 2017). The role of annulation (Ludwiczuk et al., 2017) and the type of functional chains attached to sesquiterpene lactones as the reason for the diverse antibacterial activity of these compounds has been reported (Bachelier, 2006).

The compounds isolated also inhibited the pathogenic fungus tested. Costunolide inhibited the growth of the fungus *C. albicans* at a dose of 250 µg /mL better than alantolactone and isoalantolactone which indicated inhibition at doses > 500 µg / mL. Ahmed & Abdelgaleil, (2005) reported the antifungal activity of costunolide isolated from the bark of *Magnolia grandiflora* against pathogenic fungi with costunolide showing a better antifungal activity compared to the reference drug - Fluconazole. In this study, costunolide inhibited the growth of *C. albicans* better than alantolactone, isoalantolactone and the control drug – Fluconazole (MIC 150 µg / mL).

Isoalantolactone and alantolactone exist mainly in the "U"-shape conformations and the carbonyl moiety is hindered sterically by the axial methyl groups, whereas costunolide can exist in any of the four (UU, UD, DU and DD) conformations, giving it the ability to lyse the cell membrane of the pathogenic fungus - *C. albicans*. Faraldos et al., (2007) reported the conformational structure of costunolide. The ability of costunolide to exist in the four conformational states gives it advantage over alantolactone and isoalantolactone which exist only in the “U” conformation and thus, a better antifungal activity than alantolactone and isoalantolactone against *C. albicans*.

The ability of these sesquiterpene lactones to dissolve in hexane, a low polar solvent, indicates sesquiterpene lactones isolated in this study have smaller pore sizes. This gives them the ability to pass through the cell wall of *C. albicans*, eliciting their action against this fungus. It should be noted that the sesquiterpene lactones isolated from *Inula helenium* and as purified by HPLC presented different appearances; alantolactone was isolated as a white powdery compound, isoalantolactone was isolated as a needle-like white precipitate whereas costunolide was isolated as a semi-transparent white compound. Costunolide can easily pass through the cell wall of *C. albicans* compared to other compounds isolated in this study. Barrero et al., (2000) reported that low polarity is a molecular requirement for antifungal activities of compounds. Barrero et al., (2000) reported in his findings that lactones with higher polarities were better antifungal agents. Thus, the sesquiterpene lactones - costunolide and dehydrocostus lactone (with low polarities) indicated better antifungal activities than salonitenolide and cnicin. For compounds to be effective antifungal agents, they must possess the ability to pass through the cell membrane and the sesquiterpene lactones isolated in this project meets this criterion. This explains why alantolactone, isoalantolactone and costunolide elicited weak antifungal activity against *C. albicans*. In addition to polarity, it has also been established that the α-methylene-γ-lactone moiety is responsible for biological activities of sesquiterpene lactones (Lawrence et al., 2001; Seca et al., 2014); it therefore implies that polarity, the conformation of the

sesquiterpene lactone, the small size of the molecule and the α-methylene-γ-lactone moiety are responsible for the antifungal activities indicated by the sesquiterpene lactones isolated in this study.

An important finding was made when comparing the ZOI of the fractions reported in Table 5.1 against *P. aeruginosa*, the hexane fraction indicated ZOI of 16 mm for Soxhlet extraction (SE), 13 mm for microwave-assisted extraction (MAE) and 18 mm for ultrasound-assisted extraction (UAE). MIC investigation of purified compounds isolated from the hexane fractions indicated no bacterial inhibition against *P. aeruginosa*. A look at the HPLC chromatogram (Figure 3.2) indicates the presence of traces of compounds which were biologically inactive against the bacteria tested. There is a possibility that the ZOI observed against *P. aeruginosa* may be attributed to:

i) One or more compounds eluted from 5 – 30 minutes.
ii) The potentiating effect of two or more sesquiterpene lactones isolated in this study.
iii) A potentiating effect of all compounds in the hexane fraction.

Spiridonov et al., (2005) has previously reported the potentiating effect of helenin – a combination of alantolactone (40 %) and isolantolactone (60 %), which indicated a better bioactivity compared to the positive control drug used in a certain study. The potentiating effect of compounds isolated from *Inula helenium* was not done in this study. It is therefore hypothesised that the good antibacterial activity (ZOI) recorded by the hexane fraction against *P. aeruginosa* is as a result of the hypothesis highlighted above.

5.2 Results for Antibacterial Activities of Synthesised Chrysin Derivatives

7-O-chrysin adducts made in this study were screened against the microbial species tested in this study and the zone of inhibition reported in Table 5.3. These results (Agar diffusion assay) indicates that the chrysin analogues made in this study, elicited antibacterial activities against both Gram-negative and Gram-positive bacteria. The best antibacterial activity was recorded for the Gram-positive bacteria with compounds C1, C2, C3, C6, C8 and C16 indicating zone of inhibition ranging from 12 – 19 mm on *S. aureus* with C1, C3, C2, C8 and C16 indicating inhibitory zones of 19, 17, 16, 16 and 15 mm respectively. On *E. coli,* C3, C2, C1, C5 and C16 indicated inhibitory zones of 16, 15, 13, 12 and 12 mm respectively. Compounds C7, C9 – C15 indicated inhibitory zones ≤ 2 mm and for this reason were not diluted via the micro broth dilution method to determine their MIC. Compounds indicating zone of inhibition ≥ 4 mm were diluted further via micro broth dilution method to determine their MIC (Table 5.4).

Table 5.3: Zone of Inhibition (mm) of chrysin adducts against Standard Microbial Strains

Compound abbreviation	*Escherichia coli* NCTC 13353	*Staphylococcus aureus* ATCC 25923	*Candida albicans* MTCC 227	Name of the compound used
C1	16	18	15	7-O-chrysinbutyl acetate
C2	15	16	8	7-O-chrysin-3,5dimethylbenzene
C3	17	17	14	7-O-chrysin-2,4-dinitrobenzene
C4	10	8	5	7-O-isopropylchrysin
C5	12	8	12	7-O-2-methylpropylchrysin
C6	-	12	5	7-O-butylchrysin
C8	8	16	7	7-O-hexylchrysin
C16	12	15	9	4-bromopentylchrysin
C17	-	9	-	12-bromododecylchrysin
Chrysin	6	6	9	5,7-dihydroxyflavone
Gentamycin/ Fluconazole	24	26	12	Gentamycin / Fluconazole

– - No activity

One Gram-positive bacterium (*S. aureus*) and one Gram-negative bacterium (*E. coli*) were used for disc diffusion assay prior to MIC assay. In order to study the activity in more detail, strains from representative Gram-positive and Gram-negative species were chosen for further testing. Following a review of the literature, it was found that *S. aureus* and *E. coli* are among the most commonly used species when testing novel antimicrobials. Compounds not stated above did not show any noticeable inhibition of bacterial growth at doses ≥ 500 µg/mL. C6 and C17 showed inhibition against Gram-positive bacteria only.

In order to determine the antimicrobial activities of chrysin analogues using the broth microdilution method, compounds synthesised from chrysin have been classified into three series according to their functional groups, aromaticity or aliphatic nature.

Series 1: Compounds C1, C2 and C3.

Series 2: Alkyl derivatives of chrysin (C4 – C12).

Series 3: Bromoalkylchrysin derivatives (C13 – C17).

For **Series 3** compound, C13 – C15 were inactive against the bacterial species tested. Interestingly, **C16** (a novel compound and an isomer of compound 15) indicated good inhibition against *S. aureus*, *E. faecalis, E. coli* and *MRSA* (MIC 50, 100, 100 and 125 µg /mL respectively).

Table 5.4: MIC / MBC of Chrysin adducts against Bacteria and Parasitic Fungus – *Candida albicans*

	Bacterial species tested MIC / MBC is recorded in µg / mL								**Fungus**	**Compound name**
	Gram-negative bacteria				**Gram-positive bacteria**					
Comp-pound	*E. coli NCTC 13353*	*P. aeruginosa* NCTC 13437	*K. Pneumonia* NCTC 13439	*P. Florescens ATCC 13525*	*MRSA ATCC 252*	*E. faecalis NCIMB 13280*	*S. aureus ATCC 25923*	*B. cereus ATCC 10876*	*C. albicans MTCC 227*	
C1	**50.0 / 100.0**	100.0 /200.0	125.0 / 500.0	**62.5 / 250.0**	100.0 / 200.0	**50.0 / 150.0**	**25.0 / 50.0**	**50.0 / 150.0**	**50.0**	7-O-chrysinbutyl acetate
C2	100 / 200	200.0 /800.0	250 / 500	125 / 250	250 / 500	100 / 200	**50 / 100**	100 / 200	400.0	7-O-chrysin-3,5dimethylbenzene
C3	**12.5 / 25**	**25.0 / 50.0**	**62.5 /** 250.0	**31.3 / 62.5**	**62.5 /** 125.0	**12.5 / 50.0**	**12.5 / 25.0**	**12.5 / 100.0**	**50.0**	7-O-chrysin-2,4-dinitrobenzene
C4	200.0	-	-	-	-	-	200.0	Nt	800.0	7-O-isopropylchrysin
C5	100.0	200.0	250.0	125.0	-	-	100.0	Nt	**100.0**	7-O-2-methylpropylchrysin
C6	-	400.0	-	250.0	-	-	200.0	Nt	800.0	7-O-butylchrysin
C8	400.0	200.0	-	500.0	250.0	100.0 / 400.0	**50.0 /** 200.0	Nt	400.0	7-O-hexylchrysin
C16	**100.0**	-	-	-	125.0	100.0	**50.0**	Nt	200.0	7-O-4-bromopentylchrysin
C17	-	-	250.0	-	-	200.0	100.0	Nt	-	7-O-bromododecylchrysin
Chrysin	250.0	-	-	500.0	-	250.0	125.0	125.0	200.0	5,7-dihydroxyflavone
Gentamicin Fluconazol	8.0	16.0	16.0	16.0	32.0	8.0	8.0	8.0	125.0	-

ESBL – Extended Spectrum Producing β-lactamases

Nt - Not tested.

\- - No observed inhibition at doses ≥ 500 µg / mL for bacteria and 800 µg / mL for the fungus – *C. albicans.*

C3 indicated bacterial inhibition against all bacteria tested at MIC values ≤ 62.5 µg/mL. C3 showed interesting inhibition against *S. aureus, B. cereus, E. faecalis* and *E. coli* were at MIC value of 12.5 µg/mL. C1 inhibited *S. aureus* at MIC value 12.5 µg/mL. C3 also inhibited *P. aeruginosa* at MIC value of 25.0 µg/mL. C1, C2 and C3 showed inhibition against all bacteria tested at MIC values ≤ 250 µg/mL.

C17 indicated good antibacterial activity (MIC 100 µg /mL) against *S. aureus* and moderate inhibition against *E. faecalis* (200 µg /mL and *K. pneumonia* (250 µg /mL) (Table 5.4). *P. aeruginosa* and *E. faecalis* were not inhibited by compounds in this series. For **series 2** compounds, C7, C9 – C15 were inactive against the bacterial strains tested. However, C4, C5, C6 and C8 elicited inhibition against bacteria tested with MIC values ranging from 50 – 400 µg /mL. C8 showed a wide spectrum of activity against bacteria exerting good activity against *S. aureus* and *E. faecalis* – (MIC 50 and 100 µg /mL), C8 is the only member of this series which inhibited the growth of the antibiotic resistant bacteria *MRSA* (MIC 250 µg /mL) and *E. faecalis* (Table 5.4). C8 also inhibited the growth of *P. aeruginosa* at a dose of 200 µg /mL. With exception of C4 (MIC 400 µg /mL), other compounds in this series – C5, C6 and C8 showed good antibacterial inhibition against *S. aureus* (MIC 100, 100 and 50 µg /mL). *P. aeruginosa* was susceptible to compounds (C4, C5, C6 and C8) in this series with MIC 100, 200, 400 and 200 µg /mL respectively (Table 5.4). C4 and C5 also showed good inhibition (MIC 100 and 50 µg /mL) against *E. coli*, while C6 indicated no inhibition against *E. coli* at doses ≤ 1000 µg /mL. *K*. *Pneumoniae* was not inhibited by compounds in this series except C5 which indicated moderate inhibition (MIC 250 µg /mL).

Series 1 compounds indicated a wide spectrum of good antibacterial activities against bacteria tested. C1 and C3 indicated a good antibacterial activity on all bacterial strains tested with MIC values ranging from 12.5 – 125 µg /mL). C3 showed a broad spectrum of inhibition against all bacterial strains tested with MIC values ranging from 50 – 250 µg /mL.

C1 indicated a similar antibacterial activity to C3, inhibiting all bacterial strains tested with *S. aureus* (MIC 25 µg /mL), *E. coli, E. faecalis* and *B. cereus* (MIC 50 µg /mL) being the most susceptible bacteria to inhibition by C1 which also indicated good antimicrobial activity against the antibiotic resistant bacteria – *MRSA* and *E. faecalis* at MIC of 100 µg /mL. C1 also indicated moderate inhibition against *K. pneumonia* (MIC 125 µg /mL) which was the least susceptible bacterium to inhibition by C1.

C2 also indicated a broad spectrum of antibacterial activities against all bacteria tested with *Staphylococcus aureus* (MIC 50 µg /mL)*, Bacillus cereus, E-coli* and *E. faecalis* (100 µg /mL) indicating good susceptibility to C2. C2 also showed moderate inhibition against *MRSA, P. aeruginosa* and *K. pneumonia* (MIC 250, 200 and 250 µg /mL respectively).

The main source of chrysin is from propolis. Previous reports have indicated antibacterial activity of ethanolic extracts from propolis against *S. aureus* at MIC ranging from 10 – 20 µg/ml and MBC of 20 µg/ml (Chen, et al., 2018). Propolin C characterised from propolis

indicated MIC values ranging from 1.25 to 10 µg/ml against *S. aureus*, MIC ≤ 2 µg/ml for *MRSA*. Sousa et al., (2016) also reported MIC values of 7.5, 10 and 10 µg/ml against *S. aureus*, *E. coli* and *P. aeruginosa* treated with methanolic extracts of propolis. Chrysin has been purified as the main constituent of propolis and has been reported to inhibit bacterial growth at MIC value ranging from 100 – 250 µg/ml (Li et al., 2009). This value is comparable to the findings reported in this study (MIC values of 125 µg/ml for *S. aureus* and *B. cereus*; 250 µg/ml for *E. coli* and *E. faecalis*). The novel chrysin derivatives C1, C2 and C3 showed interesting antibacterial activity against Gram-positive and Gram-negative bacteria with the former being the most susceptible bacteria to inhibition by these compounds. The significant antibacterial activity reported in this study for synthetic chrysin derivatives – C1, C2 and C3 against Gram positive bacteria may be attributed to the absence of outer cell membrane found in Gram-negative bacteria (Ramírez-Guízar et al., 2017). The absence of the outer membrane allows easy penetration of small molecules across the periplasmic space, into the bacteria (Miller et al., 1986). Li et al, (2009) had previously synthesised chrysin derivatives containing 3-carbon spacers attached to different positions of the chrysin molecule. An investigation of antibacterial activities of these compounds showed that heterocyclic compounds attached at the 7-OH position of chrysin were more effective compared to those whose attachments were elsewhere with MIC values ranging from 3.13 to 50 µg/ml against *S. aureus* and *E. coli* respectively. These findings are in agreement with our findings in this work. Furthermore, we report that heterocyclic chrysin-derivatives indicated better antibacterial activities ranging from 12.5 µg/ml for C3 against *B. cereus*¸*S. aureus*, *E. faecalis* and *E. coli*; 25.0 µg/ml against *P. aeruginosa* and 31.3 µg/ml against *P. florescens*. *MRSA* and *K. pneumoniae* were inhibited at 62.5 µg/ml. Antibacterial activity of heterocyclic compounds were better than those indicated by aliphatic compounds. Our finding correspond with those of Li et al, (2009) who also reported similar findings. Aliphatic derivatives of chrysin containing 3-carbon spacers indicated weak antibacterial inhibition compared to heterocyclic compounds (Li et al., 2009). The good inhibition shown by heterocyclic chrysin derivatives (C1, C2 and C3) might be connected with the hydrophobicity of these molecules (Comte et al., 2001). Comte et al., (2001) reported that the hydrophobicity of synthetic 7-O-Chrysin derivatives enable them to bind to P-glycoprotein, and allows for influx of drugs into the cell thus an increase intracellular drug accumulation which is necessary for bacterial inhibition and death. Long chain 7-O-chrysin derivatives synthesised in this work are less hydrophobic than their heterocyclic counterpart. This might be responsible for their reduced ability to accumulate in the bacterial cell.

Antibacterial activities indicated by C1, C2 and C3 were better than those of other chrysin derivatives. Chrysin contains two hydroxyl functional groups at position 5 and 7. The hydroxyl bond at position-5 of chrysin creates hydrogen-bonding in the chrysin molecule (Zheng et al., 2016; Lapkin et al. 2014). The hydroxyl-bond at position-7 of chrysin molecule increases its hydrophilicity. Because chrysin is more hydrophilic than synthesised chrysin derivatives made in this study (Comte et al., 2001), chrysin might lack the ability to penetrate the bacterial cell compared to C1, C2 and C3 that are mainly hydrophobic in nature.

Chrysin derivatives made in this study were most potent against Gram-positive bacteria (with exception of C3 that significantly inhibited the growth of both Gram-positive and Gram-negative bacteria).

5.3 Antifungus Activity of Synthesised Chrysin Derivatives

7-O-chrysin adducts made in this study were screened against the fungus *C. albicans* and results presented in Table 5.4 (Agar disk diffusion assay) and Table 5.4 (MIC) above. C1 (ZOI 15 mm), C2 (ZOI 14 mm) and C4 (ZOI 11 mm) indicated ZOI greater than 10 mm.

C7, C9 – C15 and C17 did not inhibit the growth of the *C. albicans* fungus at doses ≤ 800 µg / mL. Other Chrysin derivativesshowed a ZOI ranging from 5-9 mm; C16 (ZOI 9 mm), C2 (ZOI 8 mm), C8 (ZOI 7 mm), C4 and C5 (5 mm). Compounds indicating ZOI against the *C. albicans* fungus were diluted further to determine their MIC.

C1 and C3 indicated an MIC of 50 µg / mL, C5 (100 µg / mL), C16 (200 µg / mL). Other compounds (C2, C4, C6 and C8) indicated ZOI ≥ 400 µg / mL.

C1 and C3 showed better ZOI compared to the positive control drug – Fluconazole (ZOI 12 mm). ZOI indicated by C5 (12 mm) is comparable to that indicated by Fluconazole (12 mm). C1, C2 (MIC 50 µg / mL) and C5 (MIC 100 µg / mL) also indicated better MIC values compared to Fluconazole (125 µg / mL) – Table 5.4.

Chrysin is the major flavonoid present in propolis (honey), accounting for 62 – 80 % of total flavonoids (Koo et al., 2002; Uzel et al., 2005). Antifungal activity of propolis has previously been reported against *C. albicans*, *C. tropicalis* and *C. krusei* at MIC 32, 32 and 16 µg / mL (Uzel et al., 2005). Salomão et al. (2004) and Kujumgiev et al. (1999) have previously reported the antifungal activity of propolis at ZOI 18 mm and 22 mm respectively. Antifungal activities reported were due to the presence of flavonoids – chrysin, galangin and flavonol (Koo et al., 2002). Interestingly, most authors have reported antifungal activities of propolis

without mentioning the antifungal activities of chrysin. In this work, chrysin, inhibited *C. albicans* at 200 µg / mL. There are sketchy literatures (data) on the antifungal activity of chrysin as most authors only reported the antifungal activities of propolis obtained from different locations (Koo et al., 2002; Salamão et al., 2004; Uzel et al., 2005). Uzel et al., (2005) characterised apigenin, galangine, quercetin, naringenin and pinocembrin as the flavonoids present in propolis. It could therefore be inferred that the MIC of 4 µg / mL against *C. albicans* ATCC 10231 reported by Uzel et al., (2005) might be due to the presence of not just chrysin but other flavonoids. *C. albicans* ATCC 10231 is also known as a susceptible strain which shows least resistance against fungicidal agents such as fluconazole and it is often used as a positive control strain (Maebashi et al., 2001) whereas the strain used in this work (*C. albicans* MTCC 227) is known for indicate resistance to fluconazole and most other antifungal agents. These factors may be responsible for the poor antifungal activity recorded by chrysin in this study. Unlike chrysin, synthesised chrysin-derivatives (C1, C3 and C5) indicated better activities than chrysin and the positive control drug – fluconazole against the *C. albicans* fungus. Elevated levels of CDR1 and CDR2 mRNA encoding putative ATP binding cassette (ABC) transporters have been reported as being responsible for *C. albicans* MTCC 227 resistance (Maebashi et al., 2001). C1, C3 and C5 might have accumulated intracellularly within the fungus whose expression of CDR1 and CDR2 involved in the fluconazole resistance may be absent. This process is energy-dependent (Maebashi et al., 2001) and tends to reduce the lipophilicity of fluconazole across the fungal membrane as azole (in fluconazole) are less lipophilic than flavonoids (as in chrysin-derivatives) (Sheehan et al., 1999). Lipophilicity is an important criterion which allows drugs to pass through cell membranes into the intercellular space of organisms (Sheehan et al., 1999). Another mechanism of antifungal action involves the inhibition of ergosterol, an important component of fungal plasma membrane is via inhibition of the cytochrome P-450-dependent enzyme lanosterol demethylase (Hargrove et al., 2017).

5.4 Results for Anticancer Activities of Sesquiterpene Lactones isolated from *Inula helenium*.

Fractions from *Inula helenium* extracts (methanol, ethyl acetate and hexane) were tested against leukaemia cell lines (K562 and Molt-4), and colorectal cancer cell line (Caco-2). Results obtained are presented in Table 5.5 below. The hexane fraction indicated good cytotoxic inhibition (0.8 – 36.68 µg/mL) against the cancer cell lines tested. Due to poor cytotoxic inhibition shown by the methanol and ethyl acetate fractions, they were not

purified further by HPLC and the cytotoxicity of pure compounds from these fractions were not investigated. This is because the aim of this project is to discover new bioactive compounds.

Table 5.5: IC_{50} values for K562, Molt-4 and Caco-2 cell lines treated with fractions (methanol, ethyl acetate and hexane fractions) obtained from *Inula helenium* root extracts

Extraction solvent	**Cancer Cell line / IC_{50} (µg/mL)**								
	K 562			**Molt 4**			**Caco 2**		
	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h
Methanol fraction	-	-	38.02 ± 4.25	-	-	-	-	-	-
Ethyl acetate fraction	-	50.03 ± 6.92	24.36 ± 3.83	-	-	-	-	-	-
Hexane fraction	29.08 ± 0.42	21.74 ± 0.28	10.8 ± 0.96	38.63 ± 3.45	31.22 ± 4.86	14.47 ± 2.85	-	28.32 ± 2.58	12.22 ± 1.03
Chlorpromazine hydrochloride	14.28 ± 2.57	12.23 ± 1.77	5.62 ± 0.88	7.91 ± 0.29	8.17 ± 0.09	6.83 ± 0.35	8.18 ± 0.24	8.93 ± 0.28	3.08 ± 0.12

- - No cytotoxic inhibition at IC_{50} ≤ 100 µg/mL
h - hours

The anticancer activities of sesquiterpene lactones isolated from *Inula helenium* in this study has previously been reported and documented against some cell lines used in this study (see section 1.4).

Table 5.6: IC_{50} values for sesquiterpene lactones – alantolactone, isoalantolactone and costunolide against K562 cell line

K562 Cancer Cell line treated for 24, 48 and 72 hours			
	IC_{50} (µM)		
Compound	24 hours	48 hours	72 hours
Alantolactone (D1)	24.86 ± 4.16	13.48 ± 1.07	4.95 ± 0.68
Isoalantolactone (D2)	28.44 ± 3.88	21.38 ± 5.25	7.38 ± 2.01
Costunolide (D3)	6.68 ± 1.17	11.72 ± 3.02	10.98 ± 3.64
Chlorpromazine hydrochloride (CPZ)	13.66 ± 0.52	9.92 ± 1.62	4.53 ± 0.87

Table 5.7: IC_{50} values for sesquiterpene lactones – alantolactone, isoalantolactone and costunolide against Molt-4 cell line

Molt-4 Cancer Cell line treated for 24, 48 and 72 hours			
	IC_{50} (µM)		
Compound	24 hours	48 hours	72 hours
Alantolactone (D1)	-	-	-
Isoalantolactone (D2)	-	-	-
Costunolide (D3)	30.66 ± 4.72	46.26 ± 7.21	-
Chlorpromazine hydrochloride (CPZ)	8.21 ± 1.01	5.85 ± 0.36	6.43 ± 0.78

- - No bioactivity at doses ≤ 100 µM.

However, there are sketchy data on the anticancer activities of these compounds (D1, D2 and D3) against the leukaemia cell lines (Molt-4) and the colon cancer cell line (Caco-2). The anticancer activity of D1, D2 and D3 were also investigated against the leukaemia cell lines (K562 and Molt-4) and colorectal cancer cell line (Caco-2). The results are presented in Table 5.6 - Table 5.8.

Table 5.8: IC_{50} values for sesquiterpene lactones – alantolactone, isoalantolactone and costunolide against Caco-2 cell line

Caco-2 Cancer Cell line treated for 24, 48 and 72 hours			
	IC_{50} (µM)		
Compound	24 hours	48 hours	72 hours
Alantolactone (D1)	-	54.84 ± 6.37	33.81 ± 4.68
Isoalantolactone (D2)	28.34 ± 4.39	37.59 ± 8.48	46.56 ± 6.86
Costunolide (D3)	44.65 ± 12.75	34.72 ± 7.34	22.36 ± 4.76
Chlorpromazine hydrochloride (CPZ)	6.38 ± 0.72	5.93 ± 0.42	6.73 ± 0.62

- - No bioactivity at doses ≤ 100 µM.

Results of cytotoxicity of alantolactone (ALA), isoalantolactone (ISL) and costunolide (CST) against the leukaemia cell line (K562) are presented in Table 5.6. Cytotoxicity of ALA and ISL increased from 24 to 72 hours. After treatment of cell, 24 hours later, ALA and ISL indicated cytotoxic inhibition at doses of 24.86 µM and 28.44 µM. This cytotoxic inhibition (IC_{50}) against K562 cell line increased to 13.48 µM for ALA and 21.38 µM for ISL after 48 hours treatment (a decrease in IC_{50} value indicates better cytotoxicity against cancer cell line). The best cytotoxic inhibition was observed after 72 h treatment with ALA (IC_{50} 4.95 µM) and ISL (IC_{50} 7.38 µM). In contrast to the results obtained for ALA and ISL, CST indicated the best cytotoxic inhibition against K562 cell line after 24 hours treatment – IC_{50} 6.68 µM (Table 5.6). The cytotoxic inhibition elicited by costunolide decreased after 72 hours treatment (IC_{50} 10.98 µM). Cytotoxic inhibition elicited by ALA and ISO were comparable to those of the positive control – CPZ (IC_{50} 4.53 µM) after 72 h treatment. ALA indicated the best cytotoxicity against K562 cell line among the sesquiterpene lactones tested.

Results of cytotoxicity of alantolactone (ALA), isoalantolactone (ISL) and costunolide (CST) against the leukaemia cell line (molt-4) are presented in Table 5.7. Unlike the cytotoxicity of ALA and ISL on K562 cell line, these compounds were inactive against the molt-4 cell line at doses ≤ 100 µM. CST indicated cytotoxic inhibition of molt-4 cell line after 24 hours treatment (IC_{50} 30.66 µM). This inhibition decreased after 48 h treatment (IC_{50} 46.26 µM).

This cytotoxic response (inhibition) against molt-4 cell line was lost after 72 hours treatment at doses ≤ 100 µM.

Results of cytotoxicity of alantolactone (ALA), isoalantolactone (ISL) and costunolide (CST) against the colorectal cancer cell line (Caco-2) are presented in Table 5.8. ALA, ISL and CST indicated cytotoxic inhibition against Caco-2 cell line at IC_{50} ranging from 22.36 – 54.84 µM with CST indicating the best cytotoxicity (IC_{50} 22.36 µM). Interestingly, ALA did not inhibit the growth of Caco-2 cell line after 24 hours treatment at doses ≤ 100 µM. It is also observed that ISL indicated good early cytotoxicity against Caco-2 cell line (IC_{50} 28.34 µM) after 24 hours treatment which could not be sustained after 72 hours treatment (IC_{50} 46.56 µM).

ALA, ISL and CST indicated very good cytotoxicity against the K562 cell lines. Since there are sketchy data on the anticancer activities of these compounds (ALA, ISL and CST) on leukaemia cell lines (K562) in hypoxia and normoxia (during hyperglycaemia and hypoglycaemia), this activity (anticancer activities of ALA, ISL and CST) was investigated in hypoxic and hyperglycaemic conditions and results presented in Table 5.9.
Results presented in Table 5.6 (cytotoxic inhibition in normal glucose concentrations - normoglycaemia) and Table 5.9 [cytotoxic inhibition in hypoglycaemia (low glucose) and hyperglycaemia (high glucose) concentrations] indicates some interesting observations; in normoglycaemia, the IC_{50} values were higher (4.95 – 10.98 µM) than those observed during hypoglycaemia (IC_{50} 0.58 – 6.72 µM) and hyperglycaemia (IC_{50} 0.8 – 7.15 µM).

Table 5.9: anticancer activity of sesquiterpene lactones on K562 cell line exposed to high glucose and low glucose concentrations during hypoxia and normoxia

K562 Cancer Cell Line								
Compound	**IC_{50} (µM)**							
	Hypoxia (72 hours)				Normoxia (72 hours)			
	low glucose		high glucose		low glucose		high glucose	
	IC_{50}	SD	IC_{50}	SD	IC_{50}	SD	IC_{50}	SD
Alantolactone (D1)	0.58	0.05	0.80	0.03	1.80	0.09	2.60	0.24
Isoalantolactone (D2)	0.66	0.08	1.18	0.1	1.90	0.12	3.29	0.15
Costunolide (D3)	3.07	0.38	4.77	0.98	6.72	0.6	7.15	0.96
CPZ	33.55	2.74	42.88	2.82	9.36	0.56	16.21	0.39

In hypoxia, cytotoxic inhibition of K562 cell line by alantolactone (D1) and isoalantolactone (D2) were better than inhibition indicated by costunolide (D3). D3 indicated better cytotoxic inhibition in low glucose concentration compared to inhibition at high glucose concentration. Similar observation is recorded for D1 and D2 in normoxia. Cytotoxicity indicated by D3 in normoxia during low glucose and high glucose

concentrations was negligible. CPZ was less cytotoxic in hypoxia. Cytotoxicity elicited by D1, D2 and D3 was better than that of CPZ.

The anticancer activities of sesquiterpene lactones isolated from *Inula helenium* against cancer and normal cell lines during hypoxia and hyperglycaemia, were investigated and results presented in Table 5.9. In hypoxia under low glucose (LG) levels, alantolactone and isoalantolactone elicited similar cytotoxic inhibitions against K562 cell line at IC_{50} values of 0.58 and 0.66 µM respectively. Costunolide elicited cytotoxic inhibition against K562 cell line at IC_{50} value of 3.07 µM.

In hypoxia under high glucose (HG) levels, alantolactone and isoalantolactone elicited similar cytotoxic inhibition against K562 cell line (IC_{50} 0.8 and 1.18 respectively). Costunolide elicited cytotoxic inhibition against K562 cell line at IC_{50} value of 4.77 µM - Table 5.9.

In normoxia under low glucose concentration, alantolactone (D1), isoalantolactone (D2) and costunolide (D3) elicited cytotoxicity against K562 cell line at IC_{50} values 1.8, 1.9 and 2.8 µM. Under high glucose concentration in normoxia, alantolactone, isoalantolactone and costunolide elicited cytotoxicity against K562 cell line at IC_{50} values 2.6, 3.29 and 6.72 µM. Cytotoxicity elicited by these compounds was 10 – 50-fold better than that of the standard drug – CPZ (Table 5.9).

Alantolactone, isoalantolactone and costunolide elicited similar cytotoxic trend against K562 cell line exposed to similar treatment and conditions. In hypoxic conditions, alantolactone (D1), isoalantolactone (D2) and costunolide (D3) indicated the best cytotoxic inhibition against K562. When K562 cell line was exposed to treatment with high glucose concentration, cytotoxic inhibition of D1, D2 and D3 against K562 decreased. In similar hypoxic conditions (low glucose and high glucose), very interesting observations were made; CPZ which indicated IC_{50} value of 9.36 and 16.21 µM in normal oxygen concentration (in low glucose and high glucose respectively) was ineffective against the K562 cancer cell line at concentrations ≤ 33 µM (table 5.9). This observation is an indication that in hypoxia, cancer cells develop resistance to most chemotherapeutic agents (Vaupel & Mayer, 2007). Resistance to CPZ by K562 in hypoxia is best observed in hyperglycaemic (high glucose) conditions (table 5.9); CPZ elicited weak cytotoxicity (IC_{50} 42.88 µM) against the K562 cancer cell line. As the glucose concentration decreased (under hypoxic condition), the cytotoxicity elicited by CPZ increased (IC_{50} 33.55). Please note; a low IC_{50} value implies good cytotoxic (killing activities) inhibition of the cancer cells. This finding

further confirms that cancer cells proliferates faster in hyperglycaemia than in hypoglycaemia (Li et al., 2018).

In normoxia, when K562 was expose to low glucose concentration, the resistant of K562 to cytotoxic inhibition by these compounds (D1, D2 and D3) increased compared to cytotoxic resistance of K562 to D1, D2 and D3 in hypoxic conditions. This was different for CPZ whose cytotoxicity against K562 decreased under normal oxygen concentrations compared to hypoxic conditions. When K562 was exposed to high glucose concentrations, there is further increased in resistance of K562 to cytotoxicity from D1, D2 and D3. Although resistance of K562 to cytotoxic inhibition by CPZ is observed as glucose concentration increases under normal oxygen concentrations, there is no significant decrease in K562 resistance to CPZ induced cytotoxicity under high glucose concentrations (Table 5.6).

