Bioassay guided fractionation and isolation of anthelmintic compounds from natural sources

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List of abbreviations

AADs - aminoacetonitrile derivatives

CPT - carnitin palmitoyl transferase

GABA – γ-amino butyric acid

MDA - mass-drug administration

Mpro - main protease

nAChR - nicotinic acetylcholine receptor

NGM - nematode growth media

NTD - neglected tropical disease

NTS - newly transformed schistosomules

PANPL - pan African natural product library

PreSAC - pre-school aged children

SAC - school aged children

SFFC - spurious falsely labelled falsified counterfeit

STH - soil transmitted helminths

WHO - World Health Organisation

Summary

Neglected tropical diseases continue to be a global problem and their prevalence may have been worsened by the Covid 19 pandemic as well as ongoing conflict around the globe. Soil transmitted helminths are the most prevalent NTD while schistosomiasis has a very high mortality rate. Although mass drug administration remains an effective control measure against both STH and schistosomiasis, there are concerns about the development of drug resistance. There are also concerns about the effects of drug residues from MDA programs on the environment. There is therefore a need to find new natural product based drugs to help reduce global prevalence of STH and schistosomiasis.

Plants known to be used against worm infections and schistosomiasis are a good starting point when one wants to find new anthelmintic compounds with a possibly new mode of action. The fruits of *Ozoroa insignis* were investigated for their anthelmintic properties using *Caenorhabditis elegans* in bioassay guided isolation to obtain three compounds (**3.1**-**3.3**). Compounds **3.1**-**3.3** were evaluated for their antiparasitic properties by testing them against a variety of parasitic helminths. Compound **3.3** showed promising antischistosomal activity. The compounds were also evaluated for their cytotoxic properites against two cancer cell lines namely PC-3 (human prostate adenocarcinoma cells) and HT-29 (human colorectal adenocarcinoma cells), where compound **3.3** again was found to be the most active one.

Mushrooms are still under-investigated and could be a possible source of new anthelmintic compounds. In this regard the anthelmintic properties of extracts of fruiting bodies of eleven fungal species were investigated and among these *Albatrellus confluens* was active against *C. elegans*. Using bioassay guided isolation two compounds (grifolin, **4.1**; neogrifolin **4.2**) were found to be responsible for the anthelmintic activity. The isomers grifolin (**4.1**) and neogrifolin (**4.2**) demonstrate how the substitution pattern contributes to a change in activity. The effect of the length of the prenly units was investigated by synthesizing derivatives with varying prenyl chain length (compounds **4.4**-**4.6**). This revealed that shortening the length of the prenyl chain increased the activity against *C. elegans*. The isolated compounds and synthetic derivatives were tested against parasitic helminths and were also screened for antiproliferative or cytotoxic activity against the two human cancer cell lines PC-3 (human prostate adenocarcinoma) and HT-29 (human colorectal adenocarcinoma). Compound **4.4** and **4.6** showed promising activity against newly transformed schistosomula while compound **4.6** was determined to be the most effective against both human cancer cell lines.

Traditional medicine is still widely used globally and investigation into the plants used against human helminth infections could lead to the discovery of new anthelmintic compounds. In this respect we investigated the anthelmintic activity of ten plants used by traditional medicinal practitioners in Botswana. Two plant extracts, *Laphangium luteoalbum* and *Commiphora pyaracanthoides,* showed antischistosomal activity against newly transformed schistosomula and *Schistosoma mansoni*. An investigation was also carried out on the potential of herbs and spices as anthelmintics by testing various Ethiopian plants for anthelmintic activity. *Piper nigrum*, *Cinnamomum verum* and *Trachyspermum ammi* displayed the most promising anthelmintic activity and anthelmintic compounds **6.7** to **6.11** were isolated from *Echinops kebericho*

Zusammenfassung

Vernachlässigte Tropenkrankheiten (neglected tropical diseases, NTDs) sind nach wie vor ein globales Problem, welches sich durch die Covid-19-Pandemie sowie anhaltende Konflikte auf der ganzen Welt weiter verschlimmern dürfte. Dabei haben nicht lebens-bedrohende Darmwürmer, deren Eier durch verunreinigte Erde oder verschmutztes Wasser aufgenommen werden (Soil-transmitted helminths (STH) den größten Anteil unter den NTD, wohingegen die durch Süßwasserkontakt direkt aufgenommenen Parasiten (Bilharziose) durchaus zu einem lebenbedrohenden Krankheitsbild führen können. Obwohl die Massenverabreichung von Arzneimitteln (MDA) eine wirksame Kontrollmaßnahme gegen STH und Bilharziose bleibt, gibt es Bedenken hinsichtlich der Entwicklung von Resistenzen. Ebenso bedenklich sind Wirkungen von Arzneimittelrückständen aus MDA-Programmen auf die Umwelt. Es besteht daher ein Bedarf, neue Medikamente auf Naturstoffbasis zu finden, um die weltweite Prävalenz von STH und Bilharziose zu reduzieren.

Pflanzen, von denen bekannt ist, dass sie in der Volksmedizin gegen Wurminfektionen und Bilharziose eingesetzt werden, sind ein guter Ausgangspunkt, um neue anthelminthische Verbindungen zu finden. Es wurden daher die Früchte von *Ozoroa insignis* auf ihre anthelmintischen Eigenschaften gegen *Caenorhabditis elegans* untersucht. Mittels aktivitäts-geleiteter Isolierung wurden drei biozide Verbindungen isoliert (**3.1**–**3.3**). Die Verbindungen wurden im Folgenden auf ihre antiparasitären Eigenschaften gegen eine Vielzahl von Helminthen getestet wurden. Die Verbindung **3.3** zeigte eine vielversprechende antischistosomale Aktivität. Die isolierten Verbindungen **3.1**–**3.3** wurden zudem auch auf ihre zytotoxischen Eigenschaften gegen zwei Krebszelllinien, Prostata-Adenokarzinomzellen und kolorektale Adenokarzinomzellen getestet, wobei sich wiederum Verbindung **3.3** als die aktivste herausstellte.

Pilze können eine mögliche Quelle für neue anthelminthische Verbindungen sein. Von elf untersuchten Pilzarten war eine Art, *Albatrellus confluens,* gegen *C. elegans* aktiv. Die aktivitäts-geleitete Isolierung führte zu den Verbindungen **4.1** und **4.2,** die für die anthelmintische Aktivität verantwortlich sind. Diese beiden isomeren Verbindungen Grifolin (**4.1**) und Neogrifolin (**4.2**) zeigten zudem, wie die verschiedenen Positionen einer Methyl- und Hydroxylgruppe zu einer Aktivitätsänderung führen können. Der Einfluss der Länge der Prenyl-Gruppe wurde untersucht, indem Derivate mit unterschiedlicher Prenyl-Kettenlänge (Verbindungen **4.4**–**4.6**) synthetisiert wurden. Dabei zeigte sich, dass die Verkürzung der Prenylkette die Aktivität gegen *C. elegans* erhöhte. Die isolierten Verbindungen **4.1** und **4.2** und synthetischen Derivate **4.4**–**4.6** wurden gegen parasitäre Helminthen getestet, sowie ihre antiproliferative oder zytotoxische Aktivität gegen zwei menschliche Krebszelllinien Prostata-Adenokarzinomzellen und kolorektale Adenokarzinomzellen untersucht. Verbindung **4.4** und **4.6** zeigten vielversprechende Aktivität gegen neu transformierte Schistosomula, während Verbindung **4.6** sehr wirksam gegen beide menschlichen Krebszelllinien ermittelt wurde.

Pflanzen werden in der traditionellen Medizin weltweit eingesetzt. Die phytochemische Untersuchung der Pflanzen, die gegen Wurminfektionen beim Menschen eingesetzt werden, könnte zur Entdeckung neuer anthelminthischer Verbindungen führen. Wir haben daher zehn Pflanzen untersucht, die von traditionellen Heiler in Botswana als Anthelmintika verabreicht

werden. Zwei Pflanzen bzw. deren Extrakte, *Laphangium luteoalbum* und *Commiphora pyaracanthoides*, wiesen antischistosomale Aktivität gegen neu transformierte Schistosomula und *Schistosoma mansoni* auf. Zudem wurden Untersuchungen zum anthelmintischen Potenzial von äthiopischen Kräutern und Gewürzen durchgeführt. *Piper nigrum*, *Cinnamomum verum* und *Trachyspermum ammi* zeigten eine vielversprechende anthelmintische Aktivität. Zudem konnten aus *Echinops kebericho* die anthelmintisch wirkenden Verbindungen **6.7** bis **6.11** isoliert und charakterisiert werden.

1. Introduction and objectives

Helminthiases are parasitic worm infections infecting man and animals and causing morbidity to their host. They cause stunted growth and are a substantial threat to health. The continous contamination of the environment by helminths with their eggs and larvae makes helminth infection a huge challenge, both in developing and developed countries (Nalule et al., 2013). Despite their global prevalence, helminths diseases are one of the most neglected among the healthcare systems. This could be due to the chronic and asymptomatic nature of infection. However, helminth infections can cause severe debilitation, morbidity and economic losses among humans and livestock (Idris et al., 2019).

The most common helminths are human nematode infections or soil transmitted helminths (STH) as they are commonly called. They affect more than 1 billion people worldwide (King, 2019). The most common STH species are *Ascaris lumbricoides* commonly known as the round worm, *Trichuris trichiura* also known as the whip worm and the two hook worm species *Necator americanus* and *Ancylostoma duodenale* (Montresor et al., 2020).

The STH are treated using various categories of anthelmintic drugs, these are benzimidazoles, macrocyclic lactones, levamisole, piperazine and aminoacetonitrile derivatives (Idris et al., 2019). Unfortunately due to long term use of a limited number of drugs and mass drug administration programs, resistance is becoming a problem (Wit et al., 2021). Long term use of the drugs especially in the animal industry is leading to the drugs being potential environmental pollutants (Kim et al., 2017) as well as the presence of drug residues in animal products (Deshwal, 2019).

There is therefore a need to find new drugs derived from natural products to help fight these parasitic worms. Natural products have been a reputable source for drugs as they have developed secondary metabolites due to various abiotic and biotic stresses (David et al., 2015). These secondary metabolites are responsible for the various bioactivities that natural products possess. Historically most drugs were derived from natural products, for example for the period January 1981 to September 2019, 64.9% of approved drugs were either natural products or natural product based (Newman and Cragg, 2020). Due to the complexities of working with natural products including the regulations defining the need for benefit sharing with countries where the biological material originates, intellectual property issues as well as the problems with procurement, the pharmaceutical industry reduced natural product based discovery programmes (Atanasov et al., 2021). However because of the low success rate of synthetic chemistry based drug discovery programs, natural product based drug discovery is reemerging as a reputable source of drugs (David et al., 2015).

Fossil records show that plants have been used as medicines dating back to at least 60.000 years (Shi et al., 2010; Fabricant and Fansworth, 2001). It is probable that in the process of learning which plants have medicinal properties, early humans may have consumed poisonous plants leading to undesirable side effects (Yuan et al., 2016). These ‘clinical trials’ lead to the knowledge which eventually become traditional medicine (Abdullahi, 2011; Yuan et al., 2016). Mainly plants have been used in traditional medicine against various diseases in Africa (and the developed world) and are still being used extensively in areas where there are inadequate health care facilities. For example in some African and Asian countries the majority of the

population is still dependent on plant based traditional medicine as a source of primary health care (WHO, 2013). Although many man made anthelmintics have been developed in order to control the parasites, plants provide a sustainable and eco friendly substitute (Bibi et al., 2016). In addition, mushrooms have also developed secondary metabolites which may have anthelmintic properties due to their role as defence compounds (Spiteller, 2008).

