In vitro screening of *Trametes versicolor* and *Acacia nilotica* Derivatives for Antimalarial Efficacy

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Declaration

I certify that this thesis, which I submit to the University of Salford a partial fulfilment of the requirements for a Masters by research, is a presentation of my own research work. Wherever contributions of others are involved, every effort is made to indicate this clearly with due reference to the literature and acknowledgement of collaborative research and discussions. The content of this thesis has not been submitted for a higher degree at this or any other university.

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Abstract

Of the plethora of parasitic diseases that afflict mankind, malaria remains the most prevalent. The disease has caused severe global health problems, putting 3.2 billion people at risk of developing the *Plasmodium* infection. With current front-line treatments such as the artemisinins showing early warning signs of resistance, new novel drug leads are needed to ensure the safety of the populations at risk. With resistance growing, the development of new antimalarials is still slow as a result of tedious and prolonged discovery pipelines. Traditionally, antimalarial drug discoveries have derived from natural products (e.g. quinine and artemisinin derivatives). New natural product usage has diminished during the past few decades as a result of advances in molecular target detection and high-throughput screening technologies. Technical difficulties associated with the natural product extraction process, further compounded the issues. This study aims to investigate the potential antimalarial activity of *Trametes versicolor* and *Acacia nilotica* using solvent extracts in an *in vitro* study on *Plasmodium falciparum* (strain K1). Species validation from different collection sites was carried out using comparison of spectra generated with Fourier transform infra-red spectroscopy. To test drug efficiency, the parasites were exposed to the compounds for 48- and 72-hour incubations at trophozoite and ring stage. The specific doses for exposure were concentrations of 12.5 µg/ml, 25 µg/ml, 50 µg/ml, 100 µg/ml and 200 µg/ml, for each extracted compound. MTT assays were performed alongside parasite IC_{50} studies in order to define the toxicity profiles.

48 hours cycle IC_{50} values for *T. versicolor* and *A. nilotica* ethanol extractions were 45.54 ± 1.12 µg/ml and 45.54 ± 1.12 µg/ml while the 72-hour cycle was 27.47 ± 1.09 µg/ml and 37.3 ± 1.07 µg/ml on the multi-drug resistant strain K1 of *Plasmodium falciparum*. The methanol extraction on the other hand gave IC_{50} values of 20.2 ± 1.11

μg/ml after 48 hours cycle and 44.3 ± 1.06 μg/ml after 72 -hours for *A. nilotica*. The cytotoxicity assay carried out on HepG2 gave IC_{50} values of 51.6 ± 1.2 μg/ml for T. versicolor and 29.1 ± 1.07 μg/ml for *A. nilotica* ethanol extract. The cytotoxicity assay indicated a very narrow therapeutic index for both the *T. versicolor* (TI =1) and *A. nilotica* (TI = 0.6). However, given the previously published anti-cancer properties for both plant products, the use of the traditional HepG2 model for cytotoxicity is debated. This study confirmed these natural products as potential leads for new novel drug development. The evidence presented justifies further evaluation and validation of *T. versicolor* and *Acacia nilotica* as natural product leads for antimalarial drug discovery.

CHAPTER ONE

INTRODUCTION

1.1 Malaria: a historical perspective

Malaria is responsible for more deaths than any other cause in human history, including war, famine and disease. The disease, in fact, predates humanity. The oldest amber-preserved mosquito specimen containing a plasmodial infection dates back to 30 million years. Humans have been evolving alongside malaria for thousands of years, with resulting alterations to the genetic makeup of certain groups due to natural selection (Carter & Mendis, 2002).

In written history, incidents of malaria have been recorded as far back as 4000 years. Its symptoms are documented in Chinese texts (2700 BC), Egyptian papyri (1570 BC) and Indian scripts dating back to the 6th century BC (Cox, 2010). The ancient texts give a detailed description of the hallmark symptoms of malaria, including tertian and quartan fevers and hepato-splenomegaly. In 4th century BC malaria plagued ancient Greece causing major declines in populations. In addition to historical symptomatic descriptions, Egyptian mummies have shown evidence of *Plasmodium falciparum* infection from 800 BC during the Fayum depression (Lalremuata et al., 2013). Through Darwinian descent, malaria significantly impacted colonisation, exploration and development of the world we know today (Harrison, 1978). Historical figures including Alexander the Great and Pope Gregory V are suspected to have fallen victim to the disease (Carter and Mendis 2002; Harrison, 1978). During the time of the world wars, malaria alongside other infectious diseases impacted the wars significantly. For every man lost as a direct impact from the war, another life was lost due to malaria. The war

time conditions also contributed to enhancing the spread of the disease (Cox, 2002; Harrison, 1978).

The official name ‘malaria’ arose from the association of the disease with ‘bad air’ arising from marshy areas. This misguided belief associated the disease with miasmas (bad air) the Italian translation ‘mal’aria’, coming from the swamps. However, it would be many centuries and millions of deaths until the transmitter and transmission of the disease was fully understood (Stuart, 2004).

Through the middle-ages, malaria continued to spread across Europe and even into England, where it was given the name Ague (swamp fever). The disease became so popular that many literary works including William Shakespeare referred to it. Today, although malaria is a huge problem for people living in South America, the disease did not actually exist in that part of the world until after the Columbian exchange. European settlers who were already infected with the disease when they arrived, carried the disease with them, allowing native mosquitoes to become infected and thus furthering the spread of the disease.

As medical studies advanced through history, the discovery of bacteria in 1676 by Antoni van Leeuwenhoek along with the theory that microorganisms are responsible as the lead cause of infectious diseases by Louis Pasteur and Robert Koch in 1878-1879 (Cox, 2010), fuelled interest in discovering the causative agent of malaria. The discovery of the Protozoan parasite in 1880 by Charles Louis Alphonse Laveran (cox, 2010) preceded the discovery that mosquitoes were the responsible vector firstly for avian malaria in 1897 and then human malaria between 1898 and 1900 by Italian scientist Giovanni Battista Grassi (Cox, 2010). The stages of the parasite’s life cycle were finally identified in 1948 (Stuart, 2004)

1.2 Malaria today

The protozoan, parasitic disease malaria is spread through the bites of female Anopheles mosquitoes. The mosquito's saliva infected with the sporozoites of the *Plasmodium* parasites is responsible for initiating the infection in humans. The disease is found in more than 90 countries world-wide, leaving 3 billion people at high risk of infection. The disease is currently more associated with tropical and sub-tropical regions including Africa, Asia and the South Americas.

The who November (2017) report, estimates 219 million cases of malaria infections worldwide with 433000 deaths. For 1017 The highest concentration of reported malaria comes from Africa, with 200 million confirmed cases (92%) followed by south east Asia (5%). Fifteen countries in sub-Saharan Arica and India made up 80% of the world's malaria burden, with Nigeria carrying the highest burden at 19% (WHO, 2017). All WHO regions that are associated with malaria have shown signs of the reduction of mortality in 2017, compared to 2010, apart from the Americas. The most significant decline being in South-east Asia with a reduction of 54% closely followed by Africa at 40%. Although these gains seem positive the reduction rate is at its slowest since 2015 (WHO, 2017).

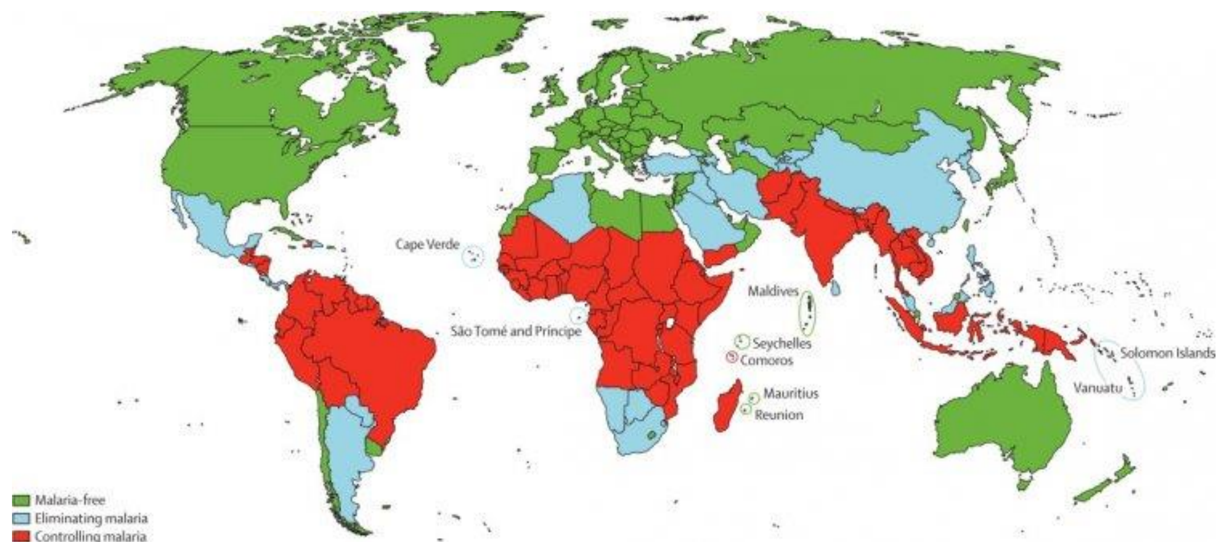


Figure 1.1 Malaria impacted areas Showing a world map of current countries that are being affected yet controlled shown in red. Countries that are on track to eliminate malaria are highlighted in blue, and the countries that are malaria free represented in green (source: Cotter *et al*., 2013).

There are five plasmodial species that are known to transmit the disease to humans. *P. falciparum,* is the most common and virulent species out of all the five mentioned, with a wide geographical distribution in subtropical and tropical areas. It is the most common due to its rapid multiplication rates once inside the host. *Plasmodium falciparum* is the most prevalent malaria parasite in the WHO African Region, accounting for an estimated 99.7% malaria cases in 2017, as well as in the WHO regions of South-East Asia (62.8%), the Eastern Mediterranean (69%) and the Western Pacific (71.9%) (WHO, 2017). *P. vivax* a second species, is found throughout Asia, parts of Africa and Latin America. *P. vivax* is the predominant parasite in the Americas, representing 74.1% of malaria cases (WHO, 2017). *P. ovale*, is found in Africa and the western pacific and *P. malariae* is found in all malaria affected countries. Finally, *P. knowlesi* – this is a zoonotic monkey malaria that has recently been found to infect humans and is found throughout southeast Asia (Mita & Tanabe, 2012) infecting humans due to the increased contact with nature as a result of deforestation. Out of the five known species mentioned, the experiments presented in this thesis

focuses on a *P. falciparum* strain named K1 that has known multi-drug resistant properties and is a genetic clone derived from the natural parasite (Gifted by Prof John Hyde, University of Manchester). K1 when cultured *in vitro* is resistant to chloroquine, pyrimethamine and Sulfadoxine, making it an ideal test subject for anti-malarial research as is it can also be used as a control to determine the sensitivity of other antimalarials and antibiotics (Agarwal et al., 2017)

1.3 Anopheles mosquito

Anopheles mosquitoes are found worldwide excluding Antarctica, with the capability to spread malaria via different species in several geographic locations. Within these regions, different environments support a specific species. There have been approximately 3,500 described species of mosquito that are divided into 41 genera groups. Malaria transmitted to humans can only be transmitted via the female mosquitos belonging to the genus *Anopheles*. There are over 400 known species of *Anopheles,* however, only 30-40 are known to be vectors of the malaria parasite. The remaining *Anopheles* mosquitoes are not able to sustain the development of the parasite (Kamareddine, 2012; Gomes *et al*., 2016). The most problematic *Anopheles* mosquito, *Anopheles gambiae* is found predominantly in sub-Saharan Africa. *A gambiae*, a known, highly efficient vector of *P.* f*alciparum,* feeds primarily on human hosts (Mattah *et al*., 2017).

All mosquito species including *Anopheles*, undergo four life stages in the completion of the life cycle (egg, larva, pupa, and adult). The first three stages are aquatic until the mosquito reaches adult stage dependent on surroundings and temperature (Beck-Johnson *et al*., 2017). The male mosquitos do not bite and therefore do not transmit

the disease. The females have a short-lived life span resulting in a small proportion of the females living the required amount of time (usually 10 days) to pass on the malaria parasite (Crans, 2004).

The adult female mosquito lays up to 200 eggs at a time. These eggs are laid one by one directly into the water supported by floats on each side of the egg (Crans, 2004). Dependant on the climate, eggs can hatch 2-3 days after being laid in more tropical regions, however in much cooler climates the eggs can take up to 2-3 weeks until they reach the larval stage.

During the larval stage, they acquire a developed head contain mouth brushes that are used for feeing on algae, bacteria and other microorganisms. They have a thorax and a segmented abdomen however at this stage possess no legs. Unlike many other mosquito species, *Anopheles* do not have a siphon used for respiratory purposes and therefore lie parallel with the water surface in order to breath (Crans, 2004). The larvae develop in 4 stages and after each stage, shed their exoskeleton in order to grow before metamorphosing into pupae.

During the pupae life stage, the mosquito does not feed for several days, instead the mosquito undergoes extreme metamorphosis. The head and thorax combine which then forms the cephalothorax resulting in the abdomen curving underneath (Crans, 2004). The pupae then use a pair of breathing trumpets that are located on the cephalothorax to breath at the surface of the water. After a few days, dependant on temperature, the adult mosquito emerges from the dorsal surface on the surface of the water (Beck-Johnson et al., 2017).

The head of the adult mosquito possess the proboscis, the part of the mosquito that is used for feeding along with two sensory palps. The head also contains the antennae

used to detect the mosquitoes host emissions. The abdomen of the mosquito specialises in the digestion of blood into proteins to sustain egg development.

The *Anopheles* life cycle will then continue repeating until the female dies. As the female usually lives up to 2 weeks, her chances of survival mainly depend of temperature and obtaining a blood meal while avoiding many host defences (Beck-Johnson *et al.*, 2017).

1.4 Plasmodium falciparum Life Cycle

P. falciparum parasite requires two hosts in order to complete its life cycle, the mosquito vector along with the human host (Gomes *et al.*, 2016). During the time spent within the human host, the parasite has two development stages, the asymptomatic pre-erythrocytic stage which is also known as the hepatic stage, along with symptomatic erythrocytic stage. The symptomatic erythrocytic stage is what causes pathologies linked with human infection (Garg et al., 2013; Fairhurst and Dondrop, 2016). Once inside the mosquito is it known as the sporogonic cycle

being in the vectors mid gut. Once the *Anopheles* mosquito ingests a blood meal from a human host the male gametocytes (microgametocytes) and female gametocytes (macrogametocytes) are also ingested. The reproduction cycle that takes place inside the mosquito is known as the sporogonic cycle. After injection, the parasites travel into the mosquito's midgut where the macro/microgametocytes form together to produce a zygote. The zygote develops into an ookinete, where they then penetrate the midgut wall forming oocyst. The oocyst continues to grow, finally rupturing releasing the sporozoites that was growing inside it. The sporozoites then travel to the mosquito's

salivary glands where it is then able to be injected into the human host (Garg *et al.*, 2013; Gomes *et al.*, 2016).

Once the female *Anopheles* mosquito take her blood meal, the sporozoites are injected into the host via the mosquito's *proboscis* (Soulard et al., 2015) where they make their way into the hosts blood stream. The sporozoites that are injected into the blood travel to the liver and become the hepatic part of the life cycle, invading the hepatocytes. Once there, they differentiate and multiply into schizonts, which when fully grown contain several thousand hepatic merozoites. The merozoites then are released back into the blood stream where they become the erythrocytic part of the life cycle after invading the red blood cells (RBCs) (Soulard et al., 2015). The merozoites start off by invading the RBCs and then transform into what is called the ring stage due to appearance under the microscope resembling a signet ring. The rings then develop into early and late trophozoites, which then develop into schizonts. The schizonts burst open releasing 32 daughter clones into the hosts blood stream, further invading the remaining non-infected RBCs (Gomes *et al.*, 2016). During this cycle some or the parasites differentiate into the gametocyte form (Soulard et al., 2015) where they are then taken up by the mosquito during a blood meal and the full cycle is repeated once more (Garg *et al.*, 2013; Gomes *et al.*, 2016).

Figure 1.2 The complete life cycle of plasmodium *showing both the human and mosquito cycle. Section A is showing the Exo-erythrocytic cycle, section B is showing the Erythrocytic cycle and Section C is showing the sporogonic cycle within the mosquito. Taken from (Centre for disease control (CDC), 2019)*

1.5 Symptoms and Diagnosis

Clinical symptoms are the first signs patients will show when infected with malaria. *P. falciparum* being the most lethal is capable of causing severe disease characterised by the parasite's ability to sequester in vital organs including the brain, resulting in cerebral malaria (coma). These symptoms include sickness, fever, headaches, tiredness which eventually progress to confusion, delirium and progressive loss of consciousness, coma and death if not treated effectively. Upon diagnosis, the patient's travel history and geographical location should always be taken into consideration. In

severe malaria, clinical symptoms could also include renal failure, severe anaemia, jaundice and respiratory difficulties. These symptoms along with the flu like ones can be enough to determine if a person has malaria, but a laboratory test should always be carried out to determine the presence of malarial parasites in the blood.

The most used method to detect malaria in the blood stream is microscopy. This procedure involves a drop of the patients' blood thinly spread out over a glass slide and stained with Giemsa. This method of detection however can prove difficult at times and requires a degree of training. Antigen detection kits are a new way of detection for the parasite. Rapid diagnostic tests (RDTs) aim to give results in a little as 2-12 minutes, however these have shown to be not as effective when the parasite count is low (Cook et al., 2015). These tests do prove effective as an alternative to microscopy when available. Tests using polymerase chain reactions (PCR) are a lot more sensitive in detecting the presence of *Plasmodium* by amplifying the parasites DNA. Although more sensitive, this method takes time and has to be conducted in the laboratory with appropriate facilities, a condition not often found in the endemic regions. Furthermore, this method is a lot more expensive due to the primers needed and the laboratory equipment (Cook *et al.*, 2015). It is however an effective method to determine the species of *Plasmodium*.

As malaria is still a dominant global concern, with a 92% mortality rate in Africa (Tambo *et al.*, 2018) new, affordable diagnostic techniques are a constant priority. A recently new alternative to PCR, Loop mediated isothermal amplification (LAMP) provides a rapid result with sensitivities as good as PCR itself, giving a more accurate reading than the leading RDTs. PCR works using a thermo-cycler that amplifies DNA at many different temperature's in order to anneal and denature the parasites DNA, detecting parasitaemia as low as 1-2parasites/μL. While LAMP uses a stable constant

temperature through at 65°C while detecting parasitaemia at the same volume (Tambo *et al*., 2018). LAMP diagnostic is also available to be conducted in the field making it much more beneficial to detecting patient malaria (Hopkins et al., 2013).

1.6 Vector control

Vector control is potentially the most important step in combatting malaria outside effective antimalarial chemotherapy. If there were no vectors to transmit the parasite, the death burden will fall. However eradicating mosquitoes would be an impossible task so controlling them is key. Vector control comes in the forms chemical, biological and mechanical, however, all are still to be perfected.

Chemical insecticides (chemical control) are currently the most used deterrent in combatting malaria (Glunt *et al.,* 2013). The most effective and successful chemical deterrents are indoor residual sprays, along with insecticide treated bed nets (ITNs), long-lasting ITNs and spraying other materials such as furniture and clothing. Using these basic control methods especially at night time when mosquitoes usually feed, it can help stop malaria upon impact (Kamareddine, 2012; Glunt *et al*., 2013; Raghavendra *et al*., 2011). The spraying of pesticides directly upon the roofs and walls, along with insecticide fogging is also effective in combating female mosquitos. This method decreases the life span that the mosquito would naturally has, thus limiting contact with humans (Raghavendra *et al*., 2011).

The chemicals used in the above chemical insecticides are pyrethroids. These are commonly used due to the low toxicity levels that are deemed safe for human use (Childs et al., 2016). Chemicals used in the past however have not always been safe for human use or beneficial to the environment. Dichlorodiphenyltrichloroethane (DDT)

was introduced in the twentieth century, leading to a new era of insect control (Raghavendra *et al*., 2011). DDT was highly efficient, leading to the eradication of malaria from Europe and was the first major insecticide used in vector control. Irrespective to DDTs success it was later banned by the Environment Protection Agency (EPA). Due to the negative environmental impacts it alongside an association with causing cancers including breast cancer in humans (Raghavendra *et al*., 2011; Kamareddine, 2012).