Cytotoxicity induced by D1, D2 and D3 was superior under hypoxia than in normoxia. Similarly, cytotoxicity indicated by D1, D2 and D3 was superior in low glucose concentration than in high glucose concentration. This suggests that these sesquiterpene lactones D1, D2 and D3 killed K562 cancer cell better during hypoxia and have the potential to kill K562 cells best when exposed to low glucose concentrations. This observation was different for CPZ which elicited better cytotoxicity against K562 in hypoxia than in normoxia. Interestingly, there is no significant difference in cytotoxicity induced by CPZ when glucose concentration changes. This suggests cytotoxicity elicited by CPZ is not dependent on glucose concentration. This is the case with D1, D2 and D3. Their cytotoxic inhibition against K562 is glucose and oxygen dependent.

Inula helenium has been conventionally used in Ayurveda for treatment of numerous ailments such as dysentery, fever etc. and its antibacterial and anticancer activities has been documented (Huo et al., 2010; Seca et al., 2014). One of the aims of this study was to investigate the anticancer activities of extracts and purified compounds isolated from *Inula helenium* against a range of cancer cell lines of different origins. In carcinogenesis, the interaction of factors affecting transcription factors, intracellular signal transduction and aberration in gene expression have been implicated for tumourigenesis (Rahman et al., 2012). The extracts of *Inula helenium* have been reported to elicit in vitro anticancer activities (Koc et al., 2018; Seca et al., 2014).

The compounds in the roots of *Inula helenium* were extracted with solvents of different polarity and the best anticancer activity was recorded for the hexane fraction (IC_{50} 0.8

µg/mL), better than the cytotoxicity indicated by Chlorpromazine hydrochloride (CPZ) – IC_{50} 2.17 µg/mL. This is consistent with results obtained from previous studies (Lawrence et al., 2001). Previous reports only investigated the cytotoxicity of extracts from *Inula helenium* after 72-hour administration (Lawrence et al., 2001; Huo et al., 2010). This study also reports the antiproliferative effect of the hexane extracts against cancer cell lines after 24 and 48-hour administration with IC_{50} values ranging from 1.74 – 2.08 µg/mL against the K562 cell line; an activity better than those shown by CPZ. Merghoub et al., (2009) reported anticancer activities on the methanolic leaves extracts of *Inula* viscoa against HeLa and SiHa cell lines at higher (low cytotoxicity) IC_{50} values (54 – 60 µg/mL). In this study, the methanolic extract indicated a better antiproliferative activity against K562 cell line (IC_{50} 38.02 µg/mL) while the ethyl acetate fraction indicated a cytotoxic inhibition at a dose of 24.36 µg/mL). The difference in cytotoxic inhibition reported in this work is because different cell lines (K562) was used in this work whereas Meghoub et al., (2009) used HeLa and SiHa cell lines. Different species of *Inula* (*Inula* viscosa) was used by Meghoub et al., (2009). The difference in cytotoxicity reported in this work to that reported by Meghoub et al., (2009) is because the phytochemical composition of *Inula helenium* used in this study differs from those present in *Inula* viscosa (Kheyar-Kraouche et al., 2018). Our findings in this research is consistent with those of Pal et al., (2010) who reported a better cytotoxic activity from the hexane extract (IC_{50} 10.25 to 17.89 µg/mL).

The difference in the cytotoxic effect of the most responsive cell line (K562) compared to the least responsive cell line (Molt-4) suggests induction of molecular and cellular apoptotic mechanisms based on the common genotoxic transformation of cancer cells (Park et al., 2014).

Inula specie constitutes a variety of bioactive secondary metabolites especially the sesquiterpene lactones that possesses anticancer activities (Huo et al., 2008, Réthy et al., 2007, Park and Kim, 1998). Most compounds isolated from *Inula* species have been tested on over twenty cancer cell lines with HL-60, HepG-2, A-549, MCF-7 and HeLa being the most frequently cited (Seca et al., 2014). However, most of these compounds are inactive or indicates an IC_{50} value higher than 10 µg/mL or 20 µM. These values are much higher than values reported for conventional cytotoxic drugs like daunorubicin (IC_{50} values ranging from 1 – 10 µM) (Seca et al., 2014). In this work, the hexane fraction met this criterion and was purified further to obtain three bioactive compounds which were investigated against the K562, Molt-4 and Caco-2 cell lines.

The anticancer activities of sesquiterpene lactones isolated from *Inula helenium* in this study – alantolactone (ALA), isoalantolactone (ISL) and costunolide (CST) against most cancer cell lines used in this work has previously been reported (Lawrence et al., 2001; Huo et al., 2010; Hu et al., 2011; Seca et al., 2014; Lin et al., 2015) except for the Molt-4 and Caco-2 cell lines. The second aspect of this work investigated the anticancer activities of ALA, ISL and CST against the leukaemia cell lines (K562 and Molt-4) and the colorectal cancer cell line Caco-2). The anticancer activities (IC_{50}) of ALA and ISL against K562 cell line after 72 hours treatment was 4.95 µM and 7.38 µM respectively. Lawrence et al., (2001) reported lower IC_{50} values for ALA (0.7 µM) and ISL (1.2 µM) against K562 cell lines. Sun et al., (2003) reported an IC_{50} of 3.5 µg/mL. These activities were reported after 72-hour treatment with compounds. In this work, we also report the cytotoxicity of these compound after 24- and 48-hours treatment. ALA and ISL showed weak inhibition after 24 (24.86 and 28.44 µM) hours treatment. After 72 hours treatment, more cancer cells were inhibited by ALA (IC_{50} 4.95 µM) and ISL (IC_{50} 7.38 µM) – (Table 5.5, page 196). This is an indication that ALA and ISL are able to inhibit the proliferation of cancer cell lines, preventing the further growth and multiplication cells after prolong treatment with drug (Liu et al., 2018). An important criterion for a potentially good anticancer therapy is its ability to inhibit cancer cells proliferation after prolonged treatment (Liu et al., 2018). Conversely, CST indicated good early cytotoxic inhibition against the K562 cell line (IC_{50} 6.68 µM). This inhibition could not be maintained after 48- and 72-hours treatment (IC_{50} 11.72 and 10.98 µM) – Table 5.5. Looking at Table 5.5, an explanation to the seemingly stable inhibition of the K562 cell line from 24 hours, to 48- and 72-hours treatment is because the K562 cell line continued to proliferate. Unlike ALA and ISL, the K562 cell line may have developed some resistance against CST (Barua et al., 2018; Vadde et al., 2017). A similar trend was observed for the treatment of Molt-4 cell line with CST (Table 5.6, page 196). After treatment for 72 hours, CST lost the ability to inhibit the proliferation of Molt-4 cell line. This is the first time the anticancer activity of CST against Molt-4 cell line has been reported although this inhibition was lost after 72 hours treatment. The loss of cytotoxicity inhibition after 72 hours could be attributed to the development of resistance mechanisms by the K562 cancer cell line (Barua et al., 2018; Vadde et al., 2017).

CST also indicated moderate cytotoxic inhibition against Caco-2 cell line after 72 hours treatment (Table 5.8, page 197), but poor inhibition (no inhibition at doses ≤ 100 µM for ALA) after 24 h treatment. Moreover, ISL lost its cytotoxicity against Caco-2 cell at IC_{50} ≤ 46 µM. ALA and CST may therefore be beneficial agents (anticancer agents) against Caco-

2 cell line due to their ability to inhibit the proliferation of Caco-2 cell line in the long run (72 hours, compared to ISL which lost its cytotoxicity after 72 hours of treatment).

The third aspect involved a novel study into the cytotoxicity of ALA, ISL and CST against K562, Molt-4 and Caco-2 cell lines in hypoxia and normoxia, and in hyperglycaemia and hypoglycaemia (Tables 5.6 – 5.8). Maintenance of oxygen homeostasis is critical for organism's survival and solid tumours have been associated with 50–60% of solid tumours consisting of hypoxic tissue areas (Vaupel et al., 2004). During hypoxia, tissues are not oxygenated adequately due to insufficient concentrations of oxygen in blood thereby initiating changes which alters the oncogenic genes in the microenvironment (Harris, 2002). Metabolism is also altered, giving rise to non-functional blood vessels and a consequent metastasis (Harris, 2002). Unlike in normal cells where oxygen supply is required for metabolism, neoplastic cells requires 2 to 10-fold oxygen supply (which is limited in supply), leading to a more hypoxic microenvironment around the cancerous cells (Vaupal et al., 2004). The cancer cells are then able to generate pseudo-blood vessels within the cancerous niche to supply the excess oxygen requirements of the tumour cells (Suda et al., 2011). This characteristic of tumour cells promotes carcinogenesis and drug resistance (Vaupel & Mayer, 2007). In this study, hypoxia increased the antiproliferative effect of sesquiterpene lactones tested with ALA and ISL indicating cytotoxicity (IC_{50}) at a dose of 0.58 and 0.66 µM compared to cytotoxicity (IC_{50}) at a dose 1.80 and 1.90 µM during normal oxygen concentration. Many cancer therapies cannot treat cancers due to the inability of these drugs to penetrate the tumour micro-environment. Cancer cells become resistant due to the hypoxic microenvironment within the tumour cells (Vaupel et al., 2004). Compounds isolated in this study indicated better antiproliferative inhibition in hypoxia than in normoxia indicating these compounds may be inhibiting the expression of hypoxia-inducible factor (HIF1α) gene (Fiegl & Spiekermann, 2011) which is associated with the development of resistance to drug in hypoxic conditions (Velasco-Hernandez et al., 2014). The antiproliferative activities of compounds isolated in this work was better than that of the positive control drug (CPZ).

Attempts have been made previously to increase the antiproliferation of the cancer stem cells in hypoxia by introduction of cytokines with no success at clinical trials in Germany (Büchner et al., 2006). In another clinical trial in Dutch, little (but not significant) success was reported (Löwenberg et al., 2003). The reason for this failure is that cytokines cannot be recruited effectively against hypoxic cells (Drolle et al., 2015). A promising approach may involve inhibition of specific pathways by combining small molecules that inhibit

different pathways such as inhibition of the apoptotic machinery / PI3K/Akt-pathways or the use of chemotherapy – as compounds isolated in this work indicated promising antiproliferative activities against K562 cell line in hypoxic conditions in-vitro. The role of the PI3K/Akt-pathway has been demonstrated to increase the anti-leukemic efficacy of ABT-737 cell line (a BH3 mimetic) thus interfering with apoptosis in a hypoxic AML model (Jin et al., 2013).

Hypoxia as a component of the leukaemic microenvironment encourages the proliferation of leukaemia cancer cells. This affects susceptibility of the leukaemia cells towards chemotherapy. In this work, we isolated bioactive sesquiterpene lactones – alantolactone, isoalantolactone and costunolide which indicated significant antiproliferative activity against the K562 (leukaemia) cell line at IC_{50} values better than that indicated by the positive control – CPZ. Further investigation especially the impact of hypoxia on certain genetically defined leukaemia sub-types and on the stem-cells might be a promising therapeutic approach in the future as the role of hypoxia (a condition of insufficient tissue oxygenation) in maintaining stemness in cancers and cancer stem cells (CSC) have been reported and has been associated with malignancy and tumourigenesis. CSC are involved in cancer degeneration and resistance especially in the leukaemia and colorectal cancers (Vadde et al., 2017).

Furthermore, the anticancer activities of sesquiterpene lactones isolated in this study were investigated against the K562 cell line in hyperglycaemic and hypoglycaemic conditions. There are reports of increased cancer incidences and mortality in patients with diabetes compared to non-diabetic patients (Barua et al., 2018; Simon & Balkau, 2010), an indication that hyperglycaemia promotes the development of cancer as cancer cells proliferate rapidly during hyperglycaemia (Li et al., 2018). It therefore implies that as cells proliferate rapidly, they are difficult to treat (Li et al., 2018). In this study, ALA, ISL and CST inhibited the proliferation of K562 cell lines in hyperglycaemic and hypoglycaemic conditions with IC_{50} values ranging from 0.80 – 7.15 µM. A comparison of cytotoxic inhibition in hyperglycaemia and in hypoglycaemia indicates that the antiproliferative activity of ALA, ISL and CST was at its best during hypoglycaemia. The difference between cytotoxic inhibition in hypoglycaemia and in hyperglycaemia is not significant. This finding suggests that hyperglycaemia increases cell proliferation (Li et al., 2018) and as cell proliferates rapidly, the drug concentration tends to deplete as new cells are constantly being formed. An IC_{50} value of 1.80 and 1.90 µM was observed in low glucose (during normoxia) for cells treated with ALA and ISL. This value almost doubled in hyperglycaemia.

This data supports previous findings that cancer cells rapidly proliferates during hyperglycaemia (Barua et al., 2018; Li et al., 2018; Simon & Balkau, 2010).

There is a link between hyperglycaemia, hypoglycaemia and hypoxia in cancer progression (Li et al., 2018). In this work, the cytotoxic effect of ALA, ISL and CST were better in hypoxia and hypoglycaemia combined. In hyperglycaemia and normoxia (combined), there was a 5-6-fold loss of cytotoxicity by these compounds (ALA, ISL and CST) against the K562 cell line. This supports the previous findings that rapidly proliferating cells (during hyperglycaemia) are difficult to treat compared to slowly proliferating cells (Barua et al., 2018; Drolle et al., 2015; Fiegl & Spiekermann, 2011; Harris, 2002; Li et al., 2018; Simon & Balkau, 2010; Vaupel & Mayer, 2007). This is the first time this finding has been reported in K562 cell lines. The efficiency associated with cytotoxic inhibition of cancer growth in hypoxia and hypoglycaemia (combined) may be attributed to induction of cell cycle arrest at G0/G1 phase of the cell cycle (Drolle et al., 2015) as cells could not transit to the S phase of the cell cycle. Drolle et al., (2015) investigated the decrease cell number observed in a study involving leukaemia cell line and reported a significant reduction of cells in S-phase after exposure to hypoxia for 48 hours treatment and reported a 50.5 % reduction of cells in the S phase from 42.5 % to 21.0 % and traced this significant reduction of cells in the S phase of the cell cycle to the upregulation of the p27Kip1 regulatory protein. Drolle et al., (2015) confirmed this observation on noticing a similar blockade of transition of cell cycle at the G0/G1 phase in NB4 cell line.

Lawrence et al., (2001) reported that ALA and ISL acts as alkylating agents, blocking the G2/M and S phase of the cell cycle. This may account for the continued cytotoxic effect elicited by ALA and ISL even in hyperglycaemic conditions in rapidly proliferating cells as alkylating agents acts best in rapidly proliferating cells (Scholar, 2007). Alkylating agents have been reported to significantly inhibit leukaemia and colorectal cancers even at the stem cell levels (Scholar, 2007).

Acute myeloid leukaemia treatment (AML) is associated with poor outcomes of treatment due to leukaemia stem cells (LSCs) which are difficult to eliminate with conventional therapy. These LSCs lead to cancer relapse in AML (Ding et al., 2016). Findings in this report suggest that sesquiterpene lactones – D1, D2 and D3 may be able to destroy LSCs even in hypoxic and hyperglycaemic conditions. Only very few small molecules have been reported to show anti-LSCs activity including D1 and D2 reported in this study (Lawrence et al., 2001).

5.5 Anticancer Activities of Synthesised Chrysin Derivatives

5.5.1 Treatment of Colorectal Cancers with Synthesised Chrysin Derivatives

5.5.1.1 HCT 116 Cell Line

The cytotoxic activity of chrysin adducts made in this study was tested on HCT 116 cell line after 24, 48 and 72 hours respectively and their cytotoxic concentration (IC_{50}): concentration required to kill 50 % of cancer cells presented in Table 5.10. IC_{50} is also represented as EC_{50}.

Table 5.10: IC_{50} values for Chrysin derivatives against HCT 116 Cancer Cell Line

HCT 116 cancer cell line treated for 24, 48 and 48 hours				
	IC_{50} (μM)			
Compound	24 hours	48 hours	72 hours	Name of compound
C1	**3.21 ± 0.11**	**3.11** ± 0.41	**1.99 ± 0.24**	7-O-chrysinbutyl acetate
C2	**3.74 ± 0.14**	**3.92** ± 0.11	**2.17 ± 0.19**	7-O-chrysin-3,5-dimethylbenzene
C3	**2.61 ± 0.08**	**1.96** ± 0.53	**1.45 ± 1.71**	7-O-chysin-2,4-dinitrobenzene
C4	42.43 ± 3.65	40.51 ± 2.25	29.59 ± 3.25	7-O-isopropylchrysin
C5	68.52 ± 6.21	65.82 ± 4.46	54.76 ± 2.77	7-O-chrysin-2-methylpropane
C6	31.93 ± 4.76	25.96 ± 4.26	21.63 ± 1.88	7-O-butylchrysin
C7	24.32 ± 5.28	21.98 ± 5.87	17.75 ± 1.53	7-O-pentylchrysin
C8	41.79 ± 6.22	41.87 ± 5.86	30.37 ± 3.81	7-O-hexylchrysin
C9	39.07 ± 2.36	36.85 ± 1.44	30.56 ± 2.04	7-O-heptylchrysin
C10	23.56 ± 4.46	24.45 ± 3.74	16.40 ± 1.18	7-O-octylchrysin
C11	26.32 ± 2.27	24.04 ± 8.22	17.53 ± 2.32	7-O-nonylchrysin
C12	23.03 ± 1.64	22.69 ± 3.64	18.91 ± 2.95	7-O-decylchrysin
C13	40.73 ± 5.63	37.16 ± 2.16	30.97 ± 4.17	7-O-3-bromopropylchrysin
C14	52.96 ± 5.37	53.04 ± 4.21	44.20 ± 2.73	7-O-4-bomobutylchrysin
C15	121.23±12.45	115.52± 3.76	96.27 ± 22.23	7-O-5-bromopentylchrysin
C16	83.86 ± 9.67	83.72 ± 3.22	69.77 ± 27.92	7-O-4-bromopentylchrysin
C17	32.91 ± 3.98	32.60 ± 1.75	27.17 ± 4.35	7-O-12-bromododecylchrysin
Chrysin	**7.33 ± 0.68**	**6.54 ± 0.54**	**4.07 ± 0.49**	5,7-dihydroxyflavone
CPZ	**9.43 ± 0.76**	**8.23 ± 0.63**	**5.90 ± 0.54**	Chlorpromazine hydrochloride

- - No activity at doses ≤ 200 μM

Chrysin derivatives synthesised in this study are classified into three series:

Series 1: Chrysin derivativesC1 to C3.

Series 2: 7-O-alkylchrysin derivatives (C4 to C16).

Series 3: 7-O-bromochrysin derivatives (C13 to C17) are the same compounds as 7-O-bromoalkylchrysin derivatives.

All compounds tested against **HCT cell line** elicited some level of inhibition against the HCT 116 cell line.

Series 3 compounds (7-O-bromoalkylchrysin derivatives) indicated inhibitory responses against the HCT 116 cell line with IC_{50} values ranging from 27.17 – 96.27 µM. 7-O-12-bromododecylchrysin and 7-O-3-bromopropylchrysin elicited good anticancer activity against HCT 116 cell line at IC_{50} values of 27.17 and 30.97 µM (Table 5.10) when the HCT cell line was treated for 72 hours. At 24 and 48 hours, these compounds indicated weak inhibition against HCT 116 cell line (Table 5.10).

The highest level of inhibition indicated by Series 3 compound is achieved after 72 hours; a characteristic also observed in chrysin and CPZ. The least inhibition was observed after 24 hours of treatment (Table 5.10).

Series 2: series 2 compounds (7-O-alkylchrysin derivatives) indicated inhibitory responses against the HCT 116 cell line better than Series 3 compounds with IC_{50} values ranging from 27.17 – 96.27 µM (Table 5.10) with IC_{50} values ranging from 17.75 to 54.76 µM. Like Series 3 compound, series 2 compounds elicited the highest level of inhibition against at 72 hours after administration of test compounds and least inhibition after 24 hours of administration of test compounds. However, 7-O-octylchrysin (C10) elicited quick cytotoxic activities against HCT 116 cell line after 24 hours of administration of C10 (IC_{50} 23.56 µM) which decreased slightly (IC_{50} 23.56 µM).

An interesting observation is the significant decrease in IC_{50} values for all series 2 compounds after 72 hours of administration of these compounds compared to the differences observed between administration for 24 and 48 hours.

C10, C11, C7 and C12 indicated significant cytotoxicity against HCT 116 cell lines after 72 hours at IC_{50} values 16.40 µM (C10), 17.53 µM (C11), C7 (17.75 µM) and C12 18.91 µM (C12).

Results obtained in this study suggests that long chain series 2 compounds (with carbon atoms ≥ 7) are more cytotoxic compared to short chain series 2 compounds.

Series 1: The most cytotoxic Chrysin derivatives synthesise in this study are the Series 1 compounds. Series 1 compounds indicated very interesting cytotoxicity against HCT 116 cell lines better than the parent compound – chrysin and much more significant than that of the positive control (CPZ) after administration at 24 h, 48 hours and 72 hours respectively (Table 5.10).

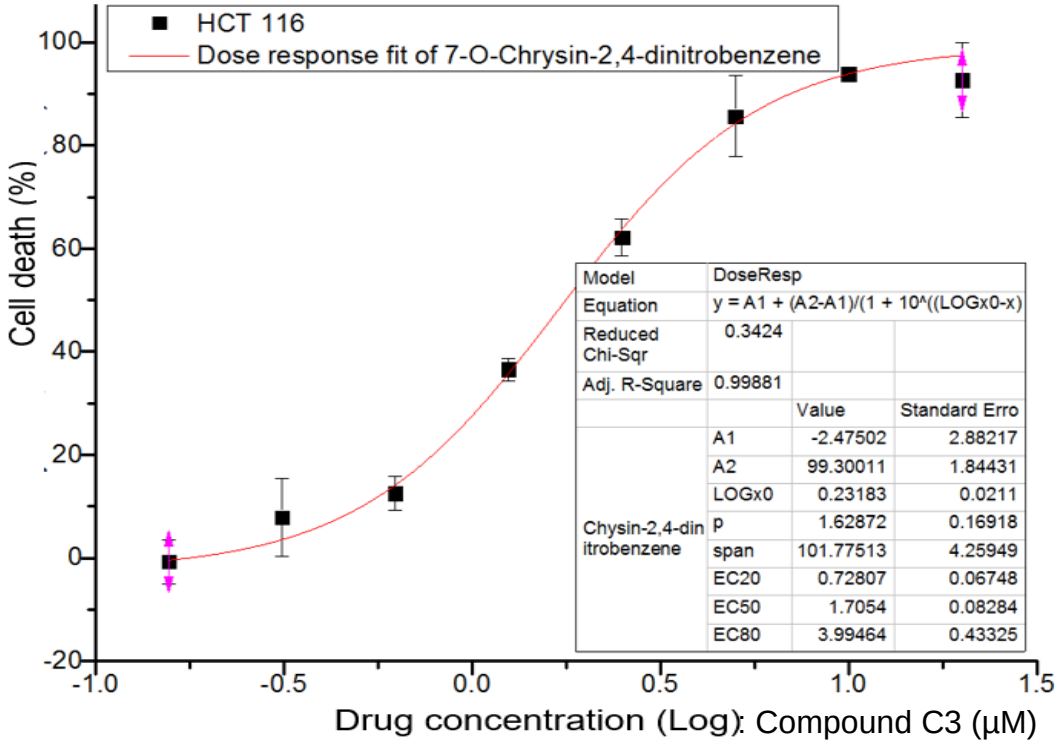


Figure 5.1: Dose response (µM) – IC_{50} plot of the most cytotoxic series 1 compound – 7-O-chrysin-2,4-dinitrobenzene **(C3)** against HCT 116 cell line after 72 hours treatment.
Error bars represent standard error (see the box on Figures 5.1 – 5.20 for details)

7-O-Chysin-2,4-dinitrobenzene (C3) indicated the best cytotoxicity (IC_{50}) against HCT 116 cell line at 24 hours (2.61 µM), 48 hours (1.96 µM) and 1.70 µM (72 h). C3 elicited its best inhibition against HCT 116 after 72 hours of administration. Cytotoxicity indicated by C3 after 72 hours was very significant compared to those indicated after 24 and 48 hours as the IC_{50} value at 72 hours (1.70 µM) doubled those indicated after 24 hours (1.96 µM) and 48 hours (2.61 µM) (Table 5.10). Inhibitory response against HCT 116 indicated by C3 was better than those of chrysin (17.33 µM at 24 h, 16.54 µM at 48 hours and 14.07 at 72 h) and CPZ (9.43 µM at 24 h, 8.23 µM at 48 hours and 5.90 at 72 h). The cytotoxicity (IC_{50}) plot for the most cytotoxic Series 1 compounds (7-O-chrysin-2,4-dinitrobenzene) after 72 hours treatment indicates an IC_{80} of 3.4 µM (Figure 5.1). The interesting thing with the Origin 9.1 software is its ability to define several other variables such as the inhibitory concentration required to kill 80% cancer cells (IC_{80}) as well as the inhibitory concentration required to kill 20 % cancer cell line. For C3, the IC_{80} suggests over 80 % of HCT 116

cancer cell line were killed at a concentration of 3.4 µM by C3 after 72 hours of treatment (Figure 5.1 above).

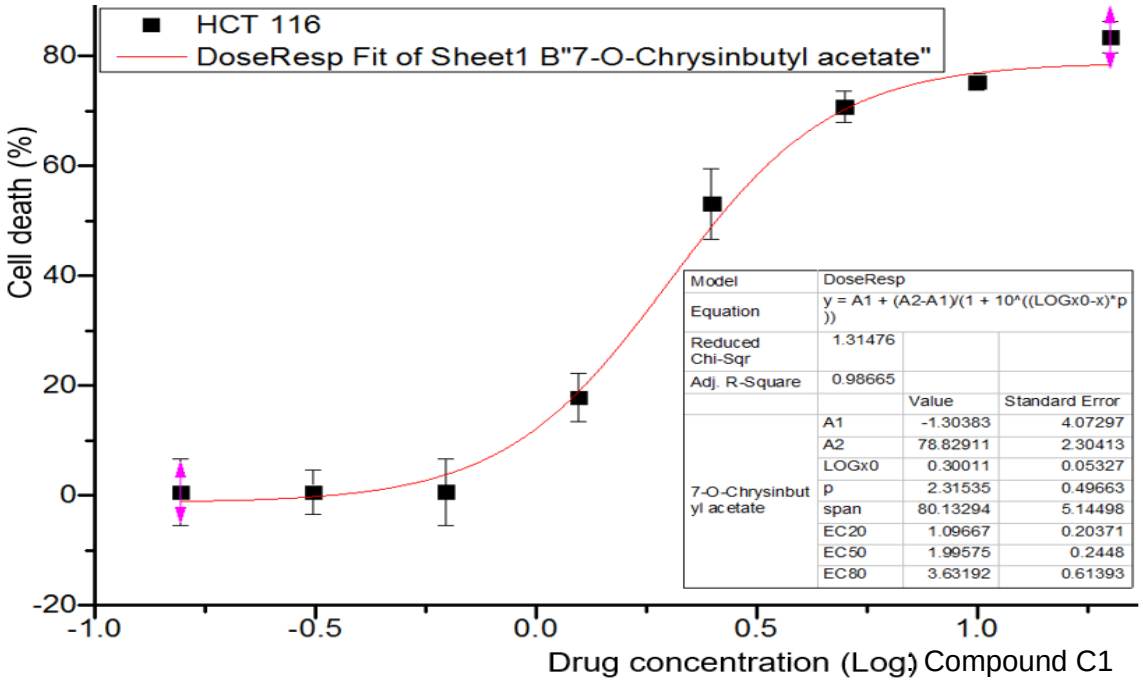


Figure 5.2: Dose response – IC_{50} plot (µM) of Series 1 compounds – 7-O-chrysinbutyl acetate (C1) against HCT 116 cell line after 72 hours treatment.

7-O-chrysinbutyl acetate (C1) indicated significant cytotoxicity comparable to that indicated by C3, against HCT 116 cell line at 24 hours (3.21 µM), 48 hours (3.11 µM) and 1.99 µM (72 h). C1 elicited its best inhibition against HCT 116 after 72 hours of administration. Cytotoxicity indicated by C1 after 72 hours was significant compared to those indicated after 24 and 48 hours as the IC_{50} value at 72 hours (1.99 µM) doubled those indicated after 24 hours (3.11 µM) and 48 hours (3.21 µM) (Table 5.9). Inhibitory response against HCT 116 indicated by C3 was better than those of chrysin and CPZ.

Cytotoxicity indicated by C2 – 3.21 µM at 24 h, 3.11 µM at 48 hours and 1.99 at 72 hours was better than those indicated by chrysin and CPZ at 24 h, 48 h and at 72 h. The best inhibitory response of C1 against HCT 116 cell line was recorded after 72 hours of administration of C1 (Table 5.10).

C2 also significantly inhibited the growth of HCT 116 cell line at IC_{50} concentrations of 3.74, 3.92 and 2.17 µM after 24 h, 48 hours and 72 hours respectively. IC_{50} indicated by C2 were better than those indicated for chrysin. The best inhibitory response of C2 against HCT 116 cell line was recorded after 72 hours of administration of C2 (Table 5.10 above).

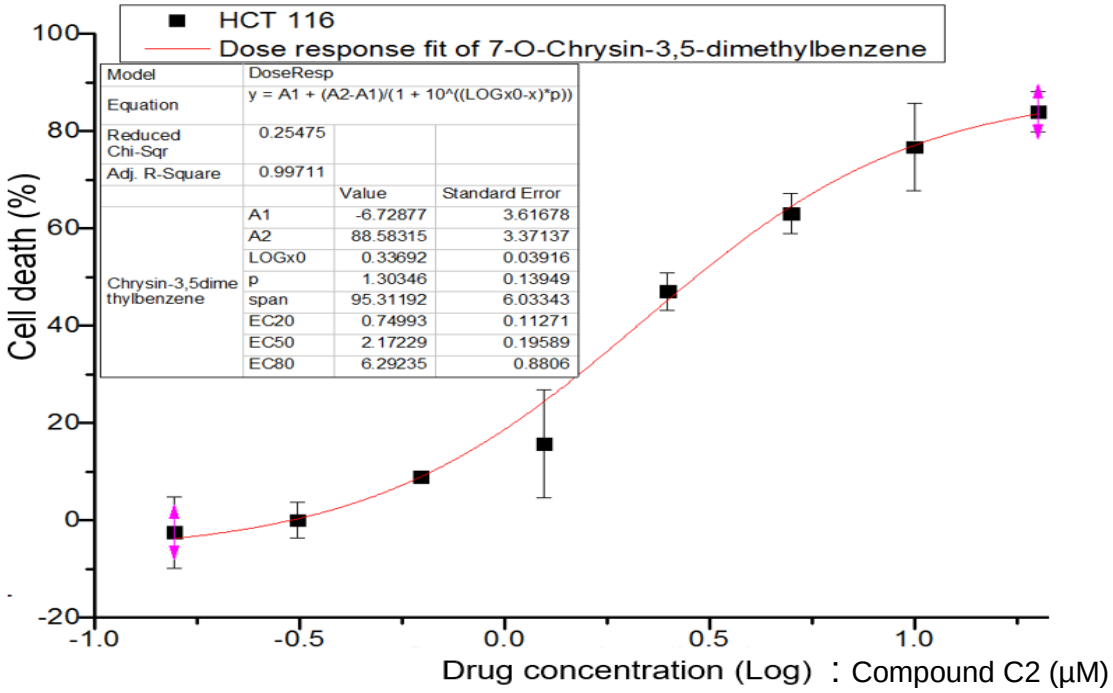


Figure 5.3: Dose response – IC_{50} plot (µM) of 7-O-chrysin-3,5-dimethylbenzene against HCT 116 cell line after 72 hours treatment.

In this study, Series 1 compounds elicited significant cytotoxicity better than the parent compound – chrysin and the positive control drug – Chlorpromazine hydrochloride (CPZ) (Table 5.10).

A summary of graphical representation of the IC_{50} of chrysin derivatives used in this study (Table 5.10) is presented in Figure 5.4 below.