The free living worm *Caenorhabditis elegans* is accepted as a good model organism for testing against parasitic worms due to its easy maintenance and fast life cycle (Burglin et al., 1997). It is metabolically similar enough to the parasites to be used as an approximate model of parasitic worms (Buckingham et al., 2014). There has also been extensive genetic work done and this has made available powerful molecular, genetic and genomic tools which can help study physiological effects and gene function as well as help identify drug targets (Sepulveda-Crespo et al., 2020).

The *C. elegans* assay is a very reliable and robust assay but the only drawback is that the counting of the worms is a tedious process and is therefore the bottleneck of the assay. There is therefore a need to develop a faster way to count the worms as this would enable more samples to be assayed and could also lead to the automation of the assay.

The objectives of this thesis is therefore to use natural products as a source of new anthelmintics to fight the prevalent STH.

This will be achieved by first of all collecting plant samples first from Botswana, and also collecting mushrooms growing in Germany. The plant samples from Botswana will be from two sources, plants from traditional healers, and other plant species collected based on either literature or randomly collected. Additionally, various Ethiopian herbs and spices will be investigated to determine their potential as anthelmintics.

The collected plant material will then be assayed for anthelmintic activity and bioassay guided fractionation will be used to isolate active compounds.

The active compounds will then be sent to the Swiss Tropical Institute for testing against the parasitic nematodes.

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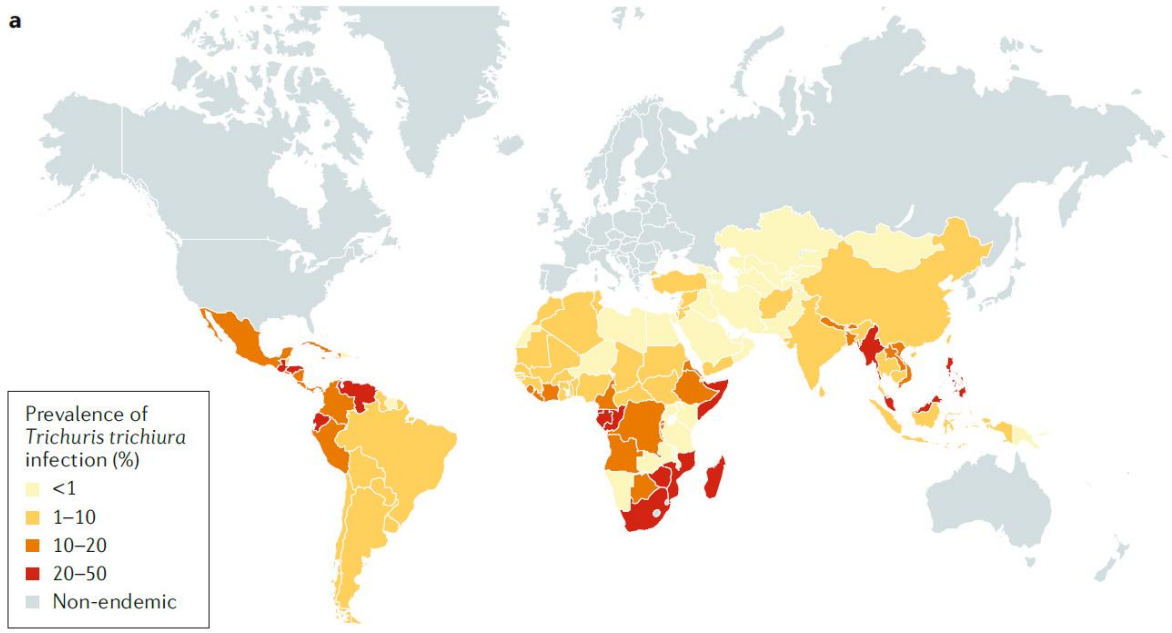
2. General part

2.1 Human nematode infections

Helminthiases are parasitic worm infections that cause morbidity to their host. They account for three out of twenty of the World Health Organisation's (WHO) recognized Neglected Tropical Diseases (NTDs) (Sharpton et al., 2020). They infect humans and animals, causing stunted growth and are a substantial threat to health. Regular control of nematode infection is necessary for efficient and welfare-friendly livestock production (Zajickova et al., 2020). In humans the most common helminths are the soil-transmitted helminths (STH) or intestinal nematodes, filarial worms, schistosomes and onchocerciasis worm (Idris et al., 2019).

STH infections are the most widespread of the NTDs, and are also the most dangerous nematodes to humans (Zajickova et al., 2020) affecting mainly marginalized populations in low- and middle-income countries (Montresor et al., 2020). This is because of unsafe and insufficient quantities of drinking water, inadequate sanitation, and poor hygiene practices (Aschale et al., 2021). STH are environmentally contagious infections that may cause nutritional impairment, cognitive impairment and may even lead to conditions requiring surgical interventions. Severe STH infections can be manifested by abdominal pain, diarrhea, blood and protein loss, and rectal prolapse (Eltantawy et al., 2021) There are an estimated 1.22 billion people with the large roundworm *Ascaris lumbricoides*, 0.74 billion with the Old World hookworm *Ancylostoma duodenale* and the New World hookworm *Necator americanus* and 0.80 billion with the whipworm *Trichuris trichiura* (Chen et al., 2021). The prevalence of the whipworm and roundworm is shown in Fig. 2.1. Even though the number of infected people is high approximately 135000 deaths occur annually due to the STH infections. The deaths are primarily due to anemia which is a result of hookworm infections, intestinal or biliary obstruction due to roundworm infections and chronic dysentery caused by whipworm infections (Tinkler, 2020). Although each of these four species has distinct characteristics, they are considered as a single group because of similarities in transmission dynamics and the prevention and control measures needed (Montresor et al., 2020).



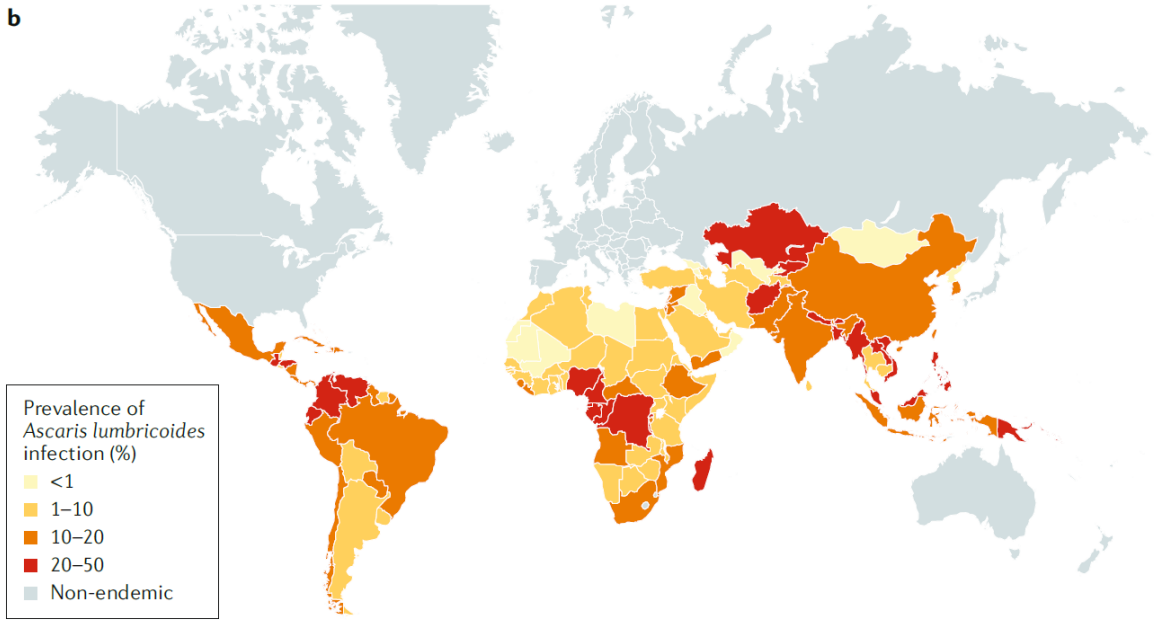


Fig. 2.1. Prevalence of *Trichuris trichiura* (a) and *Ascaris lumbricoides* (b) infection in 2010 (Else et al., 2020).

Schistosomiasis is regarded as the most important helminthiasis worldwide in terms of morbidity and mortality (Santos et al., 2021). It is caused by three different species of blood-dwelling fluke worms of the genus *Schistosoma*, namely, *S. haematobium*, which causes urinary schistosomiasis, and *S. mansoni* and *S. japonicum*, which cause intestinal schistosomiasis (Hussen et al., 2021). *S. mansoni* infection mainly affects the liver and spleen. This is characterized by hepatomegaly, splenomegaly, progressive periportal fibrosis which can lead to portal hypertension and its related sequelae, mainly ascites, liver surface irregularities, oesophageal varices and haematemesis. *S. haematobium* infection is manifested by haematuria, dysuria, nutritional deficiencies, urinary bladder lesions, hydronephrosis, urinary bladder squamous cell carcinoma and in children, growth retardation (Mazigo et al., 2021). There are an estimated 700 million people globally at risk of contracting schistosomiasis (Hussen et al., 2021) and 90% of the over 250 million cases of schistosomiasis occurring worldwide are in Sub-Saharan Africa (Mazigo et al., 2021). This is shown in Fig. 2.2 where a high prevalence is seen on the African continent. Even though historically schistosomiasis has occurred mainly in Africa there have been outbreaks in Europe affecting France, Germany and Italy with the infection originating in Corsica (France) (Boissier et al., 2015). The parasites implicated in the human cases infected in Corsica were *S. haematobium* and *S. bovis* which is a parasite that usually affects cattle. Genetic molecular analysis of the parasites indicated that their geographical origin was Senegal (Salas-Coronas, 2021). Tropical urogenital schistosomiasis is now permanently established in Corsica due to the presence of the snail *Bulinus truncates* which acts as an intermediate host for schistosomes. Vertebrate hosts also reseed the schistosomes every summer and coupled with the ability of the schistosomes to survive in their snail hosts over winter, this has lead to the establishment of schistosomiasis in Corsica since its appearance in 2013 (Mulero et al., 2019).