Larval control is a biological form of vector control. Larvicides are a proven malaria preventative but is somehow overlooked and not implemented. The aim of larviciding is to eradicate or dramatically reduce the number of vectors in known larval infested waters, before they mature into adults and become mobile. However, there are thousands of habitable places where larvae can live in a given country, therefore larviciding every one of them could lead to many different ecological problems. Larvivorous fish have been introduced in many countries in a bid to reduce adult populations, however chemical larvicides such as Paris green (copper acetoarsenite) have been banned due to high levels of arsenic (Raghavendra *et al*., 2011).

There are two known species of bacteria that have been proven to be biological larvicides. Bacillus thuringiensis i*sraelensis* (Bti) and Bacillus *sphaericus* (Bs) (Kamareddine, 2012). Both Bti and Bs work by poisoning the mosquitoes mid gut causing the cell membranes to break down, eventually killing the mosquito (Ingabire *et al*., 2017). These biological larvicides are beneficial as they have no effects on humans but have been known to affect amphibious life (Raghavendra *et al*., 2011).

The use of entomopathogenic fungus as an alternative to bacterial vector control has also been used. Fungal species in genera such as *Lagenidium* have been studied as a disease control. The benefit of using fungus, is that the fungus itself does not need

to be injected into the vector. Cuticle contact is efficient enough to infect. This method can be applied in and around larval habitats and around the homes and work places of them in high-risk areas (Kamareddine, 2012).

Biological control can be increased in the form of introducing transgenic mosquitoes (sterile males) into the atmosphere to compete with fertile males. Decreasing the population can also be effective and has been considered as a vector control strategy (Knols et al., 2007; Kamareddine, 2012). Genetic engineering of the transgenic mosquitoes could also be a vector control by altering the DNA with foreign DNA to possess anti-parasite macromolecules limiting malaria transmission (Kamareddine, 2012).

Mechanical control involves physically removing a breeding ground in order to destroy the habitat mosquitoes use to breed and grow as larvae. This could involve the removal of eutrophic waterbodies. The removal of the stagnant water bodies such a lakes, canals and ponds would limit the mosquitoes surface area. Simple strategies involving removing man-made objects that could collect rain water could also be an effective way to mechanically control the vectors (Lindblade et al., 2015).

Personal protection is an important form of vector control, preventing the bite taking place initially significantly decreases the chance of malaria transmission. This can be achieved by applying repellent oils, creams and sprays such as DEET. However, in many developing countries such items would be classed as a luxury. products would not be easily affordable, especially when they are only effective for several hours after application. Some countries prefer to use natural products as alternatives such as eucalyptus.

Malaria vaccinations are not a form of vector control, however, currently RTS,S/AS01 are the most advanced vaccinations to date in combatting P. *falciparum.* Stage 3 trials

finished back in 2009 in several countries in sub-Saharan Africa. This vaccine is currently up for stage 4 evaluation. There are also 20 other vaccines currently being researched (WHO, 2019).

1.7 History of antimalarial drugs

1.7.1 Quinine

Quinine is classified as one of the most ground-breaking discoveries of the 17th century. Quinine remains an important antimalarial 400 years after being discovered in the 1600s (Cui et al., 2013). Quinine is a natural product that is derived from the Cinchona tree and was originally referred to as the "sacred bark", the name originating in 1630 from Jesuit missionaries. There was, however, an earlier legend of quinine use that derived from an Indian who had found himself lost in a jungle located in the Peruvian Andes. The person with high fever had taken a drink from a stagnant pool that had been in contact with the surrounding cinchona trees. After initially thinking they had been poisoned, the fever disappeared. This new "fever cure" was shared with local villagers who eventually used the trees bark to cure their fevers (Achan, Talisuna et al., 2011).

Quinine was introduced to Europe after a Spanish Countess contracted a fever while in Peru. She was later cured after being administered bark from the tree, giving it the new name 'countess's bark'. The bark was then taken back to Spain, introducing Quinine to Europe in 1638 (Achan, Talisuna et al., 2011). The powdered bark was then added to drinks such as wine and taken orally (Achan, Talisuna et al., 2011; Fluckiger & Hanbury, 1874). Before being taken back to Europe it was common for Europeans to use European and Asiatic plants as fever remedies (Stuart, 2004).

Quinine was later promoted by Cardinal de Lugo, the Procurator General of the order of the Jesuits. The Jesuits then seized the opportunity and made profit by controlling

the entire supply of bark into the west, thus changing its name to 'Jesuits bark'. Due to this demand, native trees became threatened and numbers started to decline. This resulted in the need for cultivation and the Spanish doctor, Jose Celestion Bruno Mutis started this initiative (Stuart, 2004).

During the 1820s, a couple of well renowned French pharmacists were able to isolate an alkaloid from the plant that they named quinine (Stuart 2004). Quinine is a cinchona alkaloid belonging to the aryl amino alcohol group of drugs (Achan, Talisuna et al., 2011).

Quinine was the most commonly used antimalarial up until the 1920s, after this there were just as, if not more, effective synthetic antimalarials available on the market such as chloroquine and amodiaquine (Mojab, 2012), The most important of these was chloroquine (Achan, Talisuna et al., 2011).

Mefloquine was first introduced in the 1970s and is an antimalarial that is phospholipid interacting. It is effective against mild to moderate *P. falciparum* infections. Mefloquine is active against the parasites erythrocytic stage, however it is ineffective during the hepatic stage. This drug works buy enlarging the parasites food vacuole, in turn damaging the haem which destroys the membrane. Mefloquine is typically used as a prophylaxis, before travel and for several weeks after infection is suspected and works well with chloroquine-resistant strains of *P. falciparum.*

1.7.2 4-Aminoquinolines (Chloroquine, Amodiaquine and Piperaquine)

Chloroquine is an antimalarial that is part of the 4-aminiquinolines drug group. In 1934, research by a group of scientists, including Hans Anderson led to the discovery of a new synthetic substitute for quinine named chloroquine, along with a 3-methyl chloroquine (Lei et al., 2020). These new antimalarial compounds were however deemed toxic and the German research went no further. The formula was passed onto

a United States of America company after French soldiers found the German protocols on how it was to be made (Bhattacharjee, 2016).

The United States government made slight adjustments during World War II and entered the captured drug into clinical trials in 1947, as a prophylactic treatment of the malaria parasite (Bhattacharjee, 2016). Chloroquine in its heyday was classed as revolutionary, mainly due to the efficacy and safety (even during pregnancy) along with affordability (Baird, 2005; Karagoz et al., 2018). Chloroquine was typically used to treat all human *Plasmodium* strains, however, use on *P. falciparum* became more restricted after the 1950s. This was due to resistant strains arising in 3 separate regions simultaneously in Southeast Asia, Oceania, and South America.

Chloroquine was discovered to work by inhibiting the haem detoxification process. The mechanism by which it works involves drug concentrations that reach 1000-fold within the acidic food vacuole found within the parasite which in return results in parasite elimination. As chloroquine is positively charged, it is able to accumulate due to the charge being repelled by the vacuole membranes positive charge. After this chloroquine is able to accumulate, resulting in a build-up of haem. The increased build up then proves fatal to the parasite (Takala-Harrison & Laufer, 2015; Slater, 1993)

Amodiaquine is structurally related to chloroquine and was synthesised in 1948 (Whirl-Carrillo et al.,2012). However, it has been deemed toxic too for use alone, and is usually used in partnership with piperaquine (Cui et al., 2013). Amodiaquine has links to causing severe hepatitis, leading to fatalities and is recommended for treating malaria but not prophylaxis (Adjei et al., 2009). The compound also shows inhibition of the malarial heme detoxification pathway leading to a build-up of ferriprotoporphyrin IX (Whirl-Carrillo et al.,2012).

Piperaquine was synthesised in China during the 1960s, and has seen been used heavily across both China and Indochina for prophylaxis and treatment of malaria. Studies in these countries have shown the drug to be just as effective and well tolerated as chloroquine for the treatment of *P. falciparum* and *P. vivax*. Piperaquine (Davies et al 2005). The use of piperaquine dropped during the 1980s after resistant strains of *P. falciparum* emerged. More recently, China has developed a piperaquine combination with artemisinin to combat the resistant strains.

1.7.3 8-Aminoquinolines (Primaquine)

Primaquine is currently the only potent gametocidal drug commercially available on the market to date, showing positive results in the use against P. f*alciparum*. Due to its activity against hypnozoites, the drug is administered orally to hinder relapses of *P. ovale* and *P. vivax* once a completed treatment of blood schizonticide has had it full course (Ashley et al., 2014). Primaquine is also used to prevent re-infection to those who are returning to potentially high-risk areas. Once the malaria parasite breaks down the haemoglobin into two parts the haem and globin, the haem becomes toxic towards the parasite. *Plasmodium* produces a chemical that converts the haem into a non-toxic form enabling its survival. This drug has an adverse effect upon the mitochondria. This depletes the parasite of energy blocking the release of the haem, changing chemical, and thus killing the parasite due to the build-up of toxic haem. Primaquine is not classed as a prevention drug and is usually a 7-day course that is used alongside Artemisinin-based combination therapy ACT or chloroquine (Bray et al., 2005).

1.7.4 Antifolates (Sulfadoxine-pyrimethamine and Dapsone-chlorproguanil)

Antifolates are a class of compounds known to antagonise the folic acid folate pathways, thus restricting parasite DNA synthesis (Visentin et al., 2012; Desai et al., 2016). Sulfadoxine-pyrimethamine is the most used drug to combat *P. falciparum* strains that have developed a resistance to chloroquine in certain regions (Kublin et al., 2002). When chloroquine was still the first drug of choice due to low levels of resistant strains being present, Sulfadoxine pyrimethamine was usually the second drug in line for treatment (Kublin et al., 2002). The mode of action involves the pair working in synergistically to prevent two enzymes that are needed for the folate biosynthetic pathway. Sulfadoxine and dapsone work by inhibiting the dihydropteroate synthetase enzyme that is found within the parasite's folate pathway. Pyrimethamine and dapsone work by stopping the dihydrofolate reductase within the thymidylate enzyme (Hayton & Su., 2008). These drugs in partnership prevent *Plasmodium* reproduction as well as growth (Gatton et al., 2004; Cui et al., 2013). Synthesis of tetrahydrofolate is stopped by sulfadoxine working against p-aminobenzoic acid at the same time of pyrimethamine blocking the dihydrofolates (Gatton et al., 2004)

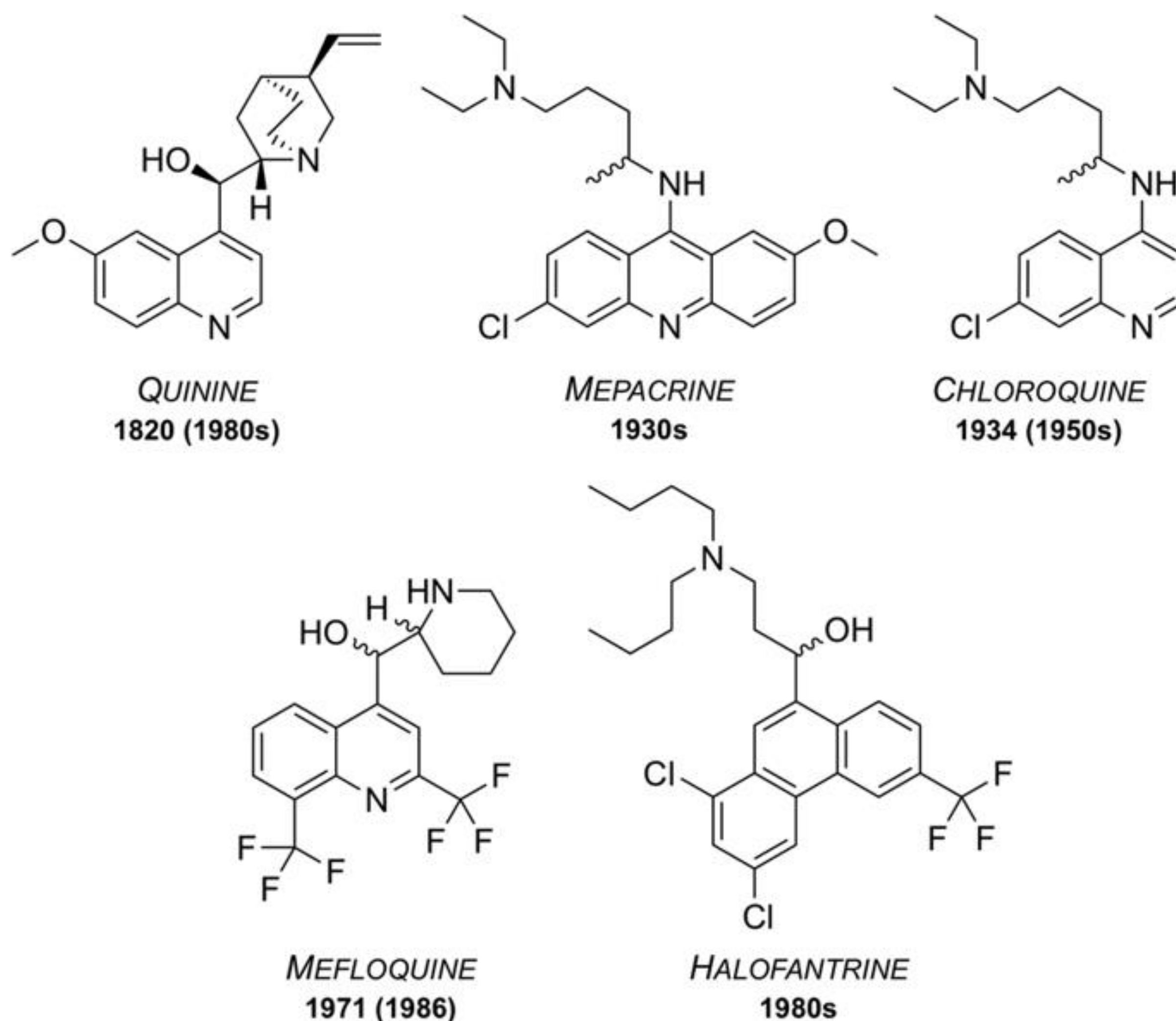


Figure 1.3 Chemical structures of know antimalarial drugs discoveries. Showing the chemical structures of Some of the better-known antimalarials are dating between 1820-1980. Some drugs such as chloroquine are still in use to date, while some deemed ineffective due to parasite resistance. The dates shown within the brackets indicate resistance (Source Edwin et al., 2019).

1.7.5 Artemisinins and Artemisinin-based combination therapy (ACT)

Artemisinin is a naturally derived product from the sweet woodworm plant (*Artemisia annua*) and originated from China. Upon its discovery it was hailed as one of the best medical discoveries of the 20th century (Krishna et al., 2008). Artemisinin is the first line drug of choice for treatment of *P. falciparum* for uncomplicated malaria (Su et al., 2015) Artemisinin is considered to be highly active and fast-acting against multiple parasites and at different development stages (Visser et al., 2014). The compound is

highly effective at lowering the transmission rate of malaria as it has the ability to destroy immature and developing gametocyte, thus lowering the chance of a mosquito contracting the parasite in a blood meal (Okell et al., 2008). The drug's activity is not however fully understood but it is thought to have its toxicity due to the haem-mediated decomposition located within the endoperoxide bridge, producing carbon-centred free radicles (Petersen et al., 2011).

Artemisinin-based combination therapy is considered to be the best current treatment for malaria. It uses two separate drugs working at the same time to ensure effective treatment. It works by combining artemisinin with partner drugs that include sulfadoxine-pyrimethamine for severe malaria (Cui et al., 2013) and drugs such as amodiaquine, lumefantrine and mefloquine (as mentioned above). Artemisinin works rapidly killing off the majority of parasites within the blood, while the partner drug works a lot slower, this prevents resistant strains developing to the compounds while dramatically lowering the transition rate (Visser et al., 2014).

1.7.6 Antibiotics

Antibiotics as treatment for malaria are recommended as they are universally already available and have passed multiple trails already making them a very cost-effective treatment and prevention for malaria when traveling to countries with a high transmission rate. Antibiotics used as antimalarials include doxycycline, clindamycin and tetracycline. These drugs are extremely useful in countries that have a high level of resistant strains to drugs like chloroquine. The downside, however, is that such antibiotics can have adverse side effects to people taking them such as limiting the amount of sun exposure a person can have, given they are in countries with extremely high temperatures (Tan et al., 2011). Furthermore, the use of these drugs would deprive their usage as anti-microbials, where drug shortages exist already.

Tetracycline is used to treat children who are not old enough to have other treatments. Doxycycline is commonly used alongside quinine and is effective for treatment for complicated *P. falciparum* infection. Clindamycin is used alongside quinine too; however, it is used when the infection of *P. falciparum* is acute and has limitations on children.

1.8 Drug resistance

The problem with *Plasmodium* parasites and why they are so difficult to eradicate *via* drug treatment is down to the parasite being genetically diverse. When the parasite passes through the mosquito vector they reproduce, and like any other reproducing organism exchange pieces of their DNA. This exchange of DNA results in mutations. These mutations enable the parasites to evade drugs and spread at a much faster rate, due to the parasite becoming less sensitive to the family of drugs (Hayton & Su., 2008).

As chemotherapy has been the front-line combat in the control of malaria, resistant strains of *Plasmodium* have become the biggest threat to malaria treatment. With all the commonly used malaria medications including artemisinin developing resistance, the need for new drugs is a top priority project (Ashley et al., 2014). With mutations being the biggest threat to resistant strains, it is important to understand that some drugs such as chloroquine requires one single mutation to develop resistance, while others may require several and this can lead to many more complications in battling drug resistance (Mohammed et al., 2013). When drugs focus on specific inhibitors that are highly potent, this means they have a specific target that can mutate and mutate quite quickly. The main mechanism used to overcome this is the advocacy of combination therapy with multiple modes of action, as practiced with ACT.

Drug resistance has been a battle for several decades. It has been observed that drug resistance often emerges in the same geographical locations such as Asia. Chloroquine, mefloquine, quinine, pyrimethamine and now artemisinins have all developed drug resistance in South East Asia. The resistant *Plasmodium* have since spread across to Africa as a result of a mutation directly linked to Asia (Price et al., 2004). Not all mutations have been directly linked to Asia, in sub-Saharan Africa. For artimisinins, the current frontline drug, a K13 propeller mutation was discovered in *P. falciparum,* which Is still yet to enter Asia (Fairhurst and Dondrop 2016). Kelch is a gene located on chromosome 13, the discovery of this gene gives hopes in detecting early the development of artemisinin resistance. Discovering the K13 marker will enable to scientist to locate resistance more quickly, avoiding a public health disaster such as what happened with chloroquine.

1.8.1 Chloroquine resistance

Chloroquine resistance in *P. falciparum* emerged from Cambodia in 1960, however failures with the drug had been happening over several years prior (Batista et al., 2009; Najera et al., 2011). In South America resistance emerged in 1959. This however, was an independent development of resistance and was not connected to the mutation in South East Asia (Dondrop et al., 2010). From Asia the resistant strain spread to East Africa and was reported there in 1978, and by 1988 chloroquine resistance had spread across all tropical countries in Africa resulting in over three million deaths (Mohammed et al., 2013).

Resistance to chloroquine caused by the mutation of two separate genes that are both located within the food vacuole. The resistant mechanism involves doubling up a reflux pump that is coded by the *P. falciparum* chloroquine resistance transporter (pfcrt) gene. This mutation means that when chloroquine enters the parasite's food vacuole

it is immediately excreted. The second gene mutation is *P. falciparum* multidrug resistance transporter-1 (pfmdr-1) this works the same way but by mutations of D1246Y, N86Y, S1034C and N1042D to remove chloroquine from the food vacuole (Mohammed et al., 2013; Koenderink et al., 2010). Resistance is caused by a single amino acid mutation from pfcrt that causes lysine to be replaced by threonine. This change results in the once positively-charged lysine to change to a neutral threonine impacting the vacuole. Due to this mutation, chloroquine is unable to concentrate in the food vacuole and block the conversion of toxic heme to inert haemozoin pigment, resulting in a loss of effectivity on the *Plasmodium* parasite (Mohammed et al 2013).

1.8.2 Antifolate resistance

At first pyrimethamine was used as an antimalarial on its own, however after only one year of use resistance was seen in *P. vivax* and *P. falciparum* and so sulfadoxine-pyrimethamine came into use (Cui et al., 2015). The significant rise of sulfadoxine-pyrimethamine resistance was reported in 1981 (Hurwitz et al., 1981), although early reports of resistance were observed in Thailand and Cambodia as early as the 1960s (Petersen et al., 2011). Regions such as Africa and South East Asia are more heavily affected by the resistant strains than any regions (Braid, 2005). As these drugs act by interference with the folate pathway, mutations in this pathway are the main cause of resistance. The antifolate drugs are effective agents in prophylaxis and therapeutics and they can also be used in combination where they act synergistically (Gregson & Plowe, 2005).