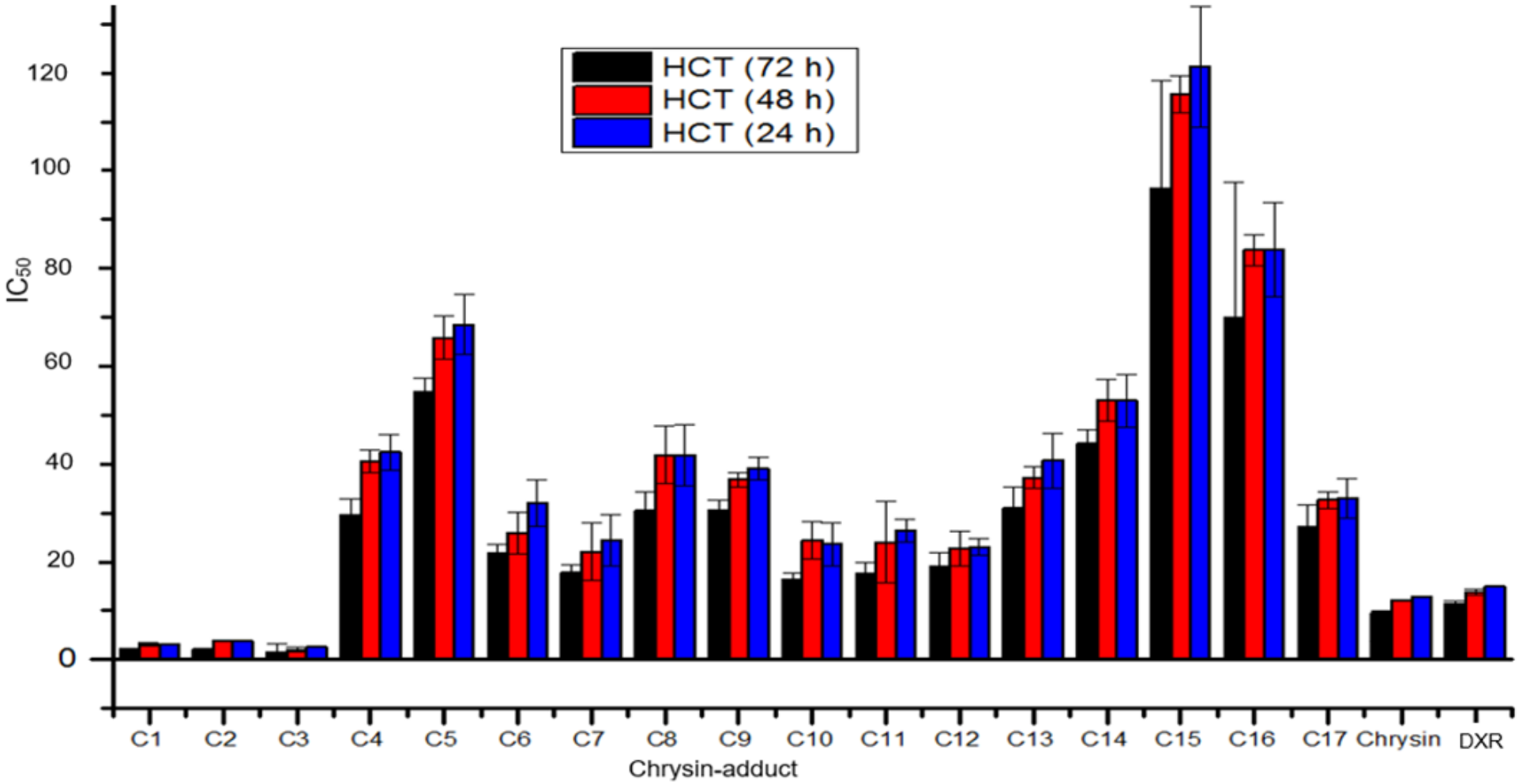


Figure 5.4: Graphical representation of IC_{50} (µM) of all Chrysin derivativessynthesised (C1 to C17) against HCT 116 cell line

An interesting observation was that all Chrysin derivativesmade in this study indicated some degree of inhibition against HCT 116 cancer cell line with Series 1 compounds indicating the most cytotoxic activity against HCT 116 cell line. All Series 1 compounds used in this study are novel compounds.

5.5.1.2 Results for Caco-2 Cancer Cell Line

Chrysin derivatives made in this study indicated significant cytotoxic activities against Caco-2 cancer cell lines Table 5.11. Unlike the HCT 116 cell lines, IC_{50} values were determined after 72 hours of treatment with test compounds. IC_{50} values are presented in Table 5.11.

Table 5.11: IC_{50} values of Chrysin derivatives against Caco-2 cancer cell line

Caco-2 cell line treated for 72 hours		
Compound	**IC_{50} (μM)**	**Name of chrysin adduct**
C1	5.89 ± 0.41	7-O-chrysinbutyl acetate
C2	4.53 ± 0.79	7-O-chrysin-3,5-dimethylbenzene
C3	4.83 ± 0.37	7-O-chysin-2,4-dinitrobenzene
C4	35.20 ± 3.66	7-O-isopropylchrysin
C5	95.54 ± 4.72	7-O-chrysin-2-methylpropane
C6	36.75 ± 3.51	7-O-butylchrysin
C7	-	7-O-pentylchrysin
C8	-	7-O-hexylchrysin
C9	-	7-O-heptylchrysin
C10	15.27 ± 1.22	7-O-octylchrysin
C11	13.05 ± 1.01	7-O-nonylchrysin
C12	18.91 ± 2.95	7-O-decylchrysin
C13	19.25 ± 3.79	7-O-3-bromopropylchrysin
C14	16.77 ± 1.45	7-O-4-bomobutylchrysin
C15	22.06 ± 3.84	7-O-5-bromopentylchrysin
C16	21.26 ± 2.21	7-O-4-bromopentylchrysin
C17	17.60 ± 6.30	7-O-12-bromododecylchrysin
Chrysin	4.84 ± 0.19	5,7-dihydroxyflavone
CPZ	5.17 ± 0.64	Chlorpromazine hydrochloride

- - No activity at doses ≤ 200 μM

Chrysin derivatives made in this study indicate significant cytotoxic activities against Caco-2 cancer cell lines (Table 5.11). Unlike the HCT 116 cell lines, IC_{50} values were determined after 72 hours of treatment with test compounds.

Series 3: The bromoalkylchrysin derivatives – C13 to C17 of chrysin adducts made in this study were tested against Caco-2 cell cancer cell line. IC_{50} values ranging from 16.77 to

22.06 µM were recorded with C14 indicating the lowest IC_{50} value (16.77 µM) – Table 5.11. All bioactive series 2 compounds recorded similar cytotoxicity against Caco-2 cell line.

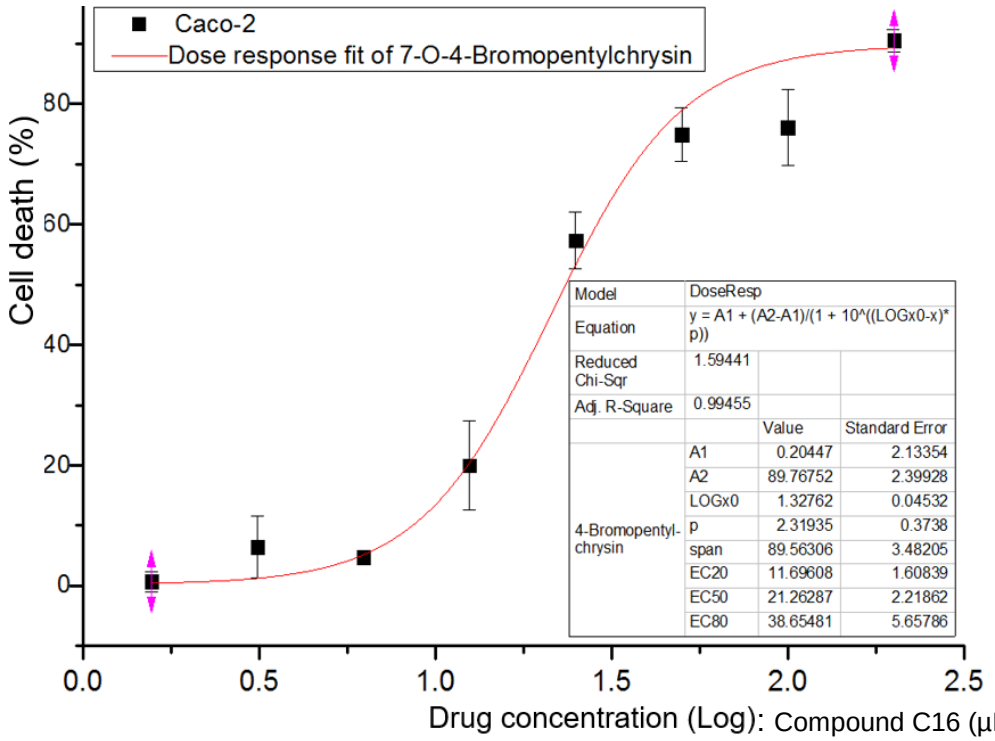


Figure 5.5: Dose response – IC_{50} plot (µM) for Caco-2 cell line treated with 4-bromobutylchrysin for 72 hours

Series 2: The series 2 chrysin adducts (C4 to C12) indicated inhibition against Caco-2 cell line with IC_{50} values ranging from 13.05 to 95.54 µM.

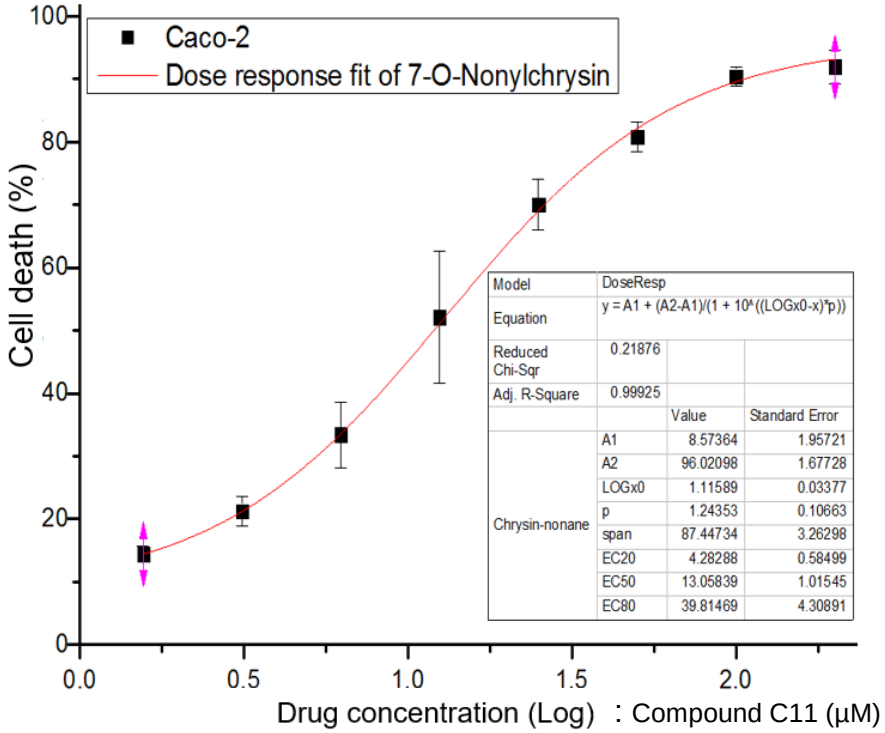


Figure 5.6: Dose response – IC_{50} plot (µM) for Caco-2 treated with 7-O-nonylchrysin for 72 hours

The most cytotoxic chrysin adducts in this series are 7-O-nonylchrysin – C11 (IC_{50} 13.05 µM) and 7-O-octylchrysin – C10 (IC_{50} 15.27 µM). 7-O-decylchrysin – C12 also indicated significant cytotoxicity against Caco-2 cell line.

C4 and C6 indicated weak cytotoxicity while C5, C7, C8 and C9 where inactive against Caco-2 cells (Table 5.11).

Results obtained for series 2 compounds suggests long chain alkyl-chrysin adducts are cytotoxic against Caco-2 cell line whereas short chain 7-O-alkylchrysin derivatives of chrysin are inactive against Caco-2 cell line at concentrations ≤ 30 µM.

Series 1: Series 1 compounds (C1, C2 and C3) elicited significant cytotoxicity against Caco-2 cell line with IC_{50} values 5.89 µM, 4.53 µM and 4.83 µM (Table 5.11) when treated with these compounds. Cytotoxicity elicited by Series 1 compounds are better than cytotoxicity indicated by chrysin and CPZ.

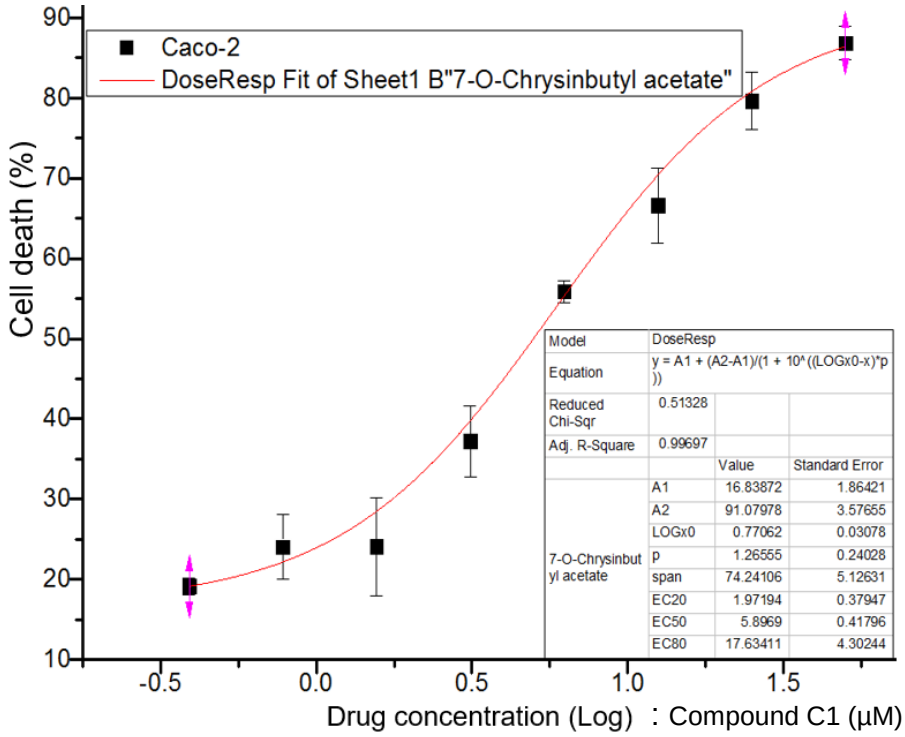


Figure 5.7: Dose response – IC_{50} plot (µM) of 7-O-Chrysinbutyl acetate against Caco-2 cell line after 72 hours treatment.

Cancer growth inhibition of C1, C2 and C3 indicates better IC_{50} values than those indicated by the positive control drug – CPZ. However, the difference in cytotoxicity between chrysin and CPZ was not significant. The dose response curve (Figure 5.7) indicates an IC_{80} of 17.63 µM suggesting that 80 % of Caco-2 cell line is susceptible to cytotoxic inhibition from C1.

The dose response curve in (Figure 5.8) indicates an IC_{80} of 12.67 µM suggesting that 80 % of Caco-2 cell line was is susceptible to cytotoxic inhibition from C2. The dose response curve in (Figure 5.9) indicates an IC_{80} of 11.68 µM suggesting that 80 % of Caco-2 cell line was is susceptible to cytotoxic inhibition from C3. The IC_{80} values indicated by Series 1 compounds is more significant than that indicated by chrysin against Caco-2 cell line.

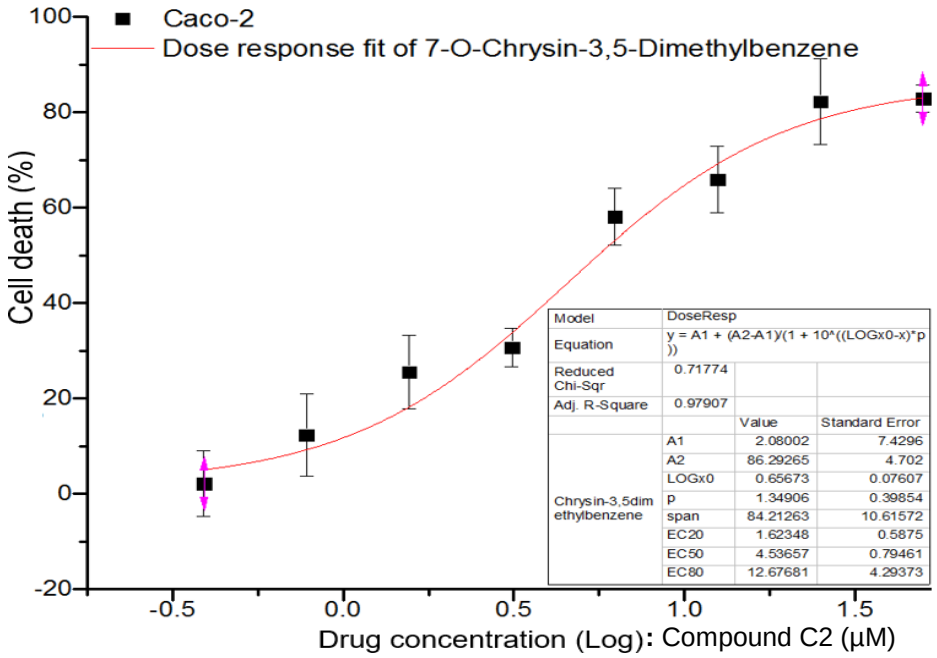


Figure 5.8: Dose response – IC_{50} plot (µM) of Caco-2 cell line treated with 7-O-chrysin-3,5-dimethylbenzene.

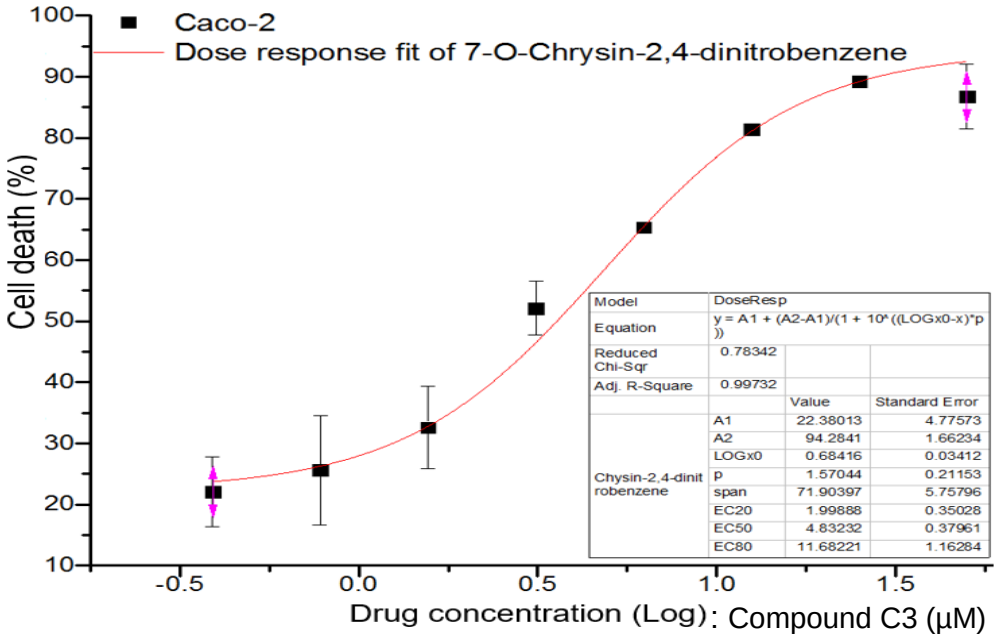


Figure 5.9: Dose response – IC_{50} plot (µM) of Caco-2 cell line treated with chrysin-2,4-dinitrobenzene for 72 hours

5.5.2 Results for Treatment of Leukaemia Cancers with Synthesised Chrysin Derivatives

5.5.2.1 Results for Molt-4 Cancer Cell Line

Chrysin derivatives made in this study were evaluated against Molt-4 cancer cell lines (Table 5.12). Unlike the HCT 116 cell lines, IC_{50} values were determined after 72 hours of treatment with test compounds. This is because any compound which cannot inhibit the growth of cancer cells after 72 hours treatment is considered inactive even if they indicate cytotoxicity within 24 – 48 hours treatment.

Table 5.12: IC_{50} values of Chrysin derivatives against Molt-4 cancer cell Line

Molt-4 cell line treated for 72 hours		
Compounds	**IC_{50} (µM) Molt-4**	**Name of chrysin adduct**
C1	7.05 ± 0.47	7-O-chrysinbutyl acetate
C2	13.87 ± 3.00	7-O-chrysin-3,5-dimethylbenzene
C3	5.41 ± 0.53	7-O-chysin-2,4-dinitrobenzene
C4	97.71 ± 70.88	7-O-isopropylchrysin
C5	17.40 ± 3.50	7-O-chrysin-2-methylpropane
C6	-	7-O-butylchrysin
C7	51.21 ± 9.23	7-O-pentylchrysin
C8	39.49 ± 3.29	7-O-hexylchrysin
C9	35.14 ± 2.38	7-O-heptylchrysin
C10	-	7-O-octylchrysin
C11	-	7-O-nonylchrysin
C12	44.74 ± 3.14	7-O-decylchrysin
C13	59.41 ± 29.05	7-O-3-bromopropylchrysin
C14	73.82 ± 65.75	7-O-4-bomobutylchrysin
C15	23.58 ± 1.67	7-O-5-bromopentylchrysin
C16	13.28 ± 2.34	7-O-4-bromopentylchrysin
C17	5.35 ± 0.47	7-O-12-bromododecylchrysin
Chrysin	5.23 ± 0.96	5,7-dihydroxyflavone
CPZ	7.05 ± 0.47	Chlorpromazine hydrochloride

- - No activity at doses ≤ 200 µM

Chrysin derivatives made in this study indicates significant cytotoxic activities against Molt-4 cancer cell lines Table 5.12.

Series 3: The bromoalkylchrysin derivatives – C13 to C17 of chrysin adducts were tested against Molt-4 cancer cell line. IC_{50} values ranging from 13.28 to 73.83 µM were recorded with **C17** indicating the lowest (best) cytotoxicity (IC_{50} 13.28 µM) – Figure 5.10. The novel Series 3 compounds synthesised in this study (C17 and C16) were the only compounds indicating significant (IC_{50} 13.28 µM for C17) and moderate (IC_{50} 23.58 µM for C16). C13, C14 and C15 indicated cytotoxicity above 40 µM. Cytotoxicity elicited by C17 is comparable to that of CPZ.

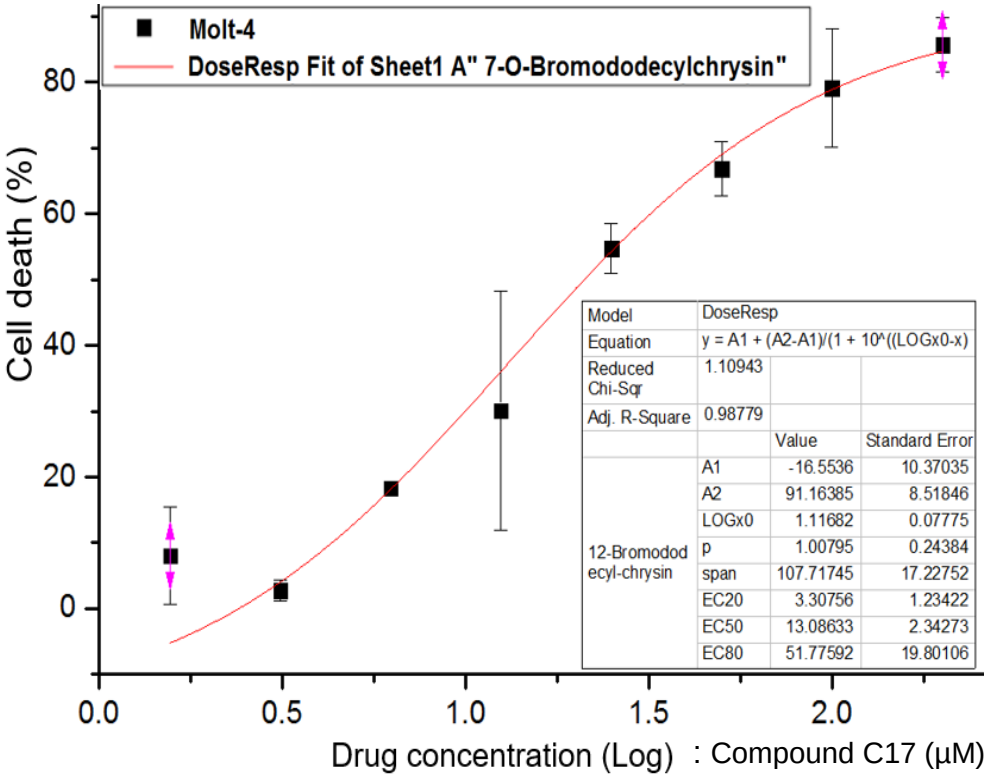


Figure 5.10: IC_{50} plot (µM) for Molt-4 cell line treated with 7-O-bromododecylchrysin for 72 hours

Series 2 compounds (C4 to C12) indicated weak cytotoxicity against Molt-4 cell line except 7-O-chrysinmethylpropane – C5 (Table 5.12), which indicated significant cytotoxicity against the molt-4 cell line. Other compounds in this group (C4, C5, C7, C8 and C9) indicated cytotoxicity (IC_{50}) at doses above 50 µM. C6, C10, C11 and C12 did not elicit cytotoxicity against Molt-4 cell line at concentration < 100 µM. Cytotoxicity indicated by C5 was comparable to that of C2 – a Series 3 compound.

Series 1 compounds indicated significant cytotoxic inhibition against Molt-4 cell line. C1 (IC_{50} 7.05 µM) and C3 (IC_{50} 5.41 µM) indicated better cytotoxicity compared to those of chrysin and CPZ (Table 5.12). C2 also indicated cytotoxicity (13.87 µM) comparable to

that of chrysin. The graphical presentation of cytotoxicity elicited by chrysin derivatives is presented in Figure 5.11 below.

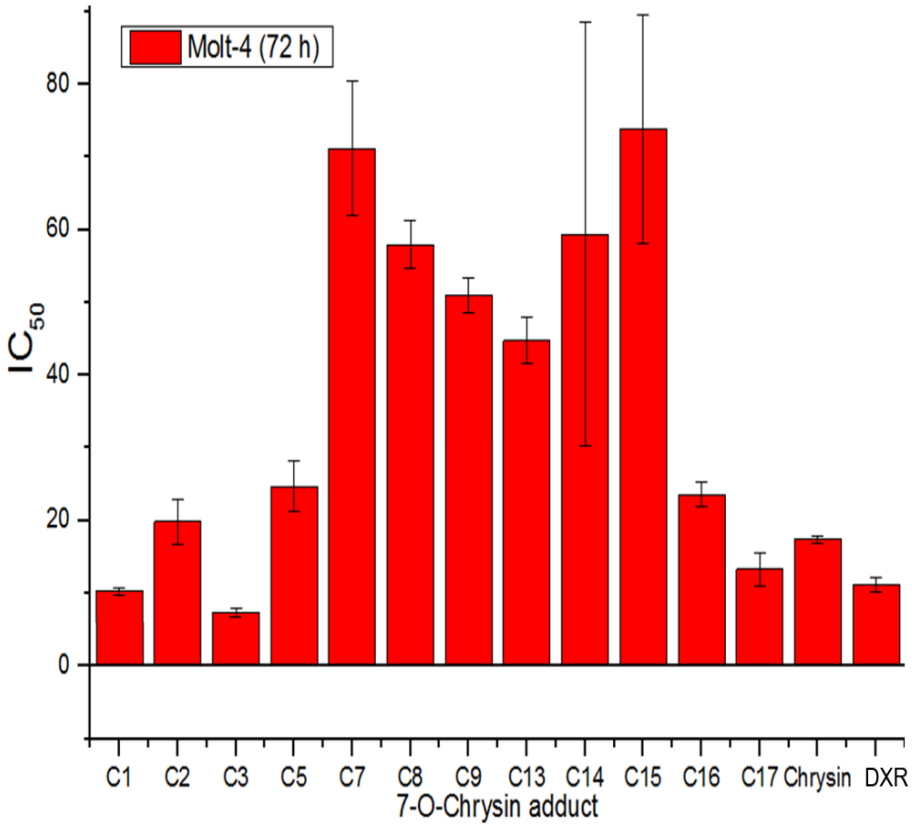


Figure 5.11: IC_{50} (µM) values of chrysin derivatives against Molt-4 cell line.

Series 3 compound (C13 to C17) indicated weak cytotoxicity (except C16 and C17), series 2 compounds (C4 to C12) indicated weak cytotoxicity except C5 which indicated significant cytotoxicity comparable to Series 3 compound.

5.5.2.2 Results for K562 Cancer Cell Line

Dose response (IC_{50} data) for chrysin adducts tested against K562 cancer cell line is presented in Table 5.13.

Table 5.13: Table showing IC_{50} values of chrysin adducts against K562 Cell Line

K562 cell line treated for 72 h		
Compound	**IC_{50} (μM)**	**Name of chrysin adduct**
C1	7.27 ± 0.71	7-O-chrysinbutyl acetate
C2	5.58 ± 0.29	7-O-chrysin-3,5-dimethylbenzene
C3	8.69 ± 2.05	7-O-chysin-2,4-dinitrobenzene
C4	-	7-O-isopropylchrysin
C5	-	7-O-chrysin-2-methylpropane
C6	-	7-O-butylchrysin
C7	-	7-O-pentylchrysin
C8	-	7-O-hexylchrysin
C9	-	7-O-heptylchrysin
C10	-	7-O-octylchrysin
C11	-	7-O-nonylchrysin
C12	15.08 ± 0.21	7-O-decylchrysin
C13	18.72 ± 2.68	7-O-3-bromopropylchrysin
C14	17.52 ± 1.60	7-O-4-bomobutylchrysin
C15	15.31 ± 2.08	7-O-5-bromopentylchrysin
C16	15.23 ± 0.15	7-O-4-bromopentylchrysin
C17	3.14 ± 0.22	7-O-12-bromododecylchrysin
Chrysin	7.38 ± 1.56	5,7-dihydroxyflavone
CPZ	7.27 ± 0.71	Chlorpromazine hydrochloride

- - No activity at doses ≤ 200 μM

Series 3 compound (C13 to C17) elicited cytotoxic inhibition against Molt-4 cell line at IC_{50} ranging from 15.31 – 36.72 with C16 and C17 indicating cytotoxicity at concentrations ≤ 23 μM. C16 elicited cytotoxic inhibition at IC_{50} 15.31 μM and C17 (22.23 μM).

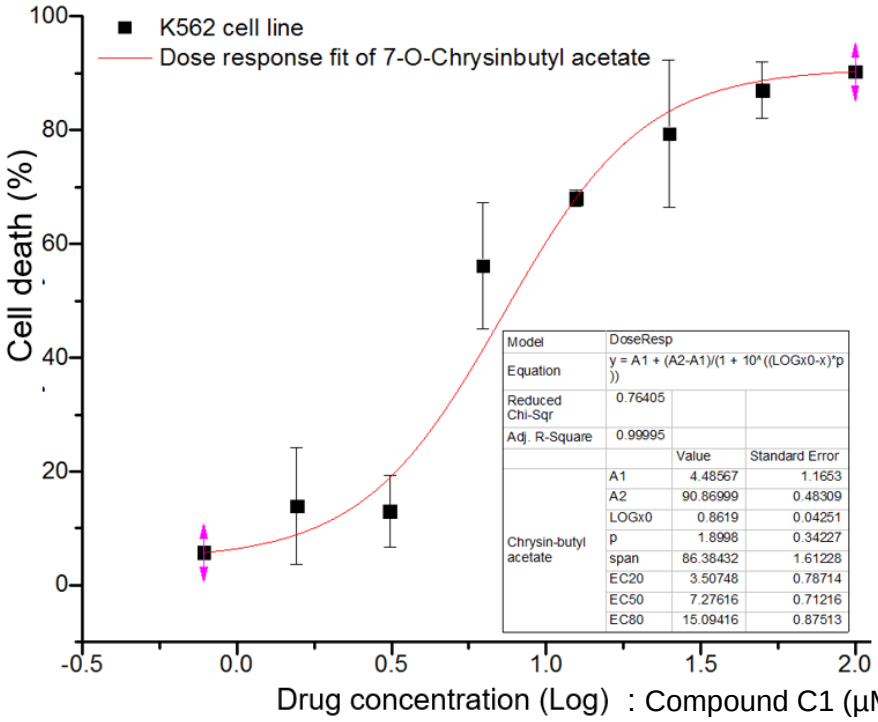


Figure 5.12: IC_{50} (µM) graph for cytotoxic activity of C1 against K562 cell line treated for 72 hours

Series 2 compounds were inactive against K562 cell lines at doses ≤ 100 µM.

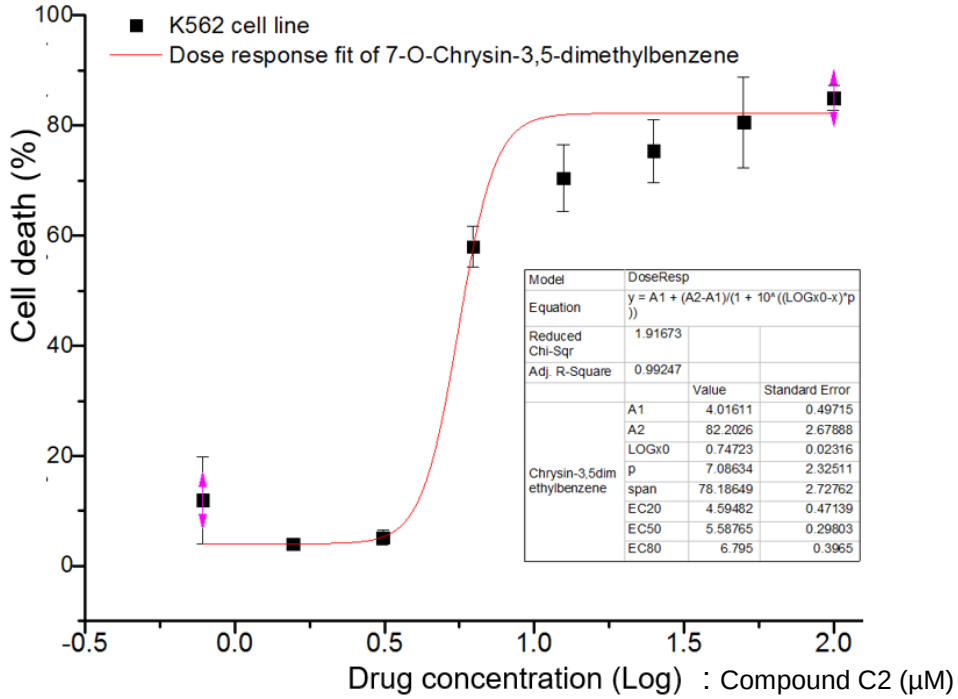


Figure 5.13: IC_{50} (µM) graph for cytotoxic activity of C2 against K562 cell line treated for 72 hours

Series 1 compounds (C1 and C2) elicited better cytotoxicity against K562 cell line compared to those of chrysin (IC_{50} 7.38 µM) and CPZ (IC_{50} 7.27 µM). C1 indicated an IC_{50} of 7.27 µM; C2 (IC_{50} 5.58 µM) and C3 (IC_{50} 8.69 µM) (Table 5.13). Cytotoxic inhibition elicited by C2 is comparable to that indicated by CPZ.