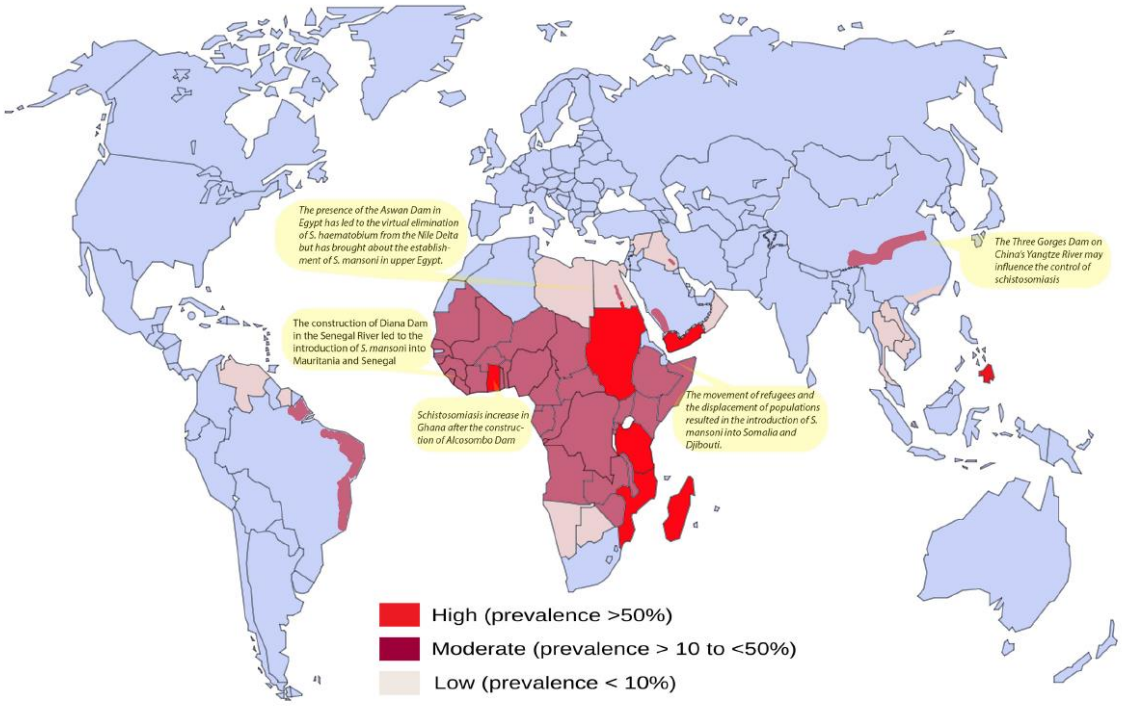


Fig. 2.2. Geographic distribution of Schistosomiasis with its approximate prevalence (Butrous, 2019).

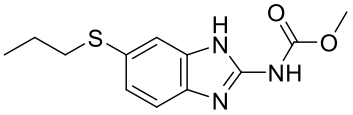
2.1.1 STH and children

Polyparasitism is a common condition in human populations whereby a person is infected by two or more concomitant, chronic parasitic infections. The causative agents are helminths and protozoan parasites (Sumbele et al., 2021). In Sub-Saharan Africa helminth coinfections with STH and *Schistosoma* are quite common (Madinga et al., 2017). This is because of the overlapping geographic distribution of the parasites. Infections with both STH and schistosomes are known to be associated with hepatosplenomegaly and portal hypertension, chronic inflammation, gastrointestinal problems, anaemia, and depletion of nutrients in children (Opara et al., 2021). Polyparasitism is most prevalent in school-aged children (SAC) (Phiri et al., 2016) who are consistently at high risk of parasitic infections due to their poor hygiene and sanitation practices (Labana et al., 2021).

The number of children requiring preventive chemotherapy for soil transmitted helminthiases was estimated to be 1,045,534,806 globally in 2019 (WHO, 2020b) while about 800 million people most of which are children are at risk of schistosomiasis (Anisuzzaman and Tsuji, 2020). In Africa about 267 million preschool-age children and 568 million school-aged children (SAC) are at risk of chronic infections (Cho et al., 2021). Chronic infection with the parasites has adverse effects on the children's growth and development, cognitive potential, and nutritional status. This has a negative impact on the SAC's school attendance, learning, and overall well-being (Labana et al., 2021).

2.1.2 Current drugs and control measures of STH

The primary defense against these parasites comprises only four major classes of anthelmintic drugs the majority of which have been leveraged from veterinary pharmaceutical research (Nixon et al., 2020). These are benzimidazoles macrocyclic lactones, nicotinic acetylcholine receptor (nAChR) agonists, and aminoacetonitrile derivatives (AADs) (Wit et al., 2021). There are only six drug options available to combat STHs and Schistosomiasis and these include albendazole (**2.1**), mebendazole (**2.2),** praziquantel (**2.3**), ivermectin containing the components **2.4** and **2.5,** oxamniquine (**2.6**), and diethylcarbamazine (**2.7**), and (Tinkler, 2020). Preventive chemotherapy is the main strategy used to control the morbidity caused by STHs in what is currently the world's largest drug administration program (Doyle and Cotton, 2019). A single, oral dose of albendazole (**2.1**; 400 mg) or mebendazole (**2.2**; 500 mg) is periodically administered to populations considered to be at risk. One of the strategies to control Schistosomiasis endemicity is also preventive chemotherapy interventions through regular mass-drug administration (MDA) of praziquantel (**2.3**) tablets (Torres-Vitolas et al., 2021). The target populations for MDA are preschool-aged (preSAC), school-aged children (SAC) and women of reproductive age (Vlaminck et al., 2020). Over 500 million children received anthelmintic drugs in MDA programs in 2016 (Doyle and Cotton, 2019). The structures of the three drugs **2.1**-**2.3** commonly used in MDA (which are all synthetic) are shown below, while the structure of the natural anthelmintic ivermectin, which is a mixture of more than 80% 22,23-dihydroavermectin B_{1a} (**2.4**) and B_{1b} (**2.5**), also commonly used against STH but mostly in mass drug administration campaigns against onchocerciasis and lymphatic filariasis, is also shown.



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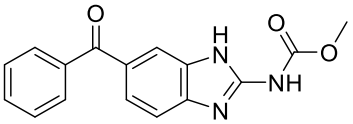

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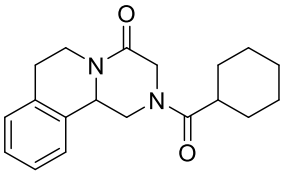

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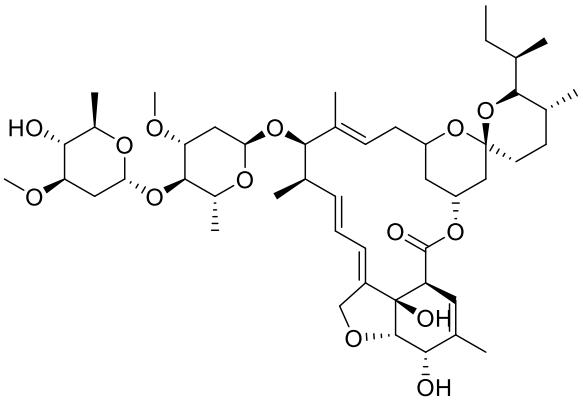

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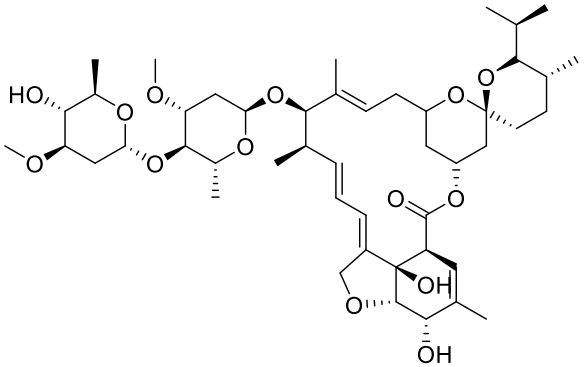

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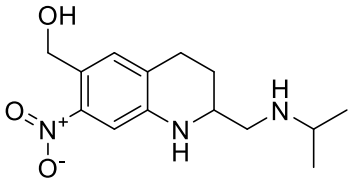

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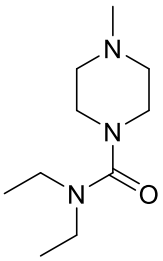

2.7

2.1.3 Drug resistance as an emerging problem

Although MDA is an effective way of controlling the prevalence of parasitic worms, there is the danger of the emergence of resistance species. This is because when parasites are repeatedly exposed to the same drug, parasites that survive, may pass on genetic variants that make the offspring more resistant to the anthelmintic drug (Mutombo et al., 2019). This can be seen in current parasite control programs in veterinary species which have moved away from mass anthelmintic treatment approaches because of the emergence of anthelmintic resistance (Tinkler, 2020). In livestock production the prevalence of anthelmintic resistance has been increasing over time. Resistance has been observed across the three major antiparasitic drug classes, including avermectins, benzimidazoles, and imidazothiazoles (Sharpton et al., 2020). For example in some areas of the United States, the prevalence of resistance of small ruminants to avermectins and benzimidazoles at herd level can range from 80 to 100% (Darby, 2021). The resistance to anthelmintic drugs in veterinary species serves as a sentinel for how anthelmintic resistance may increase within the human population (Sharpton et al., 2020). Unfortunately in humans there is no standardized surveillance protocol for monitoring anthelmintic resistance in places where MDA of anthelmintic drugs takes place (Tinkler, 2020) and the availability of only a few drugs raises the likelihood that drug resistance will develop (Schulz et al., 2018). Observations have been made linking the effectiveness of anthelmintic drugs to the duration of MDA programs. For example in Uganda the efficacy of the drug praziquantel (**2.3**) against intestinal schistosomiasis caused by *Schistosoma mansoni*, has been shown to be lower in schools that have a longer duration of MDA (Crellen et al., 2016).

Currently, the two drugs provided in the preventive chemotherapy of STH, albendazole (**2.1**) and mebendazole (**2.2**) are both benzimidazoles and they both have the same mode of action (preventing the polymerization of microtubules). Therefore, if anthelmintic resistance would arise against one of the drugs, it would probably also affect the efficacy of the other benzimidazol drug (Vlaminck et al., 2020). Poor efficacy of albendazole (**2.1**) against *T. trichiura* and the hookworm has been reported in Pemba Island which has a long history of MDA (Walker et al., 2022). Veterinary parasitologists have stated that if anthelmintic resistance develops within a certain class of anthelmintics, all drugs within this class are rendered ineffective (Tinkler, 2020). Low drug efficacy has been reported in helminth infections caused by *T. trichiura* and hookworm (Zeleke et al., 2020). Results from patients infected with schistosomiasis and not cured by multiple doses of praziquantel (**2.3**), have been

reported from different geographic locations, and this suggests that resistance to the drug may be present (Deribew and Petros, 2013). Cases of praziquantel drug resistance have been evidenced by the low sensitivity of the parasite to praziquantel (**2.3**) in areas where the drug has been extensively used (Oliveira Viana et al., 2020). Praziquantel (**2.3**) is also inactive against the juvenile stage of *Schistosoma* spp. (Panic et al., 2014).

With this emergence of drug resistance there is a need for new anthelmintic drugs. Unfortunately for humans the introduction of new anthelmintic drugs has been stagnant and an example of this is, between 1975 and 2004, 1.556 new drugs were marketed and very few were developed to treat the human helminthiases (Tinkler, 2020) and no new class(es) of anthelmintics have been approved for human use since 2000. This is due to the high costs of developing human anthelmintic medicines (Nixon et al., 2020). The same drugs have been used over a long period of time due to lack of incentives for the pharmaceutical industry to invest in anthelmintic drugs for humans as the people affected cannot afford the drugs. Since those affected by STH cannot afford the drugs, large-scale donations of benzimidazoles have been essential for STH control. The WHO coordinates the donation of anthelmintic drugs by pharmaceutical companies. A total of 600 million tablets are donated annually by Glaxo Smith Kline which donates albendazole (**2.1**) and Johnson & Johnson donating mebendazole (**2.2**) (Montresor et al., 2020). However long-term reliance on large-scale donations of anthelmintic drugs is not sustainable (Linn and Addiss, 2018). There are multiple strategies for coming up with new anthelmintic drugs including screening drugs meant for other diseases for anthelmintic activity. This is called drug repurposing and the advantage of this approach is that it can accelerate the drug development process, as the available preclinical and clinical data lowers the cost (Zajickova et al., 2020).