Resistance to these classes of drugs have however arisen, primarily due to point mutations in the dihydropteroate synthase (DHPS) and the dihydrofolate reductase (DHFR) genes. These are the two main enzymes that are responsible for the folate biosynthetic pathway targeted by the antifolates (Gregson & Plowe, 2005). However,

it is still the first front-line drug of action in some parts of sub-Saharan Africa as a safe and cost-effective alternative to chloroquine when resistance to that is high.

Once resistance to a drug has developed in a certain region it is then removed as a treatment for malaria and replaced with another, there is evidence re-introduction of the drug after several years can restore sensitivity. An example of this is in Malawi, where chloroquine resistance was discovered. After 12 years of the drug being discontinued, resistance to the drug lowered and in 2006 the drug was reinstated as an effective treatment for malaria (Laufer et al., 2006)

1.8.3 Quinine and Mefloquine resistance

Quinine resistance first became apparent in Brazil and then was later found in South east Asia. Resistance is currently found within the Western and South-East Oceania area, however, is less frequent in Africa (Schlitzer, 2008). Resistance to quinine was shown to be caused as a result of multiple gene mutations, classifying this resistance as complex (Peterson et al., 2011). Quinine resistance is heavily associated with polymorphisms in the parasite's drug transporters pfmrp1, pfcrt and pfmdr1. Studies based on the relationship between polymorphisms and pfnhe1 for parasite sensitivity to quinine have proven to be inconsistent in the results. However, most test have shown that the multiple polymorphisms are having an impact on the parasite sensitivity to the drug (Cui et al., 2015).

Mefloquine was introduced in Thailand during 1984 and was successful upon introduction. After 6 years of being a front-line choice of medication, resistant strains were reported even after careful administration and stringent regulations to avoid such a thing happening (Price., et al 2004). As resistance arose in Asia, it led to the resistance of halofantrine alongside with quinine. As resistance grew in Thailand, it also spread to neighbouring countries such as Cambodia and Burma (Hayton & Su,

2008). Due to resistance to mefloquine appearing it has since been used alongside artemisinin as a combination therapy and has since proven to be an effective form of treatment, however resistance along the Thailand-Cambodia border has been noted to this combination too (Price et al, 2004).

1.8.4 Artemisinin resistance

Artemisinins are classed as an indispensable and frontline drug in the use for malaria treatment. The increased resistance has revived worldwide attention due to the importance of the drug’s ability at combating the disease. In Thailand and Cambodia where antimalarial resistant strains are documented already, overuse and misuse of medications may have a role to play in resistance development (Dondorp et al., 2010). Artemisinin and mefloquine resistance are primarily documented along the Thai-Cambodian border. After anecdotal observations, studies documenting treatment failure were reported in 2008 (Cui et al., 2015). According to the WHO, resistance to artemisinin has now been documented in several different countries including Vietnam, Laos and Myanmar.

Several genome studies in *P. falciparum* have shown there is a mutation in regions on chromosome 13, which has been directly linked to reduced parasite clearance following artemisinin treatment. When treatment involves combined drug treatment, with a genomic approach, mutations inside the propeller domain were identified in the kelch 13 gene PF3D7_1343700 (K13) in late 2013 (Cui et al.,2015).

In the studies in Africa, the K13 gene polymorphisms are highly present in the mutation-prone propeller domain. In Asia however, such mutations are yet to be seen. When a parasite has a K13 mutation, it appears to show a higher rate of survival after a dihydroartemisinin pulse, this is then used to determine the level of resistance the

parasite has acquired. When the parasites had developed this resistance, they are also shown to have had increased expression of response pathways. With unfolded pathways and also ring stages that were considerably long-lasting (Cui et al., 2015).

1.8.5 Insecticide resistance.

The mosquitos themselves are becoming genetically resistant to some of the current insecticides used to control the vectors. The resistance is thought to be spreading at a rapid rate. It is thought that long-lasting insecticide-treated nets (LLINs) and indoor residue sprays (IRS) programs are the main reason for the emergence of insecticide resistant strains of mosquito being present in the environment (Childs et al., 2016).

1.9 Current antimalarial drugs

Antimalarial drugs combat the erythrocytic stage of the parasite which results in the parasite being eliminated from the body (Cui, et al., 2013). Artemisinins are still considered to be the current front-line treatment due to the potency they have on the parasite (Fairhurst Dondrop, 2016). Artemisinin is a leading current drug due to its efficiency in targeting all blood stages of the parasite. impacting ring, trophozoites and schizonts stages. It is also an effective gametocidal drug thereby ensure the blocking of transmission of the infection to the mosquito vector. This drug alone, however, does not have an impact on the merozoites and mature gametocytes in the parasite's life span (Fairhurst & Dondrop, 2016)

ACTs are currently used alongside the 8-aminoquinolines and the 4- aminoquinolines as recommended by WHO, to treat *P. falciparum* in high prevalence areas and are currently one of the most effective drugs for the treatment for all stages of the parasite. ACTs that are currently recommended and currently in use are artesunate/sulfadoxine-pyrimethamine, artesunate/ amodiaquine,

dihydroartemisinin/piperaquine, artesunate/ mefloquine, artemether/ lumefantrine and artesunate/ pyronaridine (Cui, et al 2013)

1.10 New antimalarial drugs

With the *Plasmodium* parasite's ability to evolve and develop resistance to every drug that has been developed, the need to discover create new drugs to combat the disease is more important now than ever. Research and drug development require several approaches to develop an effective finished product, that will tackle the malaria burden (Gamo, 2014). Denovo drug discovery refers to the discovery of new therapeutic leads (synthetic and natural product) as effective antimalarials. Another way in which drugs are developed is drug repositioning or repurposing, where drugs that have been proven to treat other diseases, for example cancers are screened against malaria, with the singular advantage of shortening the drug discovery pipeline. Another way is to reintroduce an old drug that has in the past been considered to be ineffective in the fight against malaria, to see if resistance has passed. Despite enormous financial investment, the search for a safe, effective and affordable antimalarial continues to be an elusive challenge (Hobbs & Duffy, 2001).

1.10.1 Semi-synthetic drugs

Semi-synthetic drugs that have been developed as derivatives of an effective parent compound, e.g. chloroquine from quinine, or artesunate and artemether. Drug resistance has also forged an interest in a novel series of synthetic peroxides that can be used as a new treatment against malaria (Marti et al., 2011). Compounds such as ozonide belong to a group of a synthetic trioxolane by-products. This compound Is one of the leading examples of synthetic endoperoxides undergoing research and testing to become a new synthetic antimalarial drug treatment. There have been several

studies on this possible new drug with *in vivo* animals trials showing improved oral bioavailability while possessing a prolonged half-life (Marti et al., 2011). When compared to other synthetic drugs derived from artemisinin, this new group is relatively low cost, efficient and shown to have improved biopharmaceutical properties (Griesbeck et al., 2017: Perry et al., 2006).

1.10.2 Pharmacophore hybridisation

A new approach in the development of new drugs incudes pharmacophore hybridisation. This new possibility of developing new antimalarial compounds by hybridisation is an exciting approach that involves the combination of pharmacophore compounds. When the compounds that are due to be combined possess different groups from different mechanisms of action, this can be an extremely effective way to avoid resistance in the future (Ginsberg & Deharo, 2011; Guantai et al., 2012). In a study by Dechy-Cabaret et al (2000) it was shown that when 1,2,3,4-triozane was covalently linked to a 4- aminoquinoline compound through an adequate spacer, promisingly active trioxaquines had been developed. When the study on trioxaquine-4-aminoquinoline was performed upon three different drug resistant strains of *P. falciparum* the compounds were shown to be highly effective. The IC_{50s} revealed from the study were between 2-86μM, showing effective results (Dechy-Cabaret et al., 2000) increasing expectations that this new hybrid option will produce a new novel drug. However, from evidence from recent studies indicates that the previously reported advantages of these hybrids such as low toxicity, simple formulations and improved pharmacokinetics have still to be proven and realised. In order for this new method to be passed into clinical development and late clinical trials a number of unanswered questions will have to be addressed before these new drugs are deemed of worth to the investors (Agarwal et al, 2017)

1.10.3 Protease inhibitor drugs

There are a number of drugs that fall into a group called protease inhibitor drugs which are used in the treatment of human immunodeficiency virus (HIV). Some of these drugs have been known to possess anti-parasitic properties. Some of these drugs include lopinavir, atazanavir and saquinavir (Lek-Uthai et al., 2008). During *in vitro* studies on human malaria parasites, protease inhibitor drugs have been shown to have fatal effects upon *P. falciparum's* erythrocytic stages. This method however, is not always an effective method for patients who are already immunodeficient as it could interfere with their current treatment. Antimalarials can also have an impact on the level of medication found within the blood of a patient who is already taking HIV medication (A Fehintola et al., 2011). In animal studies however, a species named *P. chabaudi,* which is a known rodent plasmodium was also shown to have an impacted erythrocytic stage after being exposed to protease inhibitor drugs *in vitro* (Hobbs & Duffy, 2011; Lek-Uthai et al., 2008)

1.10.4 Antibiotics

As previously mentioned, antibiotics have been known to possess antimalarial properties, however only certain classes of antibiotics have these properties. Classes such as macrolides, tetracyclines and lincosamides have the potential antimalarial properties that can be used to treat patients (Gaillard et al., 2016). The antibiotic mode of mechanism usually works by affecting a primitive part of the *plasmodium's* target pathways in the apoplast. (Noedl,2009). As discussed, the use of antibiotics is favourable as they are easily accessible and approved for human consumption (Tan et al., 2011; Gaillard et al., 2016). Until recently, antibiotic treatment for infections caused by *P. falciparum* was thought to be ineffective. Now, however, they are

deemed useful in conjunction with a partner drug that has known antimalarial properties (Pradel & Schlitzerr, 2010; Gaillard et al., 2016).

Antimalarials such as quinine, used alongside antibiotics such as doxycycline and tetracycline can have a positive impact on patient recovery. These drugs constitute the most common form of antibiotic combination therapy. Due to the success of this combination therapy, it has been used in South East Asia extremely successfully as a second line treatment (Gaillard et al., 2016). Quinine/ antibiotic therapy also has some down sides as it has some unpleasant side effects including nausea, headaches and abdominal pain. When new antibiotics are discovered, testing them alongside antimalarials in the future could bring new treatment opportunities (Song et al., 2016)

1.10.5 Anti-cancer agents

Anti-cancer agents such as methotrexate and trimetrexate are becoming an extremely popular topic of research as antimalarial drugs, with many repositioning trials showing shared effectivity in the two diseases. Recent studies have shown synergistic activity when anticancer drugs are used alongside artemisinin (Das, 2015). It has been discovered that these drugs have a double mode of action on the parasite. Both drugs will work together to damage the proteins that are found within the proteasome, in time building up and blocking the waste disposal system of the parasite. The hope for this possible new combination therapy is that it will also evade resistance by acting quickly while remaining within the bloodstream for quite some time.

Anti-cancer agents such as methotrexate and trimetrexate that been found to be extremely active again malaria. The problem these type of compounds presents is that they are deemed to toxic to use, therefore using them as an antimalarial at the moment is not possible. Further studies are needed in order to fully understand mechanism of action and dosages needed for ultimate response. The toxicity of anti-cancer drugs

can be lowered when the dosage is lowered, novel combination therapies with synergistic potential could achieve this objective (Nzila et al., 2010).

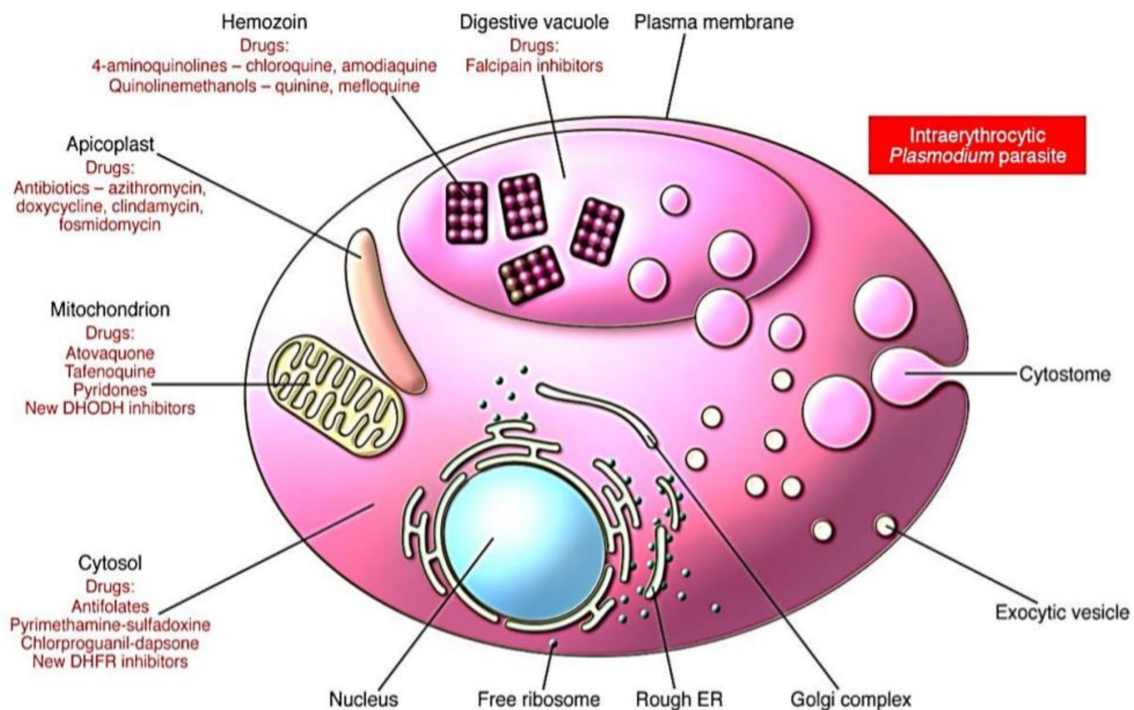


Figure 1.4. Potential drug targets. showing the intraerythrocytic plasmodium parasite and the potential targets new drugs can be directed towards to be to successful antimalarials (Source Greenwood et al., 2008)

1.11 The Drug discovery process

The traditional drug discovery process is extremely time-consuming and costly, in some cases the price exceeds $1 billon. The reason behind such a price tag and length of time is due to the number of compounds that enter the research and development sector. The high attrition rate implies that if for example, 10000 compounds that go through such research and development, only one compound will be worthy of ending

up as a consumer product. The original compound/ idea usually derives from universities or clinical and commercial sectors in a given field. With malaria, there is a high demand currently for antimalarials that impact the blood stages of the parasite to alleviate the symptoms. New drugs aimed at the liver and transition stages are in their early days, but highly desirable. Lead compounds with gametocytic activity are crucial to ensure blocking of transmission of the disease to the mosquito vector. When these drugs are put through tests, they must meet multiple stringent criteria to be classed as an effective antimalarial. New drugs need to be extremely fast acting, ensuring the disease does not progress. The new drugs must be safe for use in pregnant/ Brest feeding women, children and ideally involve one dose in the overall treatment. Due to such high costs relating to the research and development sector, many companies and organisations are not willing to fund such research and trials, due to the risk of it not being economically viable. The Bill and Melinda Gates foundation, however, have displayed an interest in the development of new antimalarials in addition to funding the impregnated bed net projects in endemic areas.

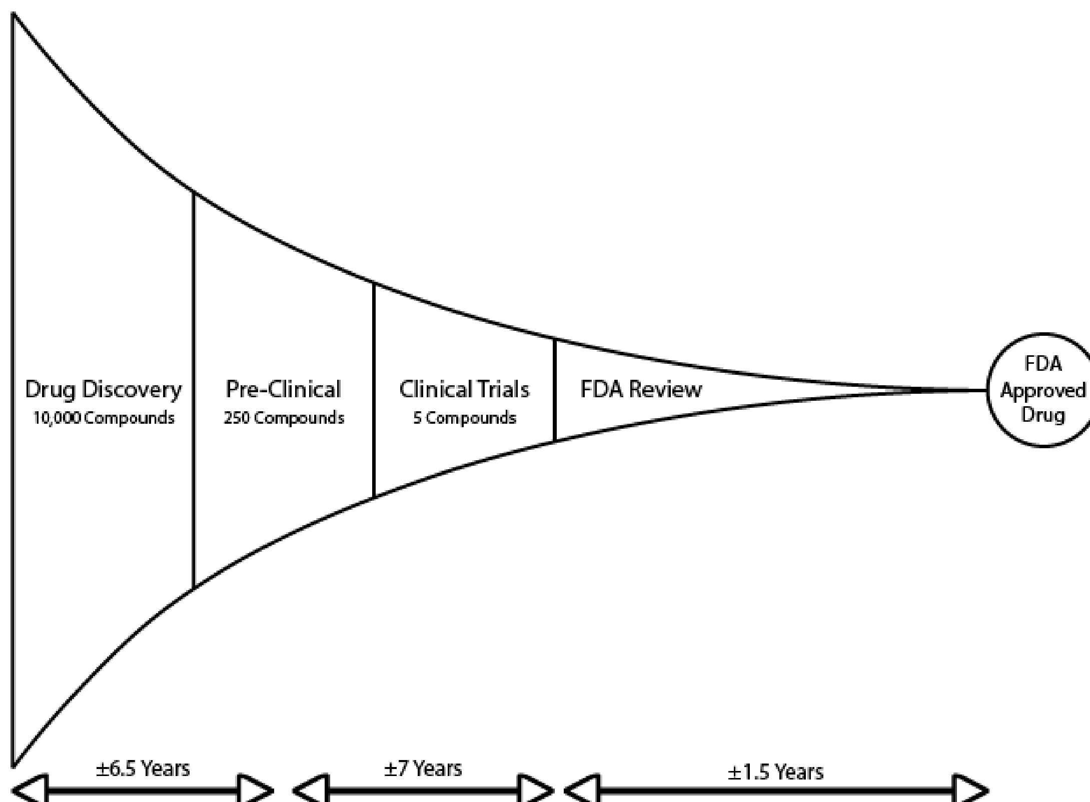


Figure 1.5 Drug discovery process showing the number of compounds that pass each stage of development along with the time scale it takes for each. (Redrawn from Trends in drug discovery and pharmaceutical research, 2002).

The main sectors behind drug development are academia and pharmaceutical companies. There are different ways a drug can be developed and these include random drug screenings of synthetic or natural product compounds, rational drug design and repositioning or re-purposing of old/existing drugs. The sequencing of the genome of the human body along with the genome of *P. falciparum* has resulted in an era with many more opportunities for drug development and related research. Genomics platforms along with bioinformatics have contributed to broadening options for drug discovery, rekindling hope that eradication of malaria in the future could become a reality (Drews, 2000).

1.11.1 Random screening of compounds

The random screening of compounds is a screening method that works by large libraries of compounds being used in screening assays against disease targets. This method uses high throughput screening and this is the typical starting point usually in the drug discovery timeline. It involves large libraries of compounds (natural and synthetic) alongside high throughput automated platforms and sophisticated software options for data analysis. A high number of drugs used on the market today have come about through random screening of these large libraries. Compounds within the library can be flexible and come from either chemically synthesised compounds or ones that have been derived from a natural product which is the focus of this thesis.

1.11.2 Rational drug design

This method involves design using computer modelling that predicts binding to proposed targets, to inform *in vitro* testing on parasite lines. For rational design to be implemented, x-ray crystallographic 3D structures of target proteins need to be available. Leads, once tested in the preliminary in vitro setting are then into pharmacokinetic, pharmacodynamic and toxicity testing (ADMETox), prior to *in vivo* testing on animal models and subsequently humans before they are deemed safe to use. Both screening and rational design are extremely time consuming and expensive with many compounds not passing the stringent test stages (Aguero et al., 2008).

1.12 Natural Products

1.12.1 Natural products as a route to drug discovery.

Natural compounds that have been extracted from any natural object, but mostly plants, have been used in traditional medicine for thousands of years. Plants have been used throughout history to prevent and cure a number of deadly infectious diseases. Primitive ancestors observed their natural surroundings and learnt to study the land and the animals around them over time, and through much trial and error, this led to the development of some of the medical plants that are still in use today. Palaeontology studies recoded in ancient Iraq indicate that Neanderthals possessed knowledge of plants with medical properties dating back to 60,000 B.C (Lietava, 1992; Ji et al., 2009) Compounds from plants that possess medical benefits have been described on ancient Assyrian tablets that date as far back as 2000 B.C. They have also been inscribed in ancient Egyptian hieroglyphics, and mentioned in ancient Indian, Chinese and European documents through history (Deharo & Ginsburg, 2011). In 2004 there were over 1,277 plants and 160 plant families that were commonly being used as traditional medicines to treat malaria alone. Since 2004 there has been extensive studies into many more natural products, increasing this number significantly, due to an increased interest in malaria research world-wide, especially in anti-malarial plants (Rasoanaivo *et al* 2011). There is a huge interest in antimalarials derived from natural products due to two of the leading malaria treatments quinine and artemisinin being able to trace their origin back to the use of traditional medicines (Graz *et al.*, 2011).