A graphical illustration of cytotoxicity elicited by bioactive chrysin derivatives is presented in Figure 5.14 below.

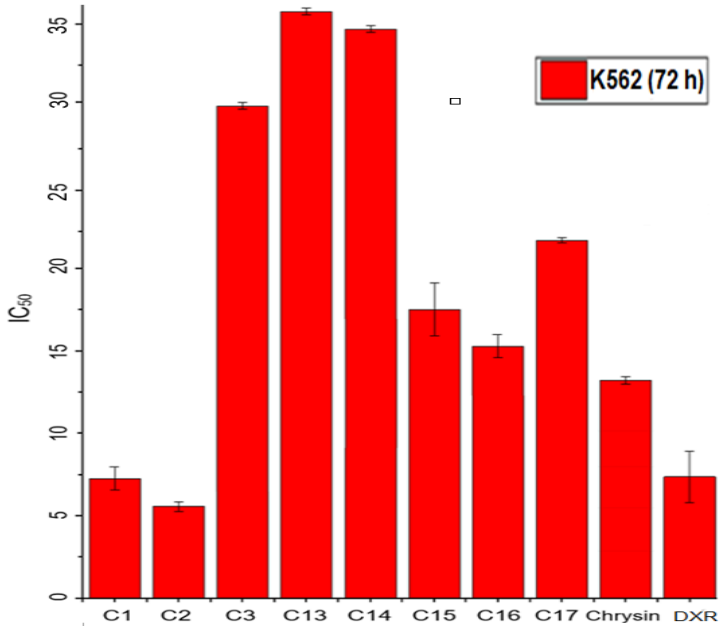


Figure 5.14: Comparison of IC_{50} (µM) values of Series 1, 2 and 3 Chrysin derivatives against K562 cell line. Series 3 compounds elicited cytotoxicity comparable to those indicated by chrysin and CPZ. Series 1 compounds were inactive against K562 cell line.

The bromoalkylchrysin derivatives (Series 3 compound) were superior cytotoxic inhibitors of K562 cell line compared to the aliphatic 7-O-Chrysin derivatives which indicated no cytotoxic inhibition against K562 cell line. Cytotoxic response indicated by Series 3 compound was comparable to that of C3 – a Series 1 compound. Generally, Series 1 compounds were the most cytotoxic inhibitors of K562 cell lines compared to all other groups. The result suggests 7-O-bromochrysin derivatives are more cytotoxic than 7-O-alkylchrysin derivatives of chrysin.

5.5.3 Results for Treatment of Breast Cancers with Synthesised Chrysin Derivatives

5.5.3.1 Results for MCF 7 Cancer Cell Line

Cytotoxic activity of 7-O-chrysin derivatives against MCF 7 cell line was investigated. Results are presented in Table 5.14.

Table 5.14: Table showing IC_{50} values of chrysin adducts against MCF 7 Cell Line

MCF 7 cell line treated for 72 h		
Compounds	**IC_{50} (μM)**	**Name of Chrysin analogue**
C1	17.6 ± 1.08	7-O-chrysinbutyl acetate
C2	-	7-O-chrysin-3,5-dimethylbenzene
C3	26.18 ± 3.34	7-O-chysin-2,4-dinitrobenzene
C4	-	7-O-isopropylchrysin
C5	46.53 ± 11.45	7-O-chrysin-2-methylpropane
C6	-	7-O-butylchrysin
C7	-	7-O-pentylchrysin
C8	-	7-O-hexylchrysin
C9	-	7-O-heptylchrysin
C10	-	7-O-octylchrysin
C11	-	7-O-nonylchrysin
C12	-	7-O-decylchrysin
C13	66.73 ± 25.61	7-O-3-bromopropylchrysin
C14	63.03 ± 7.21	7-O-4-bomobutylchrysin
C15	37.75 ± 0.14	7-O-5-bromopentylchrysin
C16	27.32 ± 4.48	7-O-4-bromopentylchrysin
C17	22.92 ± 1.89	7-O-12-bromododecylchrysin
Chrysin	4.20 ± 1.70	5,7-dihydroxyflavone
CPZ	2.17 ± 0.10	Chlorpromazine hydrochloride

- - No activity at doses ≤ 200 μM

Series 3 (C13 to C17) compound elicited weak anticancer activity against MCF 7 with IC_{50} values ranging from 22.92 to 66.73 μM. 7-O-12-Bromododecylchrysin (C17) and 7-O-4-Bromopentylchrysin (C16) elicited the best cytotoxicity against MCF 7 cell line (Table 5.14 above). Interestingly a decrease in cytotoxic activity is observed in Series 3 compounds – as the carbon atoms increased (Table 5.14). This result suggests longer chains bromoalkylchrysin adduct are more cytotoxic than shorter chain bromoalkylchrysin adducts.

Series 2 compounds (C4 to C12) were inactive against MCF 7 cell line except the 7-O-2-methylchrysin adducts which indicated a weak cytotoxicity (IC_{50} 46.53 µM) – (Table 3.12), an indication that methylation increases the cytotoxic activity of propylchrysin adduct**.**

Series 1 compounds (C1, C2 and C3): The only Series 1 compound with good cytotoxic activity against MCF 7 is 7-O-chrysinbutyl acetate (C1). C2 was inactive against MCF 7 cell lines. C3 elicited cytotoxicity at an IC_{50} value 26.18 µM.

5.5.3.2 Results for MDA-MB 468 Cancer Cell Line

Cytotoxic activity of 7-O-chrysin derivatives against MDA-MB 468 cell line was investigated. Results are presented in Table 5.15.

Table 5.15: Table showing IC_{50} values of chrysin adducts against MDA-MB 468 cell line

MDA-MB 468 cell line treated for 72 hours		
Compounds	**IC_{50} (µM)**	**Name of Chrysin analogue**
C1	7.49 ± 0.61	7-O-chrysinbutyl acetate
C2	25.41 ± 1.36	7-O-chrysin-3,5-dimethylbenzene
C3	16.59 ± 1.33	7-O-chysin-2,4-dinitrobenzene
C4	-	7-O-isopropylchrysin
C5	-	7-O-chrysin-2-methylpropane
C6	-	7-O-butylchrysin
C7	-	7-O-pentylchrysin
C8	6.06 ± 3.43	7-O-hexylchrysin
C9		7-O-heptylchrysin
C10	38.80 ± 22.90	7-O-octylchrysin
C11	-	7-O-nonylchrysin
C12	-	7-O-decylchrysin
C13	-	7-O-3-bromopropylchrysin
C14	-	7-O-4-bomobutylchrysin
C15	-	7-O-5-bromopentylchrysin
C16	8.17 ± 0.36	7-O-4-bromopentylchrysin
C17	18.24 ± 2.44	7-O-12-bromododecylchrysin
Chrysin	5.84 ± 0.67	5,7-dihydroxyflavone
CPZ	5.83 ± 0.51	Chlorpromazine hydrochloride

- - No activity at doses ≤ 200 µM

Series 3 compounds (C13 to C17) shows that short carbon chain Series 3 compounds (C13 to C15) were inactive against MDA-MB 468 cell line. As the carbon chain increases (C17), 7-O-bromododecylchrysin (C17) elicited significant anticancer activity against the breast cancer cell line – MDA-MB 468 at IC_{50} 18.24 µM. This activity is comparable to that indicated by the positive control drug – CPZ and better than that indicated by chrysin (table 5.15). Another interesting finding is the significant cytotoxicity demonstrated by the methylated 7-O-bromochrysin compound – 7-O-4-bromopentylchrysin (C16) which

indicates a superior cytotoxic effect on MDA-MB 468 (IC_{50} 8.17 µM) compared to 7-O-bromochrysin compounds without the methyl group – (Table 5.15). This novel compound indicated better anticancer activity against the triple-negative breast cancer cell line (MDA-MB 468) compared to chrysin (IC_{50} 5.84 µM) and CPZ (IC_{50} 5.83 µM). The dose response (IC_{50} plot) for C16 is presented in Figure 5.15.

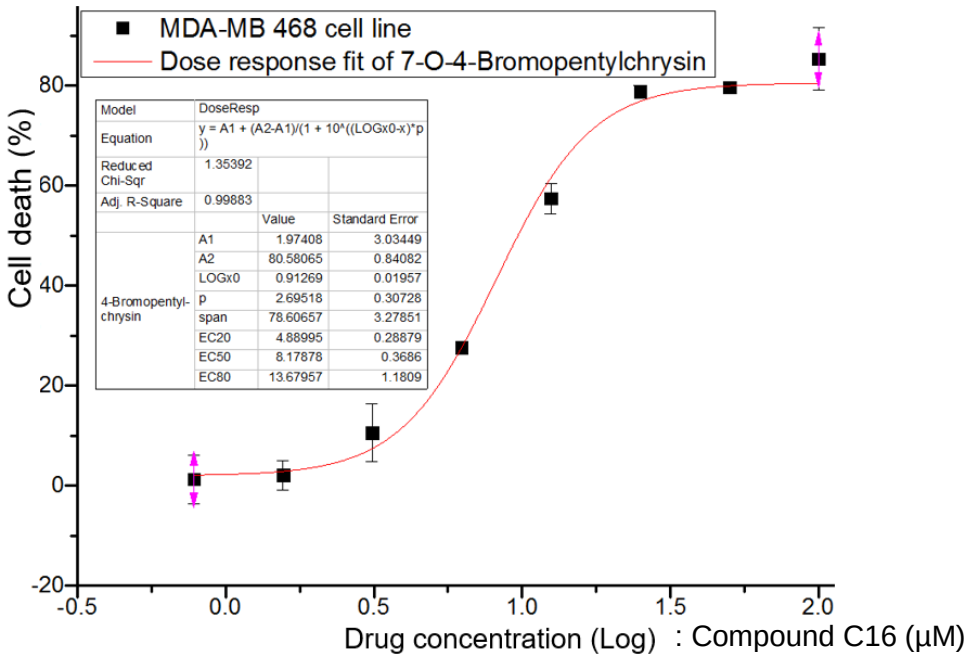


Figure 5.15: IC_{50} (µM) of 4-bromopentylchrysin against MDA-MB 468 cell line treated for 72 hours

Series 2 compounds (C4 to C12): 7-O-hexylchrysin (C7) elicited significant cytotoxic effect (IC_{50} 6.0 µM) against MDA-MB 468.

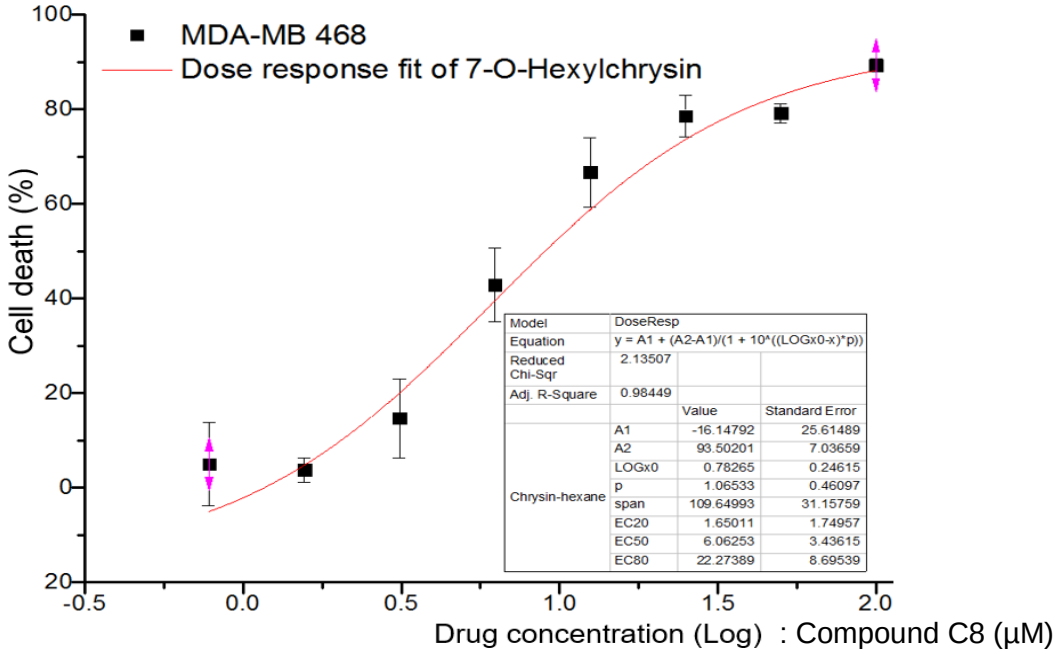


Figure 5.16: Dose response (IC_{50} plot) of C7 against MDA-MB 468 cell line after treatment for 72 hours

7-O-Hexylchrysin (C7) indicated weak cytotoxicity against MDA-MB 468 (IC_{50} 38.80 µM). Other compounds in this group were inactive against MDA-MB 468 cell line. The cytotoxic concentration (IC_{50}) of C7 is presented in Figure 5.16 above.

Series 1 compounds (C1 to C3): 7-O-chrysinbutyl acetate (C1) indicated significant cytotoxic effect against MDA-MB 468 cell line – IC_{50} 7.49 µM (Table 5.15 above). C2 and C3 were inactive against this cell line. The cytotoxicity (IC_{50}) plot for C1 against MDA-MD 468 is presented in Figure 5.17.

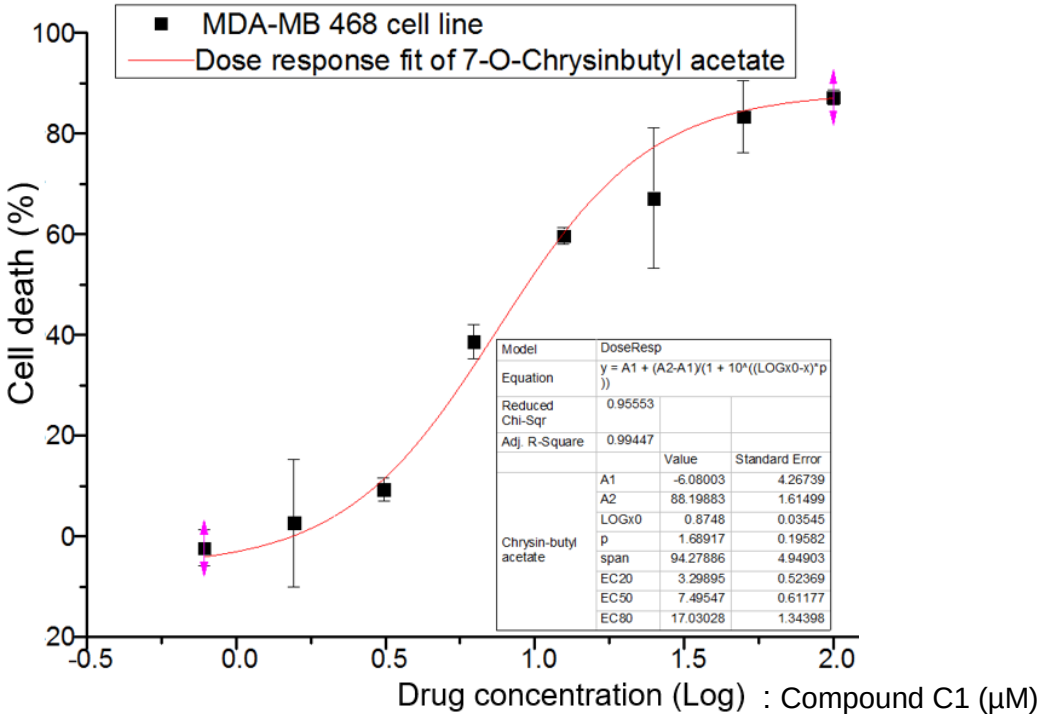


Figure 5.17: IC_{50} (µM) of C1 against MDA-MB 468 cell line

C2 elicited weak cytotoxicity (IC_{50} 25.41 µM), C3 indicated significant cytotoxicity (16.59 µM) against MDA-MB 468 cell line. A comparison of cytotoxic inhibition elicited by these compounds indicates a comparable cytotoxic inhibition between C8, CPZ, chrysin, C1 and C16 against MDA-MB 468 cell line (Figure 5.17 above).

5.5.4 Results for Treatment of Liver (Hepatocellular carcinoma) Cancer Cell Line with Synthesised Chrysin Derivatives

5.5.4.1 Results for HepG2 Cancer Cell Line

Cytotoxic activity of 7-O-chrysin derivatives against HepG2 cell line was investigated. Results are presented in Table 5.16.

Table 5.16: Table showing IC_{50} values of chrysin adducts against HepG2 Cell Line

HepG2 cell line treated for 72 hours		
Compounds	**IC_{50} (µM)**	**Name of Chrysin adduct**
C1	17.53 ± 1.01	7-O-chrysinbutyl acetate
C2	34.82 ± 1.01	7-O-chrysin-3,5-dimethylbenzene
C3	4.93 ± 0.35	7-O-chysin-2,4-dinitrobenzene
C4	-	7-O-isopropylchrysin
C5	-	7-O-chrysin-2-methylpropane
C6	-	7-O-butylchrysin
C7	-	7-O-pentylchrysin
C8	25.98 ± 3.19	7-O-hexylchrysin
C9	-	7-O-heptylchrysin
C10	-	7-O-octylchrysin
C11	-	7-O-nonylchrysin
C12	-	7-O-decylchrysin
C13	-	7-O-3-bromopropylchrysin
C14	-	7-O-4-bomobutylchrysin
C15	-	7-O-5-bromopentylchrysin
C16	-	7-O-4-bromopentylchrysin
C17	-	7-O-12-bromododecylchrysin
Chrysin	10.69 ± 0.94	5,7-dihydroxyflavone
CPZ	5.62 ± 0.83	Chlorpromazine hydrochloride

- - No activity at doses ≤ 200 µM

HepG2 cell were resistant to Series 3 (C4 to C12) and series 2 (C13 to C17) compounds except 7-O-hexylchrysin (C6) which indicated good cytotoxic inhibition against HepG2 cell line. Cancer cells inhibition shown by C6 was better than that of C2 Table 5.16.

Series 1 compounds: investigation of cytotoxic inhibition of Series 1 compounds against HepG2 cell line indicates that 7-O-chysin-2,4-dinitrobenzene was the most Cytotoxic with IC_{50} 4.93 µM, which is better than those showed by chrysin (10.69 µM) and CPZ (5.62 µM). C1 also showed good anticancer inhibition of HepG2 cells. This activity is comparable to that indicated by chrysin.

5.5.5 Results for Treatment of Lung Cancer Cell Line with Synthesised Chrysin Derivatives

5.5.5.1 Results for A549 Cancer Cell Line

Cytotoxic activity of 7-O-chrysin derivatives against A549 cell line was investigated. Results are presented in Table 5.17.

Table 5.17: Table showing IC_{50} values of chrysin adducts against A549 Cell Line

A549 cell line treated for 72 hours		
Compounds	**IC_{50} (μM)**	**Name of Chrysin analogue**
C1	20.01 ± 0.78	7-O-chrysinbutyl acetate
C2	-	7-O-chrysin-3,5-dimethylbenzene
C3	33.13 ± 2.19	7-O-chysin-2,4-dinitrobenzene
C4	-	7-O-isopropylchrysin
C5	-	7-O-chrysin-2-methylpropane
C6	-	7-O-butylchrysin
C7	-	7-O-pentylchrysin
C8	-	7-O-hexylchrysin
C9	-	7-O-heptylchrysin
C10	-	7-O-octylchrysin
C11	-	7-O-nonylchrysin
C12	-	7-O-decylchrysin
C13	-	7-O-3-bromopropylchrysin
C14	-	7-O-4-bomobutylchrysin
C15	-	7-O-5-bromopentylchrysin
C16	16.79 ± 1.73	7-O-4-bromopentylchrysin
C17	-	7-O-12-bromododecylchrysin
Chrysin	14.64 ± 1.36	5,7-dihydroxyflavone
CPZ	8.75 ± 1.24	Chlorpromazine hydrochloride

- - No activity at doses ≤ 200 μM

Most chrysin adducts made in this study were inactive against A549 cell line. For Series 3 (C13 to C17) compounds, 7-O-4-bromopentylchrysin (C16) elicited significant cytotoxic inhibition against A549 (IC_{50} 16.79 μM) (Figure 5.18) comparable to that presented by chrysin (IC_{50}14.64 μM) (Table 5.17).

Series 2 (C4 to C12) compounds were inactive against A549 cell line at IC_{50} ≤ 100 μM.

For Series 1 (C1 to C3) compounds, C1 inhibited the growth of A549 cell line at IC_{50} 20.01 µM. C2 was inactive against A549. C3 showed weak inhibition against A549 cell line (IC_{50} 33.13 µM). Nevertheless, cytotoxic inhibition indicated by C1 and C16 were comparable to those showed by the conventional drug – CPZ (IC_{50} 8.75 µM), and comparable to that indicated by chrysin (IC_{50} 14.64 µM).

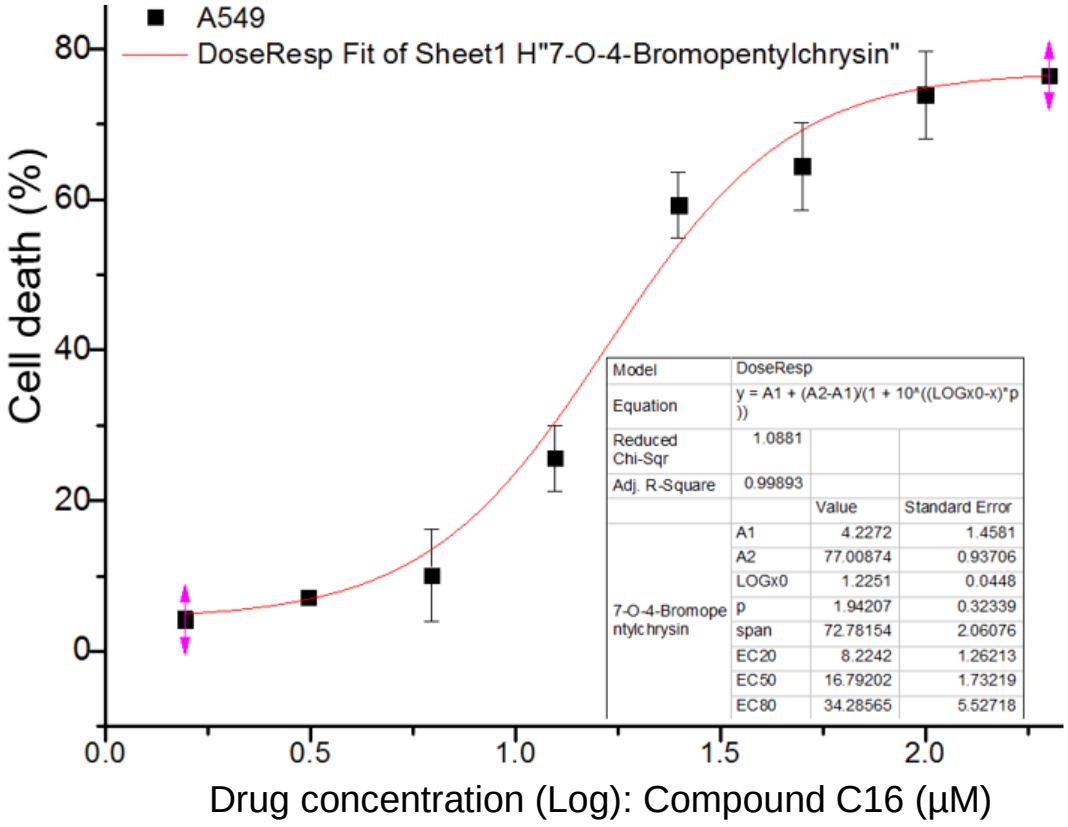


Figure 5.18: Dose response – IC_{50} (µM) plot of the most cytotoxic chrysin adduct (C16) against A549

C16 also indicated a significant IC_{20} (8.22 µM) against A549 cell line (Figure 5.18).

5.5.6 Results for Treatment of Skin Cancer (Keratinocyte) Cell Line with Synthesised Chrysin Derivatives

5.5.6.1 Results for HacaT Cancer Cell Line

Cytotoxic activity of 7-O-chrysin derivatives against HacaT cell line was investigated. Results are presented in Table 5.18.

Table 5.18: Table showing IC_{50} values of chrysin adducts against HacaT Cell Line

HacaT cell line treated for 72 hours		
Compounds	**IC_{50} (µM)**	**Name of Chrysin analogue**
C1	37.76 ± 14.56	7-O-chrysinbutyl acetate
C2	-	7-O-chrysin-3,5-dimethylbenzene
C3	-	7-O-chysin-2,4-dinitrobenzene
C4	-	7-O-isopropylchrysin
C5	21.84 ± 3.36	7-O-chrysin-2-methylpropane
C6	6.38 ± 1.09	7-O-butylchrysin
C7	26.37 ± 1.21	7-O-pentylchrysin
C8	12.81 ± 1.63	7-O-hexylchrysin
C9	12.29 ± 2.38	7-O-heptylchrysin
C10	9.62 ± 0.69	7-O-octylchrysin
C11	25.67 ± 0.12	7-O-nonylchrysin
C12	28.4 ± 1.35	7-O-decylchrysin
C13	-	7-O-3-bromopropylchrysin
C14	-	7-O-4-bomobutylchrysin
C15	-	7-O-5-bromopentylchrysin
C16	-	7-O-4-bromopentylchrysin
C17	-	7-O-12-bromododecylchrysin
Chrysin	9.8 ± 1.00	5,7-dihydroxyflavone
CPZ	12.69 ± 1.26	Chlorpromazine hydrochloride

- - No activity at doses ≤ 200 µM

Series 2: all Series 3 compound (C4 to C12) tested showed interesting cytotoxic inhibition against HacaT cell line. 7-O-butylchrysin (C6) – IC_{50} 6.38 µM (Table 5.18) and 7-O-octylchrysin (C10) – IC_{50} 9.62 µM showed very interesting cytotoxicity against HacaT cell line with C6 inducing a superior cytotoxicity compared to chrysin and CPZ. 7-O-heptylchrysin (IC_{50} 12.28 µM) and 7-O-hexylchrysin – IC_{50} 12.81 µM also elicited significant cytotoxicity against HacaT cell line. Cytotoxic inhibition elicited by C8 and C9 were better than those indicated by chrysin and CPZ.

Series 3 compounds (C13 to C17) were inactive against HacaT cell line.

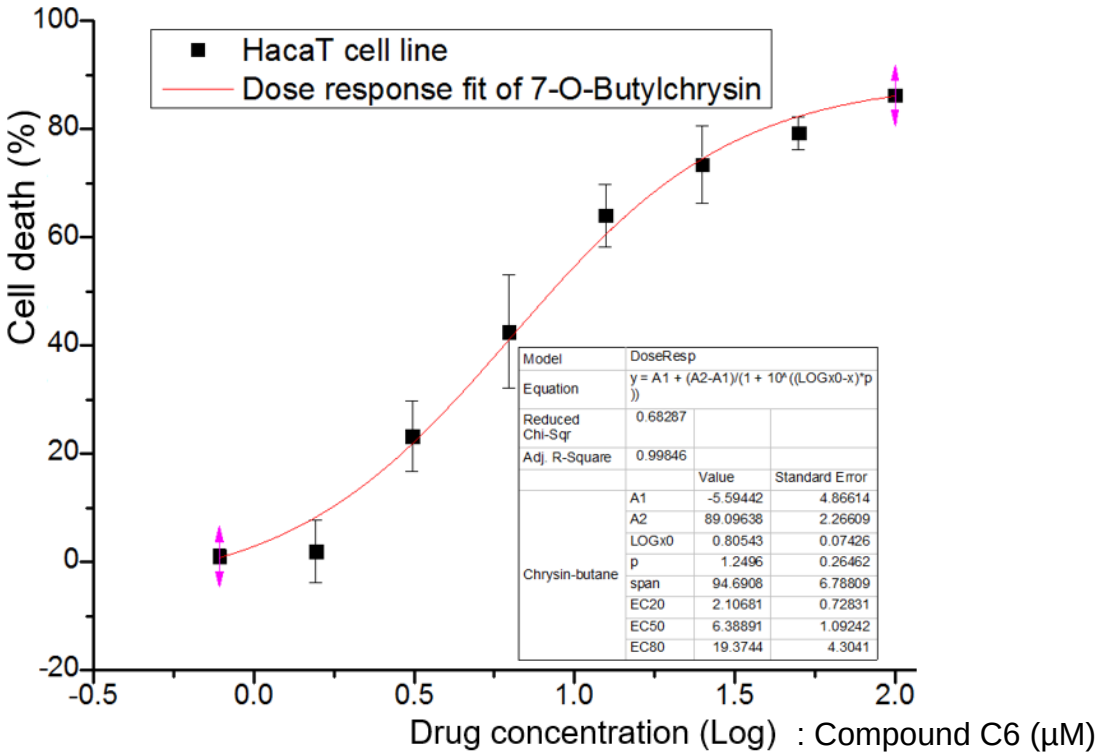


Figure 5.19: IC_{50} (µM) of 7-O-chrysinbutane against HacaT cell line treated for 72 hours

For Series 1 compounds, only 7-O- 7-O-chrysinbutyl acetate indicated weak cytotoxicity against HacaT cell line. C2 and C3 were inactive against HacaT cell line.

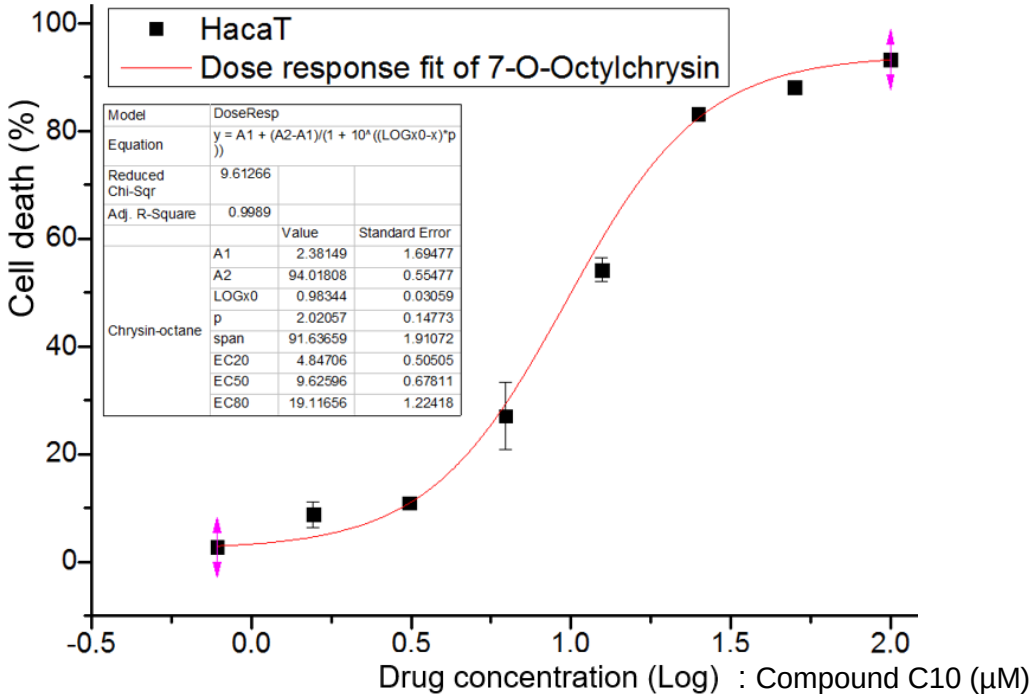


Figure 5.20: IC_{50} (µM) of 7-O-octylchrysin against HacaT cell line against HacaT cell line treated for 72 hours

5.5.7 Results for Treatment of Mesothelioma cell line with Synthesised Chrysin Derivatives

5.5.7.1 Result for Mero-14 Cancer Cell Line

Cytotoxic activity of 7-O-chrysin derivatives against Mero cell line was investigated. Results are presented in Table 5.19. It should be noted that CPZ and standard anticancer drugs are relatively inactive against the Mero-14 cell line (Mujoomdar et al., 2010). For this reason, drug repurposing and the use of chlorpromazine hydrochloride – CPZ; (a drug used in treating psychotic disorders such as schizophrenia) in cancer therapy has been reported (Mello et al., 2016; Shenoy, Biaglow, Varnes, & Daniel, 1982; Yang et al., 2019).