2.1.3.1 Substandard medicines

There is also the problem of substandard medicines in the market which is a global threat on public health (Suleman et al., 2014). There are various reasons for the presence of these poor quality medicines, which includes poor quality control during manufacturing and importing medicines, inappropriate storage and transport, chemical and/or physico-chemical instability and spurious/falsely labeled/falsified/counterfeit (SFFC) medicines (Heyman and Williams, 2011). The substandard medicines may contain an excessive dose of the active ingredient, or not enough and may even contain toxic impurities. This can result in resistance development, therapeutic failure, and occurrence of serious adverse events or even death (Juillet, 2008; Nsimba 2008). A study conducted to assess the standardized pharmacopoeia quality of anthelmintic drugs in Ethiopia found that although all samples of albendazole (**2.1**) and mebendazole (**2.2**) collected contained the active ingredient, in detail 48.0% of albendazole (**2.1**) and 45.2% mebendazole (**2.2**) samples did not meet the expected pharmacopoeial quality specifications. One of the reasons for failure to meet the pharmacopoeia quality was underdosing whereby 19.4% of the albendazole (**2.1**) and mebendazole (**2.2**) samples were underdosed and this contributes to the development of drug resistance (Suleman et al., 2014).

2.1.4 Anthelmintic drugs and the environment

There is a need for new anthelmintics because of a number of reasons. Firstly continuous use of the same drugs over a long period of time ultimately leads to the selection of resistant

organisms and this is also compounded by the use of mass drug administration as a means to deal with the intestinal worms in prevalent areas. The second reason we need new anthelmintics is that the current drugs are a potential hazard to the environment as they are widely used in rearing of cattle, sheep and other livestock (Fig. 2.5) and in MDA programs. In developed areas human drugs are usually treated by wastewater treatment plants before discharging into the aquatic environment (Li et al., 2020) but this is not the case in marginalised populations where there are usually inadequate ablution facilities let alone wastewater treatment plants. Environmental safety is becoming increasingly challenging, for both human and animal drug production because of the potential impacts of drug residues on the aquatic and terrestrial ecosystems. The residues could potentially harm other non-target organisms in the environment and could also contaminate the water table (Nixon et al., 2020). A study by Li et al (2020) showed the presence of trace amounts of 19 anthelmintic drugs in samples taken from the environment that included river water, rain water, waste water and tap water. Trace amounts of seven anthelmintics including albendazole nd praziquantel were found in marine environments in Korea which included sea water, sediment and cultured fish (Kim et al., 2017). Albendazole which is one of the most used drugs in MDA as well as livestock farming is excreted from treated animals and enters the environment. Anthelmintic drugs against parasitic worms are among the most common and frequently used drugs but not many studies have been done to investigate their phytotoxicity (Stuchlikova et al., 2020). There are some studies on the ecotoxicological effects of some classes of anthelmintics that have been conducted. An example of this is the macrocyclic lactones e.g. avermectins, which have been shown to have negative effects on dung beetle populations and also on different aquatic organisms (Mooney et al., 2021). A further example is ivermectin which has negative or even lethal effects on several terrestrial invertebrates, with the most susceptible being dung-living ones (Verdu et al., 2018). It also affects aquatic invertebrates, and *Daphnia magna* was found to be the most sensitive among the tested species (Garric et al., 2007). Ivermectin has also been observed to have a negative effect on fish (Vokral et al., 2019) and has a negative effect on soya beans as it reduces the number and weight of the beans (Navratilova et al., 2020).

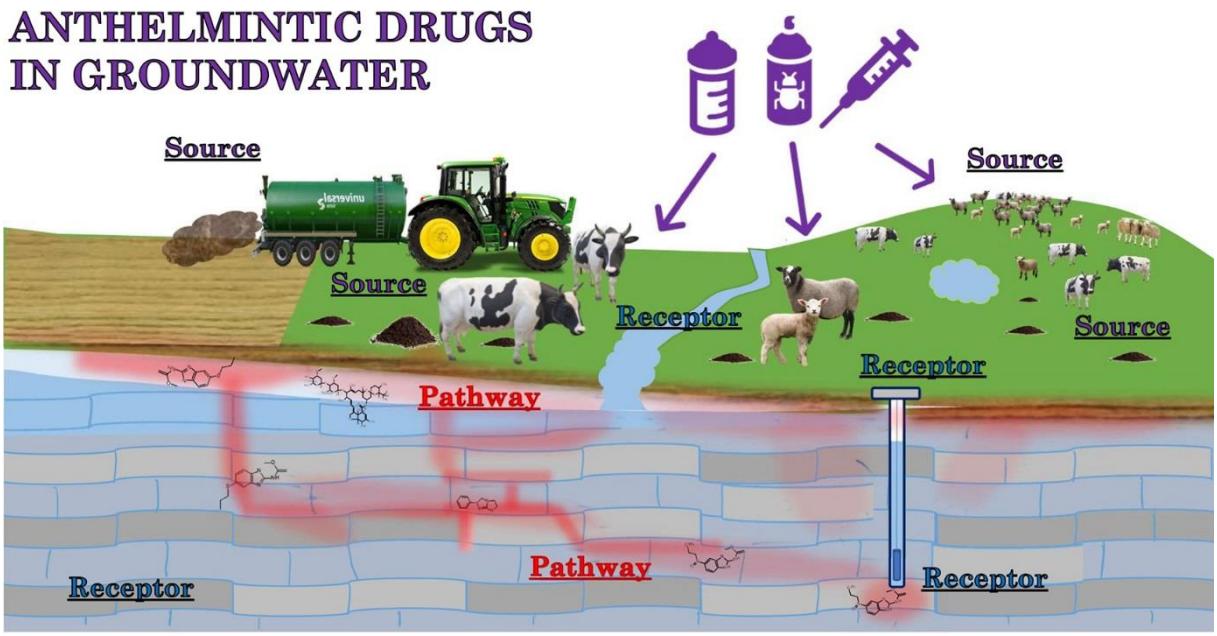

Fig. 2.5. An illustration showing how anthelmintic drugs can contaminate ground water (Mooney et al., 2021).

2.1.5 Effects of Covid 19 in the fight against NTDs

The Covid 19 pandemic has also taken the fight against neglected tropical diseases a few steps back as financial constraints, movement constraints and limitations of the healthcare sector (due to worker absence or illness) to deal with other diseases has led to reduced interventions against NTDs as the world highly focuses on Covid 19 (WHO, 2020a). The WHO recommended the postponement of all MDA programs, community based surveys and active case finding activities in April 2020. Therefore, schistosomiasis was among the diseases that will be most affected by the disruption of the programs against NTDs (Brooker et al., 2021).

2.2 Natural products

2.2.1 Natural product waste as a source of anthelmintics

An environmentally friendly solution to the intestinal parasites could be a natural product based waste product. This is in-line with sustainable development which requires incorporation of practices that include prudent use of resources and avoiding waste (Hurst and Clark, 2020). This would not only help reduce waste but also minimize the impact of anthelmintic drugs on the environment. This would also help conserve plants as instead of using a plant we would be using something which normally is thrown away. Agroindustrial by-products with anthelmintic properties have the potential to be used as nutraceutical materials. An example of this is the potential of chicory root pulp (*Cichorium intybus*), a by-product obtained from industrial chicory roots after inulin extraction, as a nutraceutical anthelmintic for livestock (Pena-Espinoza et al., 2020). Waste materials from the food industry can also be used as an initiative towards sustainable development to alleviate environmental pollution and to improve health (Akter and Rabeta, 2021). The leaves and husks of *Theobroma cacao* from the cocoa industry have shown potential to be used as anthelmintic nutraceuticals

as they have shown *in vitro* anthelmintic properties against the barber's pole worm *Haemonchus contortus* (Mancilla-Montelongo et al., 2021). In *Carica papaya,* processing the seeds make up about 12% of waste (Akter and Rabeta, 2021) and the anthelmintic activity of the seeds has been shown against various parasites in sheep, chickens and goats (Ameen et al., 2010; Ameen et al., 2012; Ameen et al., 2018). Pumpkin seeds (*Cucurbita* spp.) are also discarded as by-products in the agroindustry (Maldonade et al., 2020). Just like papaya seeds, pumpkin seeds have also shown anthelmintic activity against parasitic nematodes like *Heligmosoides bakeri* and could become an inexpensive source of anthelmintic compounds (Grzybeck et al., 2016).

2.2.2 Traditional medicine as a source of anthelmintic plants

Traditional medicine as defined by the WHO is “the sum total of the knowledge, skills, and practices based on the theories, beliefs, and experiences indigenous to different cultures, whether explicable or not, used in the maintenance of health as well as in the prevention, diagnosis, improvement or treatment of physical and mental illness” (WHO, 2013). Traditional medicine could be a likely source of relatively inexpensive drugs for symptomatic management and also has the potential to provide libraries of information for new therapeutic approaches (Jansen et al., 2021). Traditional medicine is still extensively used as there are still remote areas which do not have access to clinics and doctors or the clinics and doctors are located too far away. In Africa traditional medicine usage has been, and remains, a significant contributor to primary healthcare delivery (Andrae-Marobela et al., 2021). In Asia herbal remedies, including those for helminth infections are extensively used and studied (Valentynvna and Ivanivna, 2017). It is estimated that 80% of people in African and Asian countries use traditional medicine to meet their primary health care needs (Oyebode et al., 2016) and 88% of the WHO member states acknowledge the formal use of traditional and complementary medicines in their health systems (Lin et al., 2021). Despite these facts there has been limited funds invested in the research and advancement of herbal medicine knowledge (Thomford et al., 2018). Even with limited funding, herbal medicine has often contributed to the discovery of some of the drugs that have advanced human health. For example the drugs quinine (antimalarial drug) and acetyldigoxin and acetyldigitoxin (used in congestive heart failure) were discovered and developed under the backbone of traditional herbal medicine use of the bark of the chincona tree (Achan et al., 2011) and *Digitalis lanata* respectively (Gisvold, 1972). Traditional medicine would therefore be a good place to look for anthelmintics as the custodians of indigenous knowledge know which plants to use against which diseases. Traditional knowledge about the medicinal uses of different plants such as anthelmintic properties is generally acquired through folklore, and is passed on via word of mouth (Bibi et al., 2016). Various ethnobotanical surveys conducted in Africa and Asia have shown that traditional healers use plant species that are readily available to combat helminth infections (Bibi et al., 2016; Wintola and Afolayan, 2015). Safety is a key priority in any drug discovery program and the use of a plant in traditional medicine may provide insights regarding its efficacy and safety (Atanasov et al., 2015) and it is hoped that isolated active principles from plants used in traditional medicine will have a good safety profile. Conventional antiparasitic drug discovery using compound libraries may lead to active compounds that may prove harmful to a patient, due to both on- and off-target effects. Since

humans are eukaryotes, eukaryotic parasites in particular share significant biochemical traits with their hosts, making selectivity and safety more difficult to achieve than for antibiotics against bacteria (Nixon et al., 2020).