In rural areas in developing countries traditional medicines are often the only from of treatments that are generally trusted and accepted amongst communities. For these types of communities this is often the only source of treatment that can be accessed and afforded as they can be made locally for a fraction of the price of pharmaceuticals. People across the planet choose to use traditional medicines for many reasons, some

of them being: living in enclosed, remote areas where access to modern medical care is limited and traditional medicines are the first choice. Others choose traditional medicines because they believe they simply work better and have lost faith in the health care systems of the country they reside in. Fake drugs and side effects can add to the general mistrust in the Western medical systems (Bertrand *et al*., 2011). Some in India and in many places in China, still practice the study of ancient traditional medicines, where ancient techniques have been turned into new practises such as homeopathy, however it is important to understand that these practises have not been validated officially (Graz *et al*., 2011).

Many modern medicines derived are from a natural products, WHO records indicate that 74% of current drugs have a natural product origin. Many industrialized countries are increasing their usage of natural products in everyday life, a prime example of this would be naturopathy. Many natural products do however, have a downside, the main one being that many herbs and plants have not been through extensive scientific tests and clinical trials to determine the plant's effectiveness (Graz et al., 2011).

In recent times it is becoming crucial to understand and evaluate the properties of natural products given that 75% of the world's population is heavily dependent on their use in clinical and traditional settings (Global monitoring report, 2018).

Today most companies within the pharmaceutical industry are under immense pressure for new drugs to be available on the market. Complex discovery pipelines mean that the pharmaceutical industry faces significant challenges such as cost and time. Pharmaceutical companies today face challenges around sustainability. The cost of research and development has risen significantly since the 1980s costing up to $40 billion dollars, however, there has been no correlation between new drugs available and to the rising costs. With research and development prices exploding, companies

are searching for new approaches to drug design. Traditional medicines are becoming an attractive approach in relation to natural-based drug discoveries as drug discovery is not solely based entirely on molecular entities (Hagai & Eric, 2011; Timothy, 2011). A drug that has been rationally designed, standardized and tested scientifically deriving from natural products is named a phytodrug. They could potentially provide safer alternatives to chemically created treatments. The WHO has recognised this and has since created the Traditional Medicine Program (TMP). This program researches the use of traditional medicines from across the world, taking note of good and bad practice in order to establish useful scientific information to enable a safe dosing system of each natural medicine. The TMP also acknowledged that the role of traditional medicines for drug development is a reliable and sustainable solution for the health care industry (WHO Traditional Medicine report, 2005).

1.12.2 Historical importance of natural products as antimalarials.

The history of the chemotherapy of malaria is deeply linked with the history of herbal medicine (Timothy, 2011). Medicinal plants have throughout history played a crucially important role in the fight against malaria. From this success based on historical natural products being the mainstay of effective antimalarial chemotherapy, there is an important need to revert back to investigating new natural products as a main source for lead identification for new leads for antimalarial drug discovery. The first and most important of them all derives from the bark of a Chinchona plant, more commonly known as quinine.

1.12.3 Discovery of quinine

Quinine was introduced to Europe after a Spanish countess contracted a fever while in Peru and was cured by the bark found on the tree giving the new name of countess's

bark. The bark was then taken back to Spain introducing Quinine to Europe in 1638 (Achan, Talisuna et al., 2011). The bark of the tree was completely dried out and ground until a fine powder was achieved, the powder was then added to drinks such as wine and taken orally (Achan, Talisuna et al., 2011; Fluckiger & Hanbury, 1874). Before being taken back to Europe it was common for Europeans to use European and Asiatic plants as fever remedies (Stuart, 2004)

Quinine was later promoted by Cardinal de Lugo who was the Procurator General of the order of the Jesuits. The Jesuits then seized the opportunity and made profit by controlling the entire supply of bark into the west thus changing its name to Jesuits bark. Due to this in demand supply native trees became threatened and numbers started to decline, this the need for cultivation of the came about by Spanish doctor, Jose Celestion Bruno Mutis (Stuart, 2004).

During the 1820s, a couple of well renowned French pharmacists were able to isolate an alkaloid from the plant that they named quinine (Stuart 2004). Quinine is a cinchona alkaloid belonging to the aryl amino alcohol group of drugs, it is also cladded as a basic compound being presented as a salt (Achan, Talisuna et al., 2011).

Quinine was the most commonly used antimalarial up until the 1920s, after this there were just as effective if not more synthetic antimalarials available in the market such as chloroquine and amodiaquine (Mojab, 2012), The most important chloroquine (Achan, Talisuna et al., 2011). The discovery of quinine led to scientists wanting to synthesise the drug to make it more assessable, this resulted in the discovery of methylene blue and the dye industry. From this discovery them came the 4-aminoquinolines along with amino-alcohols which have been the main malaria treatments over the last century (Wells, 2011)

1.12.4 Other natural products as antimalarials

There are many naturally derived products that are still in use today as effective antimalarials. One of the most recent pursuits is the development of artemisinin from the plant *Artemisia annua* (see figure below). The plant has played a part in traditional Chinese medicine dating back to 168 BCE and was recommended in the 4th century as a cure for intermittent fevers (Wright et al., 2010). Artemisinins have had a longer correlation to usage and fever in comparison to quinine, however, was discovered as an antimalarial in 1971 by the Chinese army. The army screened 2000 plants in hope of finding a new antimalarial until they were successful and artemisinin was isolated from an extract from the plant. The random screening of 300,000 compounds by Walter Reed in 1970 led to the discovery of mefloquine and halofantrine. In the 1940s a natural-derived compound effective as an antimalarial named tetracycline was also discovered.

Figure 1.6 Artemisia annua showing the plant artemisinin was discovered from.

1.12.5 Natural product drug discovery processes for malaria

The image shown below (Fig 1.7) is a schematic representation of the process of natural product drug discovery. A different approach to this method is to select a natural product with potential new discovery prospects based upon its ethno-pharmacological use. This screening method involves both *in vitro* and *in vivo* assays that are used to screen against the malaria parasite. Following this, the extract is purified to isolate the active ingredient with a view to chemical synthesis and scale up. Synthesizing natural compounds has enabled researchers to modify the compounds resulting in their ability to enhance or suppress certain characteristics such as

efficiency and stability within the body. Organic chemists have been inspired by natural compounds to create novel drug synthesis (Kohan & Carter, 2005: Ji et al., 2005)

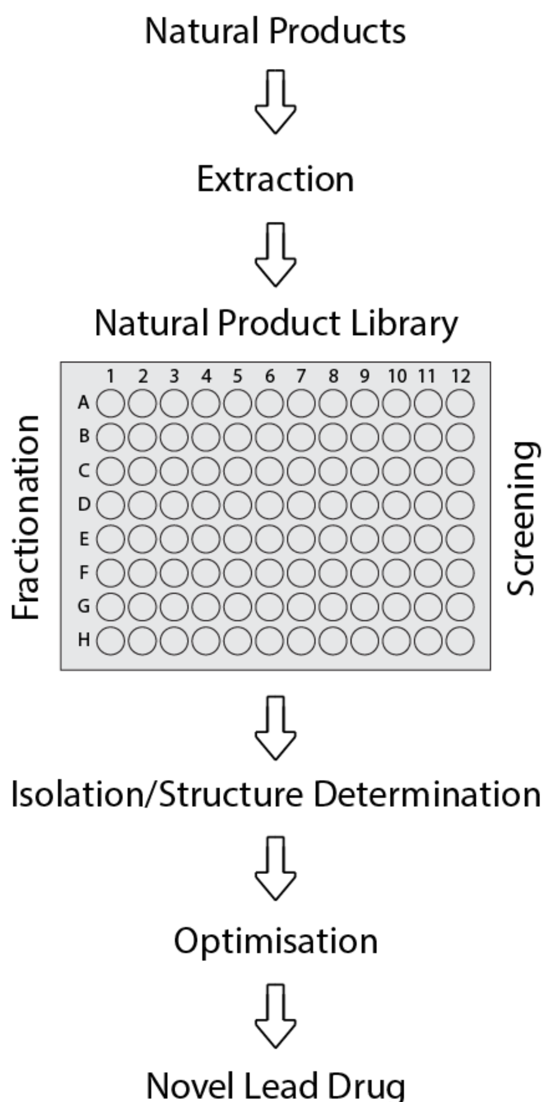


***Figure 1.7 Natural product drug discovery process** showing the process by which current natural drugs may be discovered (Redrawn from Maryam, 2016)*

1.12.6 Extraction

The extraction of the natural product is the first step when testing a product for antimalarial activity the process involves separating the compound from the raw

material. Usually, the product being extracted is carefully pre-washed, dried out and then ground. The grinding is an important step when extracting as doing so increases the surface area of product that comes into contact with the solvent being used for the extraction, thus improving the kinetics of extraction. For the extraction process many different solvents can be used depending on the natural product and the specific properties they possess. For plants that contain high levels of chlorophyll, hexane can be used beforehand to remove the chlorophyll so that the extract can be obtained. Hydrophilic compounds require solvents such as methanol, ethanol and ethylacetate while compounds that are lipophilic require solvents such as dichloromethane (Sasidharan et al., 2010), it is considered crucial that the correct solvent is used in order to maximise extraction (Singh, 2008)

There are several known extraction methods that can be used to obtain the compounds from the raw material, some of these methods include sonification and heating the raw material under reflux. The method that is widely used however is solvent extraction, this was the method used for the extraction process in this study. Solvent extraction involves introducing the solvent of choice to the ground surface of the raw product to penetrate into the solid matrix, the solutes in the product are then dissolved into the solvents used. The solute is then removed from the matrix and the solutes are collected (Singh, 2008; Sasidharan et al., 2010). Several factors can influence the extraction process some of these include the amount of time the compound was exposed to the solvent, the temperature at the time of extraction, the ratio of solvent to solid, the particle size of the materials and the properties of the solvent used (Singh, 2008).

When the extraction process is taking place, the finer the particle size, the more successful the extraction will be. Extraction is enhanced by the smaller size of the

particles due to penetration of the solvents and the diffusion of the solutes becomes smoother. However, if the particle size is too fine it will cause later problems upon filtration of the solution when drying. When the temperature is increased during extraction, it in turn increases solubility and diffusion of the particles. However, if the temperature becomes too high, it can cause the solvent to evaporate and compromise the quality of the extract. The time the compound is exposed to the solvent it crucial, however increasing this time will have no effect on extract once the equilibrium of the solute has been obtained within the solution. The amount of raw material exposed to the solution will affect the extraction results. More exposer of raw material will produce the more extract, too much solvent however till take time to concentrate upon the evaporation process (Singh, 2008).

There are other extraction methods available other than the conventional methods mentioned above as these require high solvent quantities and, in some cases, lengthy extraction times. There are more green and modern extraction techniques now available to combat these problems. These include microwave assisted extraction (MAE), pressurized liquid extraction (PLE) and super critical fluid extraction (SFC). All three of these new methods have been used to extract compounds from natural products successfully, these methods provide a low organic solvent usage with shorter extraction times with a higher selectivity (Singh, 2008)

1.12.7 Characterization and identification

There are several ways to identify and characterise specific compounds and a number of separation methods are frequently used, thin-layer chromatography (TLC), high performance liquid chromatography (HPLC), column chromatography and flash chromatography are the main methods used. TLC works in the same way as many

other types of chromatography, having a stationary and mobile phase. The compounds that are being separated interact with these two phases. The stationary phase in TLC is performed via a plate usually covered in silica gel, a small amount of compound is placed upon the gel. The mobile phase involves solvents in which the stationary phase would be placed, once placed the compounds would typically travel up the gel to later be viewed under a UV light for evaluation. The number of dots found upon the gel would represent the amount of compounds within the sample, the distance the dots travelled would determine how polar eatch compound was, the higher the dot the less polar and this could be a way of determining what compounds are present if you have an idea from previous literature on what to expect. TLC is used frequently due to the process being inexpensive, simple and rapid (Fair & Kormos, 2008; Sasidharan et al., 2010).

HPLC is much more versatile than TLC and is a common practice used within isolating natural products and is the most commonly used technique in this field. HPLC contains several different types, these include normal and reverse phase chromatography. For compounds that possess a higher polarity reverse phase chromatography is usually used. HPLC works much like TLC in which it works by using a mobile and stationary phase. The process of HPLC starts within the column of the HPLC machine, the column in this case represents the stationary phase and again usually consists of silica. The column is compact with long carbon chains, the length of the chain determines how apolar the column will be. Compounds are placed on the column where the mobile phase is started. Water and solvent are passed through the column and simultaneously elutes the compound (s). This then separates the compounds based on the polar charge where it can then be evaluated to determine the compounds

present. HPLC is the most popular method in the fingerprinting of herbal plants, it is used to separate the compounds of the natural product (Fair & Kormos, 2008).

There are techniques for identification that do not require chromatographic techniques. The main one being FTIR, but they also include techniques such as immunoassay and phytochemical screening assay. Immunoassays work well due to having high sensitivity, some techniques in immunoassays are more sensitive than the HPLC method as mentioned above. FTIR is a main technique that is used, it is a non-destructive tool that identifies chemical bonds found within compounds, it works on anything that possesses covalent bonds regardless of the sample being organic or inorganic. FTIR typically provides a molecular fingerprint of a compound using the compounds unique spectrum. The benefit of using FTIR is that it can be used on whole solid material, powders, liquids and even on whole cell analysis. In this study FTIR was used on whole sample, this FTIR technique was called Attenuated total reflection (ATR).

ATR spectroscopy, which can also be named as Internal Reflectance Spectroscopy (IRS); is mostly used for the study of surface, or the near surface of biological materials. It has been used for investigating samples which are too thick for transmission measurements or for samples that are opaque (Harrick & Beckmann, 1974). The spectra obtained from samples which have undergone ATR-FTIR, are similar to that of regular transmission spectra, but the ATR samples do not produce totally absorbing spectral bands. This is because its effective path-length is controlled by properties of the crystal which is used in the apparatus; this reduces the sample preparation time. ATR spectra have both reflection and absorption features, meaning they cannot always be used in quantitative analysis directly, but can be used to obtain information for confirmation of species (Grdadolnik, 2002).

ATR functions by measuring what changes are found from vibrations to chemical bonds when an internal IR beam is reflected when beamed onto the surface at a sample. The IR beam is reflected back into a crystal which has been placed on a specific angle, which is shown in the schematic diagram in Figure 1.8. The IR spectra is then recorded giving the wavelengths of a sample. This method is extremely easy to use and one of the main advantages of this method is that is not destructive.

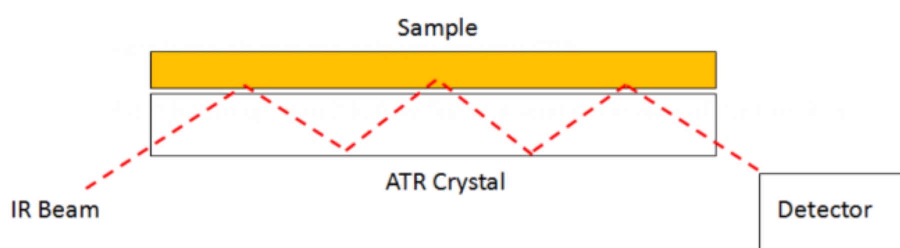


Figure 1.8 ATF-FTIR *showing a redrawn image taken from Thermo fisher. it depicts a schematic diagram of ATF-FTIR. It shows the infrared beam passing through the crystal then hitting the detector.*

1.12.8 Trametes versicolor

Mushrooms, for many years have been sought after for their many health benefits, including, increasing antioxidant activity, enhancing the immune system, managing blood sugar levels, improving brain performance and even increasing exercise levels (Zhang, Li, Liang & Reddy, 2017). Mushrooms have been found to have antifungal, anti-viral and anti-bacterial characteristics, with many commonly used antibiotics derived from fungi. Medicinal mushrooms are a plentiful source of immunoceuticals, due to the broad immune activity they possess. There are many other natural product species that have been known to possess immunologic activity, however some

mushroom species and the polysaccharides extracted from them are the most thoroughly studied. Certain mushroom species have been studied in both preclinical and clinical trials (Standish *et al.,* 2008).

There are more than 2000 edible mushroom species described while 270 have shown to possess immunotherapeutic properties due to having an effect on hematopoietic stem cells, T and natural killer cells (Guggenheim, Wright, & Zwickey, 2014)

. Of the 270, 50 of these are classified as non-toxic. 6 out of the 50 non-toxic have been used in studies on human cancers. One of these mushrooms being *Trametes versicolor,* currently classified as a medicinal mushroom, as a result of passing clinical trials (Phase I, II and III) for different specific types of cancer including stomach carcinomas in humans. Data indicates that *T. versicolor* helps patients have disease free intervals and improved overall survival rates by boosting the immune system (Standish *et al.,* 2008). *T. versicolor* has become one of the most studied medical mushrooms due to its potency (Hobbs, 1995; Stamets, 2000).

Belonging to the Basidiomycetes class of fungi, *Trametes versicolor* known more commonly as the “turkey tail” is a relatively common species of fungi found worldwide. This relatively small polypore fungus is usually found in clusters and has a ring like structure with a pale brown/ white underneath (Figure 1.9) (Hobbs, 2004). Like many other species it can be spotted virtually anywhere that dead decaying trees, hard wood and stumps are left to decompose naturally. Playing a part of a crucial recycler in the ecosystem. Like many other natural products, *T. versicolor* has been used as a traditional herbal medicine for many years in Asia, due to its immune system boosting benefits (Standish *et al,.*2008).

Figure 1.9 Trametes versicolor *Showing a cluster of Trametes versicolor growing upon decaying wood. The Colours and separate bands on the upper side of the fungus are clearly visible. (source: https://www.mushroomexpert.com/trametes_versicolor.html)*

T. versicolor was discovered to contain two related proteoglycan constituents called Krestin (PSK) and polysaccharide peptide (PSP) after a study in Japan showed cancer remission after the consumption of *T. versicolor* (Standish *et al,.*2008). PSK first got approved in Japan in 1977 due to its high molecular weight fractions (Hobbs, 2004). Krestin (PSK), was considered safe after a toxicologic assessment found that the lethal dose was extremely high. The reports of the toxicity test shown that there were no reports of abnormalities in either humans or animals following both chronic and acute tests (Standish et al*.,* 2008).

Previous studies, have shown PSP, PSK and mycelial extracts as a whole to possess a wide spectrum of immunological effects (Standish et al., 2008; Guggenheim, Wright, & Zwickey, 2014). Some of these effects are thought to be triggered by having contact with immune tissue found within the digestive tract. The cell wall components found within *T. versicolor* such as PSP are hypothesised to have an interaction with the Langerhan and dendritic cells within the small intestine. Due to these interactions with the digestive tract, many immune cells are subsequently affected. *T. versicolor* extracts*, in vitro* and *in vivo*, given orally or as an injection have been known to substantially have an impact on the stimulation activity of T cells. They have also been found to stimulate bone marrow cells along with natural killer cells and lymphocyte killer cells. Along with cells, they have also been shown to increase the level of antibodies (Hobbs, 2004)

Throughout the world, *T. versicolor* extracts are being used alongside traditional cancer-based treatments as an additional treatment. These are taken as prescribed medication or as a dietary supplement alongside conventional treatment. For people using the extracts as a supplement, they are doing so with the belief it has the ability to prevent cancer. It is also believed to prevent viral infection, improve aging and a host of problem causing ailments (Stamets, 2012; Kumar & Adki, 2018). When used as a cancer-preventive long-term, *T. versicolor* extracts are thought to have the ability to inhibit carcinogenesis via an ability to reduce effects from some known carcinogens such as asbestos and tobacco smoke on host cells (Hobbs, 2004).

When *T. versicolor* extracts are used alongside chemo and radiation therapy, some evidence shows that there is a possibility that it can reduce the risk of secondary malignancies that have been induced as a direct result of radiotherapy and

chemotherapy. *T. versicolor* extracts can also protect healthy cells while undergoing such treatments (Hobbs, 2004).

PSP extract from *T. versicolor* has shown preclinical and in clinical studies to have oncologic and immunologic activity for patients who suffer from breast, gastrointestinal and lung cancers (Standish et al., 2008).