Table 5.19: Table showing IC_{50} values of chrysin adducts against Mero 14 Cell line

Mero-14 cell line treated for 72 hours		
Compounds	**IC_{50} (μM)**	**Name of Chrysin analogue**
C1	7.65 ± 0.53	7-O-chrysinbutyl acetate
C2	26.31 ± 4.09	7-O-chrysin-3,5-dimethylbenzene
C3	8.71 ± 0.85	7-O-chysin-2,4-dinitrobenzene
C4	26.71 ± 1.47	7-O-isopropylchrysin
C5	-	7-O-chrysin-2-methylpropane
C6	-	7-O-butylchrysin
C7	-	7-O-pentylchrysin
C8	122.51 ± 8.96	7-O-hexylchrysin
C9	-	7-O-heptylchrysin
C10	-	7-O-octylchrysin
C11	-	7-O-nonylchrysin
C12	-	7-O-decylchrysin
C13	-	7-O-3-bromopropylchrysin
C14	46.13 ± 2.63	7-O-4-bomobutylchrysin
C15	32.34 ± 3.44	7-O-5-bromopentylchrysin
C16	9.56 ± 0.82	7-O-4-bromopentylchrysin
C17	25.46 ± 4.12	7-O-12-bromododecylchrysin
Chrysin	45.99 ± 3.08	5,7-dihydroxyflavone
CPZ	3.42 ± 2.87	-

\- - No activity at doses ≤ 200 μM

The results presented above indicates the 7-O-bromochrysin derivatives (C13 to C17) inhibited the growth of Mero-14 cell line. 7-O-4-bromopentylchrysin (C16) which was the most cytotoxic bromoalkylchrysin derivative (IC_{50} 9.56 μM) indicated a significant cytotoxic inhibition against the Mero-14 cancer cell line. Other than C16, other bromoalkylchrysin

derivatives showed weak cytotoxic inhibition (25.46 µM – C17, 32.32 µM –C15 and 46.13 µM – C14) - Table 5.19.

Most 7-O-alkylchrysin derivatives were inactive against Mero-14 cell lines. The only compound in this group which indicated significant cytotoxic inhibition against Mero 14 is 7-O-isopropylchrysin (C4) at IC_{50} concentration of 26.71 µM.

Series 1 compounds (C1 to C3) elicited significant cytotoxic inhibition against Mero14 cell line: C1 (IC_{50} 7.55 µM), C1 (IC_{50} 26.31 µM), C3 (IC_{50} 26.71 µM).

Methylation of Series 3 (7-O-bromochrysin derivatives) and 2 compounds (7-O-alkylchrysin derivatives) increased the cytotoxic activity of these chrysin adducts as observed in the methylated 7-O-isopropylchrysin (C4) and 7-O-4-bromopentylchrysin (C16). Despite the increased cytotoxic activity observed by the addition of a methyl group to C4 and C16, it is observed that C16 (with a bromine atom) indicated a 3-fold increase in cytotoxicity against Mero-14 cell line when compared to 7-O-isopropylchrysin (C4) without a bromine atom. The effect of increased cytotoxicity of methylated aliphatic 7-O-Chrysin adducts against Mero 14 cell line is also observed in aromatic chrysin adduct (7-O-chrysin-3,5-dimethylbenzene (C2) - Table 5.19.

Methylation of C2 also increased its cytotoxicity compared to its aliphatic counterpart – C4. However, the cytotoxic effect of C16 surpassed those of C2 and C4 three folds – an indication that methylation and bromination of 7-O-Chrysin adducts increases their cytotoxicity.

An interesting observation was seen in Series 1 compound (C1 to C3). C1 and C2 showed significant and interesting cytotoxicity against Mero 14 cell line (IC_{50} 7.65 and 8.71 µM). The cytotoxic activities of C1 and C3 were three times better than that of C2 (IC_{50} 26.31 µM). It therefore suggests the electron-withdrawing $–NO_2$ group in C3 and the ketone group (oxygen atoms in these groups) are responsible for the cytotoxicity of these compounds against Mero 14 cell line.

Another interesting observation is that bioactive cytotoxic adducts synthesised in this study were more cytotoxic against Mero 14 cell line than the parent compound – chrysin. C1, C3 and C16 indicated better cytotoxicity compared to the positive control – CPZ (IC_{50} 12.69 µM).

5.5.8 Results for Treatment of Normal Bronchial Epithelium Cell Line (BEAS-2B) with Synthesised Chrysin Derivatives

To investigate if the 7-O-chrysin adducts made in this study are cytotoxic against normal cells, we investigated the cytotoxicity of these chrysin adducts against the adherent, epithelial-like, normal human bronchial epithelial cell (BEAS-2B). The results are reported in Table 5.20.

Table 5.20: Cytotoxicity of 7-O-chrysin adducts on normal Cell Line (BEAS-2B)

BEAS-2B cell line (normal cell line) treated for 72 hours		
Compounds	**IC_{50} (μM)**	**Name of Chrysin analogue**
C1	143.22 ± 15.67	7-O-chrysinbutyl acetate
C2	59.66 ± 7.81	7-O-chrysin-3,5-dimethylbenzene
C3	112.23 ± 25.78	7-O-chysin-2,4-dinitrobenzene
C4	-	7-O-isopropylchrysin
C5	-	7-O-chrysin-2-methylpropane
C6	-	7-O-butylchrysin
C7	-	7-O-pentylchrysin
C8	122.51 ± 2.13	7-O-hexylchrysin
C9	285.67 ± 121.67	7-O-heptylchrysin
C10	-	7-O-octylchrysin
C11	156.64 ± 3.65	7-O-nonylchrysin
C12	-	7-O-decylchrysin
C13	-	7-O-3-bromopropylchrysin
C14	-	7-O-4-bomobutylchrysin
C15	-	7-O-5-bromopentylchrysin
C16	153.66 ± 45.72	7-O-4-bromopentylchrysin
C17	157.46 ± 3.74	7-O-12-bromododecylchrysin
Chrysin	45.64 ± 10.06	5,7-dihydroxyflavone
CPZ	22.36 ± 6.40	Chlorpromazine hydrochloride

- - No activity at doses ≤ 400 μM

For comparison and easy understanding of the cytotoxicity reported in this study, a summary of results indicating a comprehensive cytotoxic inhibition of 7-O-chrysin adducts against cancer and normal cell line(s) is presented in Table 5.21 below.

Table 5.21: A comprehensive overview of cytotoxicity IC_{50} (µM) indicated by chrysin derivatives against cancer cell lines and normal cell line (BEAS-2B) after 72 h of treatment with drugs Table 5.22: A comprehensive overview of cytotoxicity IC_{50} (µM) indicated by chrysin derivatives against cancer cell lines and normal cell line (BEAS-2B) after 72 h of treatment with drugs

	IC_{50} (x ± SD) µM*										
Comp.	HCT 116	Molt-4	K562	MCF 7	HepG2	A549	HaCaT	Caco-2	Mero-14	BEAS-2B	MDA-MB 468
1	1.99 ± 0.24	7.05 ± 0.47	7.27 ± 0.71	17.6 ± 1.08	17.53 ± 1.01	20.01 ± 0.78	37.76 ± 14.56	5.89 ± 0.41	7.65 ± 0.53	143.22 ± 15.67	7.49 ± 0.61
2	2.17 ± 0.19	13.87 ± 3.00	5.58 ± 0.29	-	34.82 ± 1.01	-	-	4.53 ± 0.79	26.31 ± 4.09	59.66 ± 7.81	25.41 ± 1.36
3	1.45 ± 1.71	5.41 ± 0.53	8.69 ± 2.05	26.18 ± 3.34	4.93 ± 0.35	33.13 ± 2.19	-	4.83 ± 0.37	8.71 ± 0.85	112.23 ± 25.78	16.59 ± 1.33
4	29.59 ± 3.25	97.71 ± 70.88	-	-	-	-	-	25.20 ± 2.66	26.71 ± 1.47	-	-
5	54.76 ± 2.77	17.40 ± 3.50	-	46.53 ± 11.45	-	-	21.84 ± 3.36	95.54 ± 4.72	-	-	-
6	21.63 ± 1.88	-	-	-	-	-	6.38 ± 1.09	36.75 ± 3.51	-	-	-
7	17.75 ± 1.53	51.21 ± 9.23	-	-	-	-	26.37 ± 1.21	-	-	-	-
8	30.37 ± 3.81	39.49 ± 3.29	-	-	25.98 ± 3.19	-	12.81 ± 1.63	-	24.03 ± 0.85	122.51 ± 2.13	6.06 ± 3.43
9	30.56 ± 2.04	35.14 ± 2.38	-	-	-	-	12.29 ± 2.38	-	-	285.67 ± 121.67	
10	16.40 ± 1.18	-	-	-	-	-	9.62 ± 0.69	15.27 ± 1.22	-	-	38.80 ± 22.90
11	17.53 ± 2.32	-	-	-	-	-	25.67 ± 0.12	13.05 ± 1.01	-	156.64 ± 3.65	-
12	18.91 ± 2.95	44.74 ± 3.14	15.08 ± 0.21	-	-	-	28.4 ± 2.86	18.91 ± 1.28	-	-	-
13	30.97 ± 4.17	44.74 ± 3.14	15.08 ± 0.21	66.73 ± 25.61	-	-	-	19.25 ± 3.79	-	-	-
14	44.20 ± 2.73	59.41 ± 29.05	18.72 ± 2.68	63.03 ± 7.21	-	-	-	16.77 ± 1.45	46.13 ± 2.63	-	-
15	96.27 ± 22.23	73.82 ± 65.75	17.52 ± 1.60	37.75 ± 0.14	-	-	-	22.06 ± 3.84	32.34 ± 3.44	-	-
16	69.77 ± 27.92	23.58 ± 1.67	15.31 ± 2.08	27.32 ± 4.48	-	16.79 ± 1.73	-	21.26 ± 2.21	9.56 ± 0.82	153.66 ± 45.72	8.17 ± 0.36
17	27.17 ± 4.35	13.28 ± 2.34	15.23 ± 0.15	22.92 ± 1.89	-	-	-	17.60 ± 6.30	25.46 ± 4.12	157.46 ± 3.74	18.24 ± 2.44
Chrysin	4.07 ± 0.29	5.35 ± 0.47	3.14 ± 0.22	4.20 ± 1.70	10.69 ± 0.94	14.64 ± 1.36	9.8 ± 1.00	4.84 ± 0.19	45.99 ± 3.08	45.64 ± 10.06	5.84 ± 0.67
CPZ	5.9 ± 0.54	5.23 ± 0.96	7.38 ± 1.56	2.17 ± 0.10	5.62 ± 0.83	8.75 ± 1.24	12.69 ± 1.26	5.17 ± 0.64	13.42 ± 3.50	22.36 ± 6.40	5.83 ± 0.51

- - No bioactivity at doses ≤ 200 µM for cancer cell lines and 400 for BEAS-2B

* - IC_{50} values are expressed as a mean value ± SD from two independent experiments performed in triplicate. CPZ: chlorpromazine, positive control

Multidrug resistance is associated with cancer cells due to the adaptation of cancer cells to the killing actions of chemotherapeutics (Pajak & Orzechowski, 2006). On a molecular level, those adaptations are exemplified by the overexpression of detoxifying enzymes (Moscow & Cowan, 1988), immune escape mechanisms (Begley & Ribas, 2008) or the expression of ABC-transporters which promotes the outward-transport of most commonly used cancer drugs in spite of their different chemical structures and thereby prevents the achievement of effective drug concentrations in the target cells (Lee, 2010). This causes an immense clinical problem and consequently there has been intense search for compounds which can act to kill cancer cells even after 24 – 48 hours of treatment (Lee, 2010). Amongst those compounds, many flavonoids appear to serve as substrates of BCRP, MRP-2 and P-gp (components of ABC-transporters) by competitively inhibiting these targets (Álvarez et al., 2009). Previous studies have shown that flavonoids increase the expression of P-gp *in vitro* and *in vivo* (Lohner et al., 2007). In a study by Álvarez et al. (2009) using the colorectal cancer – Caco-2, chrysin demonstrated inhibition to three apical components of the ABC-transporter – BCRP, MRP-2 and P-gp. These transporters extruded the chemotherapeutics topotecan out of HCT 116 and Caco-2 cells. The effect elicited by chrysin is similar to those reported by chrysin and its derivatives in this study against the colorectal cancer – HCT 116 and Caco-2 cell line.

At 25, 50 and 100 µM concentrations, none of the series 2 compounds (C4 – C12) containing alkyl-groups elicited inhibitory activities against the lung cancer cell line (A549). A similar observation was recorded for the liver cancer cell line (HepG2). It was also observed that most of the series 2 compounds could not inhibit the growth of leukaemia cancer cells (Molt-4 and K562), mesothelioma cancer cell (Mero-14) and the breast cancer cell lines – MCF-7 (oestrogen-receptor positive breast cancer cell line) and the MDA-MB 468 (triple-negative breast cancer cell line) at concentrations ≤ 50 µM. The failure of these compounds to elicit cytotoxicity is an indication that the series-2 compounds may be poor competitors for the enzyme active sites in the cancer cells. Furthermore, it is an indication that absence of the hydroxyl group at position-7 of the parent compound (chrysin) may have caused a conformational change in the molecule, making the cancer cell lines resistant to the 7-O-alkylchrysin derivatives synthesised in this experiment. It is an indication that the hydroxy group(s) on the chrysin molecule might be essential for their inhibitory activities and therefore we sought to synthesise brominated 7-O-chrysin adducts (7-O-bromoalkyl adducts) and to investigate whether the bromide ion could change the dynamics of the molecules (increasing its anticancer activities). The role of the hydroxyl

groups in the chrysin molecule in eliciting cytotoxicity against cancer cells has previously been reported (Che et al., 2011; Fonseca et al., 2017; Mehdi et al., 2018; Sun et al., 2012). We reasoned that brominated chrysin adducts may possess chemical dynamics similar to the chrysin molecules as alkylated derivatives failed to indicate good cytotoxicity against the cancer cell lines stated above. Solubility analysis of the series 2 compounds (7-O-alkylchrysin derivatives of chrysin) indicates that these compounds have poor solubilities compared to chrysin. It could be inferred therefore that the poor solubilities of these compounds may have prevented them from reaching and / or binding to the active sites of the enzymes that would have elicited cytotoxicity against these cancer cells.

Interestingly, brominated chrysin adducts (7-O-bromochrysin derivatives) indicated better cytotoxicities (as expected) against the leukaemia cancer cell lines (Molt-4 and K562), breast cancer cell line (MCF-7) and the mesothelioma cancer cell line (Mero-14). Surprisingly, the MDA-MB-468 breast cancer cell line was resistant to the cancer inhibitory activities of the brominated 7-O-chrysin derivatives (series-3 compounds). It is believed that the oestrogen receptor in the MCF-7 breast cancer cell line (the oestrogen receptor is lacking in the MDA-MB 468 breast cancer cell line) may play an active role in cytotoxicity of the brominated chrysin derivatives. The oestrogen receptor may therefore be the active site for the binding of the brominated 7-O-chrysin derivatives. The role of the oestrogen receptor in promoting breast cancer has been described previously (Gross & Yee, 2002). Interruption of oestrogen receptor (ER)-α function is therefore an effective strategy in breast cancer treatment. Of interest is the good anticancer activity recorded for C16 (a brominated 7-O-chrysin derivative). C16 is an isomer of C15. Despite being isomers, C15 indicated weak inhibition against the cancer cell lines stated above compared to C16. Taking a look at these compounds, it will be observed that C15 is a straight-chain compounds whereas C16 is a branched chain compound (methylated compound). It could therefore be inferred that the methyl group in addition to the bromine ion may have acted synergistically in bringing about the good anticancer activity recorded for C16 because the brominated isomer (C15) which is lacking the methyl group indicated weaker anticancer activity. The methyl group and the bromine atom may have changed the orientation of the molecule (C16), enabling it to bind to the active site that would elicit cytotoxic inhibition against cancer cell lines. It should be noted that C16 is the only series 2 compound that inhibited (significant cytotoxic activity) the growth of A549. The reason for this is unknown but it could be associated with the role played by the methyl and bromine groups attached to C16.

C17, a brominated 7-O-chrysin adduct (series 3 compounds) indicated very interesting cytotoxic inhibition against the cancer cells stated above. Cytotoxicities elicited by C17 was better than those indicated by other brominated 7-O-chrysin derivatives and comparable to C16 and chrysin. A look at the series 3 compounds shows that there all contain short chain carbons (n ≤ 5). However, C17 is a long chain 7-O-brominated chrysin adduct containing 12 carbon atoms attached to the 7-O-position of chrysin. The long aliphatic chain could provide a large degree of flexibility to the compound (C17) and its structure. It should be noted that the 12-carbon chain of C17 is nearly the size of the chrysin molecule and double the size of the A-ring of chrysin. The long aliphatic chain could bind to a hydrophobic region of the active site of an enzyme, giving rise to the cytotoxic activities of C17. The role of long chain brominated compounds has previously been investigated by Liu, (2003) when he demonstrated that long-chain theophylline derivatives elicited better anticancer activities compared to short-chain theophylline derivatives.

Series 2 (7-O-alkylchrysin derivatives) compounds and series 3 (7-O-bromoalkylchrysin derivatives) compounds were surprisingly inactive against the liver cancer cell line (HepG2) at concentration ≤ 100 µM. Surprisingly, C8 (a six-carbon series 2 compound) is about as cytotoxic as chrysin and much more potent than C2 – a series 1 compound. The anticancer activities of series 1 compounds shall be discussed shortly. We wondered why series 2 and series 3 compounds (C4 – C17) failed to inhibit the growth of the HepG2 cancer cell line and why C8 was able to inhibit the growth of this cancer cell line. Feng et al., (2017) studied the proliferation of the HepG2 cancer cell (hepatic carcinoma) using MTT assay in comparison with other cancer cell lines. They observed a significant rise in the optical density value of the HepG2 culture medium compared to the control. They also reported the presence of endothelial EA.hy926 cells in the culture medium. They concluded that the tumour microenvironment enables the HepG2 cells to proliferate and migrate faster than other cell lines and also stated that the HepG2 cells are associated with the vascular endothelial cells. This implies that wherever there is an HepG2 cell, vascular endothelial cells are also present. The failure of series 2 and series 3 compounds to inhibit the growth of the HepG2 cancer cells may be attributed to the rapid proliferation and migration of the HepG2 cells which sucked-up the compounds (C4 – C17), preventing these compounds from eliciting cytotoxic activity against them. Results obtained from the MTT-assay in this study supports the findings of Feng et al., (2017). An unusual increase in optical density was observed when culturing, growing and treating the HepG2 cancer cell lines (see appendix). It is also possible that the enzymes active sites were masked from the series 2

and series 3 compounds thus their failure to elicit anticancer activities. C8 may exhibit its cytotoxic inhibition via a different mechanism and this needs further investigation.

The series 2 compounds (C4 – C12) were surprisingly active against the aneuploid immortal keratinocyte cells – skin cancer cell line (HacaT cells); eliciting significant inhibition against the proliferation of the HacaT cells at concentrations better than the activities elicited by chrysin and the conventional drug – CPZ. Surprisingly, the series 2 compounds were the only class of 7-O-chrysin derivatives indicating significant antiproliferative activities against the skin cancer cell line. The positive control – CPZ and the parent compound – chrysin were inactive against HacaT cell line at concentrations ≤ 30 µM. This observation needs further investigation.

Studies on the anticancer activities of chrysin against different cancer types have been previously reported (Che et al., 2011; Fonseca et al., 2017; Patel et al., 2016). Similarly, the series 1 compounds (C1, C2 and C3) indicated better antiproliferative activity (better than those elicited by chrysin and the standard anticancer agent – CPZ) against HCT 116, Molt-4, K562, Caco-2 and Mero-14 cell line after 72 hours treatment. This suggests that like chrysin, synthetic chrysin derivatives made in this study could block the expression of ABC-transporters thus preventing the efflux of these compounds out of the cancer cell. A similar trend was also observed with all synthetic chrysin derivatives made in this study. They expressed better cytotoxic effects after treatment of these cancer cell lines for 72 hours. An indication that a chemotherapy agent efflux out of the cell is seen when the anticancer agent demonstrates good cytotoxic inhibition at 24 hours treatment but fail to maintain this inhibition after 48 or 72-hours treatment (Lee, 2010). Lee et al., (2010) also reported that chrysin also blocked the efflux of topotecan in HCT 116 and Caco-2 cell line, leading to accumulation of topotecan in cells thus meeting the criteria of an effective MDR-reversing agent by efficient blockade of topotecan efflux. The results in this study suggests C1, C2 and C3 could also inhibit their efflux out of the cancer cells by blocking the ABC-transporters. This hypothesis needs to be verified.

The good cytotoxic activities showed by the series 1 compounds (C1, C2 and C3) against different cancer cell lines (colorectal – HCT 116 and Caco-2, leukaemia – K562 and Molt-4, breast –MDA-MB 468, liver – HepG2, lung – A549 and mesothelioma – Mero-14) might be linked to the effect of substituted functional groups attached to these molecules (Fonseca et al., 2017; Lohner et al., 2007; Patel et al., 2016). The functional groups attached to chrysin may have changed the chemical dynamics of the series 1 compounds, making them more potent than the series 2 and series 3 counterparts. Thus, the significant

cytotoxicity indicated by C1 may be linked to the methoxy-group in C1. Previous studies on the enhanced cytotoxicity of methoxy groups in the selenium moiety of chrysin has previously been reported against Ehrlich ascetic tumour cells (Liu et al., 1992). Wen et al., (2016) reported antiproliferative activities of a series of 3-(3′-hydroxy-4′-methoxyphenyl)selenyl-5,6,7-trimethoxy-1H-indoles against three cancer cell lines – HT-29, SGC-7901 and A549 cell lines. In another study, the cytotoxic effect of methoxy groups in colorectal cancers was reported (Reddy et al., 2016). These studies support the findings in this study for the significant cytotoxicity elicited by C1 due to the presence of the methoxy moiety. The 4-carbon bridge between the 7-OH position of chrysin and the methoxy group in C1 may have been responsible for the better solubility of this compound compared to chrysin. One of the aims of this project is to synthesise chrysin derivatives with better bioactivities and solubilities compared to the parent compound – chrysin. This hypothesis may be true because compound C7 (also having a 4-carbon spacer between the 7-OH group of chrysin and the terminal carbon) indicated poor solubility and poor bioactivities against all cancer cell lines investigated. Similarly, compound C14 (also having a 4-carbon spacer between the 7-OH group of chrysin and the terminal bromine) also indicated poor solubility and poor bioactivities against cancer cell lines tested. This suggests the methoxy group is responsible for the better solubility and bioactivity of C1. C1 elicited cytotoxic inhibition against all cancer cell lines investigated.

C2 with a phenyl-group attached at 7-OH also showed significant cytotoxic inhibition against colorectal cancers (HCT 116 and Caco-2 cell lines), leukaemia cancers (K562 and molt-4 cell lines) and interestingly the mesothelioma cell line – Mero-14 which is resistant to most therapies (Sleire et al., 2017). The anticancer activities of C2 was better than those indicated by chrysin against the colorectal cancer cell lines (HCT 116 and Caco-2), leukaemia cancer cell lines (Molt-4 and K562) and the mesothelioma cancer cell line (Mero-14). C2 also indicated better cytotoxicity against HCT 116, K562, Caco-2 and Mero-14 cancer cell line compared to CPZ. C2 was also more soluble than chrysin in polar solvents. The anticancer activities of 5,7-dihydroxy-2-phenyl-6,8-bis((4-fluorophenyl)selanyl)-4H-chromen-4-one), a chrysin derivative containing a phenyl-ring has previously been reported with an IC_{50} of 19.9 µM (Fonseca et al., 2017). In another experiment by Patel et al., (2016), they investigated the anticancer activities of a series of chrysin-piperazine conjugates and reported the anticancer activities of all phenylated chrysin-piperazine conjugates against ovarian (SK-OV-3), cervical (CaSki and HeLa cell lines) at IC_{50} values 5.04 – 8.21 µg/mL. Patel et al (2016) also reported that 4-carbon spacers attached at 7-

OH did not indicate an improved cytotoxicity of chrysin derivatives. These are in agreements with our findings in this work for C2.

The role of nitrogenated organic compounds in engaging in a wide variety of intermolecular interactions such as hydrophobic forces, van der Walls interaction, metal-coordination bonding and hydrogen-bonding has been described previously (Hosseinzadeh et al., 2018). This allows them to equally match the diverse range of structural enzymes binding pockets in cancer cells. Because many enzymes have been reported to have affinity for, and interact with nitrogenated compounds, causing them to disrupt biological pathways in cancer cells (Hosseinzadeh et al., 2018), we synthesised compound C3 and investigated its effect against cancer cell lines. C3 significantly inhibited the growth of all cancer cell lines investigated (except the skin cancer cells – HacaT) at concentrations better than those of chrysin and CPZ against HCT 116, Molt-4, K562, HepG2, Caco-2 and Mero-14 cancer cell lines.

The significant cytotoxicity indicated by C3 may be associated with the role played by the nitrogen atom on a benzene ring. Patel et al., (2016) demonstrated potentiating effect of nitrogenated chrysin-piperazine conjugates with IC_{50} ranging from 5.09 to 8.05 µg/mL. The significant cytotoxicity demonstrated by C3 might also be because of the electron donating ability of nitrogen, which donates its lone pair of electrons. By donating its electrons, it may act as an alkylating agent, killing the cancer cells (Patel et al., 2016).

Although series 1 compounds indicated significant cytotoxicity against cancer cell lines, C2 did not indicate cytotoxic inhibition against breast cancer (MCF 7), lung cancer (A549) and HacaT cell lines at $IC_{50} \leq 100$ µM. A look at the positioning of atoms on C2 and C3 at the point of attachment of the phenyl ring to 7-OH of chrysin indicates an ortho-bonding of the dimethyl-groups attached to the benzene ring of C2. For C3, an ortho and para conformation is seen. The role of ortho, meta and para-bonds on the cytotoxicity of chrysin derivatives has been previously demonstrated by Patel et al., (2016). Patel et al., (2016) demonstrated that para-substitution at the 7-OH groups increased the cytotoxicity of chrysin derivatives better than ortho or para-substitutions. This explains why C3 (with one ortho and one para-substitution) indicated better cytotoxicity than C2. Our findings suggest that an ortho and para-substitution is important for optimal bioactivity for phenylated chrysin derivatives as the para position increases the polarity and electronic density of the compounds (Fonseca et al., 2017).

Apart from series 1 compounds (C1, C2 and C3) which demonstrated significant cytotoxicity against cancer cell lines, series 2 compounds (brominated 7-O-alkyl chrysin derivatives) – C13 to C17 also indicated good to moderate cytotoxicity against cancer cell lines. Structure-activity data from experiments indicates that compounds having one halogen (bromine, fluorine or chlorine) are more cytotoxic against cancer cell lines including the lung cancer cell line – A549 (Reddy et al., 2015). This explains why brominated 7-O-chrysin derivatives were more cytotoxic compared to non-brominated 7-O-chrysin derivatives made in this study.

An interesting finding in this work is the role played by methyl-groups and halogens in cytotoxicity of molecule(s) made in this study. For instance, the novel compound made in this study – C16 which has both the methyl and halogen functional groups maintained its cytotoxicity against eight cancer cell lines (HCT 116, Caco-2, Molt -4, K562, MCF 7, MDA-MB 468, A549 and Mero-14) with IC_{50} values ranging from 9.56 – 27.32 µM), except for HCT 116 cell line (IC_{50} 69.77 µM). This cytotoxic potential was reduced in C4 and C5 (novel compound) with only the methyl functional group. C4 and C5 indicated moderate cytotoxicity against four cancer cell lines – HCT 116, Caco-2, Molt-4, Mero 14 (for C4) and (MCF 7 for C5) with IC_{50} values ranging from 17.4 – 97.71 µM. These findings demonstrate that methylation in addition to halogenation of chrysin derivatives increases their cytotoxicity better than compounds with just only a methyl or a halogen group. The role of the methyl group in increasing the cytotoxicity of chrysin-selenium derivatives has been reported (Barreiros et al., 2006). The effect of methylation was expressed significantly in the treatment of the lung cancer cell line – A549 with synthesised chrysin derivatives. Except for C1, only methylated derivatives – C3 and C16 inhibited the proliferation of the lung cancer cell line.

The increase in cytotoxic effect of halogenation of 7-O-alkylchrysin is best expressed on the K562 cell line. Series 3 compounds (C13 to C17) showed cytotoxic inhibition against K562. Series 2 compounds (which are similar to series 3 compounds except for the absence of the bromine atom) did not indicate cytotoxicity against the K562 level at concentrations ≤ 100 µM. This further confirms the role of the terminal bromine atom in increasing cytotoxicity of the 7-O-bromoalkylchrysin compounds (Reddy et al., 2015).

Beyond apoptosis, another crucial parameter which has been shown to be diminished in cancer cells exposed to an effective chemotherapeutics is diminished differentiation of cancer cells (van der Flier & Clevers, 2009). In this study, untreated cancer cells indicated 23 – 48 % increase in concentration (from readings made using the spectrophotometer)

compared to treated cancer cells. This finding is an indication that chrysin derivatives (C1, C2 and C3) made in this study effectively inhibited the differentiation of HCT 116 and Caco-2 cell line in the early (24 hours), mid (48 hours) and later (72 hours) stages.

The apoptotic effect of chrysin derivatives (C1, C2 and C3) might be due to inhibition of expression of ABC-transporters. In a study by Tainton et al. (2004) where mutant cells were transfected into cells, there was a reduction in caspase activities in the transport deficient P-gp. Multidrug-resistant tumour cell lines expresses high levels of P-gp, which inhibits caspase-activation mediated by ligation of Fas (Smyth et al., 1998). It therefore suggests that inhibition of P-gp leads to a better drug performance (Lohner et al., 2007). The role of chrysin derivatives made in this study against p-gp should be investigated in future research.

The differences observed in the cytotoxicity shown by chrysin derivatives synthesised in this work is an indication that large biochemical differences that exist between cancer cell lines which influences their level of interaction with chemotherapeutic agents. It is also an indication that some cancer cell lines have developed resistant mechanisms to some chemotherapeutic agents.

5.6 Selectivity Index (SI)

Selectivity index (SI) is defined as the inhibitory concentration of the drug required to kill 50 % of normal cells (BEAS-2B) compared to the inhibitory concentration required to kill 50 % cancer cells (Seca et al., 2014).

$$SI = \frac{IC_{50} \text{ of normal cell}}{IC_{50} \text{ of cancer cell}}$$

BEAS-2B was the normal cell line used in this study. The selectivity index for the various cancer cell lines was evaluated and the results stated below. Seca et al., (2014) noted that many interesting literatures fail to investigate / evaluate the SI index of novel compounds reported in their literature thus making it difficult to evaluate the safety index of a promising novel anticancer agents. A higher SI is a pointer that a potential anticancer agent will kill cancer cells and not normal cell and vice versa. The selectivity index of sesquiterpene lactones isolated from *Inula helenium* has previously been reported against leukaemia cancer cell line (Ding et al., 2016; Seca et al., 2014). This section therefore focuses on investigating the selectivity indices of synthetic chrysin-derivatives.

5.6.1 Selectivity Index (SI) of Chrysin-Derivatives on Breast Cancer

The SI of 7-O-chrysin adducts was evaluated against breast cancer cell lines (MCF 7). Results are presented in Table 5.22 below. C2 was not cytotoxic against MCF 7 and for this reason; no SI was evaluated for C2.

C2 indicated a very narrow selectivity index against MDA-MB 468 (SI = 2) - Table 5.22. This implies that although C2 indicated good cytotoxicity against MDA-MB 468 (IC_{50} 25.41 µM), its safety may need be monitored due to the slim SI indicated by C2. 7-O-4-bromopentylchrysin (C16) was highly selective cytotoxic against MDA-MB 468 with SI = 19 despites eliciting significant cytotoxic inhibition against this cell line (IC_{50} 8.17 µM).

Chrysin indicated selective cytotoxicity against MCF 7 but marginal selectivity against MDA-MB 468. Both chrysin and CPZ indicated a slim SI against MDA-MB 468. With exception of cytotoxicity shown by C2 against MDA-MB 468, these results suggest 7-O-chrysin adducts synthesised in this study selectively elicited cytotoxicity against breast cancer cell lines but not normal cells.

Table 5.23: Selectivity index (SI) of chrysin adducts on breast cancer cell lines. BEAS-2B is the normal cell line used in this study.

Selectivity Index (SI) for normal cell line and Breast Cancer Cell Lines						
	IC_{50} (µM)					
Abbrev	**BEAS-2B IC_{50} (µM)**	**MCF 7 IC_{50} (µM)**	**SI (MCF 7)**	**MDA-MB 468**	**SI (MDA-MD 468)**	**Name of compound**
C1	143.22± 5.67	17.6 ± 1.08	8	7.49 ± 0.61	19	7-O-chrysinbutyl acetate
C2	59.66 ± 7.81	-	NT	25.41 ± 1.36	2	7-O-chrysin-3,5-dimethylbenzene
C3	112.23 ± 25.78	26.18 ± 3.34	4	16.59 ± 1.33	7	7-O-chysin-2,4-dinitrobenzene
C4	-	-	NT	-	NT	7-O-isopropylchrysin
C5	-	46.53 ± 11.45	NT	-	NT	7-O-chrysin-2-methylpropane
C6	-	-	NT	-	NT	7-O-butylchrysin
C7	-	-	NT	-	NT	7-O-pentylchrysin
C8	122.51 ± 2.13	-	NT	6.06 ± 3.43	20	7-O-hexylchrysin
C9	285.67± 121.67	-	NT		NT	7-O-heptylchrysin
C10	-	-	NT	38.80 ± 22.90	NT	7-O-octylchrysin
C11	156.64 ± 3.65	-	NT	-	NT	7-O-nonylchrysin
C12	-	66.73 ± 25.61	NT	-	NT	7-O-decylchrysin
C13	-	63.03 ± 7.21	NT	-	NT	7-O-3-bromopropylchrysin
C14	-	37.75 ± 0.14	NT	-	NT	7-O-4-bomobutylchrysin
C15	-	27.32 ± 4.48	NT	8.17 ± 0.36	NT	7-O-5-bromopentylchrysin
C16	153.66 ± 45.72	22.92 ± 1.89	7	18.24 ± 2.44	8	7-O-4-bromopentylchrysin
C17	157.46 ± 3.74	4.20 ± 1.70	37	5.84 ± 0.67	27	7-O-12-bromododecylchrysin
Chrysin	45.64 ± 10.06	2.17 ± 0.10	21	5.83 ± 0.51	8	5,7-dihydroxyflavone
CPZ	22.36 ± 6.40	17.6 ± 1.08	1	7.49 ± 0.61	3	Chlorpromazine hydrochloride

NT - Not evaluated
– - No activity
The lower the selectivity index, the more toxic the compound is on normal cell lines.