2.2.3 Natural products as a drug source

Historically natural products have been the source of most drugs (Newman and Cragg, 2020) but there was a shift by the pharmaceutical industry in the last few decades to libraries of synthetic compounds and high throughput screening as drug discovery sources because of the complexities of working with natural products (David et al., 2015). These include intellectual property issues as gaining intellectual property rights for unmodified natural products exhibiting bioactivities can be challenging. This is because it is not always possible to patent naturally occurring compounds in their original form (Harrison, 2014). Changes in the metabolite profiles of the starting plant material due to factors such as environmental variations in the location at which the plants were collected is also an obstacle in the drug development process (Atanasov et al., 2015). There are also problems in sourcing material as accessing sufficient material to isolate and characterize a bioactive compound may also be challenging (Cragg et al., 1993). Natural products are also not easy to synthesise as they often appear to be structurally too complex (Lachance et al., 2012). Even though there may be challenges, plants have always been a major source for the discovery of new therapeutic agents. This is because plants produce a broad spectrum of secondary metabolites which are responsible for or play a part in biological activities including defending the plant against pests and diseases (Idris et al., 2019). These secondary metabolites cover a wider area of chemical space compared with synthetic small molecule libraries (Lachance et al., 2012). As a result of the production of these secondary metabolites, many diverse active compounds with anthelmintic activities have been isolated from plants (Zajickova et al., 2020). Plants are therefore still a good source for anthelmintic compounds. Studies have shown that besides being a good alternative to synthetic drugs in reducing the effects of worm infections, plants can also be used as a source of anthelmintic drugs which have fewer side effects than synthetic drugs (Cheraghipour et al., 2019). Bacteria can also be a source of anthelmintic compounds. The avermectins from which ivermectin was derived were also isolated from the vegetative mycelia of *Streptomyces avermitilis* (Miller et al., 1979). More recently, macrolides (milbemycines) active against *C. elegans,* were isolated from *Streptomyces microflavus* (Xiang et al., 2010).

Fungi could also be a source of secondary metabolites with anthelmintic properties. In Asia, mushrooms have been used for promoting and maintaining a good state of health and for the treatment of diseases since ancient times (Venturella et al., 2021) while in Central and Southern Africa they contribute significantly to diets (Cheung, 2008). Mushrooms have been known to possess medicinal properties in different cultures around the world. They have therefore been used to treat various human diseases (Zeb and Lee, 2021). Mushrooms have also been recognized as a food source which can be used for the development of medicines and nutraceuticals (Alves et al., 2012). The presence of bioactive metabolites in the mycelium but most importantly in the fruiting body gives mushrooms various biological activities. Studies have shown that some mushrooms contain anthelmintic properties for example the hydroalcoholic extracts from *Pleurotus djamor* showed antiparasitic effects against

Haemonchus contortus eggs (Pineda-Alegría et al., 2017). Fungal metabolites known to have bioactivity are phenolic compounds, fatty acids, antioxidants and laccases (Elkhateeb, 2020).

Natural product compound libraries are a good source to look for anthelmintic compounds as secondary metabolites have many biological activities, so testing a natural compound library is quite efficient in that it saves one the time of sourcing plant material and having to do the isolation of the compounds and structure elucidation. The Pan African Natural Product Library (PANPL) is a consortium of natural product collections isolated from African biota and owned by scientists working in African institutions (Ntie-Kang et al., 2015). It represents the largest physical collection of natural products from African medicinal plants (Tietjen et al., 2015).

2.2.4. Helminths

The word "helminth" is a general term which means "worm," but helminths are made up of parasites, roundworms (Nematodes), and flatworms, which are subdivided into tapeworms (Cestodes) and flukes (Trematodes) (Sepúlveda-Crespo et al., 2020). Whole organism screening is often costly, resource-intensive, low-throughput and requires specialist training. This is because parasitic helminths must be passaged through a host, maintained *in vivo* and then isolated for testing (Nixon et al., 2020) and this greatly complicates quantitative experiments done in their natural habitat (Holden-Dye and Walker, 2014). The free living nematode *C. elegans* is an ideal model organism as it has enough similarities to the parasitic worms to act as an approximate test model (Buckingham et al., 2014). For example the animal parasitic suborder *Strongylida* including the human hookworms *Ancylostoma duodenale* and *Necator americanus* are closely related to *C. elegans* and it is therefore an excellent model organism for these pathogens (Aboobaker and Blaxter, 2000). Although the life-style of *C. elegans* is very different to that of the parasites (Holden-Dye and Walker, 2014) and there may be genomic diversity between *C. elegans* and other parasitic nematodes, similarities in physiology, pharmacology, and genetic, genomic, and molecular tools make *C. elegans* an ideal candidate for direct experimentation and characterization. Commercially available anthelmintics have also been shown to be effective against *C. elegans* and therefore compounds that have an anthelmintic effect against *C. elegans* could potentially be effective against a wide range of parasitic nematodes (Sepúlveda-Crespo et al., 2020). Genes that are unique to *C. elegans* and nematodes or show specific differences from vertebrates and insect genes can be suitable drug targets (Burglin et al., 1998). *C. elegans* was the first complex eukaryote to have its genome sequenced (The *C. elegans* sequencing consortium, 1998) and this provided vital information for genomic approaches. The genomic analysis revealed that *C. elegans* has around 20 000 protein-coding genes of which 48% have assigned gene names. This lead to the availability of powerful molecular, genetic and genomic tools that can be used to detect targets and mechanisms of action of approved anthelmintics that were unknown upon their discovery (Sepúlveda-Crespo et al., 2020).

The *C. elegans* assay is a very robust and reliable assay with many advantages as maintenance of *C. elegans* in the laboratory and culturing is very simple. Unlike assays involving adult parasites where the host animal has to be killed, *C. elegans* can be grown quickly in large numbers and because worm viability is assessed microscopically, colour of extracts or compounds does not influence the results (Thomsen et al., 2012). One minor draw back is the counting of the worms which is tedious and time consuming and limits the number of samples

that can be tested at once. It would therefore be advantageous to come up with an automated system of counting the worms. This would enable more samples to be tested and bring about the possibility of developing a high through put version of the assay.

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3. Anthelmintic activity and cytotoxic effects of compounds isolated from the fruits of *Ozoroa insignis* Del. (Anacardiaceae)

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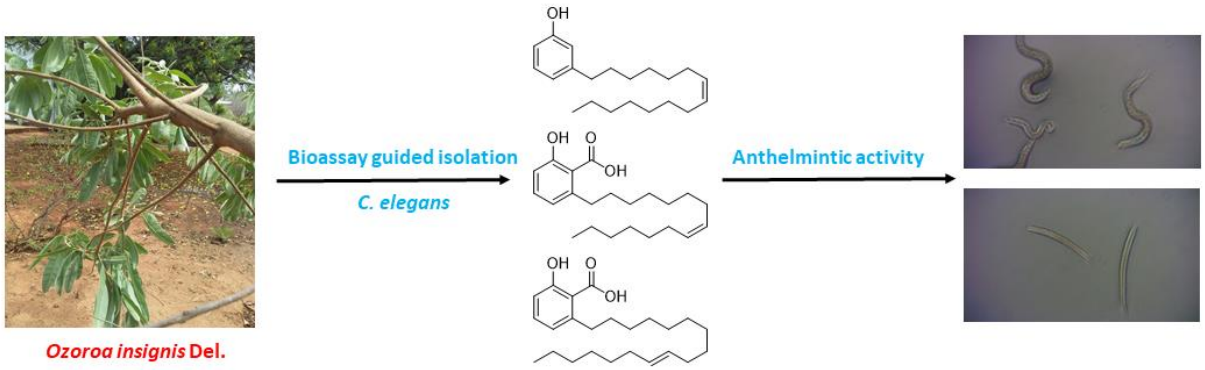


Abstract: *Ozoroa insignis* Del. is an ethnobotanical plant widely used in traditional medicine for various ailments, including schistosomiasis, tapeworm, and hookworm infections. From the so far not investigated fruits of *Ozoroa insignis*, the anthelmintic principles could be isolated through bioassay-guided isolation using *Caenorhabditis elegans* and identified by NMR spectroscopic analysis and mass spectrometric studies. Isolated 6-[8(Z)-pentadecenyl] anacardic (**3.1**), 6-[10(Z)-heptadecenyl] anacardic acid (**3.2**), and 3-[7(Z)-pentadecenyl] phenol (**3.3**) were evaluated against the 5 parasitic organisms *Schistosoma mansoni* (adult and newly transformed schistosomula), *Strongyloides ratti*, *Heligmosomoides polygyrus*, *Necator americanus*, and *Ancylostoma ceylanicum*, which mainly infect humans and other mammals. Compounds **3.1**–**3.3** showed good activity against *Schistosoma mansoni*, with compound **3.1** showing the best activity against newly transformed schistosomula with 50% activity at 1µM. The isolated compounds were also evaluated for their cytotoxic properties against PC-3 (human prostate adenocarcinoma) and HT-29 (human colorectal adenocarcinoma) cell lines, whereby compounds **3.2** and **3.3** showed antiproliferative activity in both cancer cell lines, while compound **3.1** exhibited antiproliferative activity only on PC-3 cells. With an IC_{50} value of 43.2 µM, compound **3.3** was found to be the most active of the 3 investigated compounds.**3.1.**

Introduction

Human nematode infections are the most prevalent infections, affecting 1.5 billion people or 24% of the world's population (WHO, 2019). Despite this high global prevalence, medical research into parasitic diseases remains neglected. In Africa, multiple ethnic groups have used traditional medicine for centuries to treat nematode infections (Cock et al., 2018). *Ozoroa insignis* Del. (Anacardiaceae) is used as a traditional medicinal plant for many indications, including intestinal worms. For example, in Botswana, people use a decoction of the roots or add fresh crushed fruits to porridge to eliminate the parasites (J. Mlilo, personal communication, 17 December 2019). In spite of this information, we did not find any literature pertaining to the anthelmintic properties or chemical constituents of the fruits of *O. insignis*.

The anthelmintic effect of the roots, leaves, and bark of *O. insignis*, but not the fruits, have been evidenced *in vitro* against *Schistosoma mansoni* and *Hymenolepis diminuta* (Mwihaki et al., 2009). Additionally, the bark has also shown *in vitro* cytotoxic activity against HEP-G2 (human hepatocellular carcinoma), MDA-MB-231 (human mammary adenocarcinoma), and 5637 (human bladder carcinoma) cell lines (Rea et al., 2003). In order to isolate the active phytoconstituents of *O. insignis*, studies have been performed on different organs of the plant, which led to the identification of a variety of structurally diverse metabolites. From the roots of the plant, several tirucallane triterpenes have been recognized (Liu et al., 2006). From the same organ, a macrolide named ozoroalide was isolated (Abreu and Liu, 2007). Several closely related anacardic acids (compounds consisting of a salicylic acid moiety with an additional saturated or unsaturated alkyl chain) were recognized from *O. insignis*. From the twigs, the anarcadic acid derivative, 6-pentadecylsalicylic acid, was isolated (He et al., 2002). Furthermore, from the root bark of the plant 6-tridecyl anacardic acid, 6-[8(Z)-pentadecenyl] anacardic acid, 6-[10(Z)-heptadecenyl] anacardic acid, and 6-[nonydecyl] anacardic acid, a flavonoid named ozoranone, as well as triterpenes, for instance, _α-amyrin, magnificol, betulonic acid, and betulinic acid were isolated (Mwihaki et al., 2009; Rea et al., 2003). Other chemical investigations have shown that plants containing anacardic acids like *O. insignis* commonly have a mixture of related phenolic lipids including cardols and cardanols (Schultz et al., 2006). To the best of our knowledge, there is no report on the constituents of *O. insignis* fruits. We herein report the identification of 6-[8(Z)-pentadecenyl] anacardic acid (**3.1**), trivially named ginkgolic acid C15:1 (CAS 22910-60-7), 6-[10(Z)-heptadecenyl] anacardic acid (**3.2**), trivially named ginkgolic acid C17:1 (CAS 111047-30-4) and 3-[7(Z)-pentadecenyl] phenol (**3.3**; CAS 936109-15-8) from the fruits of *O. insignis* through bioassay-guided isolation using the well-established test system with the model organism *Caenorhabditis elegans* (Caffrey, 2012; Hahnel et al., 2021; Holden-Dye and Walker, 2014). The anthelmintic properties of **3.1**–**3.3** were next evaluated against the 5 parasitic organisms *Schistosoma mansoni* (adult and newly transformed schistosomula), *Strongyloides ratti*, *Heligmosomoides polygyrus*, *Necator americanus*, and *Ancylostoma ceylanicum* (all larval stages). These parasites mainly infect humans and other mammals like rodents, dogs, cats, and baboons. In addition, the cytotoxic and antiproliferative potential, respectively, of **3.1**–**3.3** was evaluated against PC-3 (human prostate cancer) and HT-29 (human colorectal adenocarcinoma) cell lines.