Syndromes such as septic shock are a complex syndrome resulting from lipopolysaccharide gram-negative bacteria binding to immune cells. Currently, a large number of patients are being diagnosed with septic shock daily, because of such large numbers new therapeutic possibilities are necessary. Fruiting body extracts from *T. versicolor* have shown to inhibit *in vitro* binding of lipopolysaccharide. These extracts could possess lead structures for further drug development against lipopolysaccharide-mediated septic shock (Lindequist, Niedermeyer, & Jùluch, 2005)

Figure 1.10 Trametes versicolor dried *showing a close up of the visible rings on the upper side if the Trametes.*

1.12.9 Acacia nilotica

Acacia nilotica is considered an important medical plant belonging to the family *Fabaceae* of the genus *Accacia* (babul) (Rather *et al*., 2015*)*. It is commonly found abundantly scattered across sub-tropical and tropical regions such as Africa, south Africa and India. The plant is scented and usually grows between 5 to 20 meters with a spherical crown (Ali *et al*., 2012), branches and stems have a deep dark appearance, with a grey/ pink bark. The plant has thick spines for protection and has bright yellow flowers when in bloom located at the end of the branches usually between 2 to 3 centimetres long (Ali et al., 2012). The pods found upon the tree are strongly constricted with a hardened exterior, white grey appearance that are hair like and thick (Baravker *et al*., 2008).

All parts of the tree have been known to have medical benefits in many different medical fields. The roots have been used on cancers and tumours, particularly cancers of the eye, testicular and ear (Kalaivani & Mathew, 2010). The leaves found upon the tree have been used as anti-bacterial, anti-cancer to treat diarrhoea, Alzheimer's and even as dressing for wounds (Kalaivani & Mathew, 2010). The gum on the tree has been found to contain anti-asthmatic compounds (Baravker *et al*., 2008). The bark of the tree is most commonly used as an antibiotic. it has been shown to have antimicrobial activity especially on *E. coli*, *S. aureus* and *Salmonella typhi*, curing food poisoning in many tropical regions (Banso, 2009). It has also shown signs of being anti-fungal. The pods found on *A, nillotica* have shown to inhibit HIV 1 and also Hepatitis C. Extracts of the pod along with crude extract on the root have shown to possess high antimalarial activity on *P. falciparum*. Root extracts have the same effect as chloroquine on *P. berghei* in mice (Jigam, 2010). The pods are also used to treat malaria in Nigerian communities (Alli et al., 2016).

Phytochemical screening of *A. nilotica* bark confirmed the presence of glycosides, alkaloids, terpenoids and saponins. Steroids and flavonoids were not found to be present in the bark extract, however flavonoids are presentt elsewhere. The bark extractions used upon the agar diffusion method has shown antimicrobial activity against several different bacterial species, including the main ones as mentioned above. Decrease in patients' blood pressures have been reported after use of a methanol extract from the pods. This indicates anti-hypertensive activities that are autonomous of muscarinic receptor stimulation (Alli et al., 2016).

Extracts obtained from the powder of the bark using different solvents in a study by Del (2009), found free-radical scavenging activity via maceration extraction (Alli et al., 2016). In a similar study by Amos et al (1999) revealed *A. nilotica* to be a source of accessible antioxidants. which can be used as additional supplements in the fight against free radical mediated diseases including cancer, inflammation and diabetes. It is thought the high scavenging properties could be caused by hydroxyl groups (Alli et al., 2016).

Figure 1.11 Acacia nilotica pods *showing the pods from A. nilotica that have previously been dried out.*

1.13 Aims and objectives

This study aims to investigate the potential prospects of natural products as effective antimalarials for future use. Using multidrug resistant *Plasmodium falciparum* K1, *Trametes versicolor* and *Acacia nilotica* extracts were investigated to determine the level of antimalarial activity. Same extracts were also tested upon HEPG2 and MDBK cells to determine the cellular toxicity levels in order to investigate their potential as novel natural product antimalarial drug leads.

CHAPTER TWO

2.1 Materials and methods

2.1.1 Collection of samples

The samples of *Trametes versicolor* were collected from three different woodland locations around Manchester. The samples were harvested and left to dry over several days.

2.1.2 Fourier-transform infrared spectroscopy (FTIR)

Once dry the samples were placed in Fourier-transform infrared spectroscopy (FTIR) machine to determine that *Trametes* were of the same species. Tests were performed in a Nicolet 5700 FTIR testing machine with a range of 4000 to 800 cm^{-1}, with a resolution of 4, a scan rate of 32 and an absorbance mode from which CO_2 was omitted. Before testing, a blank background was collected as a correction. Files were saved as cvs. and spa. The same tests were performed on the extracted product along with *Acacia nilotica*.

2.1.3 Extraction

The dried samples were blended until only a powder was remaining, the powder was then transferred into a container where the stock was built up. The powder was then added to 50 ml falcon tubes containing 40 ml ethanol and was left to soak at room temperature for 48-hours. The solvent extract was removed using a Pasteur pipette and added to falcon tubes and stored in a refrigerator at 4°C until a sufficient amount of stock was gathered. The extract was then placed into a 50 ml round bottom flask and ethanol removed on a rotary evaporator. The process was repeated until there was a sufficient extracted compound for analysis. The flask was then given an ethanol wash and the solvent was placed in four Eppendorf tubes with the lids open to enable

to ethanol to evaporate leaving behind only the extract. The extract was then stored in a refrigerator at 4°C. Methanol and water extractions were previously attempted, during method optimisation after process, initial compensations of 0.0204 mg of yield showed ethanol to be more effective (Kerry Leigh Dreyer, 2018).

2.1.4 Stock concentration

The extracted materials were dissolved in 1ml DMSO where it was then further diluted using complete media. The dilutions used for all experiments were 200, 100, 50, 25 and 12.5 µg/ml and all concentrations contained <1% DMSO to avoid toxicity to *Plasmodium falciparum* cultures.

2.1.5 In vitro culture of Plasmodium falciparum

All experimental work was carried out at the pathogen laboratory in the University of Salford. Experiments were performed under a sterile hood (ESCO class 11 Biological safety cabinet) using sterilised techniques and pre-sterilised equipment. All surfaces and equipment were sterilized using 70% ethanol. Virkon (Antec international, UK) was used for disinfection of waste before autoclave. All protocols by Read and Hyde (1993) were followed.

2.1.6 Preparation of complete media

In a 50 ml falcon tube 2.5g of Albumax was added along with 15-20 ml of RPMI 1640 (containing 25 mM HEPES buffer, sodium bicarbonate, 0.3/ L l-glutamine, 10% human serum and 1 ml of washed erythrocytes) and placed upon a vortex until fully dissolved. In the falcon tube 2.5 ml of hypoxanthine (1mg/ml), 500 µl of gentamycin (50 mg/ml)

and 2.5 ml of glucose (40%). The final mixture was then syringe-filtered into the newly opened bottle of RPMI. 10 ml of the complete media were then added to a small culture flask and paced into the incubator.

2.1.7 Washing media

A 500 ml bottle of pre-sterilised RPMI 1640 1x (+) L-Glutamine (+) 25mM Hepes (Gibco, Life Technologies, UK) without additives was used as washing media throughout the study and stored at 2-8°C for up to 2 weeks.

2.1.8 Preparation of human blood culture of Plasmodium falciparum

Human O+ blood was obtained from the Manchester blood bank. 200 ml of the whole blood were transferred into sterile falcon tubes and the remainder into a sterile flask and stored in the fridge. The whole blood was centrifuged for 5 minutes at 30,000 – 31,000 rpm. The supernatant, containing plasma and leukocytes, was removed and discarded, leaving behind only the red blood cells (RBCs). RPMI was then added, resuspending the RBCs well. The mixture was then centrifuged as mentioned above and the process repeated three more times until the RPMI appeared clean. 5-10 ml of complete media was then added, resuspended and centrifuged. The final top layer was then removed leaving behind RBCs that were at 100% haematocrit. An equal amount of complete media was added to give 50% haematocrit. Once washed 0.5 ml of the blood was added to the flask containing complete media and mixed well. The remaining blood was stored in the fridge at 4 ° for future use.

2.1.9 Preservation in liquid nitrogen

When predominantly at ring stage with a parasitaemia >10% the culture was selected for preservation in liquid nitrogen. The culture was centrifuged at 3,400 rpm for 5 min. the supernatant was then removed and the culture brought back to a 50 % haematocrit by adding an equal amount of warm complete media. Aliquots of the culture were transferred into 2 ml cryotubes and 0.5 ml 20 % dimethyl sulphoxide (sterile filtered DMSO, Sigma, UK) in Ringer's solution (9 g NaCl, 0.42 g KCl and 0.25 g $CaCl_2$/ Litre) was added. The cryotubes were then immediately placed into the liquid nitrogen for later use.

1.1.10 Revival from liquid nitrogen

The method used to culture the drug resistant K1 strain of P. *falciparum* was *In vitro* cultivation. The parasites in frozen form are stored in liquid nitrogen. The Cryo vial was removed from the liquid nitrogen and placed into an Eppendorf tube. The parasites were centrifuged for 90 seconds at 14,000 rpm. The DMSO supernatant was then removed and 1 ml of 10% sorbitol was added. The mixture was then resuspended and centrifuged for 90 seconds at 14,000 rpm and then supernatant was removed. 1 ml of complete media was then added and centrifuged as above, the supernatant removed and a final 1 ml of complete media added, mixed well and then placed into the culture flask containing the washed blood and RPMI. The flask was then gassed for fifteen seconds with a gas mixture of 5% O_2, 5% CO_2 and 90% N_2 and placed into the incubator where it was incubated at 37 ^{o}C for 48 hours.

2.1.11 In vitro Plasmodium falciparum culture

To begin a new culture, 10 ml of complete media and 0.5 ml of washed blood was added to a 50 ml culture flask and warmed to 37°C prior to the addition of the parasites. Approximately 0.5 ml of parasitised blood retrieved from liquid nitrogen was then added to the warmed culture media to give a final haematocrit of 5 %. Following inoculation, the parasite culture was gassed with a 5 % CO_2, 5 % O_2 and 90 % N_2 gas mixture (BOC Limited, UK) and placed in the incubator at 37°C. The culture was left for periods of 48 hours in the incubator at 37 °C. To avoid the culture overgrowing frequent media changed as fresh non-infected RBCs was needed to maintain a healthy culture, along with gas. Generally, the culture was maintained at around one – three %.

2.1.12 Synchronisation of Plasmodium falciparum

Sorbitol (5 % w/v) was prepared in distilled water and filtered through a 0.22 µm filter. The sorbitol solution was then added directly to pelleted parasite culture (9 ml to 1 ml of culture pellet) and incubated for 5 mins at room temperature. Following this, the culture was centrifuged at 3,400 rpm for 5 mins and the supernatant was discarded. The parasite pellet was then subjected to three washing steps in complete media, before re-suspension in complete media at 50% haematocrit. The synchronised parasite culture was used to set up a new culture as described previously.

2.1.13 Slide preparation and estimating parasitaemia

100 µl of the culture were removed from the flask and transferred into Eppendorf tubes. The tubes were then centrifuged at 3500 rpm for two - five minutes. The supernatant was then removed leaving a small amount of media remaining to mix the pellet. Once mixed a small droplet was added to the glass slide and thinly smeared using the end

of another slide, leaving behind a clean thin blood smear. The slides were then left to air dry in the incubator. Once the slide was completely dry 100% methanol was applied to the slide to fix the cells, and once again left to completely dry. The slides were then covered in 10% Giemsa stain and left for twenty minutes. The Giemsa stain was then washed off where the slide was then left to dry for a final time.

The parasites were examined using the immersion oil lens at 100x magnification. The parasites were then observed to check the life stage and level of parasitaemia. The total number of infected RBC was divided by the total number of RBC then X100. This was repeated three times in three different fields and an average was taken to determine the final percentage of parasitaemia. Upon this the formula $C_1 V_1 = C_2 V_2$ was used to obtain the desired level of parasitaemia for an experiment or cultivation.

1.2.14 Plate assay for Plasmodium falciparum

The parasites were first washed to remove any dead or fragmented parasites to avoid inaccurate results. The parasitaemia then diluted to 1% using freshy washed blood and complete media. The haematocrit was maintained at 2.5%. The experiment was set up on a black bottom 96 well plate. Firstly, 100 µl of the drug dilutions 200, 100, 50, 25 and 12.5 µg/ml of each compound was added in triplicates on the plate. 100 µl of the blood, parasite and media were then added to each well. A positive control was used containing 100 µl of media and 100 µl of the parasitized blood along with a negative control containing blood and media. The outer well on the plate was filled with 200 µl PBS to avoid the plate drying out. The plates were then placed into chambers, along with a small cap of distilled water, where there were gassed for three minutes with the 5% O_2, 5% CO_2 and 90% N_2 gas mixture and incubated for either 72 hours (trophozoite) or 48 hours (ring stage).

2.1.15 SYBR Green Microtiter plate assay

The SYBR Green solution was prepared by adding 3.3 µl of SYBR Green into 10 ml of PBS. After the incubation period 140 µl of the media was removed from each of the wells and 140 µl of the SYBR Green solution was added. The plate was then left in a dark room for 40 minutes while the SYBR green stained the parasites. The plate was then taken to the microplate fluorescence reader with the measure parameters set to gain 70 and set to read plate.

2.2 MTT HEPG2/ MDBK

2.2.1 Media preparation

From a fresh bottle of RPMI containing L- glutamine, 60 ml of the RPMI was removed as set aside. To the bottle of RPMI 50 ml of fetal bovine serum, 5 ml of penicillin at 1% and 5 ml of L- glutamine were added to make the complete media.

2.2.2 Culturing of cells

First the flask was placed under the microscope to determine the level of cells in the flask. The media was then removed and the flask was washed with 1x PBS, the flask was washed three times. Once washed, 3-5 ml of trypsin was added and the flask was incubated at 37°C for three minutes. After incubation the flask was gently tapped and place under the microscope to ensure the cells were fully detached from the flask. 8 ml of media were then added to the flask and the entire contents transferred into a falcon tube. The falcon tube was then centrifuged for five minutes at 1500 rpm. The supernatant was then removed. In a fresh flask 14 ml of media and 1 ml of culture were added and then left to incubate once again.

2.2.3 MTT Plate assay

The cells were diluted accordingly in 96 well plates to give 4 x 103 cells (100µl) per well. On a 96 well clear flat bottom plate 100 µl of the culture were added to each well, with 200 µl of media placed on the outer wells to avoid the inner well drying out. The plates were then left in the incubator for 24 hours. The next day the compound dilutions were added 200, 100, 50, 25, 12.5 and 6.25 µg/ml. The plates were then incubated for five days.

Once the plates were ready to read 50 µl of MTT solution was added to each well and the plates were incubated in a dark environment for a further three hours. After the three hours all the media plus MTT solution was removed from each well. In each of the wells 150 µl DMSO was added and the plate was immediately placed into the plate reader

2.3 Data analysis

Microsoft Excel was used to transfer the data from the plate readers and calculate parasite growth and inhibition. Graph Pad was another programme used to generate the graphs and acquire the IC_{50} values from each of the experiments. The tables were made using Microsoft Word.

Chapter 3

3.1 Results

3.1.1 Extract of *Trametes versicolor*.

Briefly two methods were attempted: ethanol and methanol extraction. The methanol extraction failed to yield adequate extracts and further experiments were confined the ethanol extracts for *Trametes.*

Initial ethanol extraction methods reliant on maceration of the samples in ethanol and ethanol evaporation overnight resulted in poor yield (Fig. 3.1). Subsequently, solvent from the macerated ethanol suspension was removed by rotary evaporation which yielded better powder extracts of 0.0204 mg (Fig. 3.2 & 3.3). The methanol and ethanol extracts of *Acacia nilotica* produced good yields of 0.0394 mg.

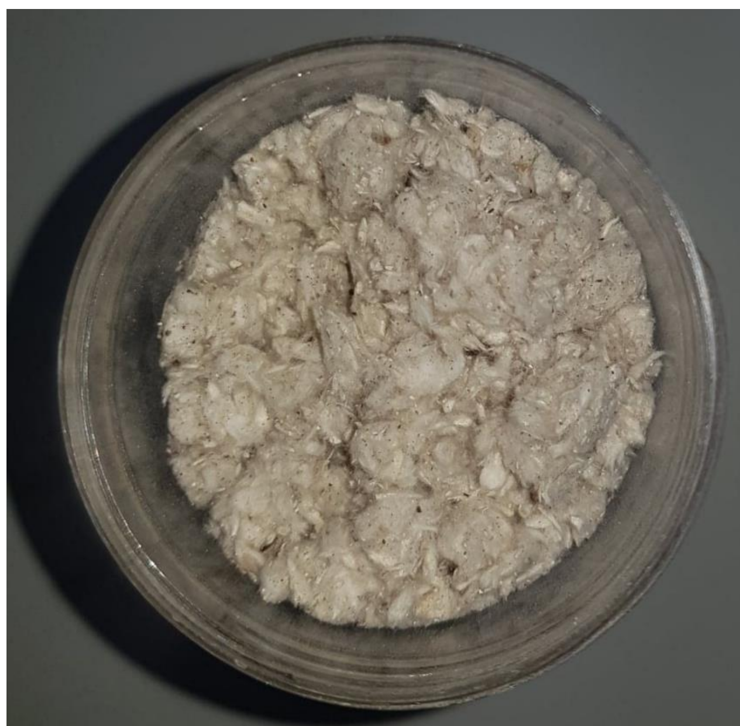


Figure 3.1 Ground and whole Trametes versicolor *showing the Trametes versicolor once ground and before being exposed to the ethanol for extraction.*

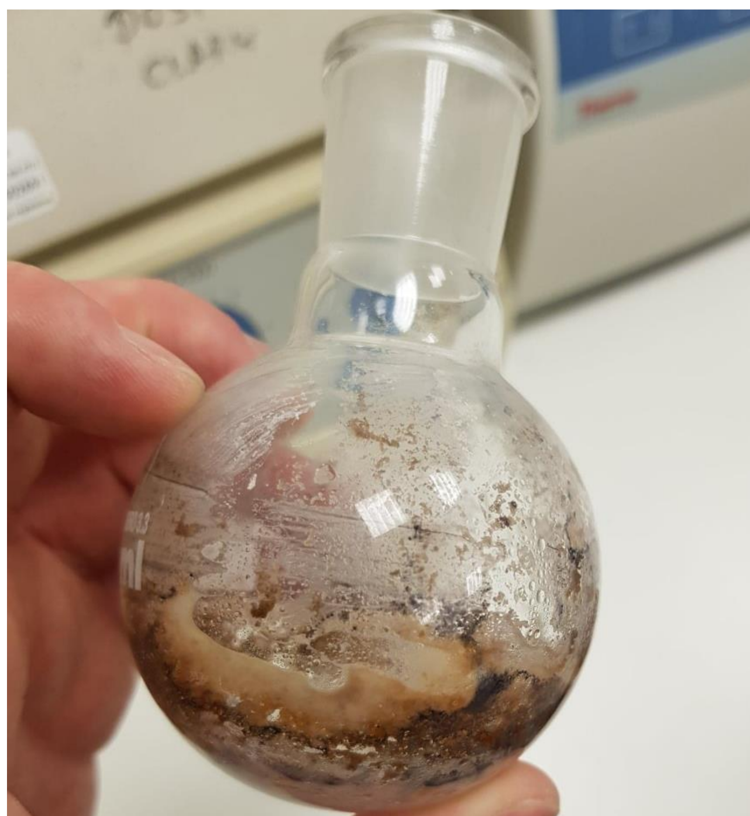


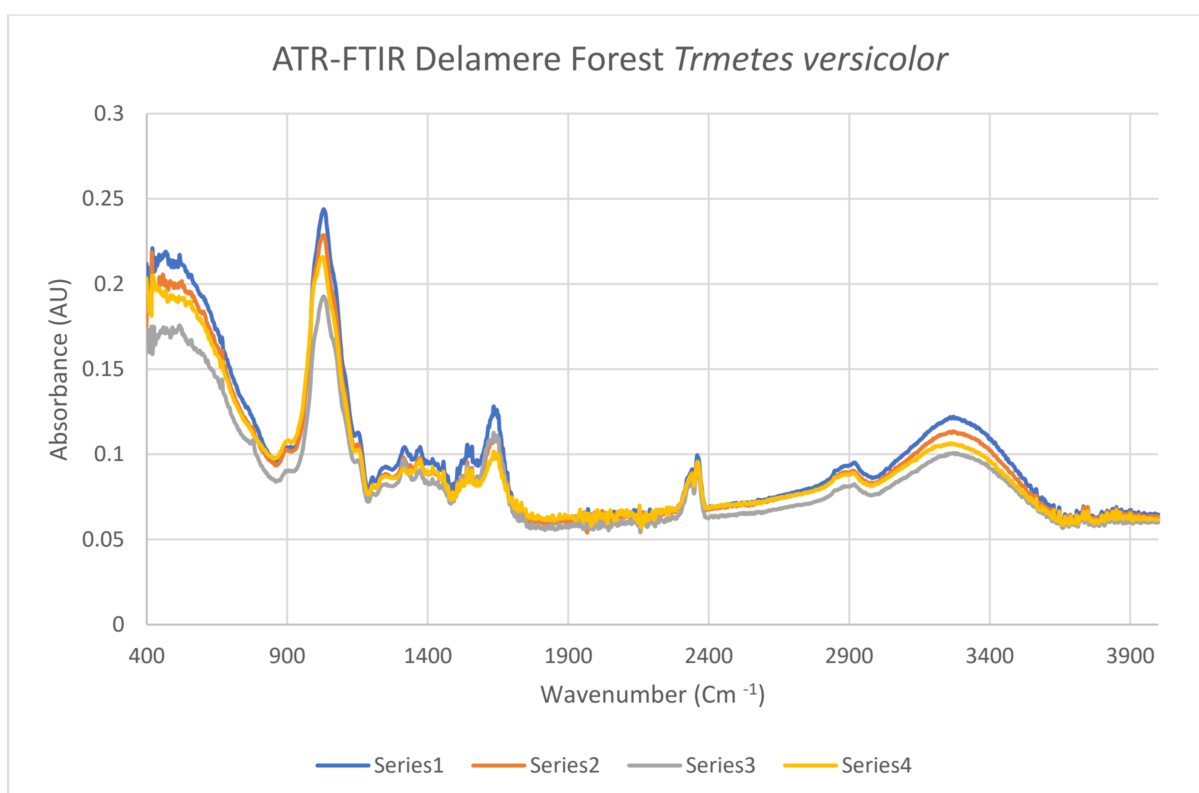
Figure 3.2 Auto evaporation of Trametes versicolor *showing the ethanol extract after auto-evaporation.*