5.6.2 Colorectal Cancer Cells

The SI of 7-O-chrysin adducts was evaluated against breast cancer cell lines. Results are presented Table 5.23 below. Colorectal cancer cell lines were the most susceptible to cytotoxic inhibition by 7-O-chrysin adducts synthesised in this study. The SI presented indicates that most 7-O-alkylchrysin adducts - series 2 compounds (C4 to C12) were inactive against BEAS-2B cell line (Table 5.23). Their SI was therefore not evaluated.

Despite significant cytotoxicity elicited by Series 1 compounds, they were relatively inactive against BEAS-2B cell lines. C1 indicated SI of 72 and 24 against HCT 116 and Caco-2 cell lines. C2 indicated SI of 27 and 13 against HCT 116 and Caco-2 cell lines. C3 indicated SI of 77 and 23 against HCT and Caco-2 cell lines. Chrysin indicated SI of 3 and 3 against HCT and Caco-2 cell lines.

CPZ indicated SI of 1 and 2 against HCT and Caco-2 cell lines. Although the standard drug – CPZ indicated significant cytotoxicity against K562 cell line, its SI was low compared to those of chrysin and Chrysin derivatives made in this study.

These results (Table 5.23) suggest suggests 7-O-chrysin adducts made in this research, elicited selective cytotoxic inhibition against colorectal cancer cell lines used in this study.

Table 5.24: SI of chrysin adducts on colorectal cancer cell lines

Selectivity index for normal cell line and breast colorectal cell lines						
	IC_{50} (µM)					
Compound	**BEAS-2B IC_{50} (µM)**	**HCT 116 IC_{50} (µM)**	**SI (HCT 116)**	**Caco-2 IC_{50} (µM)**	**SI (Caco-2)**	**Name of compound**
C1	143.22 ± 15.67	1.99 ± 0.24	72	5.89 ± 0.41	24	7-O-chrysinbutyl acetate
C2	59.66 ± 7.81	2.17 ± 0.19	27	4.53 ± 0.79	13	7-O-chrysin-3,5-dimethylbenzene
C3	112.23 ± 25.78	1.45 ± 1.71	77	4.83 ± 0.37	23	7-O-chysin-2,4-dinitrobenzene
C4	-	29.59 ± 3.25	NT	25.20 ± 2.66	NT	7-O-isopropylchrysin
C5	-	54.76 ± 2.77	NT	95.54 ± 4.72	NT	7-O-chrysin-2-methylpropane
C6	-	21.63 ± 1.88	NT	36.75 ± 3.51	NT	7-O-butylchrysin
C7	-	17.75 ± 1.53	NT	-	NT	7-O-pentylchrysin
C8	122.51 ± 2.13	30.37 ± 3.81	4	-	NT	7-O-hexylchrysin
C9	285.67 ± 121.67	30.56 ± 2.04	9	-	NT	7-O-heptylchrysin
C10	-	16.40 ± 1.18	NT	15.27 ± 1.22	NT	7-O-octylchrysin
C11	156.64 ± 3.65	17.53 ± 2.32	9	13.05 ± 1.01	12	7-O-nonylchrysin
C12	-	30.97 ± 4.17	NT	19.25 ± 3.79	NT	7-O-decylchrysin
C13	-	44.20 ± 2.73	NT	16.77 ± 1.45	NT	7-O-3-bromopropylchrysin
C14	-	96.27 ± 22.23	NT	22.06 ± 3.84	NT	7-O-4-bomobutylchrysin
C15	-	69.77 ± 27.92	NT	21.26 ± 2.21	NT	7-O-5-bromopentylchrysin
C16	153.66 ± 45.72	27.17 ± 4.35	6	17.60 ± 6.30	9	7-O-4-bromopentylchrysin
C17	157.46 ± 3.74	4.07 ± 0.29	37	4.84 ± 0.19	33	7-O-12-bromododecylchrysin
Chrysin	45.64 ± 10.06	5.9 ± 0.54	8	5.17 ± 0.64	9	5,7-dihydroxyflavone
CPZ	22.36 ± 6.40	1.99 ± 0.24	11	5.89 ± 0.41	4	Chlorpromazine hydrochloride

NT - Not evaluated
– - No activity

5.6.3 Leukaemia Cell Lines

The SI of 7-O-chrysin adducts was evaluated against leukaemia cell and results presented in Table 5.24 below. 7-O-bromochrysin derivatives of chrysin indicated SI – 10 and 7 (C16 and 17 respectively) against K562 cell line, and 7 and 12 (C16 and C17) against Molt-4 cell line. 7-O-alkylchrysin derivatives (C4 to C12) were inactive against BEAS-2B, thus no SI for these compounds was evaluated. C1 selectively killed leukaemia cells (K562 and Molt-4) at SI 20 respectively. C2 was selective against K562 (SI = 11) but indicated a narrow SI (4) margin for Molt-4. C3 also selectively inhibited the leukaemia cancer cells growth in K562 (SI = 13) and Molt-4 (SI = 21). Chrysin selectively killed leukaemia cell line (SI = 4 and 3 for K562 and Molt-4 respectively) whereas CPZ indicated a slim SI margin against K562 and Molt-4 cell lines (3 and 2). Chrysin adducts synthesised in this study selectively inhibited the growth of K562 and Molt-4 while the standard drug – CPZ maintained a slim safety margin (SI index).

Table 5.25: SI of chrysin adducts on leukaemia cell lines

Selectivity index for normal cell line and leukaemia cancer cell lines						
	IC_{50} (µM)					
Abbrev	**BEAS-2B IC_{50} (µM)**	**K562 IC_{50} (µM)**	**SI (K562)**	**Molt-4 IC_{50} (µM)**	**SI (Molt-4)**	**Name of compound**
C1	143.22 ± 15.67	7.27 ± 0.71	20	7.05 ± 0.47	20	7-O-chrysinbutyl acetate
C2	59.66 ± 7.81	5.58 ± 0.29	11	13.87 ± 3.00	4	7-O-chrysin-3,5-dimethylbenzene
C3	112.23 ± 25.78	8.69 ± 2.05	13	5.41 ± 0.53	21	7-O-chysin-2,4-dinitrobenzene
C4	-	-	NT	97.71 ± 70.88	NT	7-O-isopropylchrysin
C5	-	-	NT	17.40 ± 3.50	NT	7-O-chrysin-2-methylpropane
C6	-	-	NT	-	NT	7-O-butylchrysin
C7	-	-	NT	51.21 ± 9.23	NT	7-O-pentylchrysin
C8	122.51 ± 2.13	-	NT	39.49 ± 3.29	3	7-O-hexylchrysin
C9	285.67 ± 121.67	-	NT	35.14 ± 2.38	8	7-O-heptylchrysin
C10	-	-	NT	-	NT	7-O-octylchrysin
C11	156.64 ± 3.65	-	NT	-	NT	7-O-nonylchrysin
C12	-	15.08 ± 0.21	NT	44.74 ± 3.14	NT	7-O-decylchrysin
C13	-	18.72 ± 2.68	NT	59.41 ± 29.05	NT	7-O-3-bromopropylchrysin
C14	-	17.52 ± 1.60	NT	73.82 ± 65.75	NT	7-O-4-bomobutylchrysin
C15	-	15.31 ± 2.08	NT	23.58 ± 1.67	NT	7-O-5-bromopentylchrysin
C16	153.66 ± 45.72	15.23 ± 0.15	10	13.28 ± 2.34	7	7-O-4-bromopentylchrysin
C17	157.46 ± 3.74	3.14 ± 0.22	50	5.35 ± 0.47	12	7-O-12-bromododecylchrysin
Chrysin	45.64 ± 10.06	7.38 ± 1.56	6	5.23 ± 0.96	3	5,7-dihydroxyflavone
CPZ	22.36 ± 6.40	7.27 ± 0.71	3	7.05 ± 0.47	2	Chlorpromazine hydrochloride

NT - Not evaluated
– - No activity

5.6.4 Liver Cancer – HepG2 Cell Line

The selectivity index of chrysin adducts on HepG2 cells was investigated and reported in Table 5.25 below.

Table 5.26: SI of chrysin adducts on HepG2

Selectivity index for normal cell line and liver cancer cell lines				
Comp-ound	BEAS-2B IC_{50} (µM)	HepG2 IC_{50} (µM)	SI (HepG2)	Name of compound
C1	143.22 ± 15.67	17.53 ± 1.01	8	7-O-chrysinbutyl acetate
C2	59.66 ± 7.81	34.82 ± 1.01	2	7-O-chrysin-3,5-dimethylbenzene
C3	112.23 ± 25.78	4.93 ± 0.35	23	7-O-chysin-2,4-dinitrobenzene
C4	-	-	NT	7-O-isopropylchrysin
C5	-	-	NT	7-O-chrysin-2-methylpropane
C6	-	-	NT	7-O-butylchrysin
C7	-	-	NT	7-O-pentylchrysin
C8	122.51 ± 2.13	25.98 ± 3.19	5	7-O-hexylchrysin
C9	285.67 ± 121.67	-	NT	7-O-heptylchrysin
C10	-	-	NT	7-O-octylchrysin
C11	156.64 ± 3.65	-	NT	7-O-nonylchrysin
C12	-	-	NT	7-O-decylchrysin
C13	-	-	NT	7-O-3-bromopropylchrysin
C14	-	-	NT	7-O-4-bomobutylchrysin
C15	-	-	NT	7-O-5-bromopentylchrysin
C16	153.66 ± 45.72	-	NT	7-O-4-bromopentylchrysin
C17	157.46 ± 3.74	10.69 ± 0.94	NT	7-O-12-bromododecylchrysin
Chrysin	45.64 ± 10.06	5.62 ± 0.83	4	5,7-dihydroxyflavone
CPZ	22.36 ± 6.40	17.53 ± 1.01	3	Chlorpromazine hydrochloride

NT - Not evaluated
– - No activity

C3 indicated a good SI on HepG2 cell (23). Other bioactive compounds indicated narrow SI (8, 2, and 5) for C1, C2 and C8.

Chrysin and CPZ indicated a very narrow SI of 4 and 3 respectively.

5.6.5 A549 Cell Line

The selectivity index of chrysin adducts on A549 cells was investigated and reported in Table 5.26 below.

Table 5.27: SI of chrysin adduct on A549 Cell Line

Selectivity index for normal cell line and leukaemia cancer cell lines				
Compound	BEAS-2B IC_{50} (µM)	A549 IC_{50} (µM)	SI (A549)	Name of compound
C1	143.22 ± 15.67	20.01 ± 0.78	7	7-O-Chrysinbutyl acetate
C2	59.66 ± 7.81	-	NT	7-O-Chrysin-3,5-dimethylbenzene
C3	112.23 ± 25.78	33.13 ± 2.19	3	7-O-Chysin-2,4-dinitrobenzene
C4	-	-	NT	7-O-Isopropylchrysin
C5	-	-	NT	7-O-Chrysin-2-methylpropane
C6	-	-	NT	7-O-Butylchrysin
C7	-	-	NT	7-O-Pentylchrysin
C8	122.51 ± 2.13	-	7	7-O-Hexylchrysin
C9	285.67 ± 121.67	-	NT	7-O-Heptylchrysin
C10	-	-	NT	7-O-Octylchrysin
C11	156.64 ± 3.65	-	NT	7-O-Nonylchrysin
C12	-	-	NT	7-O-Decylchrysin
C13	-	-	NT	7-O-3-Bromopropylchrysin
C14	-	-	NT	7-O-4-Bomobutylchrysin
C15	-	16.79 ± 1.73	NT	7-O-5-Bromopentylchrysin
C16	153.66 ± 45.72	-	9	7-O-4-Bromopentylchrysin
C17	157.46 ± 3.74	14.64 ± 1.36	11	7-O-12-Bromododecylchrysin
Chrysin	45.64 ± 10.06	8.75 ± 1.24	5	5,7-dihydroxyflavone
CPZ	22.36 ± 6.40	20.01 ± 0.78	1	Chlorpromazine hydrochloride

NT - Not evaluated
– - No activity

The SI of chrysin adducts on A549 was marginal. C1 and C16 showed a SI of 7 and 9; C3, chrysin indicated a very narrow SI of 5 whereas the SI on A549 indicates it is lethal.

5.6.6 HacaT Cell Line

The selectivity index of chrysin adducts on HacaT cells line was investigated and reported in Table 5.27 below.

Table 5.28: SI of chrysin adduct on HacaT Cell Line

Selectivity Index for normal cell line and leukaemia Cancer Cell Lines				
Compound	BEAS-2B IC_{50} (µM)	HacaT IC_{50} (µM)	SI (HacaT)	Name of compound
C1	143.22 ± 15.67	37.76 ± 14.56	4	7-O-chrysinbutyl acetate
C2	59.66 ± 7.81	-	NT	7-O-chrysin-3,5-dimethylbenzene
C3	112.23 ± 25.78	-	NT	7-O-chysin-2,4-dinitrobenzene
C4	-	-	NT	7-O-isopropylchrysin
C5	-	21.84 ± 3.36	NT	7-O-chrysin-2-methylpropane
C6	-	6.38 ± 1.09	NT	7-O-butylchrysin
C7	-	26.37 ± 1.21	NT	7-O-pentylchrysin
C8	122.51 ± 2.13	12.81 ± 1.63	10	7-O-hexylchrysin
C9	285.67 ± 121.67	12.29 ± 2.38	23	7-O-heptylchrysin
C10	-	9.62 ± 0.69	NT	7-O-octylchrysin
C11	156.64 ± 3.65	25.67 ± 0.12	6	7-O-nonylchrysin
C12	-	-	NT	7-O-decylchrysin
C13	-	-	NT	7-O-3-bromopropylchrysin
C14	-	-	NT	7-O-4-bomobutylchrysin
C15	-	-	NT	7-O-5-bromopentylchrysin
C16	153.66 ± 45.72	-	NT	7-O-4-bromopentylchrysin
C17	157.46 ± 3.74	9.8 ± 1.00	16	7-O-12-bromododecylchrysin
Chrysin	45.64 ± 10.06	12.69 ± 1.26	5	5,7-dihydroxyflavone
CPZ	22.36 ± 6.40	37.76 ± 14.56	1	Chlorpromazine hydrochloride

NT - Not evaluated
– - No activity

The selectivity index of chrysin adducts on HacaT cells line was investigated.

The 7-O-bromoalkylchrysin adducts were inactive against BEAS-2B cell lines thus no SI was evaluated for the 7-O-bromochrysin compounds against HacaT cell line. The 7-O-alkylchrysin adducts indicated good SI ≥ 6. C1 indicated a SI of 4, chrysin 5. CPZ indicated a lethal SI (1) against HacaT cell line.

5.6.7 Mero 14 Cell Line

The selectivity index of chrysin adducts on Mero 14 cells line was investigated and reported in Table 5.28 below.

Table 5.29: SI of chrysin adduct on Mero 14 Cell Line

Selectivity index for normal cell line and Mero 14 cancer cell lines				
Abbrev	BEAS-2B IC_{50} (µM)	Mero 14 IC_{50} (µM)	SI (Mero 14)	Name of compound
C1	143.22± 15.67	7.65 ± 0.53	19	7-O-Chrysinbutyl acetate
C2	59.66 ± 7.81	26.31 ± 4.09	2	7-O-Chrysin-3,5-dimethylbenzene
C3	112.23 ± 25.78	8.71 ± 0.85	13	7-O-Chysin-2,4-dinitrobenzene
C4	-	26.71 ± 1.47	-	7-O-Isopropylchrysin
C5	-	-	-	7-O-Chrysin-2-methylpropane
C6	-	-	-	7-O-Butylchrysin
C7	-	-	-	7-O-Pentylchrysin
C8	122.51 ± 2.13	24.03 ± 0.85	10	7-O-Hexylchrysin
C9	285.67 ± 121.67	-	-	7-O-Heptylchrysin
C10	-	-	-	7-O-Octylchrysin
C11	156.64 ± 3.65	-	-	7-O-Nonylchrysin
C12	-	-	-	7-O-Decylchrysin
C13	-	46.13 ± 2.63	-	7-O-3-Bromopropylchrysin
C14	-	32.34 ± 3.44	-	7-O-4-Bomobutylchrysin
C15	-	9.56 ± 0.82	-	7-O-5-Bromopentylchrysin
C16	153.66 ± 45.72	25.46 ± 4.12	6	7-O-4-Bromopentylchrysin
C17	157.46 ± 3.74	45.99 ± 3.08	3	7-O-12-Bromododecylchrysin
Chrysin	45.64 ± 10.06	13.42 ± 3.50	3	5,7-dihydroxyflavone
CPZ	22.36 ± 6.40	7.65 ± 0.53	3	Chlorpromazine hydrochloride

NT – Not tested
\- - No activity

C1, C3 and C16 indicated good SI (19, 13 and 16) Mero 14 cell line. C2 and C8 indicate very narrow SI (2 and 1). Chrysin indicated a lethal SI (1). CPZ indicated a narrow SI of 2. Interestingly, the chrysin adducts synthesised in this study – C1 and C3 indicated good safety index against Mero 14 while chrysin, the parent compound from were these compounds were made indicated a lethal safety index. CPZ also indicated a narrow safety index (sensitivity index).

Chapter 6 General Discussion / Conclusion

This work was divided into two distinct sections that explored the antibacterial and anticancer activities of bioactive sesquiterpene lactones isolated from the root extracts of Inula helenium and, investigation of the antibacterial and anticancer activities of synthesised chrysin derivatives.

Natural products, such as plants extracts (either as crude extracts, standardised products or as pure compounds), provide unlimited opportunities for novel drug discoveries because of the presence of numerous phytochemicals in them which can be used to treat diseases (Catalano et al., 2013; Croteau et al., 2000; Seca et al., 2014). The World Health Organisation estimates that about 80% of the world population depends on ethnomedicine for their healthcare needs (Catalano et al., 2013). The first crucial and outcome determining step in drug discovery is extraction. Extraction is important because further separation and characterisation depends on good extraction of desired and targeted molecules. In this work, several basic operations were done including steps such as grinding and drying of homogenised roots of Inula helenium, extraction of bioactive components using three extraction methods – Soxhlet extraction, microwave-assisted extraction (MAE) and ultrasound-assisted extraction (UAE) using methanol. Three solvents were used in fractionation of extracted components – methanol, ethyl-acetate and hexane. Although methanol offered the highest yield of extracted compounds, followed by ethyl-acetate, bioactive compounds were isolated mainly from the hexane fractions. This finding is consistent with those of Sassi et al., (2008), Afifi-Yazar et al. (2011) and Talib and Mahasneh (2010). Other authors have reported that the highest yield of bioactive components was isolated from petroleum ether and / or acetone fractions (Al-Dissi et al., 2001; Sassi et al., 2008). This is still consistent with the findings in this work as the polarity index of petroleum ether (0.1) is the same as that of hexane (Al-Dissi et al., 2001). UAE offered the best extraction method for bioactive components of Inula helenium. This is consistent with the findings of Wang et al., (2013). Abu-Lafi et al., (2018) and Lawrence et al., (2001) used maceration technique in extraction of compounds from Inula helenium. They reported poor yield (5 – 28.2 %) compared to the yield obtained in this work (15.6 – 44.1%) of fractionated sample. This is an indication that maceration technique is less efficient compared to the techniques used in this study. In addition to alantolactone and isoalantolactone – which have been previously isolated from Inula helenium (Konishi et al., 2002; Lawrence et al., 2001; Seca et al., 2014), costunolide, a known sesquiterpene lactone – first isolated from Costus speciosus (Eliza et al, 2010), was also isolated from

Inula helenium. This is the first time this compound has been isolated from Inula helenium. A careful search of the literature reveals that authors used different extraction and isolation methods, but none centrifuged the extracted compounds. Centrifugation involves the application of centrifugal force to separate particles from a solution according to their densities, shapes, sizes and viscosities of the medium and rotor speed. This forces light / floating molecules unto the base of the centrifuged. Costunolide was isolated in minute quantity (1.48 %) compared to alantolactone (36.3 %) and isoalantolactone (32.14 %) from fractionated extracts. This minute quantity of costunolide was detected because the extraction and isolation procedures were optimised such that the hexane fractions which contained the bioactive components were freeze-dried and purified using an automated high-performance liquid chromatography (HPLC) containing an auto-sampler and an auto-sample collector. Other authors used crude methods of extraction, followed by column chromatography (Moghadam et al., 2012; Cheng et al., 2012; Lawrence et al., 2001). One can therefore infer that the use of optimised extraction, isolation and purification techniques is necessary in the isolation of novel compounds. Alantolactone, isoalantolactone and costunolide showed moderate to weak antibacterial activities against Gram-positive bacteria. A significant observation in this work is the significant antibacterial activity recorded by the hexane fraction of Inula helenium extracts against ESBL producing Gram-negative bacteria: E. coli (NCTC 13353) and P. aeruginosa NCTC 13437, and Gram-positive bacteria: S. aureus ATCC 25923 and B. cereus ATCC 10876 when susceptibility assay (disc diffusion assay) was done. This activity was not observed when the hexane fraction of Inula helenium were purified. This might be because alantolactone, isoalantolactone and costunolide acted in a synergistic or additive manner against the bacterial strains tested. The synergistic effect of helenin (a combination of 40% alantolactone and 60% isoalantolactone) has been reported previously as eliciting better bioactivity against the Raji lymphoblastoid cells (Spiridonov et al., 2005; Seca et al., 2014). The HPLC chromatograms also reveals there are some minute compounds which did not indicate any antibacterial activity individually. These compounds might play a role in the better antibacterial activity seen in disc diffusion assay. The preliminary data obtained by Liu et al., (2001) are consistent with the findings above. However, they observed that isoalantolactone is a poor inhibitor of bacterial growth (E. coli, P. aeruginosa and B. subtilis). Their result also suggests a stronger anti-fungus activity of isoalantolactone. This is consistent with findings in this research.

Alantolactone, isoalantolactone and costunolide also indicated significant anticancer activities against the leukaemia cell line – K562 in hypoxia and hyperglycaemic conditions.

The anticancer activity of alantolactone, isoalantolactone and costunolide has previously been reported (Eliza et al., 2009; Eliza et al., 2010; Hu et al., 2018; Lawrence et al., 2001; Seca et al., 2014). Our findings suggest that sesquiterpene lactones isolated from Inula helenium (alantolactone, isoalantolactone and costunolide) could potentially be useful for the development of therapeutic agents for the treatment of leukaemia cancer. This is the first-time the anticancer activities of these sesquiterpene lactones against leukaemia cells have been reported in hypoxia and hyperglycaemia. This finding is important because hypoxia influences leukaemic cells differentiation, proliferation, and resistance to chemotherapy (Deynoux et al., 2016). Resistance of the K562 cancer cell line to the conventional anticancer therapy – CPZ in hypoxia was recorded in this study. A significant discovery in this research is the fact that the sesquiterpene lactones isolated in this research inhibited the growth of the leukaemia cancer cell line (K562) in a 10 – 50-fold manner (increased activity). It could be inferred that the sesquiterpene lactones (D1, D2 and D3) might be acting by inhibiting angiogenesis. Angiogenesis increases the resistance of cancer cells to chemotherapeutics (George et al., 2018). Amyloid leukaemia is the most common myeloid leukaemia in adults. Several strategies have been applied in clinical treatments for adults younger than 30 years. However, increasing the cure and survival rate for older patients (above 60 years) has increasingly become challenging due to drug-resistance and recurrence after treatment (Estey, 2014). Finding novel amyloid leukaemia therapies is presently an active area for clinical trials (Medina, 2018). Despite some improvements in leukaemia cancer treatment, cancer cell relapse after treatment has significantly increased (Deynoux et al., 2016). It has been hypothesised that by selectively targeting leukaemia stem cells, drug-resistance and tumour relapse without damage done to normal cells could be achieved (Ding et al., 2016). This therapeutic limitation may be overcome by potential treatment with alantolactone, isoalantolactone and costunolide. Alantolactone selectively ablates acute myeloid leukaemia progenitor and stem cells (Ding et al., 2016). The significant cytotoxic inhibition of the K562 leukaemia cells observed in this work indicates that these cancer cells may not be resistant to these sesquiterpene lactones. Alantolactone, isoalantolactone and costunolide have been studied as potential anticancer agents in several cancer cell lines (Eliza et al., 2010; He et al., 2018; Lawrence et al., 2011; Seca et al., 2014). Costunolide elicits its anticancer activitiy via activation of p53 and inhibition of proliferation of cancer cells (Hu et al., 2018), binding to thioredoxin reductase-1 (Zhuge et al., 2018), inhibition of NF-κB etc. The mechanism of action of alantolactone and isoalantolactone has been hypothesised to include induction of apoptosis and improvement of chemosensitivity of cancer cells by improving the activities

of the autophagy-lysosome pathway (He et al., 2018), suppression of STAT 3 (transcription factor that is a potent regulator of tumorigenesis) activity (Chun et al., 2015), inhibition of the cell cycle at the G2/M and S phase; thus, they act as alkylating agents (Lawrence et al., 2001; Nandakumar et al., 2017). In this study, it could be hypothesised that alantolactone, isoalantolactone and costunolide elicits cytotoxic inhibition against the K562 cell line via inhibition of angiogenesis because a 10-50-fold increase in anticancer activities was recorded for these compounds in hypoxia; a characteristic absent when the cancer cells (K562) were treated with CPZ. Alantolactone inhibits the expression of P-glycoprotein in drug-resistant leukaemia cell line – K562 (that showed resistance to Adriamycin) (Yang et al., 2013) and idarubicin (Liu et al., 2013). The observations in this work (Table 5.6, page 196) indicates that K562 cell line is less resistant to cytotoxic inhibition by alantolactone and isoalantolactone as the IC50 value was observed to decrease (a decrease in IC50 value signifies better cytotoxicity of a compound) from 24.66 µM after 24 h treatment to 4.96 µM after 72 h treatment with alantolactone. Similarly, there was a significant decrease in IC50 values from 28.44 µM to 7.38 µM after treatment for 24 h and 48 h with isoalantolactone. With costunolide, the observation was different; there was a noticeable mild resistance of the K562 cancer cell line after treatment for 72 h (Table 5.6 page 196). The observations with alantolactone and isoalantolactone are consistent with those of Ding et al., (2016) and it shows that K562 cancer cell line is less-resistant to cytotoxic inhibition by these compounds. These compounds (alantolactone and isoalantolactone) also indicated better cytotoxicity compare to the positive control drug – CPZ. The α-methylene-γ-butyrolactone moiety of sesquiterpene lactone isolated in this work is responsible for the cytotoxicity of these compounds (Lawrence et al., 2001; Seca et al., 2014; Yan et al., 2013).

The second aspect of this work explored the antibacterial and anticancer activities of derivatives of a known flavonoid – chrysin. Chrysin is a small molecule containing two-hydroxyl (-OH) functional groups at positions-5 and -7 of the chrysin molecule (Cheng et al., 2014). Authors have reported that synthesis of chrysin derivatives at the 5-OH position will result in a strong hydrogen-bonding that will prevent interaction of the chrysin-derivative with the cellular components (Cai et al. 2017; Walle et al., 2001). Others have reported that the 5-OH position of chrysin is the active site of this molecule (Zhou et al., 2012), implying that synthesizing derivatives at the 5-OH position will eliminate the biological activities of this molecule. This research work therefore focussed on synthesizing chrysin-derivatives at the 7-OH position with better biological activities. The synthesis of chrysin derivatives at other positions has previously been reported (Che et al., 2011; Chen

et al., 2014; Fonseca et al., 2017). Different methods including the use of a microwave-reactor, stirring of reaction mixture under regulated conditions of temperature and / or enzymatic reaction were used. Microwave-irradiation was time-saving as the reaction conditions were optimised to bring forth the reaction yield in less than 15 minutes. The yield recorded was low compared to the yield recorded when the reaction mixture was stirred under regulated reaction conditions. This synthesis is an important milestone as there is little literature on the synthesis of flavonoids derivatives using microwave irradiation. This methodology can be included within the concept of green chemistry because the strong absorption of microwave irradiation led to shorter reaction times and improved energy efficiency. In this work, eight novel chrysin derivatives were synthesised. 7-O-chrysinbutyl acetate (C1), 7-O-chrysin-3,5-dimethylbenzene (C2) and 7-O-chysin-2,4-dinitrobenzene (C3) (novel compounds) indicated significant to moderate antimicrobial activity against the microbial strains tested especially the Gram-positive bacteria and the pathogenic fungus C. albicans. In addition to C1, C2 and C3, 7-O-butylchrysin (C6 - novel) and 7-O-4-bromopentylchrysin (C16 - novel) also showed significant to moderate inhibition against the extended spectrum β-lactamase (ESBL) producing E. coli NCTC 13353. This is the first time the antibacterial activity of these compounds has been reported. The straight chain 7-O-alkylchrysin derivatives (C7 to C12) indicated weak to no activity against the microbial strains tested. This finding (weak / no activity of 7-O-alkylchrysin derivatives) is consistent with previous findings (Li et al., 2009; Li et al., 2017). With exception of C6 (7-O-alkylchrysin derivative), these observations indicate that 7-O-alkylchrysin derivatives are weak antimicrobial agents. Similarly, straight chain 7-O-bromoalkylchrysin derivatives are weak antimicrobial agents. It should be noted that C16 is a branched-chain 7-O-bromoalkylchrysin derivatives. Although only one branched-chain 7-O-bromoalkylchrysin derivatives was synthesised in this work, more synthetic branched-chain 7-O-bromoalkylchrysin derivatives should be synthesised in future research, and their antimicrobial activity investigated. This will enable an inference / conclusion to be made regarding the potency of the branched-chain 7-O-bromoalkylchrysin derivatives as potential antimicrobial agents. The site and number of hydroxyl and carbonyl or O-alkyl groups on the phenol ring of compounds are thought to be responsible for their toxicity to micro-organisms (Lawrence et al., 2009). This might account for the significant antibacterial toxicity of C1 which contains a hydroxyl-group and an O-alkyl functional group. Phenolics (phenol-ring containing compounds) also elicit bactericidal toxicity by disrupting cell membranes and denaturing proteins (Lawrence et al., 2009). This could account for the significant toxicity of C2 and C3 that contains additional phenolic rings

attached to the 7-OH position of chrysin. The role of phenolic nitrites in disrupting cell membranes and denaturing proteins have previously been described (Dykhuizen et al., 1996). C3, a nitrite analogue of chrysin synthesised in this study indicated a broad spectrum of significant to moderate antibacterial activity against all bacteria strains tested. C3 indicated the overall best antibacterial inhibition. This role of nitrite, in addition to the hydroxyl group and the phenol rings might be acting to disrupt the cell wall and denaturing proteins. C3 could be acting at multiple sites and there might be numerous possible targets for inhibition in the bacterial cells, including inhibition of metalloproteins, iron–sulphur proteins and the respiratory chain (Lawrence et al., 2009). Phenolic compounds containing nitrites are also thought to inhibit the enzymatic activity of the micro-organisms (Duke et al., 1992) and inhibit glucose uptake and ATP production in the resting cells of bacteria (Weir et al., 2004).

ESBL are a group of rapidly evolving β-lactamases which can hydrolyse certain drugs such as the third generation aztreonam and cephalosporins. β-lactam antibiotics are susceptible to hydrolysis by these enzymes (Paterson and Bonono 2005). It is difficult to treat ESBL producing bacteria using most antibiotics such as the penicillin, cephalosporins etc. However, compound which significantly inhibited the growth of ESBL producing bacteria at concentrations ≤ 50 µg / mL were synthesised in this research. Interestingly, chrysin failed to inhibit the growth of all bacteria tested especially the ESBL producing bacteria at concentrations < 250 µg / mL. The reason for this failure of chrysin to inhibit the growth of these bacteria could be because these bacteria may have hydrolysed the hydroxyl group at the 7-OH position of chrysin. C1, C2 and C3 may have elicited their inhibitory activities due to the present of functional groups (attached to the 7-OH position of chrysin) which prevented hydrolysis of these compounds. For instance, the nitrites attached to the benzene ring in C3, the methoxy group in C1 and the dimethyl groups attached to the benzene ring in C2 are relatively stable compounds compared to the hydroxyl groups in chrysin and these compounds (nitrites, methoxy, alkylated benzene rings) are known to be inhibitors of bacterial growth (Duke et al., 1992; Dykhuizen et al., 1996; Lawrence et al., 2009).