3.2. Materials and methods

3.2.1. General methods

Column chromatography for fractionations or purifications was performed either on silica gel (0.040–0.063 mm, Merck, Germany) or Sephadex LH 20 (Fluka, Steinheim, Germany). Analytical TLCs were performed on pre-coated silica gel F254 aluminum sheets (Merck, Darmstadt, Germany), and spots were detected by their color, their absorbance under a spectrophotometer, or after spraying with vanillin and heating.

NMR spectra were recorded with an Agilent DD2 400 MHz NMR spectrometer (Varian, Palo Alto, CA, USA) operating at a proton NMR frequency of 400 MHz using a 5-mm inverse detection cryoprobe. 2D NMR spectra were recorded using standard CHEMPACK 8.1 pulse sequences (^{1}H,^{1}H zTOCSY, ^{1}H,^{13}C gHSQCAD, ^{1}H,^{13}C gHMBCAD) implemented in Varian VNMRJ 4.2 spectrometer software (Varian, Palo Alto, CA, USA). The mixing time for the TOCSY experiments was set to 80 msec. The HSQC experiment was optimized for ^{1}JCH = 146 Hz with DEPT-like editing and ^{13}C-decoupling during the acquisition time. The HMBC experiment was optimized for a long-range coupling of 8 Hz; a two-step 1JCH filter was used (130–165 Hz). ^{1}H chemical shifts are referenced to internal TMS (^{1}H δ = 0 ppm), while ^{13}C chemical shifts are referenced to $CDCl_3$ (^{13}C δ = 77.0 ppm).

The negative ion-electron spray ionization high-resolution mass spectra (ESI-HRMS) were obtained from an API 3200 Triple Quadrupole System (Sciex, Framingham, MA, USA) equipped with a turbo ion spray source, which performs ionization with an ion spray voltage on 70 eV. Sample introduction was performed by direct injection through an Agilent- HPLC 1200 (Agilent, Santa Clara, CA, USA) syringe pump. During the measurement, the mass/charge range from 5 to 1800 was scanned.

3.2.2. Plant material

The stem bark, leaves, roots, and fruits of *Ozoroa insignis* Del. (Anacardiaceae) were collected in October 2018 in Sehithwa in northwest Botswana. All the samples were authenticated by Mr. Bongani Sethebe from the University of Botswana, where a voucher specimen was deposited with the registration number MD1-10/2018-Fil.

3.2.3. Extract preparations and preliminary anthelmintic screening

Air-dried plant organ (root bark, stem bark, leaves, and fruits, each 1 g) was extracted by sonication 3 times for 15 min with 10 mL of 80% MeOH at room temperature. The resulting solutions were evaporated to dryness under reduced pressure using a rotary evaporator maintained at 40 °C to afford crude extracts (root bark 122.7 mg, stem bark 98.5 mg, leave 32 mg, and fruits 83.9 mg). From each extract, a stock solution of 1 mg/mL in 4% DMSO was prepared. Each stock sample was screened at the final concentration of 500 µg/mL. Ivermectin (10 µg/mL) was used as a positive control.

3.2.4. Isolation

A total of 86 g of air-dried ground fruits of *Ozoroa insignis* Del. were extracted 3 times by sonication for 15 min with (3 × 400 mL) of 80% MeOH. Filtrates were obtained by using filter papers (Whatman no.1) and were evaporated to dryness under reduced pressure using a rotary evaporator maintained at 40 °C to yeild 28.48 g of crude extract.

This extract, showing 90% anthelmintic activity, was dissolved in 200 mL of water and partitioned between *n*-hexane (500 mL × 3), EtOAc (300 mL × 5), and *n*-butanol (200 mL × 3).

The resulting fractions were evaporated to dryness at 40 °C to yield 22.58 g of *n*-hexane extract, 4.16 g of EtOAc extract, 2.73 g of *n*-BuOH extract, and 1.4 g of the remaining aqueous fraction.

A total of 20.82 g of the most active fraction (*n*-hexane fraction) was adsorbed on an equivalent mass of silica gel and chromatographed over a silica gel column (8 × 36 cm) using *n*-hexane-EtOAc and EtOAc-MeOH gradients as eluent systems. The column was monitored by UV lamp (254 and 366 nm). Fractions of 400–500 mL were collected as follows: [(1–5), *n*-hexane-EtOAc (90:10)], [(6–27), *n*-hexane-EtOAc (8:2)], [(28–35), *n*-hexane-EtOAc (7:3)], [(36–39), *n*-hexane-EtOAc (6:4)], [(40–41), *n*-hexane-EtOAc (1:1)], [(42–43), *n*-hexane-EtOAc (4:6)], [(44), *n*-hexane-EtOAc (3:7)], [(45–46), *n*-hexane-EtOAc (2:8)] [(47–48), *n*-hexane-EtOAc (1:9)] [(49), EtOAc (100%)][(50), EtOAc-MeOH (97.5:2.5)] [(51), EtOAc-MeOH (95:5)] [(52–53), EtOAc-MeOH (92.5:7.5)] [(54–58), EtOAc-MeOH (9:1)] [(59–65), MeOH (100%)]. These fractions were pooled according to their TLC profiles into 5 subfractions F1 to F5 as follows: F1 (1–5), F2 (6), F3 (7), F4 (8–59), and F5 (60–65). Compound **3.1** precipitated in F4 as a white amorphous solid. 460 mg of F3 was separated by size exclusion chromatography on a Sephadex LH 20 column (1.7 × 40 cm) using DCM-MeOH (7:3) as eluent to afford 35 fractions. Fractions 8–14 were combined to give compound **3.2** which precipitated as a white amorphous solid. Next, 240 mg of F1 was further separated by Sephadex LH 20 column chromatography (1.7 × 40 cm) using DCM-MeOH (7:3) as eluent. A total of 15 fractions were collected and fractions 4–8 were combined to yield compound **3.3**.

3.2.5. *In vitro* anthelmintic bioassay

3.2.5.1. *Caenorhabditis elegans* assay

The Bristol N2 wild-type strain of *Caenorhabditis elegans* was used in the anthelmintic assay. The nematodes were cultured on NGM (Nematode Growth Media) Petri plates using the uracil auxotroph *E. coli* strain OP50 as a food source according to the methods described by Stiernagle (2006). The anthelmintic bioassay was carried out following the method developed by Thomsen et al. (2012). In all the assays, the solvent DMSO (2%) and the standard anthelmintic drug ivermectin (10 µg/mL) were used as negative and positive controls, respectively. All the assays were carried out in triplicate. LC50 values were calculated using SigmaPlot 14.0.

3.2.5.2. Parasitic helminths

In vitro studies were carried out in accordance with Swiss national and cantonal regulations on animal welfare under permission number 2070 at the Swiss Tropical and Public Health Institute (Swiss TPH). The drug sensitivity assays with *Schistosoma mansoni* [adult and newly transformed schistosomules (NTS)] and *Strongyloides ratti*, *Heligmosomoides polygyrus*, *Necator americanus*, and *Ancylostoma duodenale* were carried out as described in previous publications (Lombardo et al., 2019; Keiser and Haeberli, 2021) to test the activity of 6-[8(Z)-pentadecenyl] anacardic acid (**3.1**), 6-[10(Z)-heptadecenyl] anacardic acid (**3.2**), and 3-[7(Z)-pentadecenyl] phenol (**3.3**) at 100 µM and 10 µM.

3.2.5.3. *In vitro* tests on *A. ceylanicum, H. polygyrus*, *N. americanus*, and *S. ratti* L3

The life cycles of the assayed nematodes are maintained at the Swiss TPH. *A. ceylanicum*, *H. polygyrus*, and *N. americanus* larvae (L3) were obtained by filtering the feces of infected

hamsters (*A. ceylanicum* and *N. americanus*) and mice (*H. polygyrus*) and cultivating the eggs on an agar plate for 8–10 days in the dark at 24 °C. *S. ratti* L3 were acquired as summarized by Garcia and Bruckner (1997). For the drug assay, 30–40 L3 were placed in each well of a 96-well plate for each compound. Larvae were incubated in 175 µL of culture medium with the test drugs at concentrations of 10 and 100 µM. RPMI 1640 (Gibco, Waltham, MA, USA) medium supplemented with 5% amphotericin B (250 µg/mL; Sigma-Aldrich, Buchs, Switzerland) and 1% penicillin 10,000 U/mL (Sigma-Aldrich, Buchs, Switzerland), and streptomycin 10 mg/mL solution (Sigma-Aldrich, Buchs, Switzerland) was used for the assays with *H. polygyrus* L3. Phosphate-buffered saline (PBS; Sigma-Aldrich, Buchs, Switzerland) supplemented with 1% penicillin (10,000 U/mL) and streptomycin (10 mg/mL) solution was used to incubate *S. ratti* L3. *A. ceylanicum* and *N. americanus* L3 stages were incubated in Hanks' balanced salt solution (HBSS; Gibco, Waltham, MA, USA) supplemented with 10% amphotericin B and 1% penicillin (10,000 U/mL) and streptomycin (10 mg/mL) solution. Larvae were kept in the dark at room temperature for 72 h, after which the drug effect was evaluated. For this, the total number of L3 per well was determined. Then, 50–80 µL of hot water (≈80°C) was added to each well, and the larvae that responded (the moving worms) were counted. The proportion of larval death was determined.