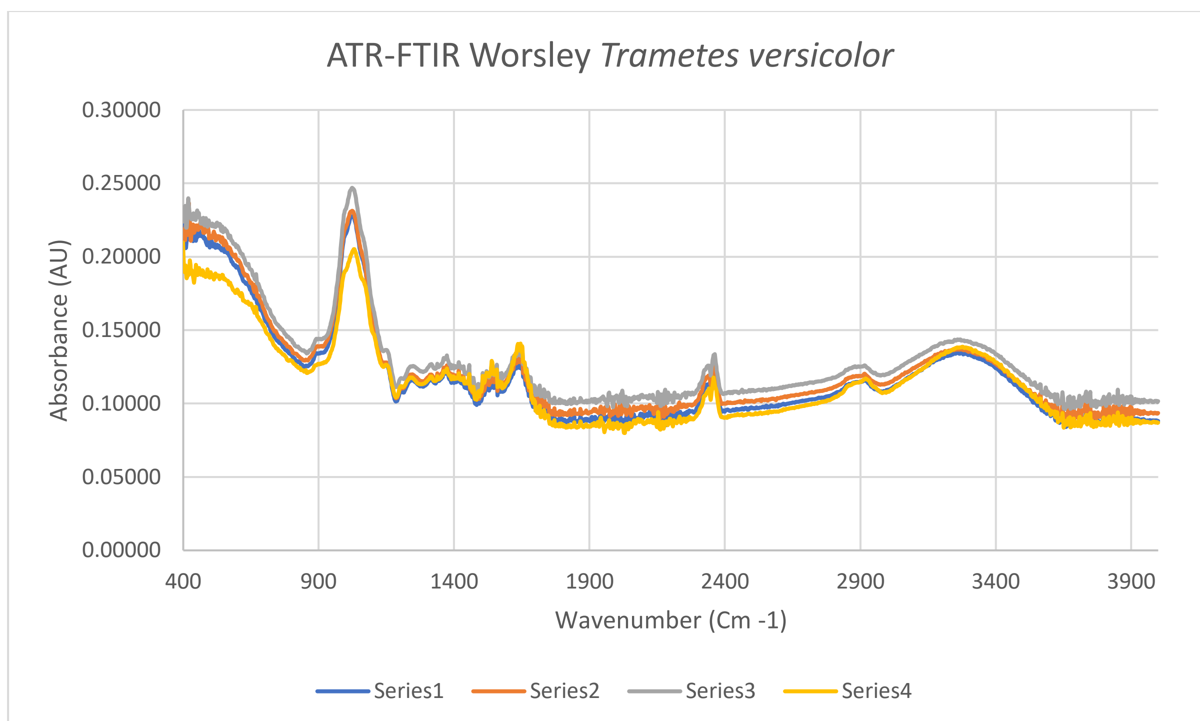
Figure 3.3 Final ethanol extract *after having a wash with DMSO and and left to dry. This was the extract used in this study.*

3.2 Fourier-transform infrared spectrometry (FTIR)

3.2.1 FTIR of Trametes versicolor

The samples of *Trametes versicolor* were collected from three different locations in the North West of England (Delamere forest, Worsley woods and Ellesmere park Eccles). In order to confirm they were of the same species, each sample was characterised by Fourier-Transform infrared spectrometry (FTIR) before extraction to avoid mixing different species. It was observed that all the stretching modes were similar amongst all the samples. FTIR fingerprint was also performed on the ground and extracted *T. versicolor (Fig. 3.4)*. All figures show 4 series per graph.





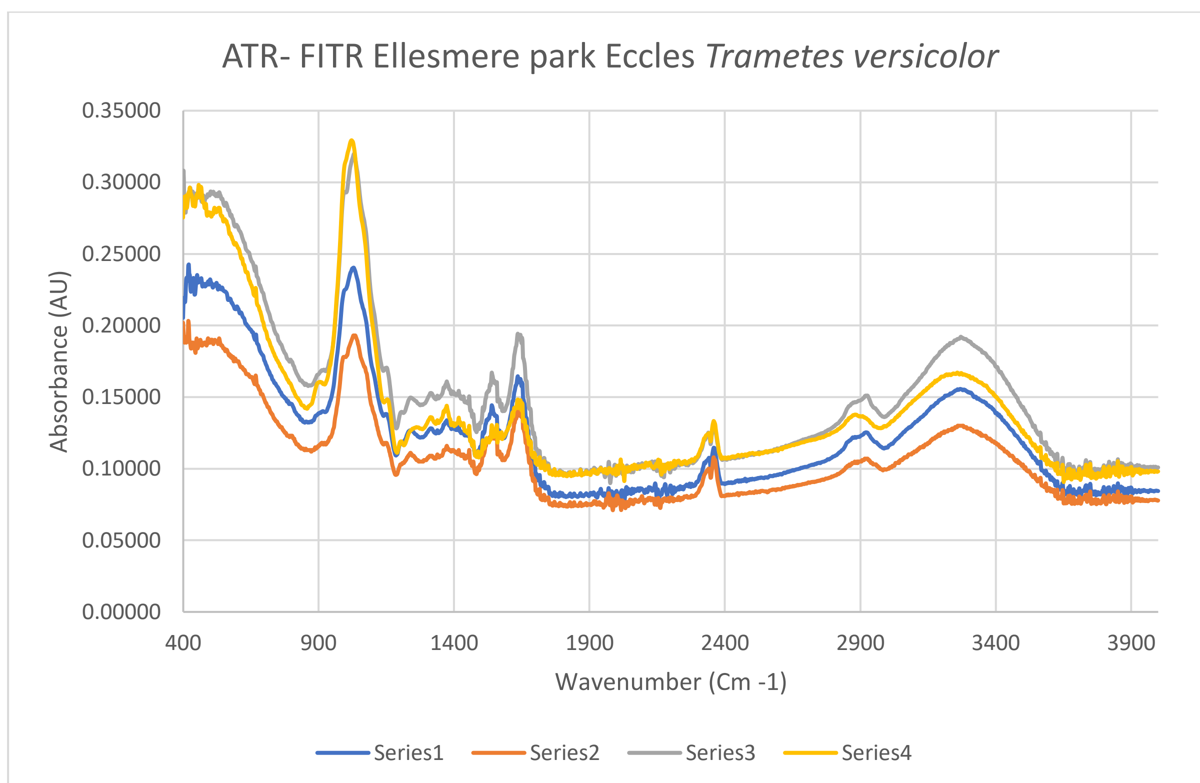


Figure 3.4. FTIR analysis of Trametes versicolor *found in Delamere forest, Worsley forest and Ellesmere park Eccles. All three were repeated four times to give more accurate reading.*

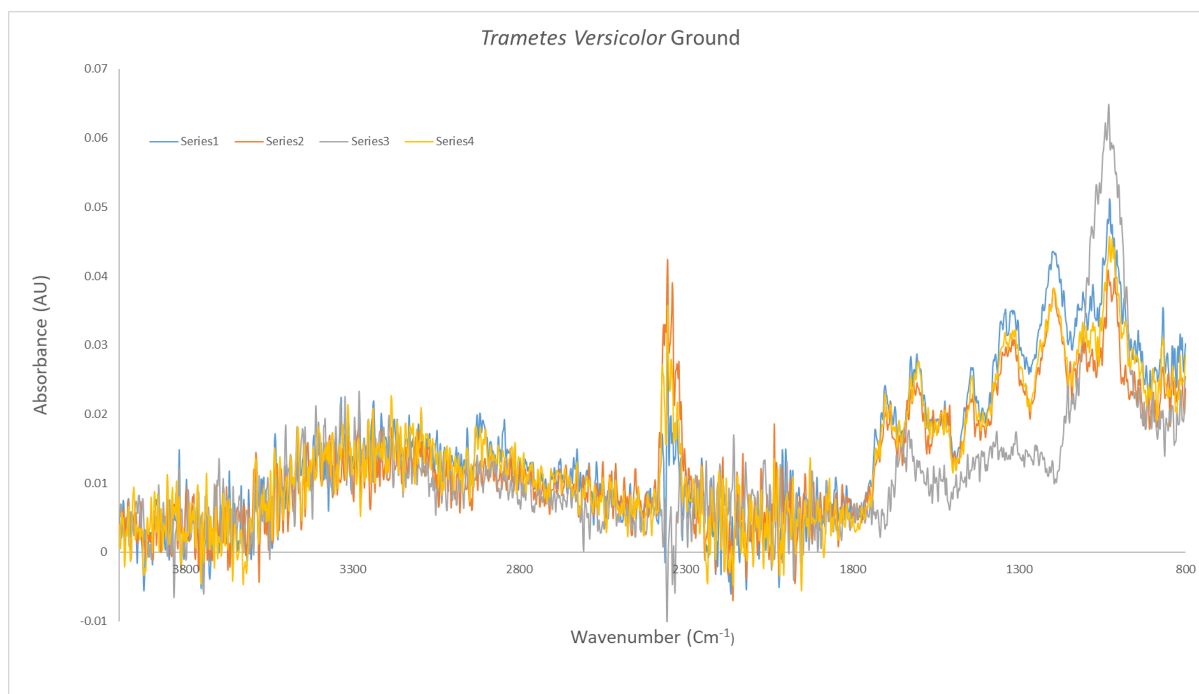


Figure 3.5 FTIR ground Trametes versicolor *showing wavenumber alongside absorbance of the FTIR fingerprint on the sample once ground into a powder.*

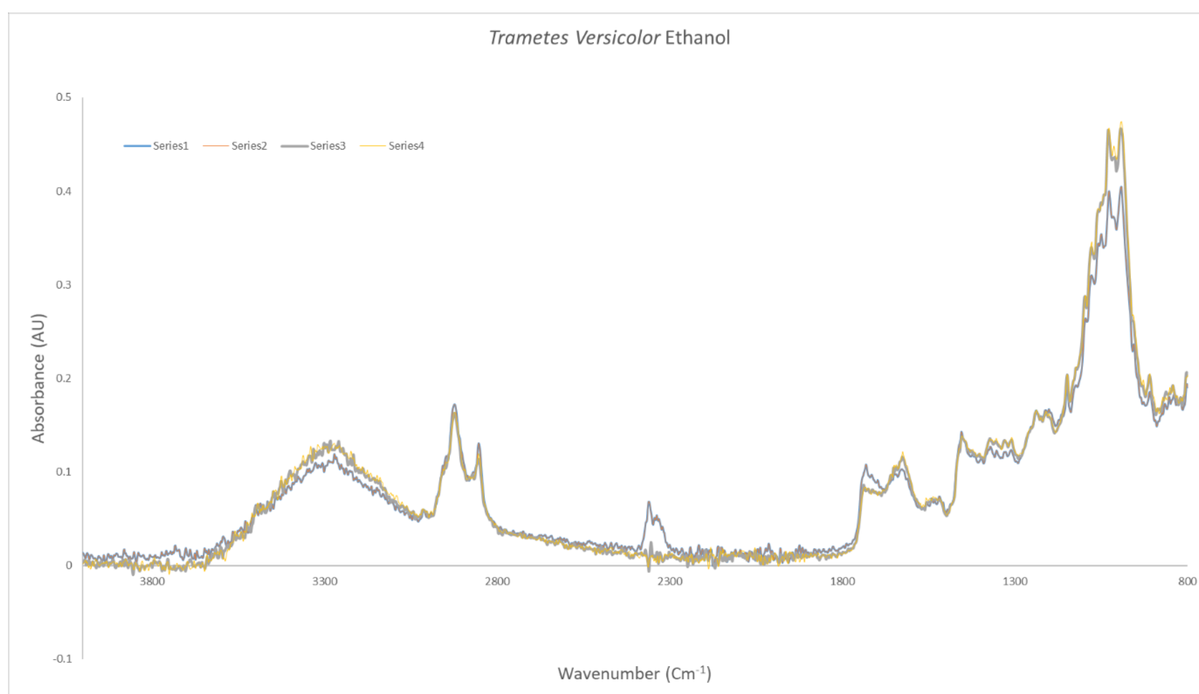


Figure 3.6 FTIR of the Ethanol Trametes versicolor *showing wavenumber alongside absorbance of the FTIR fingerprint of the final extract.*

3.2.2 FTIR of Acacia nilotica

FTIR fingerprint spectra analyses were also carried out for *A. nilotica*. The first FTIR fingerprint performed was on the whole pod (Fig. 3.7) and after grinding (Fig 3.8). FTIR fingerprints were then produced for the ethanol and methanol extractions (Fig 3.9 & 3.10). All figures show four series per graph.

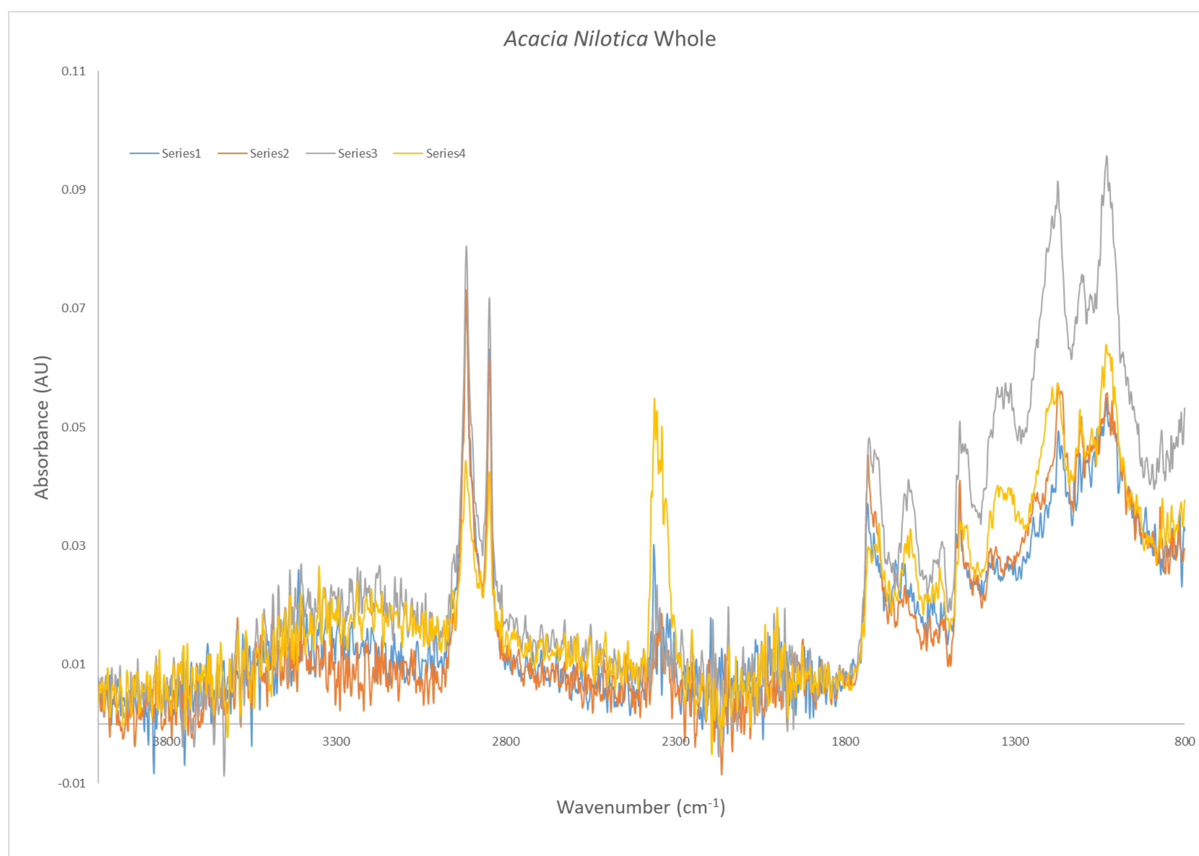


Figure 3.7 FTIR of Acacia nilotica whole *showing the wavenumber alongside absorbance of the FTIR fingerprint of the whole A. nilotica pod.*

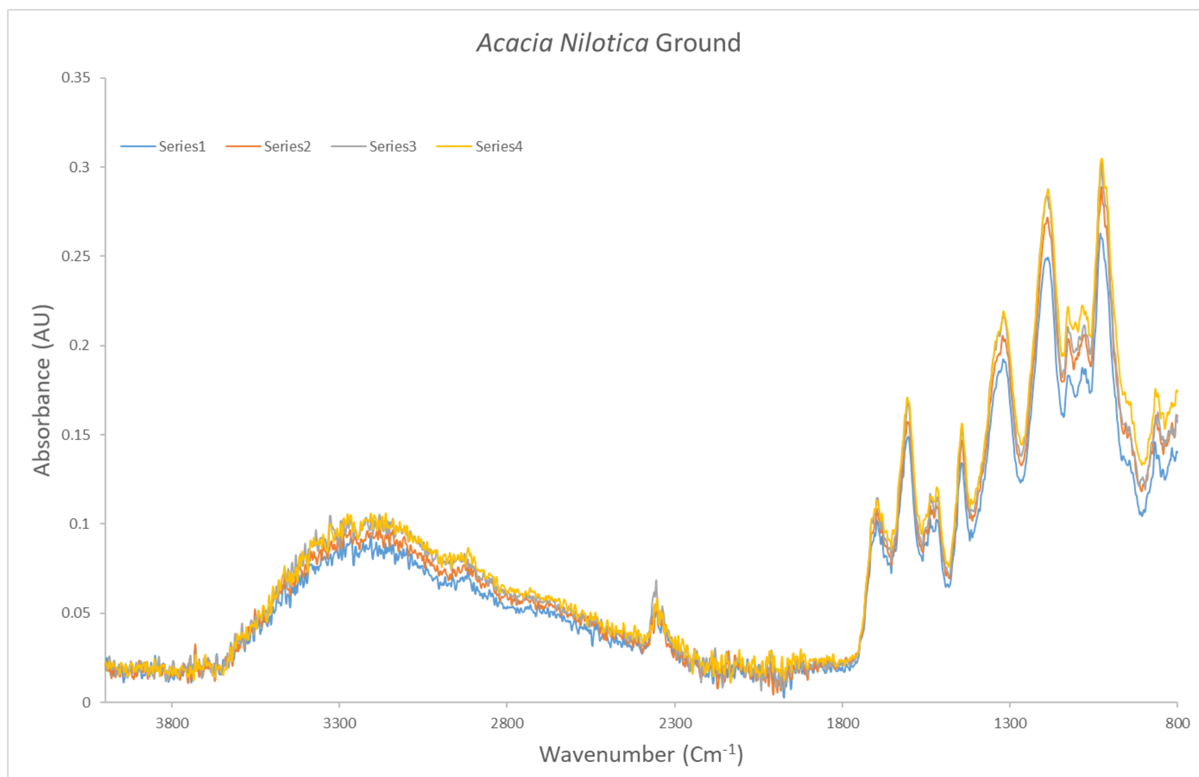


***Figure 3.8 FTIR of Acacia nilotica ground** showing wavenumber alongside absorbance of A. nilotica once ground into a powder.*