Similarly, the anticancer activity of novel 7-O-chrysin-derivatives synthesised in this work were investigated in vitro against ten cancer cell lines and one normal cell line (BEAS-2B). C1, C2 and C3 indicated significant to moderate anticancer activity against the cancer cell lines investigated. Other synthetic chrysin derivatives such as the 7-O-bromochrysin adducts C13 to C17 (showed significant anticancer activity against leukaemia cell line –

K562), 7-O-chrysin-2-methylpropane– C5 (novel compound), 7-O-4-bromopentylchrysin – C16 (novel compound) and 7-O-bromododecylchrysin (C12) indicated significant to weak anticancer activities against cancer cell lines. It is also worthy to note that 7-O-hexylchrysin also indicated significant anticancer activity against the lung cancer cell line – A549 whereas the 7-O-alkylchrysin compounds – C5 to C12 showed significant to moderate anticancer activities against skin cancer cell line (HacaT cell line) – the only class of compounds that indicated anticancer activities against HacaT cell line (with exception of C1). 7-O-butylchrysin (C6) and 7-O-hexylchrysin (C8) stands out in this class of synthesised chrysin derivatives (7-O-alkylchrysin) with anticancer activity better than those indicated by chrysin and CPZ against hacaT cell line (C6) and the breast cancer cell line – MDA-MB 468 (C8). The anticancer activities indicated by these compounds (especially C1, C2, C3, C6, C8 and C16) were comparable to those indicated by chrysin and CPZ and in some cases, better than those indicated by chrysin and CPZ. Interestingly, synthesised chrysin derivatives showed a high degree of selectivity towards human normal lung cell line (BEAS-2B) compared to CPZ which indicated marginal selectivity. The high degree of selectivity and sensitivity indicated by these compounds enhances their possibility and potentials as prospective pharmacological agents. Zheng et al., (2003) synthesised a series of chrysin-derivatives prepared by trifluoromethylation, acetylation, methylation, nitration, halogenation and alkylation. Of these compounds, only nitrated chrysin derivatives (8-nitrochrysin) and 8-iodomethoxychrysin indicated cytotoxicity against colorectal adenocarcinoma cell line (HT-29) and human gastric adenocarcinoma cell line (SGC-7901). As observed in this work, halogenated, acetylated and alkylated chrysin derivatives indicated weak / no cytotoxicity against the cell lines investigated. This agrees with the findings of Zheng et al., (2003). Other synthetic chrysin derivatives made at positions 6 and 8 of the chrysin molecule were inactive against cancer cell lines at IC50 < 50 µM (Zheng et al., 2003). Liu et al., (2018) also reported that aliphatic chrysin derivatives indicated no cytotoxicity against cancer cell lines at IC50 < 50 µM. Nevertheless, methylated chrysin derivatives synthesised at the 7-OH position indicated cytotoxic inhibition against cancer cell lines at IC50 ranging from 24 – 40 µM. This result agrees with the findings in this work as methylated 7-O-chrysin derivative (C6) and brominated-methylated 7-O-chrysin derivative (C16) indicated similar cytotoxicity against cancer cell lines. C1, an O-alkylated compound, indicated significant cytotoxic inhibition against cell lines tested in this work. Methoxylated compounds synthesised by other authors also indicated good cytotoxicity against cancer cell lines (Fonseca et al., 2017; Liu et al., 2018; Zheng et al., 2003). However, the cytotoxic inhibition of C1 reported in this work was better

than those reported by Fonseca et al., (2017). This discrepancy might be as a result of the development of hydrogen bonding at positions 5-OH and 7-OH of the chrysin molecule because they synthesised chrysin derivatives at positions other than the 5-OH and 7-OH positions. Patel et al., (2016) has previously reported good cytotoxicity of chrysin-piperazine conjugates synthesised at the 7-OH position of the chrysin molecule. The effect of hydrogen-bonding in limiting the bioactivity of the chrysin molecule has been described previously (Lapkin et al., 2014; Zheng et al., 2016). It could therefore be hypothesised that nitrated, oxygenated and methylated 7-O-chrysin-derivatives tend to elicit better cytotoxic inhibition against cancer cell lines compared to halogenated or alkylated compounds.

One of the aims of this project was to isolate novel bioactive compounds from Inula helenium. Costunolide, a sesquiterpene lactone first isolated from Costus speciosus was isolated for the first time from Inula helenium in addition to alantolactone and isoalantolactone which have been previously isolated from this plant. These compounds elicited moderate antibacterial activity against B. cereus ATCC 10876 and S. aureus ATCC 25923. Extracts from Inula helenium indicated significant synergistic activity against a wide spectrum of microbial agents tested, including the pathogenic fungus – C. albicans. Sesquiterpene lactones isolated from Inula helenium also showed significant cytotoxicity against the leukaemia cell line K562 in hypoxia, normoxia, hyperglycaemia and hypoglycaemic conditions In-vitro. Likewise, synthesised chrysin derivatives (especially C1, C2 and C3) also indicated promising anticancer activity against most cancer cell lines used in this study. Cytotoxicity of synthesised chrysin derivatives was selective against normal bronchial cell line – BEAS-2B. Other aim(s) of this project was to synthesise chrysin derivatives with better bioactivities and solubilities compared to chrysin. C1 and C3 (potent bioactive compounds synthesised in this study), indicated better bioactivities and solubilities than chrysin. Similarly, C13 showed comparable lipophilicity to chrysin. Likewise, synthesised 7-O-chrysin derivatives C1, C2 and C3, indicated significant to moderate antibacterial activity against bacteria tested with C1 and C3 showing very promising bacterial inhibition against ESBL producing E. coli, and other bacteria, including inhibition of the pathogenic fungus – Candida albicans MTCC 227. Bioactivities indicated by synthesised chrysin derivatives were better than those shown by chrysin and in some cases, comparable to those shown by conventional therapies / drugs used as positive controls. Although the biological activity of chrysin derivatives was not investigated in-vivo to determine the bioavailability of these compounds, the hydrophilic index indicates that C1, a novel chrysin derivative was more soluble in polar solvents than chrysin. Similarly, C3 and C13 indicated similar solubility in polar solvents to chrysin. A better lipophilic index

is an indication that a compound is likely to make it into the cellular components, thus a better bioactivity in-vivo (Bae et al., 2012; Beretta et al., 2012). Nevertheless, hydrophobic compounds could also be important anticancer agents individually, or in combination with other anticancer agents where their role could be in increasing internal concentration of anticancer agents via inhibition of the P-glycoprotein (Pgp), thus preventing drug-efflux from cells (Abdallah et al., 2015; Mohana et al., 2016). The use of nanoparticles in delivering hydrophobic agents to targeted cells could also overcome this challenge. Also, some hydrophobic drugs effectively bind to targets in-vivo, thus they could still elicit significant bioactivity against their targets (Amin, 2013; Mason et al., 2008). The calculated masses of all novel chrysin derivatives was similar to the actual masses recorded.

In this project, a novel compound (costunolide) was isolated from Inula helenium. Novel chrysin derivatives which elicited a broad spectrum of antibacterial activities against Gram-positive and Gram-negative bacteria (including ESBL producing bacteria) were synthesised. These compounds also elicited better anticancer activities than chrysin and the conventional anticancer drug – Chlorpromazine hydrochloride. Some synthesised chrysin derivatives were also more soluble than chrysin.

The present study has given some insight into the interaction of novel 7-O-chrysin derivatives with cancer cells. However, further work is necessary to determine the mechanism of action of these compounds.

In the first instance, several new analogues will have to be synthesised in other to carry out more detailed structure-activity relationship. The synthetic route developed in this synthesis is suitable for the generation of these analogues. These structures should include branched and shorter chain analogues targeting the 7-OH position of chrysin: 7-O-bromoalkylchrysin and 7-O-alkylchrysin analogues with N = 1 and 2, 7-O-bromoalkylchrysin with N = 6, 7, 8, 9, 10 and 11, 7-O-alkylchrysin analogues with N = 11 and 12. Branched chain analogues may also be made. Apart from 7-O-bromoalkylchrysin analogues, 7-O-chloroalkyl, 7-O-Fluoroalkyl and 7-O-iodoalkyl chrysin derivatives should also be made and their cytotoxicity investigated.

A significant omission from this thesis was data that will prove that 7-O-chloroalkyl derivatives may indicate a better anticancer activity compared to their 7-O-bromoalkylchrysin counterparts. This will likely be achieved by the synthesis and purification of 7-O-chloroalkyl derivatives of chrysin as 7-O-chloroalkyl chrysin derivatives

made in this study were impure; and their bioactivities investigations were discontinued during biological studies.

Further studies could also include synthesis and examining 5-O-bromoalkyl, and 5-O-alkyl derivatives of chrysin; comparing their cytotoxicity to those of their 7-O-counterparts.

An interesting further study could include the synthesis of 7-O-thioalkyl chrysin derivatives as the sulphur ion can completely change the dynamics and activity of molecules.

The lipophilic nature of some chrysin adducts made in this study might present difficulty in drug delivery to patients; however, rapid advances in phospholipid vesicles (liposomes) based delivery systems should provide adequate formulations of the drugs. This is highly recommended for synthesised chrysin derivatives that indicated significant biological activities compared to chrysin and/or the positive control agents used in this study.

In the disc diffusion assay study involving the antibacterial activities of hexane fraction of Inula helenium on bacteria, the data suggests a significant inhibition of the growth of P. aeruginosa. This was not replicated during the minimum inhibitory concentration (MIC) studies. From the HPLC chromatograms, the hexane fraction contained other compounds (in addition to alantolactone, isoalantolactone and costunolide) that were inactive (when tested individually) against bacteria tested at MIC ≤ 500 µg/mL. It is possible that the inhibitory response might be associated with the activities of two or more compounds in the hexane fraction. It might also be that a combination of two or more bioactive sesquiterpene lactones isolated in this study (alantolactone, isoalantolactone and costunolide) were responsible for this activity observed in the disc diffusion assay. The potentiating effect of these compounds should therefore be investigated in the future. Minimum doubling time / growth curve (MDT) and antimicrobial synergy testing / checkerboard assay should be done using compounds C1, C2, C3, D1, D2 and D3 on bacterial species that are susceptible to inhibition by these compounds. MDT testing is used in determining the doubling time of the bacteria population. This assay shows the effect of antibacterial agents on bacterial growth by comparing growth in broth culture with and without the antimicrobial compound. Optical density (OD) values are recorded at multiple timepoints and calculated by comparing the OD at different timepoints in exponential phase of growth (Gibson et al., 2018). Synergy testing evaluates the interaction of two antimicrobial agents when used in combination. MIC and MBC values of test compounds alone and in combination against each bacterial strain being evaluated is used to calculate the antagonistic, synergistic or additive effect of compounds isolated /

synthesised in this work. The promising antibacterial and antifungus activity of C1 and C3 warrants in-vivo investigation in mouse or albino Wistar rat model infected with bacterial or fungal species. Other bacterial species which are susceptible to antibacterial inhibition should also be investigated.

In-vitro and in-vivo studies involving the anticancer activities of sesquiterpene lactones isolated in this work against the leukaemia cell line (K562) and their mechanism of action should be investigated. This should include activities on hypoxia-inducible factor-1α and the relationship between inhibition of cancer in hypoxia by these compounds (alantolactone, isoalantolactone and costunolide).

The mechanism of action of synthesised chrysin derivatives that indicated cytotoxicity ≤ 20 µM should be investigated. This might include the free radical scavenging activity, cell cycle analysis, histone deacetylase (HDAC) activity, Western Blotting, Real Time Quantitative polymerase-chain-reaction (PCR) analysis and in-vivo analysis using xenograft model (such as albino Wister rats or nude mice). These analyses will aid in the understanding of the mechanism of action of these compounds.

Molecular docking of bioactive chrysin derivatives synthesised in this study, especially those of C1, C2, C3, C4, C5, C8 and C16 with the aim of determining the domain of binding fitness of these molecules and how these molecules could form complex on protein receptors should be investigated. The role of hydrogen-bonding as postulated by some authors could also be investigated by investigating the binding affinity of these molecules with amino acids. Molecular docking will reveal the structure-activity relationship between compounds and the receptor proteins. An investigation should be done to ascertain the active site of the chrysin molecules by synthesising chrysin-derivatives at the 5-OH position and then synthesising chrysin derivatives at the aromatic ring as well. Zhou et al., (2012) have postulated that the 5-OH position of chrysin is the active site, whereas Babu et al., (2006) have postulated that the aromatic ring is the active site of this molecule. This is confusing, and verification is needed as this will aid future synthesis of useful bioactive chrysin derivative. A synthetic plan could include synthesis of compounds at the 5-OH position of the chrysin molecule while allowing the 7-OH position and the aromatic ring to be vacant. Another approach could include synthesising chrysin derivatives at the aromatic ring while allowing the 5-OH and 7-OH positions to be vacant. Another approach could be synthesising derivatives at both the 5-OH positions and at the aromatic ring and their anticancer activity investigated. A loss of activity when 5-OH is occupied, or when the

aromatic ring is occupied and vice versa will give an insight into the actual active site of the chrysin molecule especially if such investigation is followed by molecular docking.

7-O-bromoalkyl chrysin derivatives synthesised in this study indicated weak anticancer activity. This trend could change if chlorinated and fluorinated chrysin derivatives are synthesised at the 7-OH position of the chrysin molecule as highly electronegative atoms such as chlorine and fluorine could change the thermodynamics of molecules. Also recommended for further studies include; the free radical scavenging activity, cell cycle analysis, histone deacetylase (HDAC) activity, Western Blotting, Real Time Quantitative polymerase-chain-reaction (PCR) analysis and in-vivo analysis using xenograft model (such as albino Wister rats or nude mice). These analyses will aid in the understanding of the mechanism of action of synthesised chrysin derivatives. The histone deacetylases (HDACs) catalyse deacetylation of the ε-amino group of lysine in the N-terminal tails of histones. Histone deacetylation and acetylation plays crucial roles in gene expression in eukaryotic cells, causing chromatin remodelling (Hadnagy et al., 2008). Imbalance in histone acetylation can cause changes in chromatin conformation, leading to dysregulation of genes involved in apoptosis, cell differentiation and cell cycle progression (McLaughlin & La Thangue, 2004).

Western blot is a procedure used to separate and identify proteins from a mixture of proteins base on the molecular weight and size of these proteins, using gel electrophoresis (Mahmood & Yang, 2012). The results obtained are then transferred to a membrane producing a band. These membranes are then incubated with labels antibodies specific to the protein of interest. Unbound antibodies are washed off, leaving only the antibodies of interest bound on these membranes (Mahmood & Yang, 2012). A film of the bound antibodies is then developed, and the bound antibodies detected. Antibodies only bind to proteins of interest and this is highly specific. Thus, only one band should be visible per protein of interest. The thickness of the band corresponds to the amount of protein present; thus, doing a standard can indicate the amount of protein present (Mahmood & Yang, 2012).

the antitumor activity of chrysin derivatives in a nude mice or albino Wistar rat xenograft model implanted with cancer cells used in this study (especially those with increase susceptibility to treatment by compounds made in this study) should be investigated to determine the interaction of these compounds (synthesised chrysin derivatives) with other cellular components. Despite the promising anticancer activity of the chrysin molecule, the literature review indicates that the absorption and bioavailability of this molecule is very

poor and less than 5% has been found in the serum (Mani & Natesan, 2018; Sabzichi et al., 2017). This is also an indication of poor solubility of this compound within biological systems. One of the aims of this project was to synthesise chrysin derivatives with improved solubility and bioavailability in biological systems. In-vivo anticancer activity using xenograft model will ascertain the bioavailability of bioactive chrysin derivatives synthesised in this work.

The relationship between hypoxia, hyperglycaemia and cancer should be investigated by studying the anticancer activities of synthetic chrysin derivatives – C1, C2 and C3 against leukaemia cancer cell line (K562) and other cell lines in hypoxia and hyperglycaemia. This is necessary because chrysin derivatives indicated good antiproliferative activity against the K562 cell line. Chrysin derivatives synthesised in this work could be studied in addition to a standard investigation of the mechanism of action of chrysin derivatives against Pgp in K562 cell line. This could offer new insight on a possible relationship between cancer and disease.

Multidrug resistance (MDR) of tumour cells is associated with overexpression of P-glycoprotein (Pgp). Pgp are responsible for the efflux of chemotherapeutic drugs out of the cells using ATP hydrolysis as an energetic source (Amin, 2013). Many cancer cells are resistant to anticancer agents including epipodophyllotoxins, taxanes, vinca alkaloids and anthracyclines (Amin, 2013). Pgp is able to efflux drugs out of the cells due to its ability to bind most compounds, transporting them out of the cell such as corticosterone, dexamethasone etc (Amin, 2013). Interestingly, more hydrophobic compounds such as antiprogestin RU 486 and progesterone acts as efficient modulators of cellular MDR. This is because these compounds are hydrophobic in nature (Mason et al., 2008). They therefore inhibit the efflux of anticancer drugs because they act as efficient modulators of cellular MDR (Amin., 2013; Mason et al., 2008). However, antiprogestin RU 486 and progesterone cannot be used at therapeutic levels because of the negative effect associated with using excessive hormones (Amin., 2013). Findings in this project indicates that chrysin and most of its derivatives are hydrophobic agents. The role of flavonoids as modulators of Pgp have previously been reported (Abdallah et al., 2015; Mohana et al., 2016). Future research should focus on investigating the role of chrysin and its derivatives (synthesised in this project), as potential Pgp modulators in tumour cells in-vitro and in-vivo in leukaemia cancer cell (K562) as well as other cancer cells. Characterisation of the inhibitory flavonoid-binding site on Pgp at the cellular and molecular levels will enhance the development of more potent Pgp inhibitors. The success of such research could

revolutionize cancer treatment because the discovery of Pgp inhibitors implies that anticancer agents can accumulate in cancer cells, killing them thus overcoming the challenge associated with MDR.

In summary, in the extraction and isolation work, costunolide – a novel isolate from Inula helenium, was isolated. This was possible via the method developed in this study which include centrifugation of supernatants in order to obtain minute quantity of bioactive compounds such as costunolide. On the synthetic work we developed a rapid method (microwave irradiation) for the synthesis of chrysin derivatives in less than 12 minutes. Conventionally, it takes between 24 - 48 hours or more to synthesise similar molecules. Novel biologically active small molecules that significantly inhibited the growth of cancer cells and pathogens were also synthesised. Some compounds were specific (i.e. inhibited the growth of either Gram-positive or Gram-negative bacteria) only. Some compounds indicated broad-spectrum antibacterial inhibition of both Gram-positive and Gram-negative bacteria including the extended-spectrum beta-lactamases (ESBL) producing bacteria as well as the multi-drug resistant (MDR) such as the ESBL producing Escherichia coli, Pseudomonas aeruginosa and Klebsiella pneumoniae. The MDR Gram-negative bacteria as well as the MDR Gram-Positive bacteria (MRSA) are serious health concerns in hospitals, clinics and the society. As their names suggest, there are difficult to treat because they have developed resistance to almost all drugs currently in the market. Compounds synthesised in this work significantly inhibited the growth of the MDR pathogens. Antibacterial activities of synthesised chrysin derivatives were better than those of chrysin. Antifungal activity of synthesised chrysin derivatives (C1 and C3 were better than those of chrysin and the standard antifungal drugs – fluconazole. We also reported the cytotoxic inhibition of the K562 cancer cell by sesquiterpene lactones isolated from Inula helenium in and in hyperglycaemia. We also reported the anticancer activities of novel chrysin derivatives against a range of cancer cell lines. The anticancer activities of novel chrysin derivatives was selective; killing only the cancer cells but not normal cells.

Additional work on the mechanism of action of these compounds (chrysin derivatives) where undertaken such as apoptosis studies and growth inhibition studies. Additional work done is reported in the appendix.

Novel chrysin derivatives synthesised in this study especially compounds C1, C2, C3, C8 and C16 are potential antimicrobial and anticancer drug candidates; likewise, the sesquiterpene lactones isolated from Inula helenium (alantolactone, isoalantolactone and costunolide).

APPENDIX

The spectral data of novel compounds are presented below:

Appendix I: Spectral data (high resolution mass spectrometry data) of novel compounds.

Appendix II: Spectral data (high resolution mass spectrometry data) of novel compounds.

APPENDIX I

Appendix I-A: TOF MS ES of C1 (7-O- Chrysinbutyl acetate).

Appendix I-B: TOF MS ES of C2 (7-O-Chrysin-3,5-dimethylbenzene).

Appendix I-C: TOF MS ES of C3 (7-O-Chysin-2,4-dinitrobenzene).

Appendix I-D: TOF MS ES of C5 (7-O-Chrysin-2-methylpropane).

Appendix I-E: TOF MS ES of C10 (7-O-Octylchrysin).

Appendix I-F: TOF MS ES of C11(7-O-Nonylchrysin).

Appendix I-G: TOF MS ES of C12 (7-O-Decylchrysin).

Appendix I-H: TOF MS ES of C16 (7-O-4-Bromopentylchrysin).

Appendix I-I: TOF MS ES of D3 Costunolide.

APPENDIX II

Appendix II-A: ^{1}H NMR assignment of C1 (7-O- Chrysinbutyl acetate).

Appendix II-B: ^{1}H NMR assignment of C2 (7-O-Chrysin-3,5-dimethylbenzene).

Appendix II-C: ^{1}H NMR assignment of C3 (7-O-Chysin-2,4-dinitrobenzene).

Appendix II-D: ^{1}H NMR assignment of C5 (7-O-Chrysin-2-methylpropane).

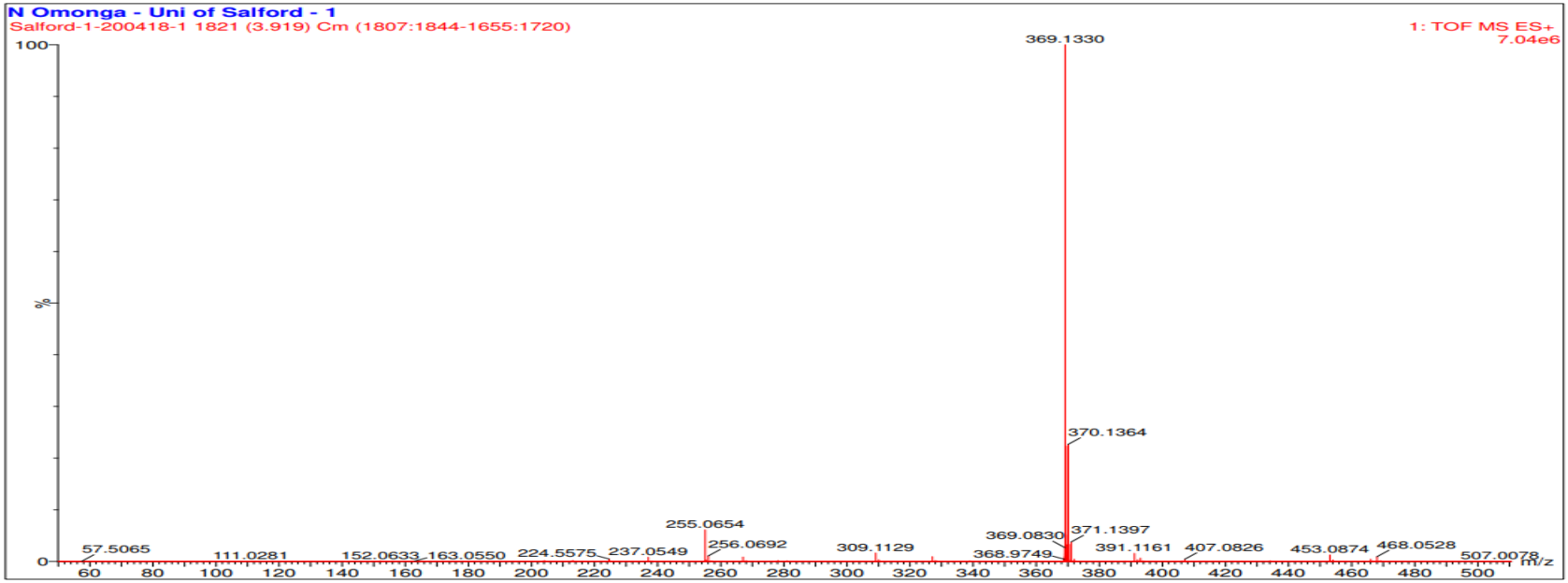
Appendix II-E: ^{1}H NMR assignment of C10 (7-O-Octylchrysin).

Appendix II-F: ^{1}H NMR assignment of C11 (7-O-Nonylchrysin).

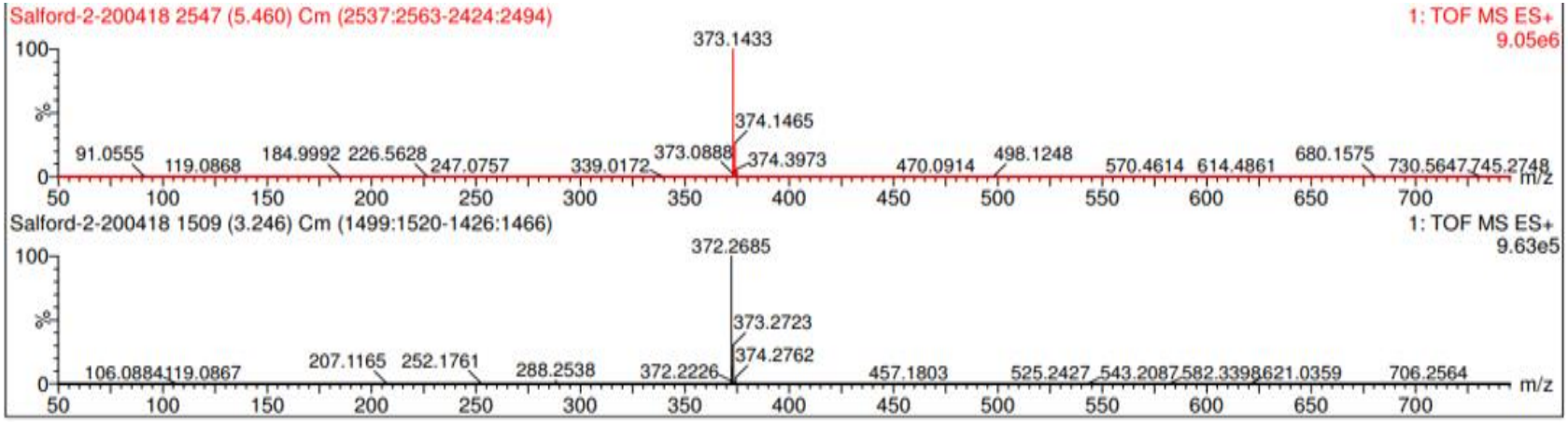
Appendix II-G: ^{1}H NMR assignment of C12 (7-O-Decylchrysin).

Appendix II-H: ^{1}H NMR assignment of C16 (7-O-4-Bromopentylchrysin).

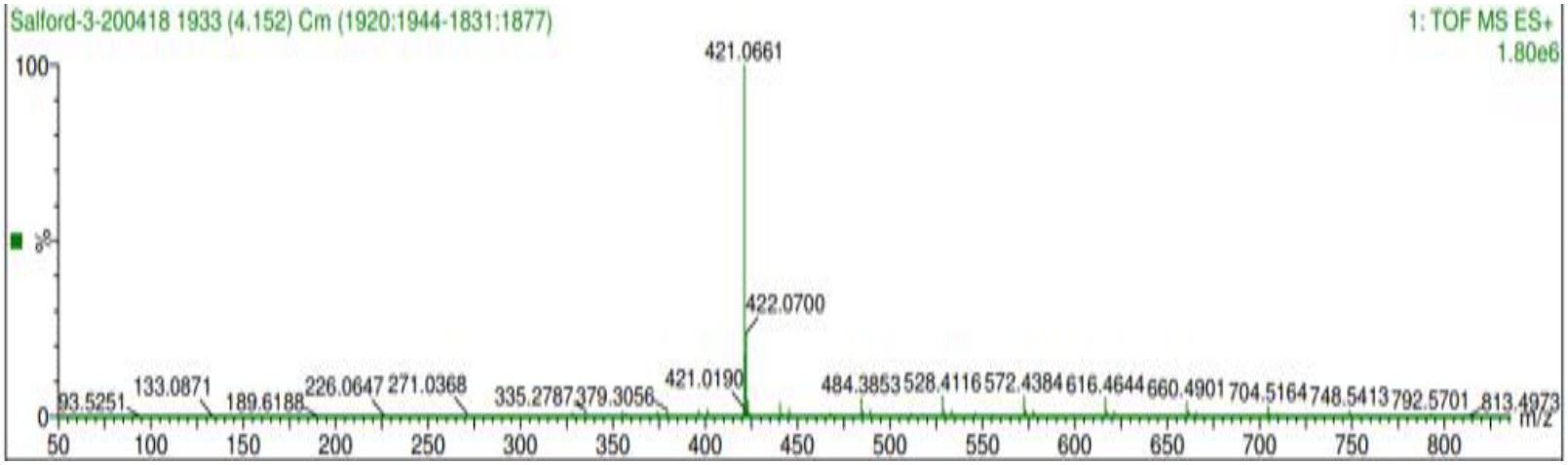
Appendix II-I: ^{1}H NMR assignment of D3 Costunolide.



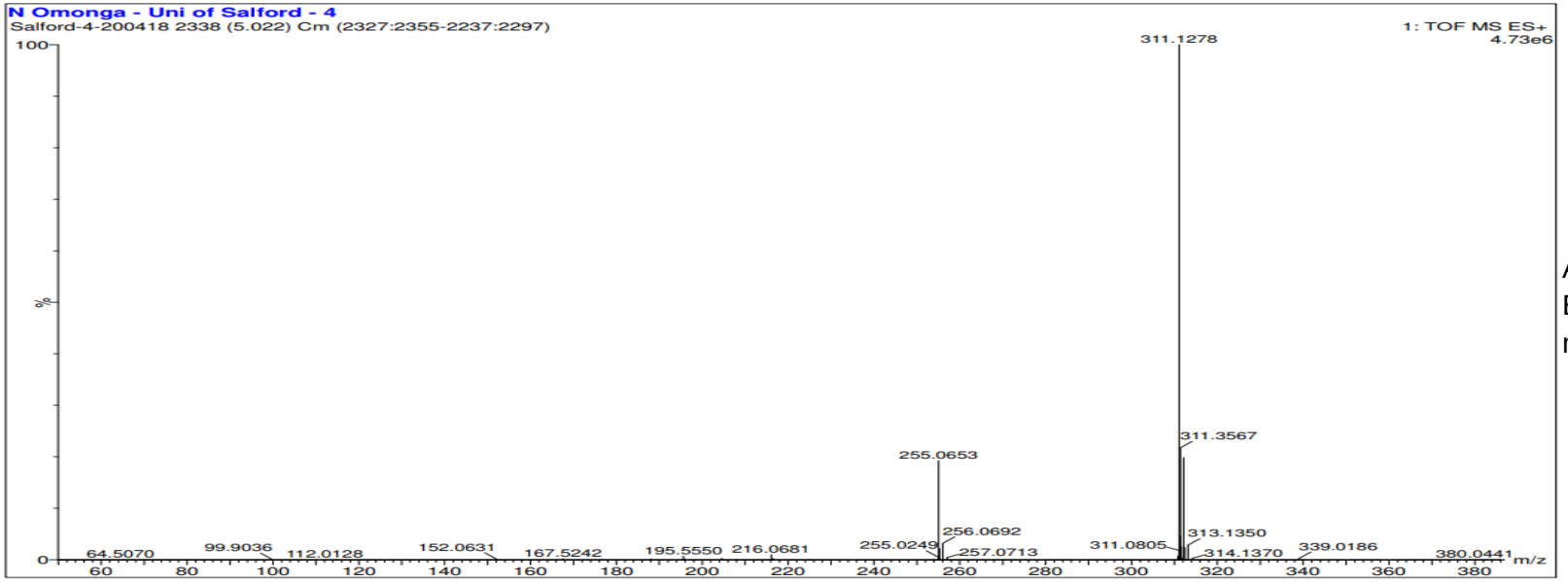
Appendix 1-A: TOF MS ES of C1 (7-O-Chrysinbutyl acetate)



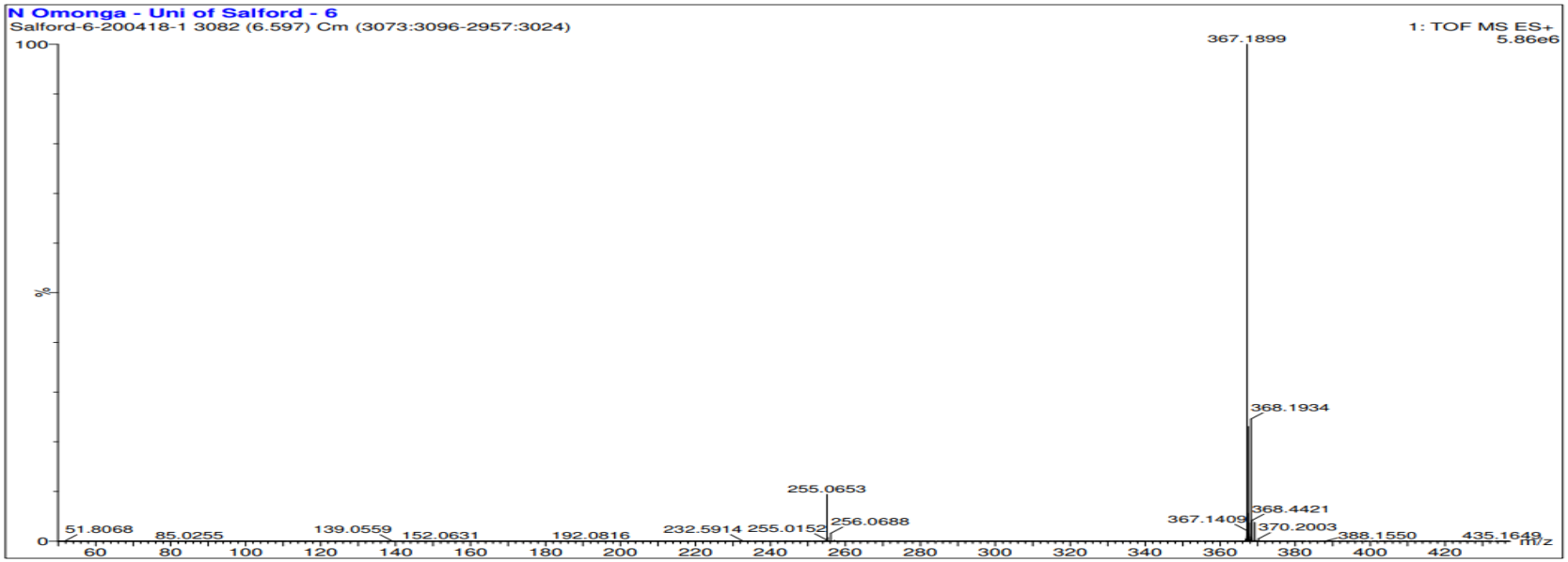
Appendix 1-B: TOF MS ES of C2 (7-O-Chrysin-3,5-dimethylbenzene)