3.2.5.4. *In vitro* tests on *S. mansoni*

The *in vitro* tests on *S. mansoni* were carried out as described in literature (Lombardo et al., 2019). Briefly, to obtain newly transformed schistosomula (NTS), cercariae were collected from infected *Biomphalaria glabrata* snails (maintained at Swiss TPH) and were mechanically transformed. The NTS were kept in the incubator (37° C and 5% CO_2) in medium 199 (Gibco, Waltham, MA, USA), supplemented with 5% fetal calf serum (FCS; Bioconcept, Allschwil, Switzerland) and 1% penicillin/streptomycin and 1% (v/v) antibacterial/antifungal solution until usage. Adult *S. mansoni* worms were collected by dissecting the mesenteric veins of infected mice at day 49 post-infection. For NTS and adult *S. mansoni*, transparent flatbottom 96- and 24-well plates were used, respectively (Sarstedt, Nürmbrecht, Germany). 30–40 NTS were incubated with the test drug (1, 10, and 100 µM) in 250 µL of M199 medium (Gibco, Waltham, MA, USA) supplemented with 5% (v/v) FCS (Bioconcept, Allschwil, Switzerland), 1% (v/v) penicillin/streptomycin solution (Sigma-Aldrich, Bruch, Switzerland) for up to 72 h at 37 °C and 5% CO_2. The experiment was conducted in triplicate. For the adult *S. mansoni* assay, at least 3 worms (both sexes) were incubated in a final volume of 2 mL RPMI 1640 supplemented with 5% (v/v) FCS and 1% (v/v) penicillin/streptomycin at 37 °C and 5% CO_2 for 72 h and the test drug at 10 and 100 µM. The experiment was conducted in duplicate. NTS and adult worms were judged via microscopic readout 72 h after incubation; they were scored according to motility, morphology, and granularity (scores from 0 to 3).

3.2.6. Cytotoxic effects on human cancer cell lines

The investigated cell lines, PC-3 (human prostate adenocarcinoma) and HT-29 (human colorectal adenocarcinoma), were purchased from ATCC (Manassas, VA, USA). The cell culture medium RPMI 1640, the supplements FCS and L-glutamine, as well as PBS and trypsin/EDTA, were purchased from Capricorn Scientific (Ebsdorfergrund, Germany). Culture flasks, multi-well plates, and further cell culture plastics were from Greiner Bio-One (Frickenhausen, Germany) and TPP (Trasadingen, Switzerland), respectively. Resazurin used for the cell viability assays was purchased from Sigma-Aldrich (Taufkirchen, Germany).

3.2.6.1. Cell culture

The compounds of interest were studied for their cytotoxic and cytostatic, respectively, impact on 2 human cancer cell lines, PC-3 (prostate adenocarcinoma) and HT-29 (colorectal adenocarcinoma). Both cell lines were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, and 1% penicillin/streptomycin, in a humidified atmosphere with 5% CO_2 at 37° C. Routinely, cells were cultured in T-75 flasks until reaching subconfluency (~80%). Subsequently, cells were harvested by washing with PBS and detached by using trypsin/EDTA (0.05% in PBS) prior to cell passaging and seeding for sub-culturing and assays in 96-well plates, respectively (Khan et al., 2020).

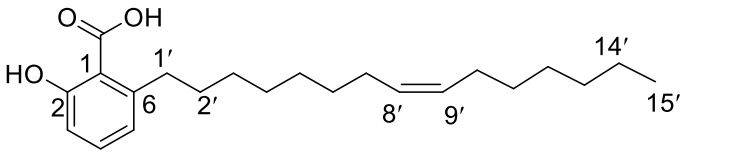
3.2.6.2. *In vitro* cell viability assay

The antiproliferative, i.e., cytotoxic or cytostatic effect of the compounds **3.1**, **3.2** and **3.3,** was investigated by using a fluorometric resazurin-based cell viability assay as described previously (Kufka et al., 2019). For that purpose, cancer cells were seeded in low density in 96-well plates -6000 cells/100 µL/well in case of PC-3 cells, -10,000 cells/100µL/well in case of HT-29 cells. Subsequently, the cells were allowed to adhere overnight, followed by a 48 h treatment with the compounds of interest. For that purpose, dilution series (12.5, 25, 50, and 100 µM) of the compounds **3.1**, **3.2,** and **3.3** were prepared in standard culture medium, starting from 20 mM DMSO stock solutions. For control measures, cells were treated in parallel with 0.5% DMSO (negative control, representing the final DMSO content of the highest, 100 µM test concentration), and 100 µM digitonin (positive control, for data normalization, set equal to 0% cell viability), both in standard growth medium. As soon as the 48 h incubation was finished, cells were treated with a final resazurin concentration of 50 µM (based on 2.5 mM aqua bidest. stock) for 4 h under standard growth conditions. During that time, just viable, metabolically active cells were able to convert resazurin to its reduced and fluorescent derivative resorufin that was measured (λ_{exc} = 540 nm, λ_{em} = 590 nm) by using a SpectraMax M5 multi-well plate reader (Molecular Devices, San Jose, CA, USA). Cell viability data were determined with technical quadruplicates in biological triplicates. GraphPad Prism 8 (GraphPad Software, San Diego, CA, USA), Statsdirect software version 3.2.8 (Statsdirect, Wirral, UK), and Microsoft Excel 2013 (Microsoft, Redmond, WA, USA) were used for data analyses.

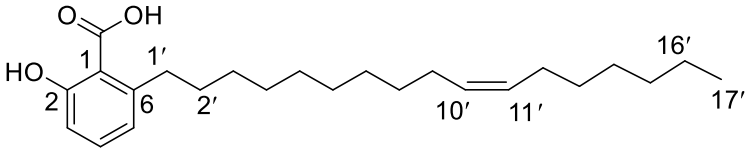
3.3. Results

The anthelmintic activity of 80% methanol crude extracts of stem bark, leaves, roots, and fruits of *O. insignis* was evaluated using *C. elegans* as a model organism and revealed that the extract of fruits was the most active with mortality activity of 91.73 ± 6.05% compared to the extracts of roots, leaves and stem bark which had 35.11 ± 2.91%, 17.58 ± 3.28% and 16.42 ± 7.86% percentage mortality, respectively, at a concentration of 500 µg/mL (Table S1 Supplementary Materials). After partitioning of the fruit extract between water and different organic solvents, it was observed that the *n*-hexane fraction was the most active with 92.54 ± 0.81% mortality at a concentration of 500 µg/mL. Other fractions from partitioning had activity below 5% as follows: EtOAc (4.54 ± 4.01), *n*-butanol (4.28 ±1.07), and the remaining aqueous fraction (4.90 ± 1.70) (Table S2; Supplementary Materials). The *n*-hexane fraction was subjected to column chromatography on silica gel using *n* hexan /ethyl acetate (increasing polarity) as solvents. Using bioassay-guided fractionation, compound **3.1** (Fig. 3.1) was isolated as a white amorphous solid and identified as 6-[8(Z)- pentadecenyl] anacardic acid (**3.1**) based on its

spectral data, mainly HRMS, 1D, and 2D NMR (Fig. S1–S9, Tables S3–S4; Supplementary Materials), and by comparison with detailed reported data (Mwihaki et al., 2009; Bastos et al., 2019). Compound **3.2** (Fig. 3.1) was isolated as a white amorphous solid and identified as 6-[10(Z)-heptadecenyl] anacardic acid (**3.2**) by comparing its spectral data (Fig. S10–S18, Tables S3 and S4; Supplementary Materials) with that reported in literature (Itokawa et al., 1987; Chen et al., 1998; Corthout et al., 1994). Compound **3.3** (Fig. 3.1) was isolated as a clear oily liquid and determined to be 3-[7(Z)-pentadecenyl] phenol (**3.3**) based on detailed HR-MS, 1D, and 2D NMR studies (Fig. S19–S28, Tables S3–S4; Supplementary Materials) and comparison with reported spectral data (Liu and Abreu, 2006).



3.1



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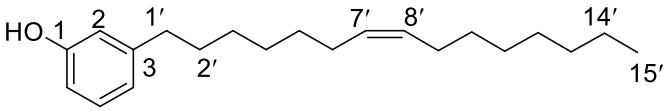

3.3

Fig. 3.1. Structures of isolated compounds **3.1**–**3.3**.

The isolated pure compounds **3.1** and **3.2** were re-tested against *C. elegans* and exhibited 100% activity at the tested concentration of 500 µg/mL. The LC_{50} value of **3.1** was determined to be 51.9 µM, while the LC_{50} of **3.2** was determined to be 93.4 µM (Tables S5 and S6; Fig. S29; Supplementary Materials) (positive control ivermectin 10 µg/mL). Re-testing of **3.3** and salicylic acid (data not shown) exhibited no anthelmintic activity against *C. elegans*. Compounds **3.1**–**3.3** were next tested against 5 parasitic helminths *Schistosoma mansoni* (adult and newly transformed schistosomules (NTS)), and the larval stages of *Strongyloides ratti*, *Heligmosomoides polygyrus*, *Necator americanus*, and *Ancylostoma ceylanicum*. The compounds were tested at 2 concentrations, 100 µM and 10 µM (Table 3.1). Compounds **3.1**, **3.2,** and **3.3** showed good activity against adult *S. mansoni* and newly transformed schistosomula, killing 100% of the organisms at 100 µM. At the same test concentration of 100 µM, 76.3% of *S. ratti* were killed by compound **3.1,** while compounds **3.2** and **3.3** had a mortality rate of less than 30%. All 3 compounds showed weak activity against *N. americanus* at 100 µM, killing only 26.7% to 35.8% of the larvae. The activity of **3.1**–**3.3** against *H. polygyrus* was weak at 100 µM, with less than 30% of the larvae dying. Compounds **3.1** and **3.3** showed moderate activity against *A. ceylanicum*, killing 50.9% and 46.1% of the parasites, respectively, at 100 µM, while compound **3.2** killed only 12.6% of the larvae at the same concentration. At the reduced test concentration of 10 µM, compounds **3.1** and **3.2** showed weak anthelmintic activity against *S. ratti*, *H. polygyrus*, *N. americanus*, and *A. ceylanicum*, killing less than 30%

of the larvae. Also, at 10 µM, compounds **3.1** and **3.2** had reduced activity against adult *S. mansoni* and NTS (< 30% activity). Compound **3.3** showed a high activity of 95% against NTS at 10 µM. Compound **3.3** also showed moderate anthelmintic activity against *A. ceylanicum*, killing 41% of the organisms at the test concentration of 10 µM. Testing at a further reduced concentration of 1 µM against NTS resulted in a mortality rate of 50% for **3.3**, while compounds **3.1** and **3.2** were inactive.

Table 3.1. Anthelmintic activity against parasitic organisms

Organism	**3.1** Activity %*	**3.2** Activity %*	**3.3** Activity %*
NTS** (100 µM)	100 ± 0***	100 ± 0***	100 ± 0***
NTS** (10 µM)	14.0 ± 2	30.0 ± 3.3	95.0 ± 5***
NTS** (1 µM)	0	0	50 ± 0***
S. mansoni adults (100 µM)	100 ± 0***	100 ± 0***	100 ± 0***
S. mansoni adults (10 µM)	25.3 ± 0	0	25.0 ± 0
S. ratti L3 (100 µM)	76.3 ± 0.4***	19.7 ± 4.8	23.8 ± 8.3
S. ratti L3 (10 µM)	7.0 ± 11.9	2.2 ± 4.3	16.4 ± 8.1
N. americanus L3 (100 µM)	27.2 ± 12.8	35.8 ± 6.9	26.7 ± 0.1
N. americanus L3 (10 µM)	14.4 ± 3.4	28.7 ± 2.1	21.2 ± 5.3
H. polygyrus L3 (100 µM)	21.6 ± 17.6	25.9 ± 3.1	21.4 ± 0.7
H. polygyrus L3 (10 µM)	1.8 ± 7.2	24.6 ± 0.4	8.7 ± 3.3
A. ceylanicum L3 (100 µM)	50.9 ± 6.1***	12.6 ± 2.5	46.1 ± 7.4***
A. ceylanicum L3 (10 µM)	12.2 ± 3.2	12.3 ± 5.6	41 ± 10.4

*mortality % based on three replicates; ** NTS = newly transformed schistosomula; *** compound activities determined to be significant using Kruskal Wallis non parametric test (Statsdirect software version 3.2.8) with $p < 0.05$.