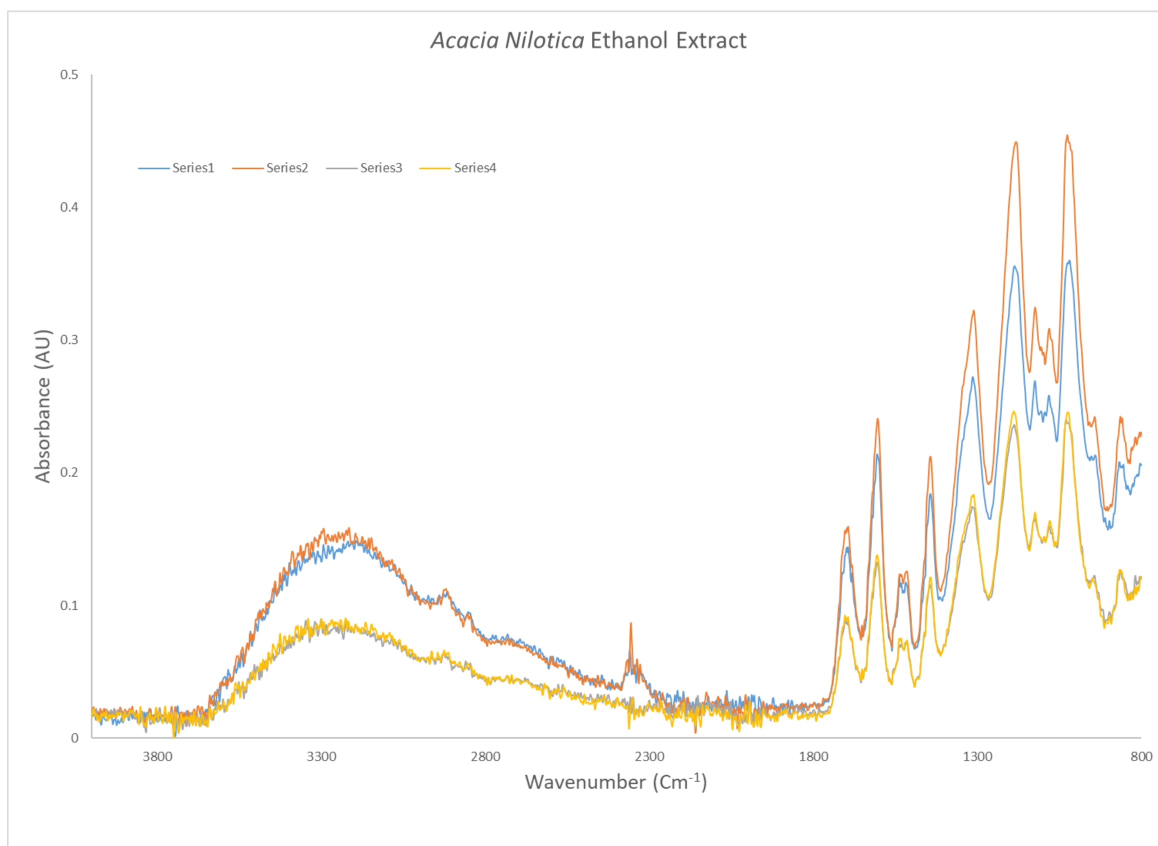


Figure 3.9 FTIR analysis of Ethanol Acacia nilotica *showing wavenumber alongside absorbance of the ethanol extraction of A. nilotica.*

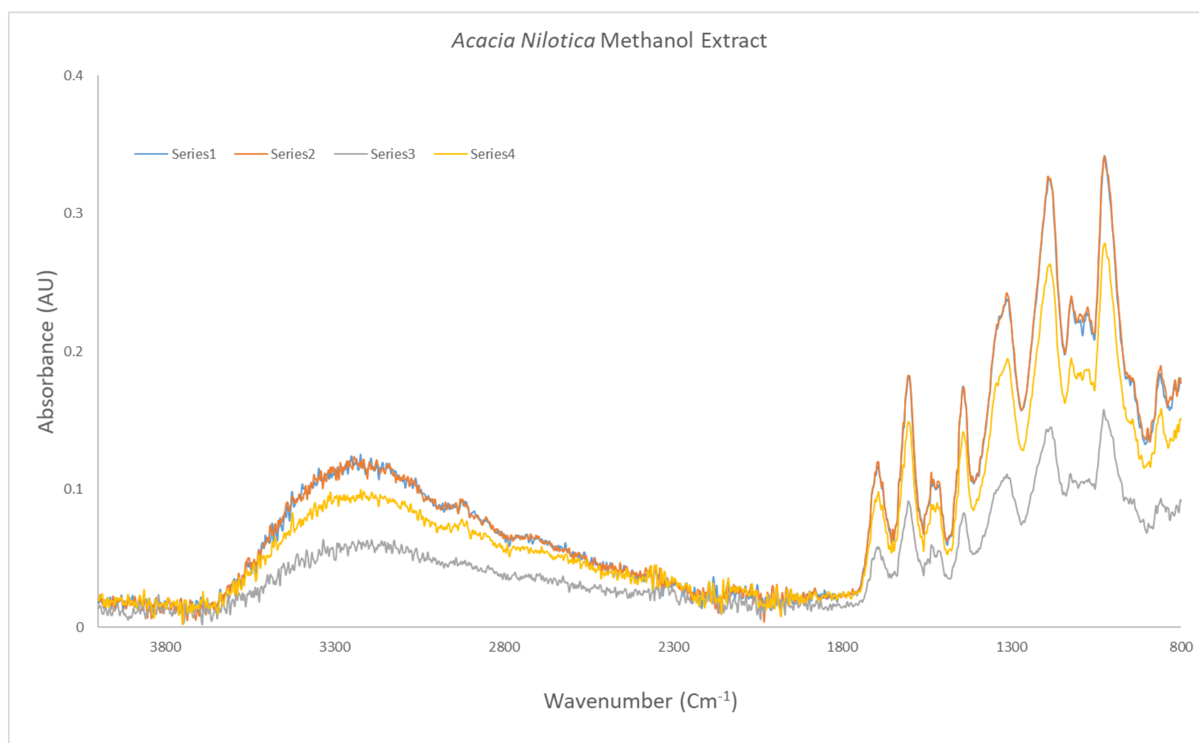


Figure 3.10 FTIR analysis of methanol Acacia nilotica *showing wavenumber alongside absorbance of the methanol extraction.*

Similarity of the FTIR spectrum for the different collections was used to confirm species similarity prior to further analysis.

3.3 Drug efficacy

Experiments to test the efficacy of the *Trametes versicolor* were performed as described in the methodology section in Chapter 2. Briefly, 2-fold serial dilutions of the *Trametes* ranging from 200, 100, 50, 25 and 12.5 μg/ml were tested against *P. falciparum* K1 strain with synchronised cultures initiated at trophozoite or ring stages for 48- or 72-hour incubations respectively. All experiments were carried out in duplicated and the averaged IC_{50} values for each were calculated.

For the *Trametes* ethanolic extract, the 48-hour value was IC_{50} 45.5 ± 1.12 μg/ml (Fig. 3.11) and the 72-hour producing an average IC_{50} of 27.47 ± 1.09 μg/ml (Fig. 3.12).

For *Acacia* extractions the 48-hour IC_{50} value ethanol extract of *A. nilotica* was 49.38 ± 1.06 μg/ml (Fig. 3.13) and the 72-hour reached achieved 50 % growth inhibition at 37.3 ± 1.07 μg/ml (Fig 3.14). For *A. nilotica* methanolic extract, the final IC_{50} of the 48-hour averaged at 20.2 ± 1.11 μg/ml (Fig 3.15), the 72-hour gave an IC_{50} of 44.3 ± 1.06 μg/ml (Fig 3.16).

3.3.1 48-hour Trametes versicolor

The *T. versicolor* ethanol extraction incubated for 48-hours produced an average IC_{50} of 45.54 ± 1.12 μg/ml. Despite the increase in IC_{50} values, the compound still shows potency prospects. The first IC_{50} value giving 58.71 ± 1.04 μg/ml and the second 32.37 ± 1.20 μg/ml (Fig 3.11).

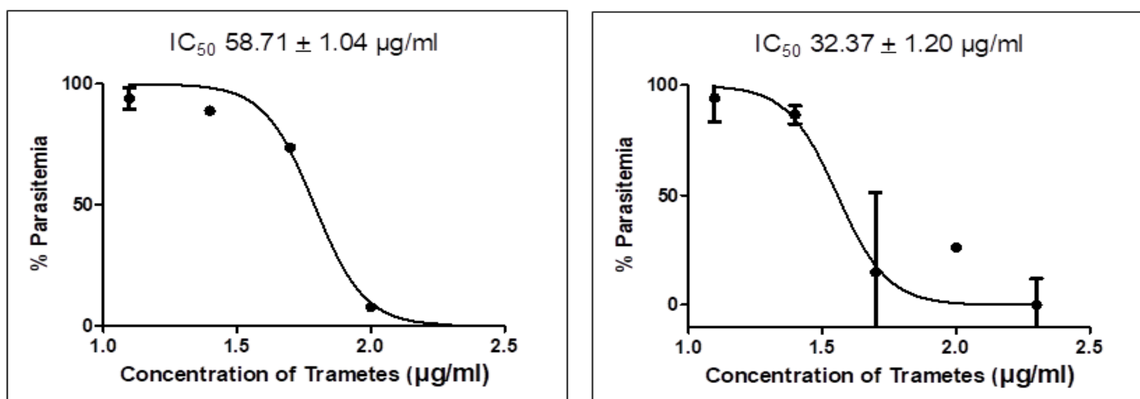


Figure 3.11 48-hour Trametes versicolor *showing parasitaemia against drug concentrations. Each line represents thee triplicate experements.*

3.3.2 72-hour Ethanol Trametes versicolor

The two sets of data shown below produce an average IC_{50} of 27.47 ± 1.09 µg/ml. Both sets indicate the compounds are active with antimalarial properties. The first IC_{50} value giving 27.50 ± 1.15 µg/ml and the second 27.45 ± 1.10 µg/ml (Fig 3.12).

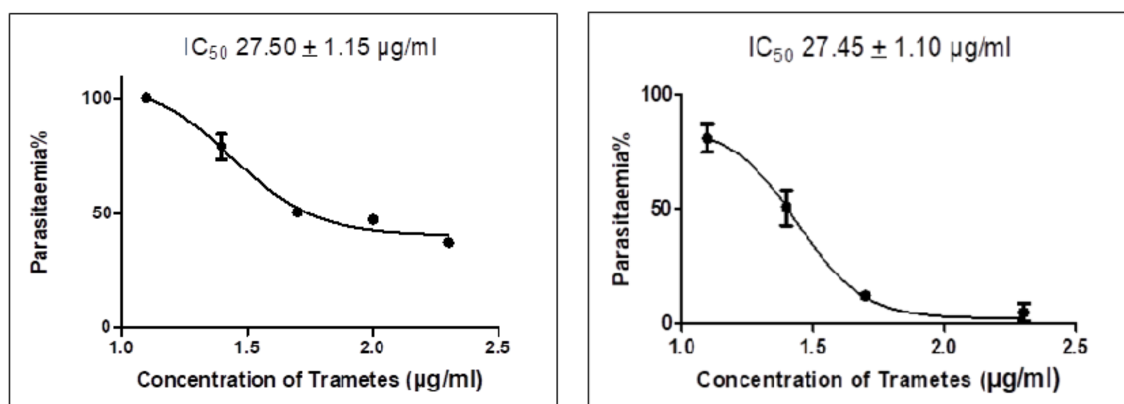


Figure 3.12 72-hour Trametes versicolor *showing parasitaemia against drug concentrations. Each line represents three triplicate experiments.*

3.3.3 48-hour Ethanol Acacia nilotica

The average IC_{50} produced from the ethanolic extract of *A. nilotica* after 48-hour incubation is 49.38 ± 1.06 µg/ml, giving a higher IC_{50} than that of the 72-hour. Similar

to that of *T. versicolor*, there was an increase in IC_{50}, however antimalarial properties were still present (Fig 3.13).

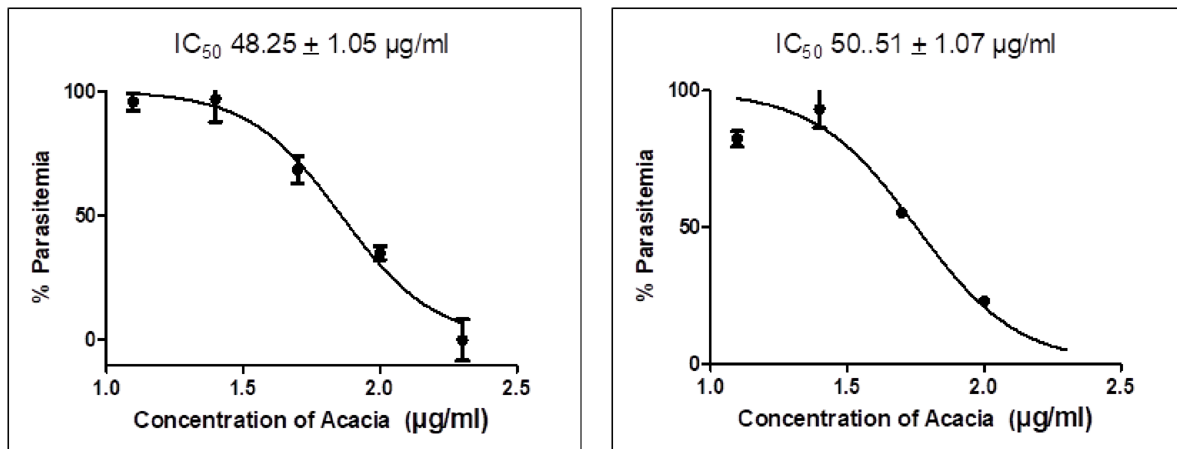


Figure 3.13 48-hour Ethanol Acacia nilotica *showing parasitaemia against `drug concentrations. Each line represents three triplicate experiments.*

3.3.4 72-hour Ethanol Acacia nilotica

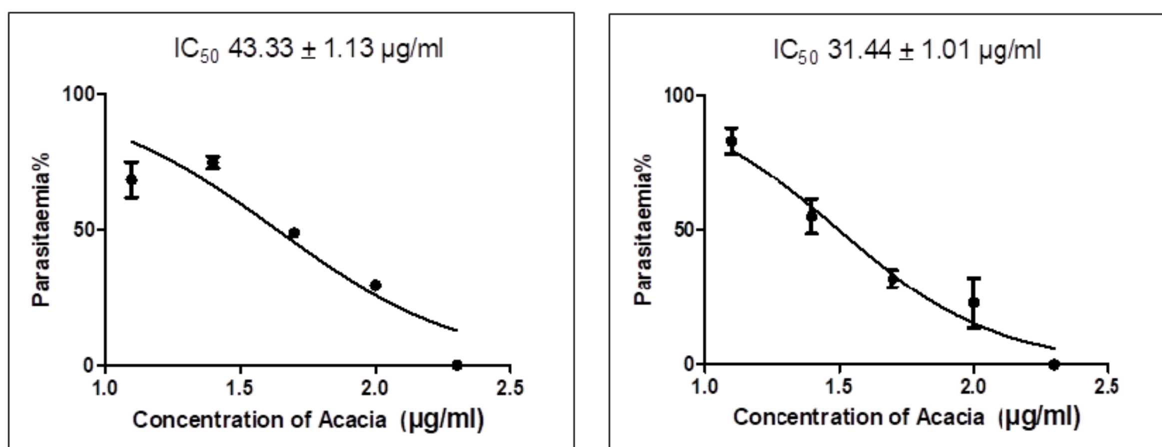


Figure 3.14 72-hour Ethanol Acacia nilotica *showing parasitaemia against drug concentration. Each line represents three triplicate experiments.*

The two graphs below produce an average IC_{50} of 37.3 ± 1.07 µg/ml. The average value shows antimalarial activity, however slightly lower than that of *Trametes*. The first IC_{50} value giving 43.33 ± 1.13 µg/ml and the second 31.44 ± 1.01 µg/ml (Fig 3.14).

3.3.5 48-hour Methanol Acacia nilotica

The results obtained from the 48-hour incubated methanol extract of *A. nilotica* produced the most promising results found in this study. The average IC_{50} obtained was 20.2 ± 1.11 µg/ml, which is significantly lower than the 72-hour incubation. Both data sets produced almost identical results with the first IC_{50} value being 20.25 ± 1.07 µg/ml and the second 20.24 ± 1.15 µg/ml (Fig 3.15).

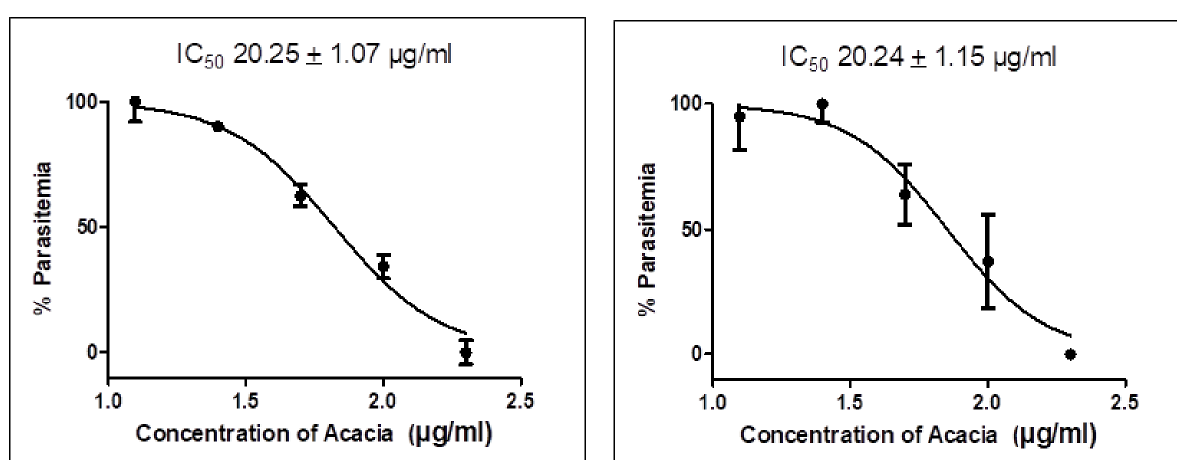


Figure 3.15 48-hour Methanol Acacia nilotica *showing parasitaemia against drug concentrations. Each line represents a triplicate experiment.*

3.3.6 72-hour Methanol Acacia nilotica

The average IC_{50} results were 44.3 ± 1.06 µg/ml. Although the highest average IC_{50} value of the 72-hour the results still show to possess antimalarial activity. The first IC_{50} value giving 48.26 ± 1.01 µg/ml and the second 40.49 ± 1.11 µg/ml (Fig 3.16).

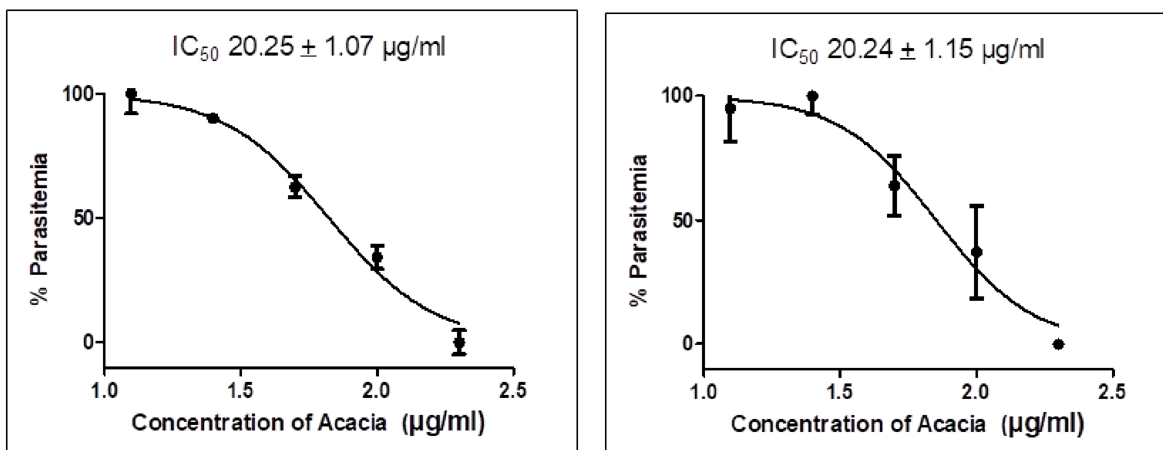


Figure 3.16 72-hour Methanol Acacia nilotica*. showing parasitaemia against drug concentrations. Each line represents three triplicate experiments.*

3.4 MTT assay

The standard MTT (3-(4. 5-dimethyltrazol-2-yl)-2,5-diphenyl terrazolium bromide assay was performed to investigate the toxicity of the compounds. The HepG2 cells were incubated with dose range (200, 100, 50, 25 and 12.5 µg/ml) of the different extracts. Cisplatin was used as a control alongside the compounds to validate the MTT assay (Fig. 19). Due to better yield and more promising results the ethanol extracts were carried forward as the methanol extract for *A. nilotica* was not reached. The following results were obtained after a five-day incubation period upon exposure to the compounds.