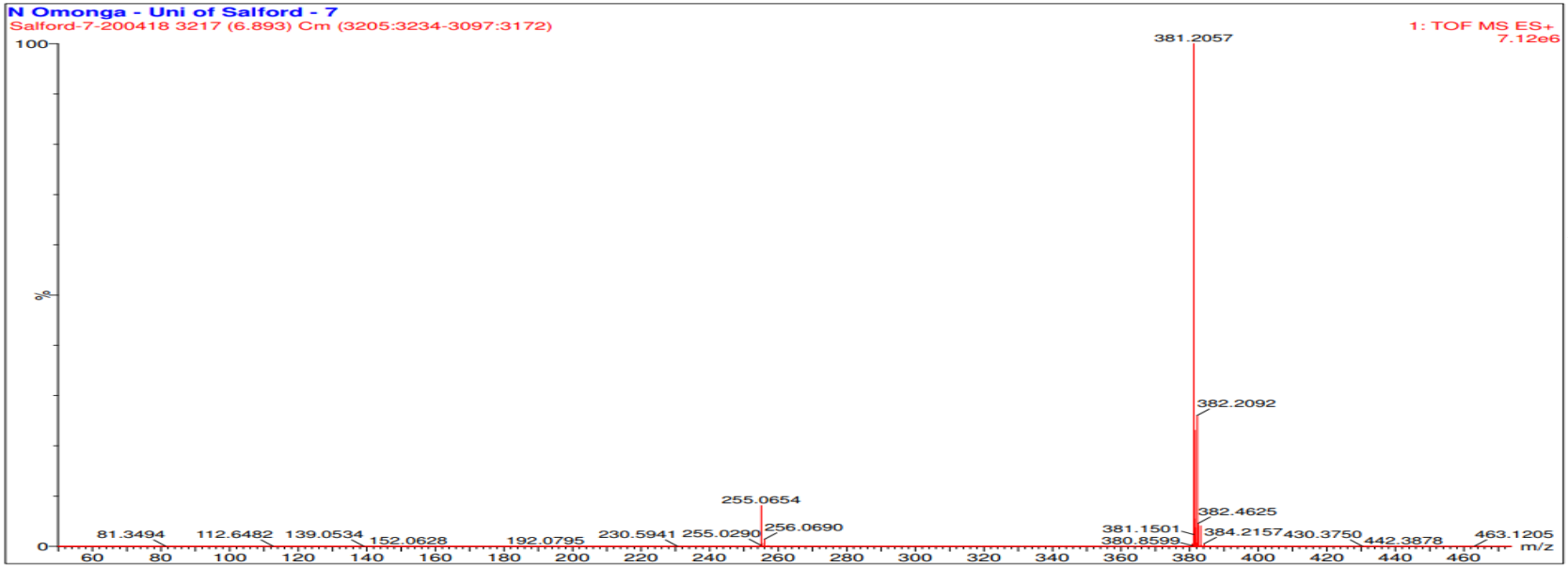
Appendix 1-C: TOF MS ES of C3 (7-O-Chysin-2,4-dinitrobenzene)



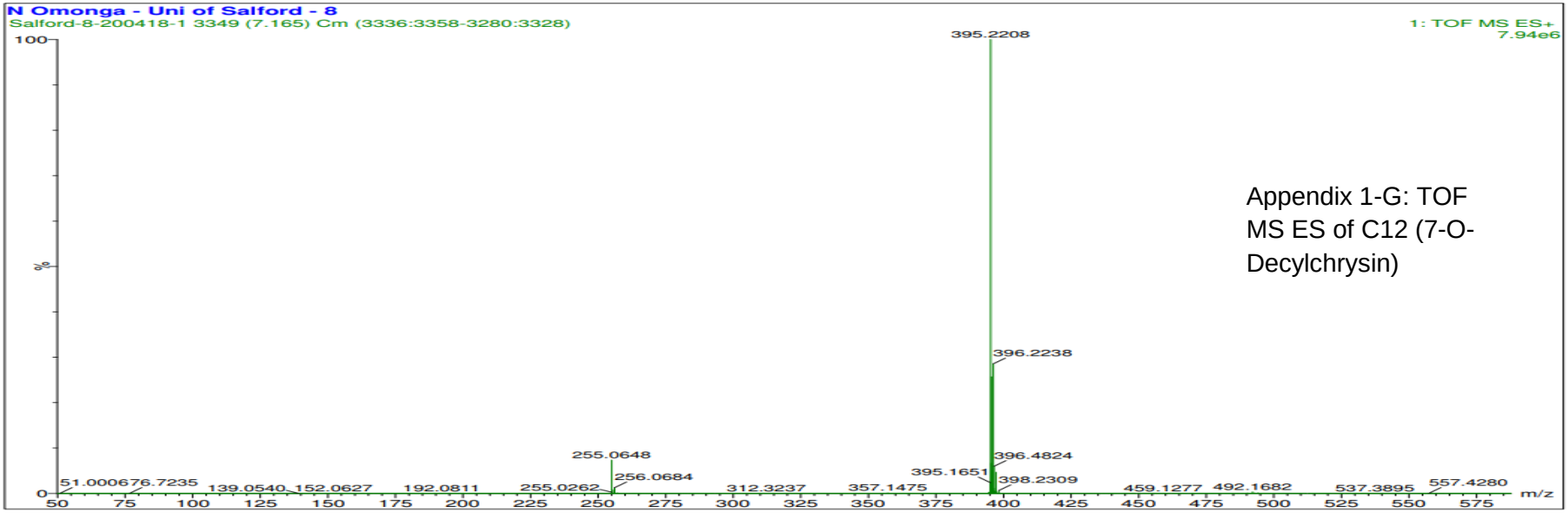
Appendix 1-D: TOF MS ES of C5 (7-O-Chrysin-2-methylpropane)



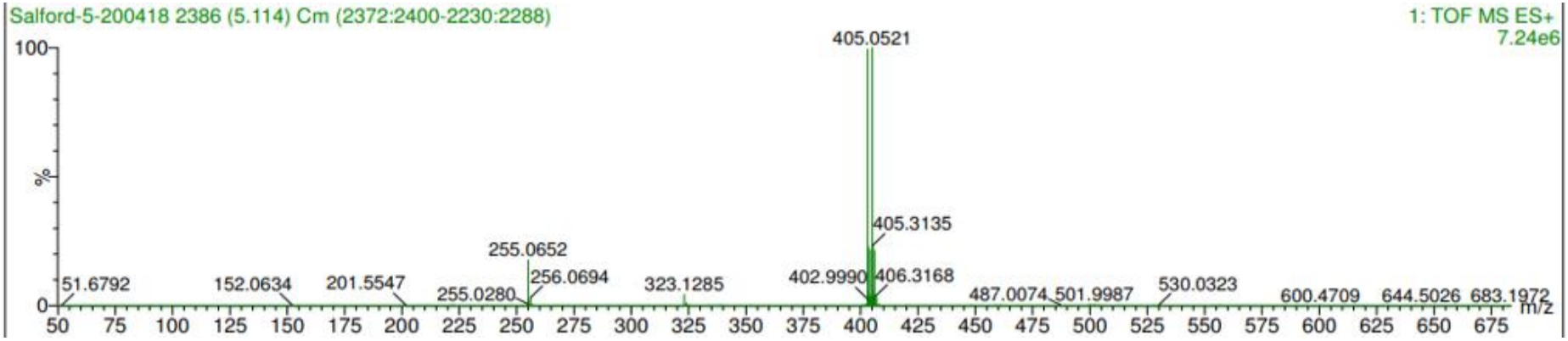
Appendix 1-E: TOF MS ES of C10 (7-O-Octylchrysin)



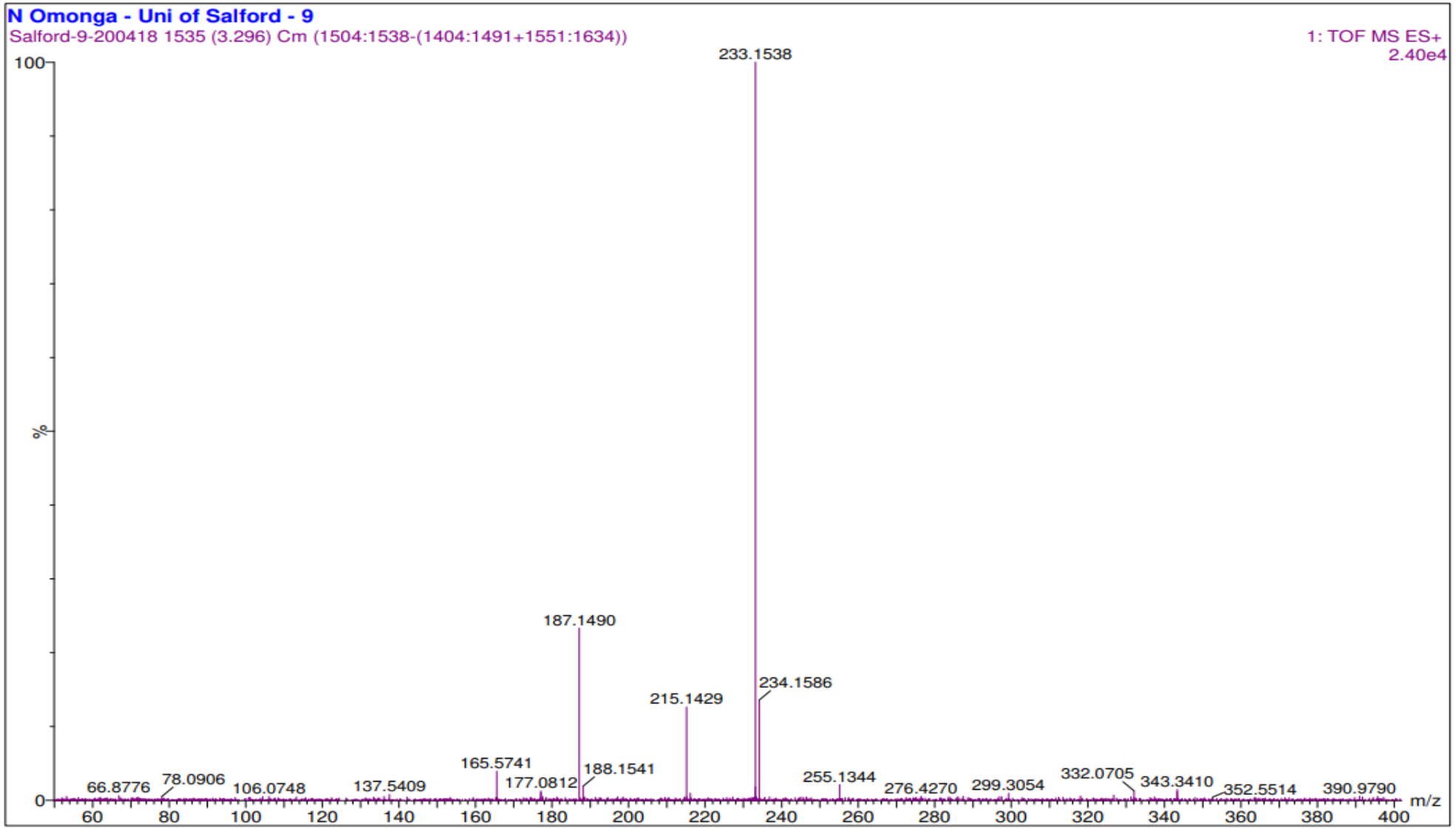
Appendix 1-F: TOF MS ES of C11 (7-O-Nonylchrysin)



Appendix 1-G: TOF MS ES of C12 (7-O-Decylchrysin)

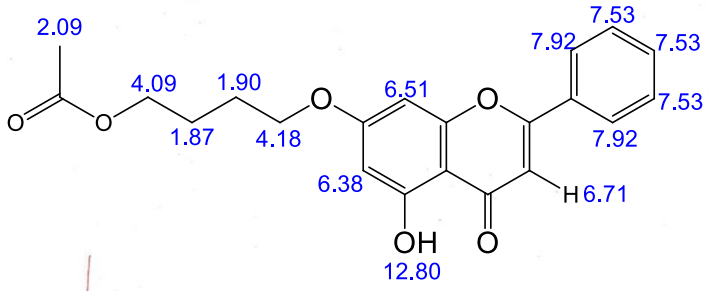


Appendix 1-H: TOF MS ES of C16 (7-O-4-Bromopentylchrysin)



Appendix 1-I: TOF MS ES of D3 (Costunolide)

^{1}H NMR ASSIGNMENT OF C1

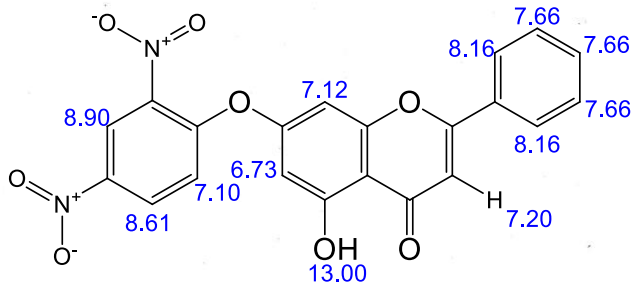


University of Salford MANCHESTER

NAME	Feb17-2018-NO
EXPNO	20
PROCNO	1
Date_	20180217
Time	19.15
INSTRUM	spect
PROBHD	5 mm BBO BB-1H
PULPROG	zg30
TD	56290
SOLVENT	CDCl3
NS	64
DS	0
SWH	8802.817 Hz
FIDRES	0.156383 Hz
AQ	3.1973219 sec
RG	456.1
DW	56.800 usec
DE	6.50 usec
TE	296.2 K
D1	2.59999990 sec
TD0	1
======== CHANNEL f1 ========	
NUC1	1H
P1	10.05 usec
PL1	-2.00 dB
SFO1	400.1333626 MHz
SI	32768
SF	400.1300086 MHz
WDW	EM
SSB	0
LB	0.30 Hz
GB	0
PC	1.00

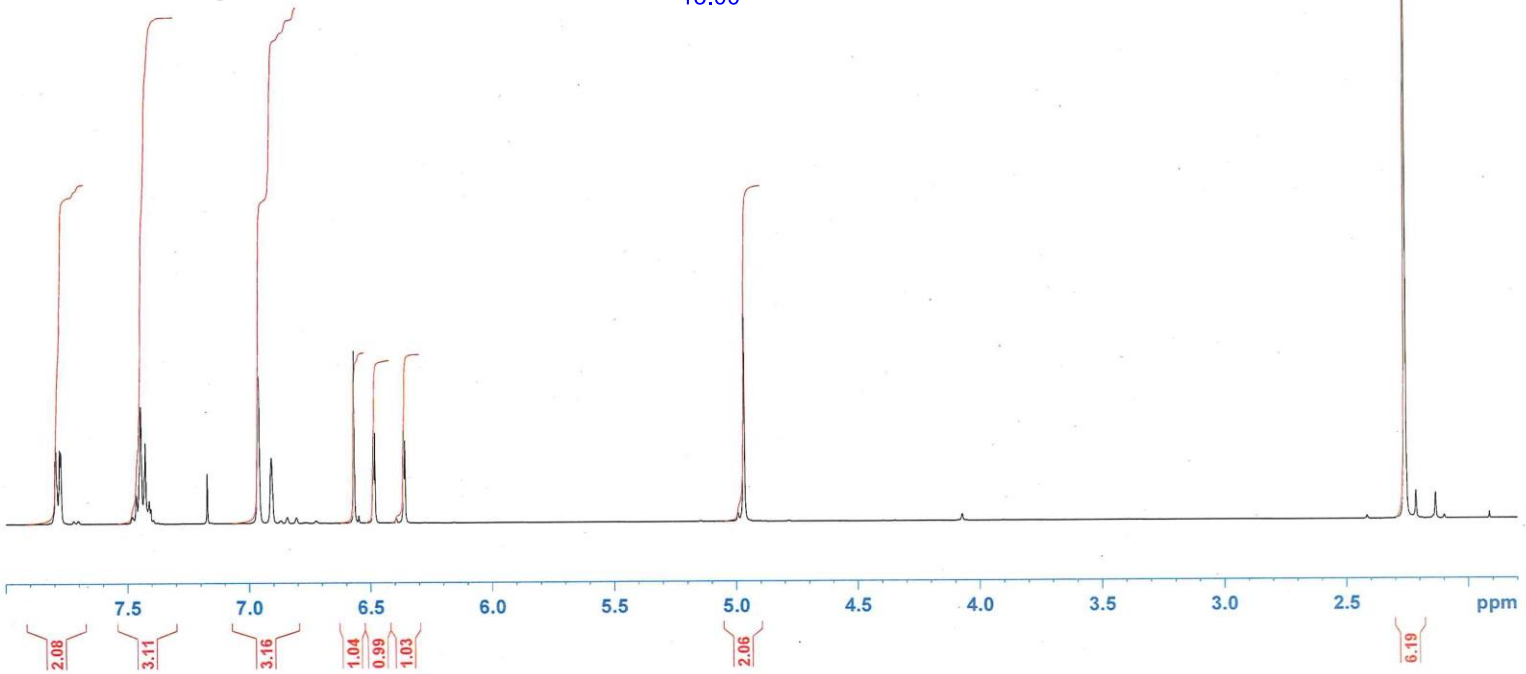
TMF

^{1}H NMR ASSIGNMENT OF C2

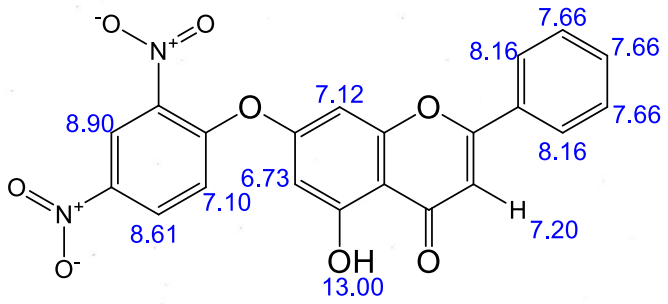


University of Salford MANCHESTER
1967-2017 50 YEARS

NAME	May17-2017-NO
EXPNO	10
PROCNO	1
Date_	20170518
Time	5.59
INSTRUM	spect
PROBHD	5 mm BBO BB-1H
PULPROG	zg30
TD	56290
SOLVENT	CDCl3
NS	64
DS	0
SWH	8802.817 Hz
FIDRES	0.156383 Hz
AQ	3.1973219 sec
RG	181
DW	56.800 usec
DE	6.50 usec
TE	296.2 K
D1	2.59999990 sec
TD0	1
======== CHANNEL f1 ========	
NUC1	1H
P1	10.50 usec
PL1	2.00 dB
SFO1	400.1333626 MHz
SI	32768
SF	400.1300451 MHz
WDW	EM
SSB	0
LB	0.30 Hz
GB	0
PC	1.00

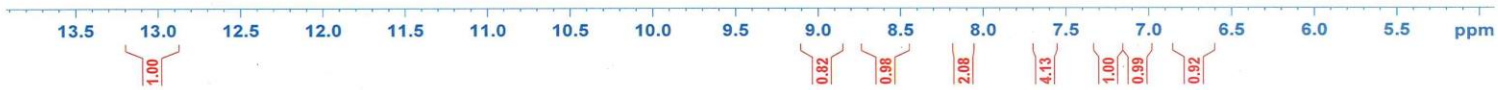


^{1}H NMR ASSIGNMENT OF C3

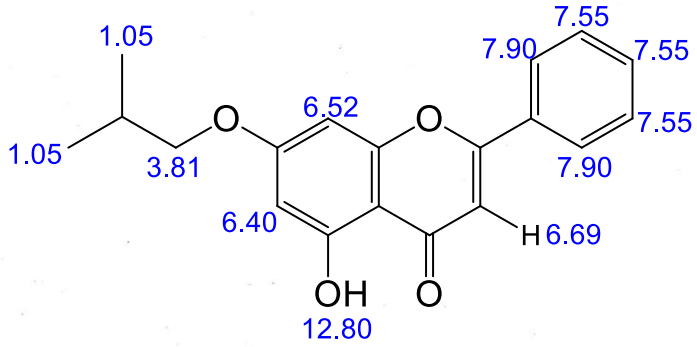


University of Salford MANCHESTER
1967-2017 50 YEARS

NAME	Oct14-2017-NO
EXPNO	10
PROCNO	1
Date_	20171014
Time_	14.36
INSTRUM	spect
PROBHD	5 mm BBO BB-1H
PULPROG	zg30
TD	56290
SOLVENT	DMSO
NS	192
DS	0
SWH	8802.817 Hz
FIDRES	0.156383 Hz
AQ	3.1973219 sec
RG	362
DW	56.800 usec
DE	6.50 usec
TE	296.4 K
D1	2.59999990 sec
TD0	1
======== CHANNEL f1 ========	
NUC1	1H
P1	10.50 usec
PL1	2.00 dB
SFO1	400.1333626 MHz
SI	32768
SF	400.1300000 MHz
WDW	EM
SSB	0
LB	0.30 Hz
GB	0
PC	1.00



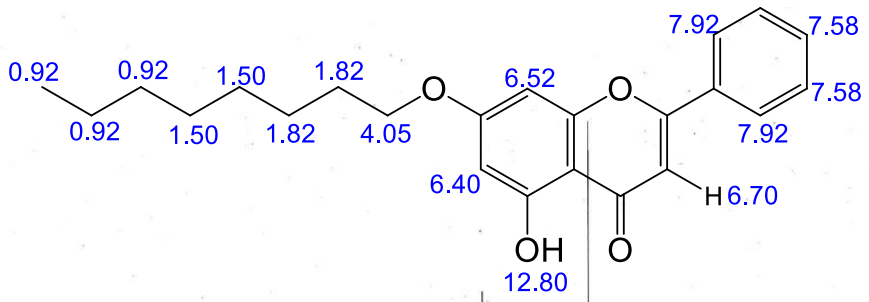
^{1}H NMR ASSIGNMENT OF C5



University of Salford MANCHESTER

NAME	Apr04-2018-NO	
EXPNO	10	
PROCNO	1	
Date_	20180405	
Time	5.22	
INSTRUM	spect	
PROBHD	5 mm BBO BB-1H	
PULPROG	zg30	
TD	56290	
SOLVENT	CDCl3	
NS	192	
DS	0	
SWH	8802.817	Hz
FIDRES	0.156383	Hz
AQ	3.1973219	sec
RG	362	
DW	56.800	usec
DE	6.50	usec
TE	296.2	K
D1	2.59999990	sec
TD0	1	
======== CHANNEL f1 ========		
NUC1	1H	
P1	10.05	usec
PL1	-2.00	dB
SFO1	400.1333626	MHz
SI	32768	
SF	400.1300000	MHz
WDW	EM	
SSB	0	
LB	0.30	Hz
GB	0	
PC	1.00	

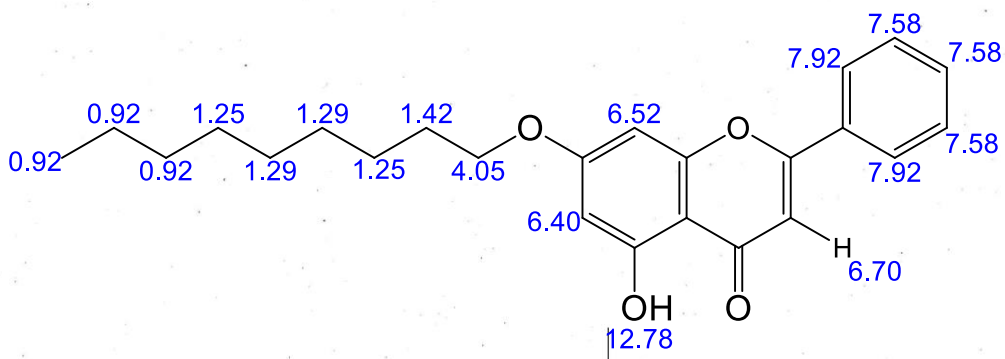
^{1}H NMR ASSIGNMENT OF C10




```
NAME          Apr14-2018-NO
EXPNO                   52
PROCNO                   1
Date_             20180414
Time                 17.54
INSTRUM              spect
PROBHD   5 mm BBO BB-1H
PULPROG                zg30
TD                    56290
SOLVENT               CDCl3
NS                      256
DS                        0
SWH                8802.817 Hz
FIDRES             0.156383 Hz
AQ                 3.1973219 sec
RG                     161.3
DW                  56.800 usec
DE                    6.50 usec
TE                   296.2 K
D1              2.59999990 sec
TD0                       1

======== CHANNEL f1 ========
NUC1                     1H
P1                   10.05 usec
PL1                  -2.00 dB
SFO1           400.1333626 MHz
SI                     32768
SF             400.1300000 MHz
WDW                      EM
SSB                       0
LB                    0.30 Hz
GB                        0
PC                     1.00
```

^{1}H NMR ASSIGNMENT OF C11

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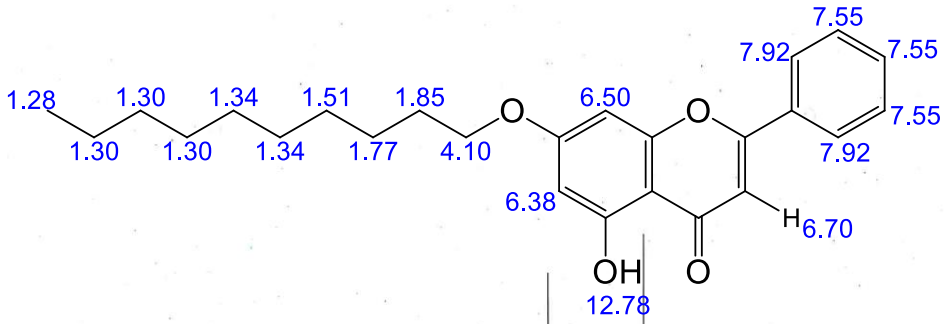
NAME          Jan05-2018-NO
EXPNO                   53
PROCNO                   1
Date_             20180106
Time                 18.41
INSTRUM              spect
PROBHD   5 mm BBO BB-1H
PULPROG               zg30
TD                   56290
SOLVENT              CDCl3
NS                     266
DS                       0
SWH               8802.817 Hz
FIDRES            0.156383 Hz
AQ                3.1973219 sec
RG                   812.7
DW                  56.800 usec
DE                   6.50 usec
TE                  300.0 K
D1                2.59999990 sec
TD0                      1

======== CHANNEL f1 ========
NUC1                    1H
P1                   10.50 usec
PL1                   2.00 dB
SFO1          400.1333626 MHz
SI                   32768
SF            400.1300087 MHz
WDW                     EM
SSB                      0
LB                    0.30 Hz
GB                       0
PC                    1.00
```

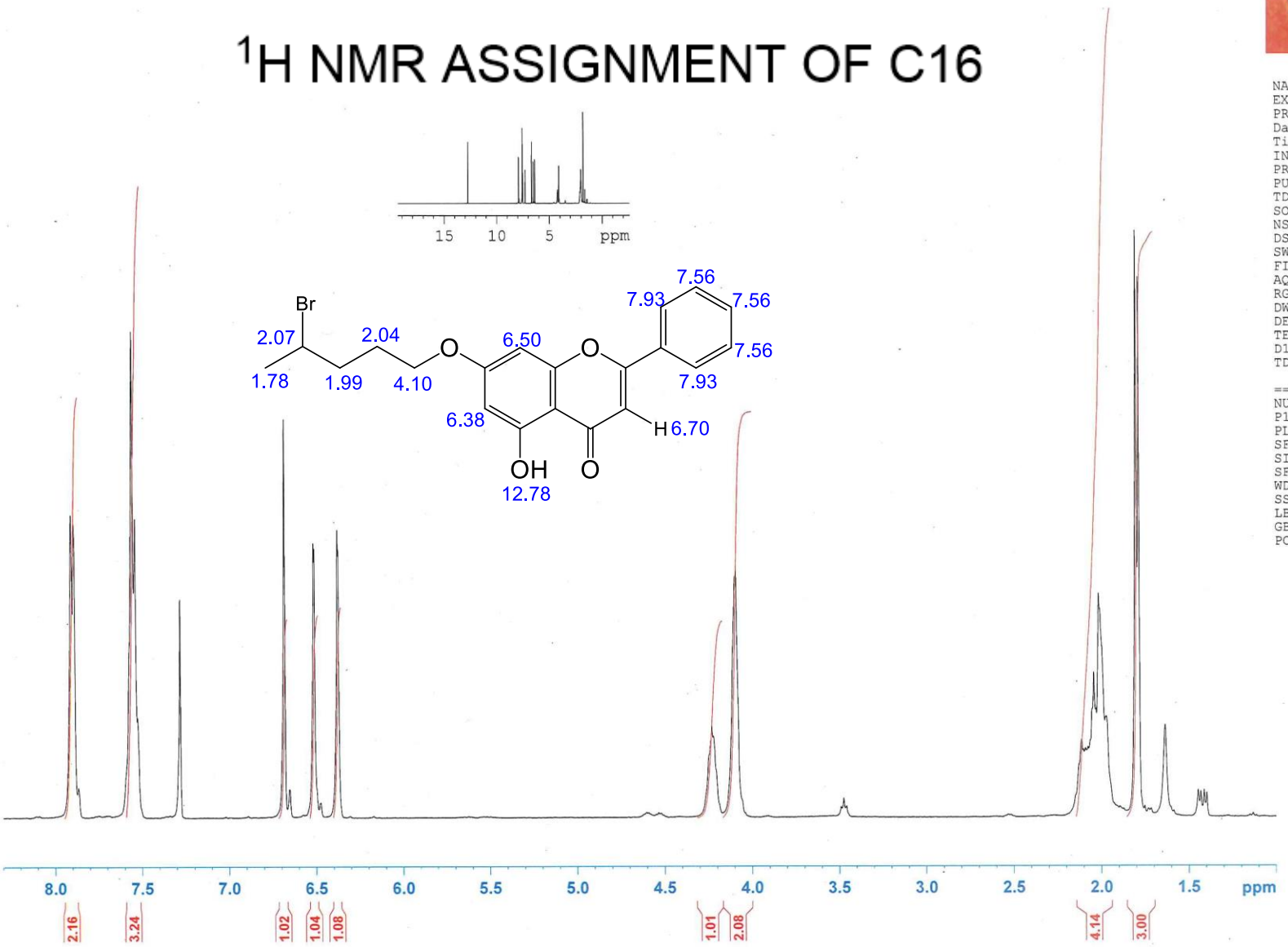
^{1}H NMR ASSIGNMENT OF C12




```
NAME        Apr14-2018-NO
EXPNO                 20
PROCNO                 1
Date_           20180414
Time               14.24
INSTRUM            spect
PROBHD   5 mm BBO BB-1H
PULPROG             zg30
TD                 56290
SOLVENT            CDCl3
NS                   128
DS                     0
SWH           8802.817 Hz
FIDRES        0.156383 Hz
AQ            3.1973219 sec
RG                 161.3
DW              56.800 usec
DE               6.50 usec
TE               296.3 K
D1            2.59999990 sec
TD0                    1

======== CHANNEL f1 ========
NUC1                  1H
P1              10.05 usec
PL1             -2.00 dB
SFO1         400.1333626 MHz
SI                 32768
SF           400.1300000 MHz
WDW                   EM
SSB                    0
LB              0.30 Hz
GB                     0
PC                  1.00
```

^{1}H NMR ASSIGNMENT OF C16



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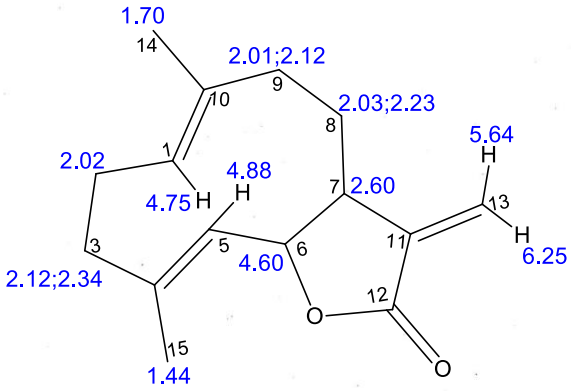
NAME	Jul15-2017-NO
EXPNO	10
PROCNO	1
Date_	20170715
Time	21.57
INSTRUM	spect
PROBHD	5 mm BBO BB-1H
PULPROG	zg30
TD	56290
SOLVENT	CDCl3
NS	64
DS	0
SWH	8802.817 Hz
FIDRES	0.156383 Hz
AQ	3.1973219 sec
RG	362
DW	56.800 usec
DE	6.50 usec
TE	296.5 K
D1	2.59999990 sec
TD0	1
======== CHANNEL f1 ========	
NUC1	1H
P1	10.50 usec
PL1	2.00 dB
SFO1	400.1333626 MHz
SI	32768
SF	400.1300000 MHz
WDW	EM
SSB	0
LB	0.30 Hz
GB	0
PC	1.00

^{1}H NMR ASSIGNMENT OF COSTUNOLIDE



University of Salford MANCHESTER
1967-2017 50 YEARS

NAME	SASNO
EXPNO	10
PROCNO	1
Date_	20170322
Time	14.02
INSTRUM	spect
PROBHD	5 mm BBO BB-1H
PULPROG	zg30
TD	56290
SOLVENT	CDCl3
NS	64
DS	0
SWH	8802.817 Hz
FIDRES	0.156383 Hz
AQ	3.1973219 sec
RG	71.8
DW	56.800 usec
DE	6.50 usec
TE	296.2 K
D1	2.59999990 sec
TD0	1
======== CHANNEL f1 ========	
NUC1	1H
P1	10.50 usec
PL1	2.00 dB
SFO1	400.1333626 MHz
SI	32768
SF	400.1300009 MHz
WDW	EM
SSB	0
LB	0.30 Hz
GB	0
PC	1.00

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