The ethanol extraction of *T. versicolor* produced the highest IC_{50} value for the HEPG2 cell line providing a value of 51.6 ± 1.2 µg/ml (Fig. 3.17). While the ethanol extract of *A. nilotica* was found to be more toxic with an IC_{50} of 29.1 ± 1.07 µg/ml Fig. 3.18).

3.4.1 HEPG2 Trametes versicolor

The average IC_{50} obtained from the ethanol extraction of *T. versicolor* on the HEPG2 cell line was 51.6 ± 1.2 µg/ml. The first IC_{50} value giving 50.11 ± 1.15 µg/ml and the second 58.18 ± 1.34 µg/ml (Fig 3.17).

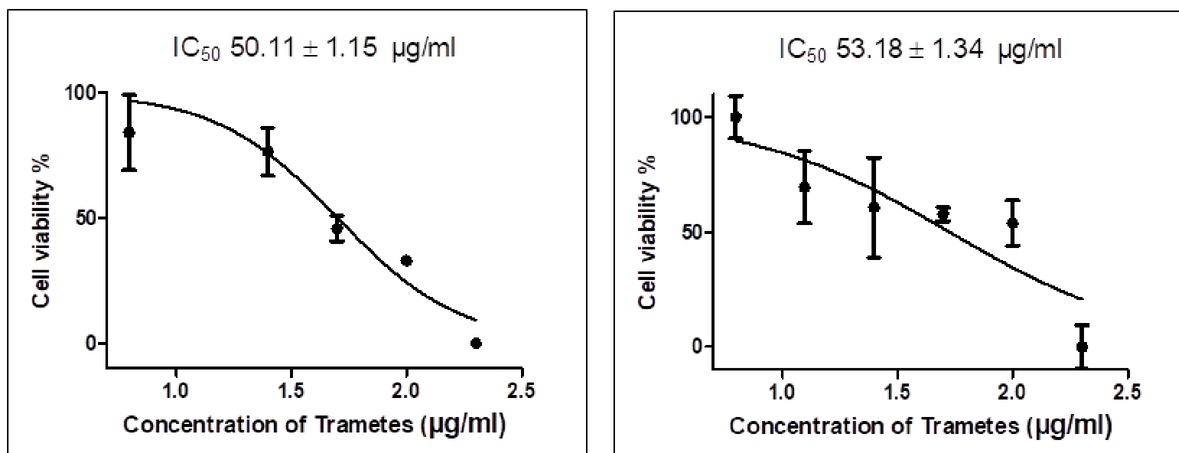


Figure 3.17 HEPG2 Trametes versicolor *showing cell viability against T. versicolor drug concentration. Each line represents a triplicate experiment.*

3.4.2 HEPG2 Acacia nilotica

From the two data sets of the ethanol extrication of *A. nilotica* on the HEPG2 cell line, an average IC_{50} of 29.1 ± 1.07 µg/ml was obtained. The first IC_{50} value giving 34.54 ± 1.28 µg/ml and the second 23.85 ± 1.25 µg/ml (Fig 3.18).

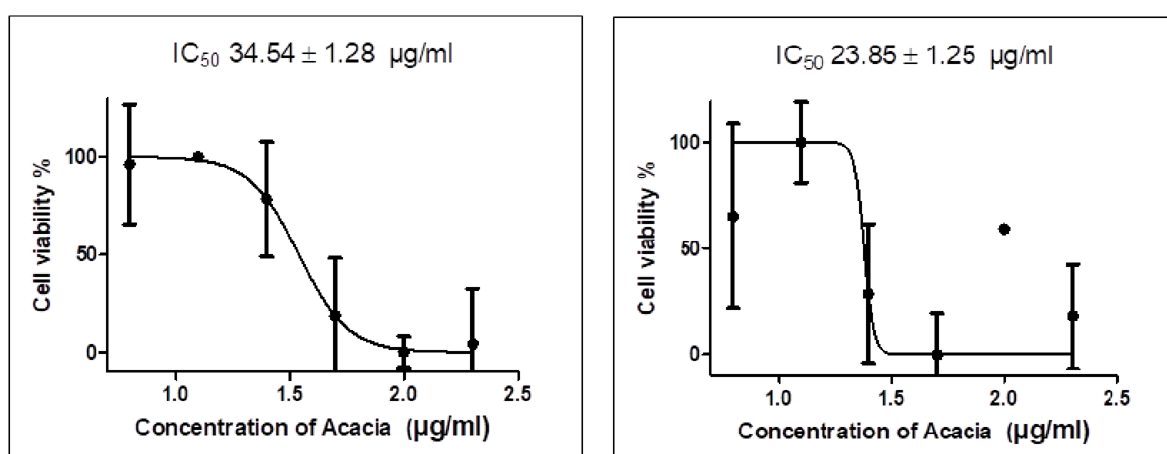


Figure 3.18 HEPG2 Acacia nilotica *showing cell viability against A. nilotica drug concentration. Each line representing a triplicate experiment.*

3.4.3 HEPG2 Cisplatin

Cisplatin was used alongside *T. versicolor* and *A. nilotica* as a control, the average IC_{50} obtained for the HEPG2 cell line was 40.29 ± 1.07 µM/ml. The first round gave an IC_{50} of 44.13 ± 1.4 µM/ml. The second round have a final IC_{50} of 36.46 ± 1.10 µM/ml (Fig 3.19).

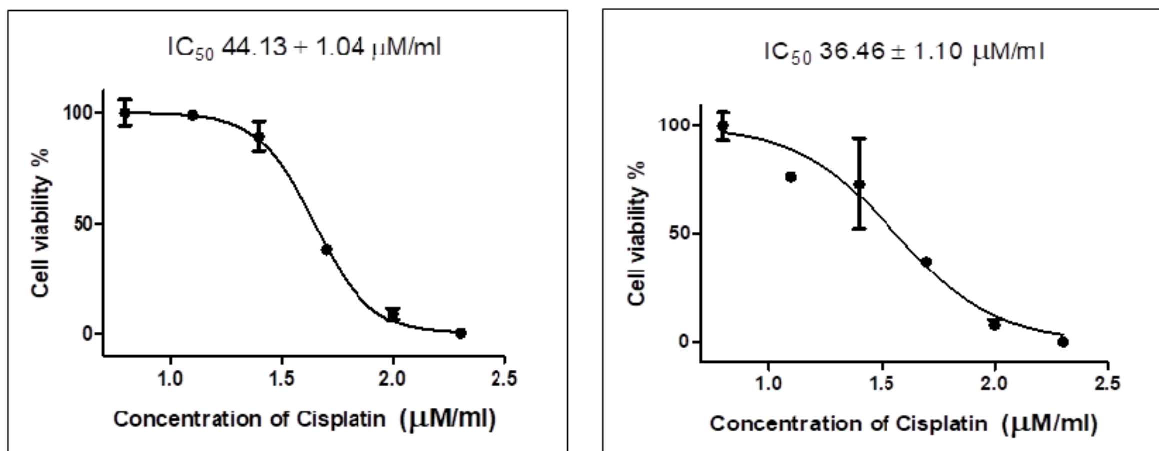


***Figure 3.19 HEPG2 Cisplatin** showing cell viability against cisplatin drug concentration. Each line represents a triplicate experiment.*

3.5 Therapeutic index.

The therapeutic index is used to determine the compounds toxicity, this determines whether or not the compound is going to be passed to the next stage of testing.

For the 72-hour set of experiments alongside HEPG2 *T. versicolor* produced a therapeutic index of 1.87 while *A. nilotica* gave 0.7. The 48-hour produced similar results with *T. versicolor* giving 1.13 and *A. nilotica* 0.58.

	72-hour	*48-hour*
Trametes versicolor	*1.87*	*1.13*
Acacia nilotica	*0.7*	*0.58*

Table 1. Therapeutic index *showing the final therapeutic indexes of the compounds tested alongside the MTT assays.*

Chapter 4

Discussion

Fortuitously discovered natural product leads have been the mainstay of antimalarial chemotherapy for centuries. Much of these discoveries were informed through the keen observations of traditional ethnopharmacological practices. The structural diversities and complexities of natural product-derived scaffolds together with their innate affinity for biological receptors offer an advantage, yielding the most effective antimalarials known to date. Unfortunately, reliance on newly-evolved high-throughput screening and discovery platforms has led to decreased focus on natural product leads, resulting in a significant decrease of such leads in the past 3 decades.

This study, focusing on *A. nilotica* and *T. versicolor* has confirmed the exciting potential of natural product extracts to contain novel antimalarial leads for future drug design. The study focused on exposing the extracts to multidrug resistant *P. falciparum* K1 strains. The studies were carried out in the pathogen lab at the University of Salford, using *in vitro* study alongside non-parasitized human blood as a control. The results of the whole extract *in vitro* evaluation offer promising potential for natural product lead discovery and further optimisation through active compound analysis. The high IC_{50} values obtained in this study could be down to several factors, a main one being that whole extract were used which could show varied results dependant on the nature of the natural product and appropriate solvent used. The solvents used in this study were ethanol and methanol, as previous studies had shown water extractions to be of little effect. From literature and previous studies, IC_{50} values that have been obtained to be over 100 µg/ml are classed as ineffective as an anti-plasmodial drug. 50-100 µg/ml is deemed to be of low activity and 10-50 is considered to be of moderate activity. An

IC_{50} value ≤ 10 μg/ml is classed as highly active again *Plasmodium* and would be considered for future research and development (Clarkson et al., 2004).

The 72-hour ethanol extract of *T. versicolor* produced the lowest IC_{50}, with an average IC_{50} of 27.47 ± 1.09 μg/ml, and the 48-hour incubation had an average IC_{50} value of 45.5 ± 1.12 μg/ml. While both incubation times produced IC_{50} values of moderate *Plasmodium* activity, this natural product could be taken further for investigation. The results were corroborated in a study at Salford University by Kerry Leigh Dreyer (2018), showing the ethanol extraction *T. versicolor* IC_{50} value to be 46 μg/ml (personal communication, unpublished data).

The *T. versicolor* component used in this study was the whole extract of the fruiting bodies. A recent study by Benson et al., (2019), found there was a highly distinguished immune activating effect in the mycelium of *T. versicolor* when compared to its fruiting body. Furthermore, the mycelium and fruiting body have distinctly different biological and immune modulating effects on the body. Mycelium helped in triggering the immune cell activation whereas the fruiting body increased cytokine induction. Investigating the mycelium effects on the *P. falciparum* as oppose to the fruiting body could possibly give improved IC_{50} values and would be a recommended course for analysis in future experiments (Benson et al., 2019).

Fungi along with plants and bacteria are known to produce secondary metabolites. These metabolites provide a crucial source of effective pharmaceutical products. Secondary metabolites found within fungal endophytes have been described as pharmaceutically useful crops. Bioactive metabolites within fungi possess a wide range of biological activities including antibiotic, antitumor and anti-inflammatory. Public interest in secondary metabolites is growing resulting in drug discovery

research including bio-chemical and molecular techniques from higher medicinal fungi (Devi et al., 2020; (Kivrak, & Karababa, 2020).

For further development of pharmacologically interesting mushrooms and mushroom compounds, cultivation is a crucial step to ensure demand is met sustainably and in a standardised manner. Whole fruiting bodies, extracts derived from mycelium and fruiting bodies along with isolated compounds are all suitable for cultivation. Foraging in the wild, mushroom farming, harvest and cultivation of mycelium are all ways of gathering adequate supplies. Extraction methods can be performed with dry or fresh as long as suitable solvents are used. There is a possibility that pure compounds can be gathered from the natural product, cultivated and later by chemical induced or altered synthesis. It is often known for a natural compound to serve as a lead compound, with the prospect of a high variety of derivatives. As most mushroom species are easily and economically cultivated already, the need for mushroom compounds to be genetically modified is not needed at this time. One of the most used polysaccharides from mushrooms, which have been developed for therapeutic agents, have been derived from *Trametes versicolor* which has been used in this study. Intracellular protein polysaccharides (IPS) which are isolated from *Trametes versicolor* has high efficiency as an immunomodulator and anti-tumour agent (Cui *et al.*, 2007; Wang *et al.*, 2017). Polysaccharide peptide and polysaccharide keratin from *T. versicolor* are examples of mushrooms being collected from the wild/farmed and chemically synthesized. After production of such extracts, simple capsules, tablets, shots and teas can be commercialised for human use (Lindequist, Niedermeyer, & Jùluch, 2005; (Pinheiro, Michelin, Vici, de Almeida, & de Moraes, 2020).

The best anti-plasmodial activity from the experiments was seen with ethanol extracted *Acacia nilotica,* yeilding average IC_{50} values of 37.3 ± 1.07 µg/ml at 72-hour

incubations, and methanol extracted *A. nilotica*, yielding average IC_{50} values of 20.2 ± 1.11 µg/ml at 48-hour incubations. This produced the lowest IC_{50} out of all experiments regardless of incubation time, indicating peak antimalarial activity. This value indicates a successful level of anti-plasmodial activity and would need further studies using fractionation techniques to identify active compound and further reduction in IC_{50} value.

The current experiments were conducted with whole extracts of seed pods. Published literature on the medicinal value of *A. nilotica*, suggests differential medicinal potential in the different parts of the tree (e.g. bark, leaves, roots) and there is a great need and demand for isolation and identification of new compounds from each part of the tree. Henc, expansion of the current experiments to investigate the whole extract from the other parts of the tree are likely to yield more avenues for investigation. In further studies on *A. nilotica*, it was seen to have possible interactions between serotonin receptors that are linked with hypertension (Gilani et al., 1999; Alli et al., 2016; Omara, 2020. This could indicate a potential mode of action for this natural product lead in malaria. Drug repositioning studies (Matthews et al., 2013) have reported antimalarial activity in patent expired drug library screens on serotonin inhibitors, among other classes of drugs. This does provide a further line of investigation for the next phase of optimisation

As *A. nilotica* displays high radical scavenging properties, indicating there is high activity due to the occurrences of phenolic compounds. These individual compounds still require further research for identification. Until then, their introduction into the pharmaceutical industry is not possible (Kalaivani & Mathew, 2010). Some antioxidants that have been isolated from *A. nilotica* such as Umbelliferone, have been used in assisting the prevention of cancer and ageing. This antioxidant eliminates

oxidative species responsible for carcinogenesis via DNA alteration. (Singh et al., 2009).

There have been previous studies on *A. nilotica*, using aqueous extractions of the root. These extractions were eluted and its antimalarial properties were tested on mice that had been infected with *P. berghei*. The extractions produced positive results showing the reduction of parasite prevalence in relation to dose response. The mice treated with the extracted root compound shown a significant increase in overall survival to that compared with the control subjects (Alli et al., 2016). In a relatively recent study, the leaves found upon the *Acacia* tree once extracted using an ethanol solvent, were found to have an average IC_{50} value of 1.29 μg/ml after a 48-hour incubation period. Significantly lower than the IC_{50} obtained in this study, however this study focused on the pods, which averaged at 37.3 ± 1.07 μg/ml. The results obtained from the 48-hour incubated methanol extract of *A. nilotica* produced the most promising results found in this study. The average IC_{50} obtained was 20.2 ± 1.11 μg/ml.

Cultivation of *A. nilotica* plants in a sustainable way would be needed to achieve drug development on a pharmaceutical level. Further in-depth research is still needed to reveal the full potential this medical plant has to offer to mankind (Alli et al., 2016)

The *T. versicolor* and *A. nilotica* extracts were further tested on HEPG2 cell lines to test compound toxicity. Cisplatin was used as a control alongside the compounds ensuring the experiment worked accordingly.

The therapeutic index is used to determine the compounds toxicity, to determine whether or not the compound is going to be passed to the next stage of testing. The therapeutic index is worked out by dividing the IC_{50} of the MTT assay by the Plasmodium IC_{50}.

For the 72-hour set of experiments alongside HEPG2 *T. versicolor* produced a therapeutic index of 1.87 while *A. nilotica* gave 0.7. The 48-hour produced similar results with *T. versicolor* giving 1.13 µg/ml and *A. nilotica* 0.58. All compounds in this study would be deemed too toxic for human consumption when used in the whole extract form. A non-cancerous cell line and repeated experimental would be needed to obtain a more reliable therapeutic index outcome.

T. versicolor extracts are being used alongside traditional cancer-based treatments as an additional treatment. PSP extract from *T. versicolor* have shown preclinical and in clinical studies to have oncologic and immunologic activity for patients who suffer from breast, gastrointestinal and lung cancers. When *T. versicolor* extracts are used alongside chemo and radiation therapy, some evidence shows that there is a possibility that it can reduce the risk of secondary malignancies that have been induced as a direct result of radiotherapy and chemotherapy (Habtemariam, 2020). Similarly, the roots of *A. nilotica* have been used on cancers and tumours, particularly cancers of the eye, testicular and ear (Kalaivani & Mathew, 2010). *A. nilotica* is an easily accessible source of antioxidants, which can be used as additional supplements in the fight against free mediated diseases including cancer.

B-glucans and their protein derivatives, mainly protein-polysaccharides, attract the most attention due to their potential biological functions and non-toxic characteristics. They have been found to have vital roles in anti-tumour, anti-oxidant, anti-viral and immunomodulating activities (Gan, Lie Li 2015; Wang *et al*., 2017).

Interestingly, HepG2 cell lines have been used as the gold standard to decipher antimalarial *in vitro* for most published work. HepG2 cell lines themselves are derived from hepatic carcinoma. Given that both *T. versicolor* and *A. nilotica* possess anti-cancer properties as stated above, the increased IC_{50} values from this study may be

a result of the confounding effect the antcancer properties of thisextract may have on the HepG2 cell line. Hence the low selectivity index should be further investigated using a non-cancerous human cell line.

Therefore, exposing both *T. versicolor* and *A. nilotica* extracts to cancer cell lines such as HEPG2 to determine toxicity has proven ineffective in obtaining a reliable therapeutic index. Non-cancerous human cell lines would be needed for further study in order to obtain a toxicity level required for next stage testing in a development for a novel drug. Toxicity could also be a direct factor of the DMSO used within this study, while extractions were taking place. Both *T. versicolor* and *A. nilotica* are natural products used on a daily basis across the world for many different reasons. Therefore, a toxic result should not have been the outcome of this study.

It is vital that selected natural products have a therapeutic index that is favourable. The parasite must be completely eliminated or inhibited from the body while having minimal toxicity to the host. Such discoveries have proven difficult in the past. Multiple natural products have displayed the ability to inhibit protozoal growth, with only a small number having selective toxicity to the parasite only. Drugs that act upon the biochemical target unique to the *Plasmodium* parasite are needed as these have little to no effect on the host, therefor being deemed safe for consumption *(Singh, Krishna & Kumar 2020)*.

To conclude, further studies on both *T. versicolor* and *A. nilotica* is required given their potent antimalarial activity. The isolation of the active compounds within the natural products could be an exciting new research idea for the future. Using HPLC analysis would be a useful technique in determine the compounds present that have these antimalarial properties. As this *in vitro* study focused on multidrug resistant K1 stain of *P. falciparum*, there is a potential to test the same compounds upon different strains

in the future to determine its effectiveness. The same principle could also be applied to different species of *Plasmodium*. A study on all five *Plasmodium's* currently known to affect humans would be of interest to compare the effectiveness of the natural products on the different species. Finally, given that many antimalarials also possess anti-cancer activity *in vitro*, the use of the gold standard HepG2 cell lines to test cellular toxicity and derive selectivity indices may result in important leads being discarded due to erroneous inferences resulting from an inappropriate testing platform. A safe policy would be to defer such decisions until the *in vivo* activity of the compounds are verified.

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