Compounds **3.1**, **3.2,** and **3.3** were tested for their effects on the viability of two human cancer cell lines, namely PC-3 prostate adenocarcinoma cells and HT-29 colorectal adenocarcinoma cells (Fig. 3.2). The *in vitro* cell viability and cytotoxicity assays were measured by using a fluorometric resazurin-based read-out after 48 h cell treatment. The saponin digitonin (100 µM), a very potent permeabilizer of cell membranes, was used as positive control compromising the cells to yield 0% cell viability after 48 h. The compounds were tested with at least 4 concentrations, i.e., 12.5 µM, 25 µM, 50 µM, and 100 µM.

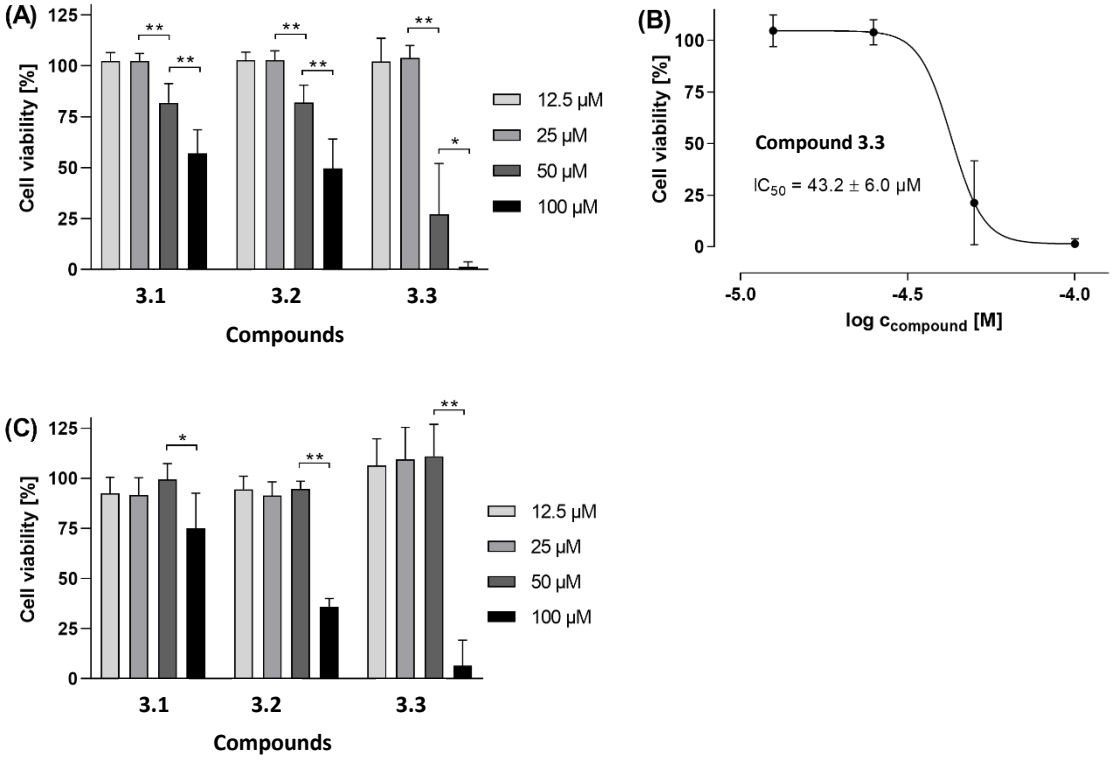

Fig. 3.2. Effects of compounds **3.1**, **3.2,** and **3.3** on the viability of (**A**) PC-3 (human prostate cancer) and (**C**) HT-29 (human colorectal cancer) cells, respectively. The IC_{50} curve of compound **3.3** in PC-3 cells is shown in (**B**). After 48 h of compound treatment, the cell viability was measured by using a fluorometric resazurin-based assay. Data represent biological triplicates, each with technical quatruplicates. Digitonin (100 µM) was used as positive control compromising the cells to yield 0% cell viability after 48 h. Data were analyzed by using GraphPad Prism 8. Statistical significances were evaluated by using Brown-Forsythe and Welch one-way ANOVA tests, including Tamhane's multiple comparison test as well as ordinary one-way ANOVA including Sidak's multiple comparison test; in all cases with ** $p < 0.0001$, and * $p < 0.05$.

3.4. Discussion

The free-living nematode *C. elegans* is a good model organism due to its ease of laboratory maintenance, and it has enough similarities to the parasitic worms to act as an approximate test model (Buckingham et al., 2014). For example, the animal parasitic suborder Strongylida including the human hookworms *Ancylostoma* and *Necator*, is closely related to *C. elegans* and, therefore, the latter is an excellent model organism for these pathogens (Aboobaker and Blaxter, 2000). The bioassay-guided fractionation of the fruit extract of *O. insignis* showed that the activity against *C. elegans* was found in the *n*-hexane fraction while the ethyl acetate, *n*-butanol, and aqueous fractions have much lower activity against *C. elegans*. Compound **3.3** showed low activity against *C. elegans* but moderate to strong activity against some of the parasitic helminths, e.g., *Schistosoma* and NTS. This demonstrates that although *C. elegans* is suitable for bioassay-guided isolation, there may be compounds that are weakly active against *C. elegans* but may be more active against parasitic helminths. This may be due to *C. elegans* innate physical and enzymatic defenses to xenobiotics, which are factors important for its

survival in the natural environment. This makes it difficult for some chemicals to access the worm and the compounds therefore, require high concentrations to bring about observable changes in phenotype (Partridge et al., 2020).

Compounds **3.1** and **3.2** showed strong anthelmintic activity against *C. elegans*, while compound **3.3** showed no activity. The low activity of compound **3.3** against *C. elegans* may have been due to the absence of the carboxylic acid functional group, which was absent in compound **3.3** but present in both compounds **3.1** and **3.2**. When *C. elegans* was tested against salicylic acid (data not shown), this substance showed no activity against the free-living worm, giving an indication that the alkyl chain in compounds **3.1** and **3.2,** which is absent in salicylic acid, is necessary for the anthelmintic activity against *C. elegans*. Remarkably, compound **3.3** showed better activity against NTS and *A. ceylanicum* than compounds **3.1** and **3.2**.

The strong anthelmintic activity of compounds **3.1**, **3.2,** and **3.3** against *S. mansoni* and NTS gives evidence to the reported use of *O. insignis* roots in the treatment of schistosomiasis (Mwihaki et al., 2009). The strong activity of 6-[8(Z)-pentadecenyl] anacardic acid (**3.1**) is in accordance with data published by Wang et al. (2009) on the activity of ginkgolic acids C13:0 and C15:1 on *Pseudodactylogyrus*, a parasite of the gills of aquacultured European eels. 6-[10(Z)-heptadecenyl] anacardic acid (**3.2**) has been shown to be toxic against the citrus red mite *Panonychus citri* by Pan et al. (2006). To the best of our knowledge, there are no reports on the anthelmintic activity of 3-[7(Z)-pentadecenyl] phenol (**3.3**), but the compound has shown activity against the brine shrimp *Artemia salina* (Liu and Abreu, 2006a). This study provides scientific evidence of the anthelmintic activity of *O. insignis* fruits extract, which was far more active than root, stem bark, and leaf extracts.

As shown in Fig. 3.2 A, C, compounds **3.2** and **3.3** showed antiproliferative activity in both PC-3 human prostate adenocarcinoma cells and HT-29 human colorectal adenocarcinoma cells, while compound **3.1** exhibited antiproliferative activity only on PC-3 cells. Whereas 100 µM of both compound **3.1** and **3.2** reduced the viability of PC-3 cells by approximately 50% (Fig. 3.2A), compound **3.3** showed enhanced antiproliferative activity against PC-3 cells with a calculated IC_{50} value of 43.2 ± 6.0 µM (Fig. 3.2B). In HT-29 cells (Fig. 3.2C), compound **3.3** was less active, with an estimated IC_{50} between 50 and 100 µM. In the same cells, the IC_{50} of compound **3.2** is estimated to be just below 100 µM, since this concentration of compound **3.2** reduced the HT-29 cell viability by more than 50% (down to 36% viability). Contrarily, compound **3.1** did not cause a 50% reduction (IC_{50}) of HT-29 viability even with a 100 µM concentration. Taken together, compound **3.3** is the most active of the tested compounds, permitting an antiproliferative effect with an IC_{50} value of 43.2 µM in human PC-3 prostate adenocarcinoma cells. Furthermore, since in Fig. 3.2B, the lower curve plateau reached 0% cell viability, it can be stated that the antiproliferative effect is caused by cytotoxicity of compound **3.3**, i.e., induction of cell death, not just cytostatic cell growth arrest. For compound **3.1**, these findings are in accordance with data published by Rea et al. (2003) on the bioactivity-directed (cytotoxicity) chromatographic separation and isolation of compounds named anacardic acid and ginkgoic acid (= 6-[8(Z)-pentadecenyl] anacardic acid, **3.1**), the main active constituents of *O. insignis* bark extract. The authors determined the IC_{50} values against the human cell lines Hep-G2 (human hepatocellular carcinoma) as 385 ± 71 µM, MDA-MB-231 (human mammary adenocarcinoma) as 289 ±54 µM, Hs 578T (human mammary ductal carcinoma) as 88.9 ± 8.7 µM, MCF-7 (human mammary adenocarcinoma) as >300 µM, SK-MEL-28 (human melanoma) 199 ± 11 µM, and 5637 (human primary bladder carcinoma) as 131.3 ± 1.0 µM. However, Rea et al. (2003) did not obtain compounds **3.2** and **3.3** from *O.*

insignis bark, either through bioactivity-directed (*Artemia salina* lethality) fractionation or bioactivity-directed (cytotoxicity) chromatographic separation as we could demonstrate here for the fruits of *O. insignis*. Itokawa et al (1987) also reported compound **3.1** had potent activity against Sarcoma 180 ascites in mice using the total packed cell volume method (grow ratio 17.4%).

Compounds **3.1** –**3.3** are also constituents of *G. biloba*, the most valued and ancient among medicinal plants (Liu et al., 2021). The growth inhibitory effects of **3.1** –**3.3** isolated from *G. biloba* were examined on several human cancer cell lines and a normal cell line. All compounds inhibited the growth of human cancer cells such as HCT-15 (colon), MCF-7 (breast), A-549 (lung), HT-1197 (bladder), and SKOV-3 (ovary). Interestingly, compounds **3.1** and **3.3** were less cytotoxic on the normal colon cell line (CCD-18-Co) than on the corresponding colon carcinoma (HCT-15). Unfortunately, data for compound **3.2** on CCD-18-Co are not presented (Lee et al., 1998). Very recently, it was reported that compound **3.1** inhibited cell proliferation, migration, epithelialmesenchymal transition, and overall protein SUMOylation in BGC823 and HGC27 cells. Additionally, **3.1** hindered the progression of gastric cancer by inhibiting the SUMOylation of IGF-1R (Liu et al., 2021a).

In general, compounds **3.1** and **3.2** possess a wide range of bioactive properties and can exert diverse pharmacological activities (Liu and Abreu, 2006a; Cui et al., 2020). Compound **3.1** has been shown to inhibit HIV protease activity in a cell-free system and HIV infection in PBMCs without significant cytotoxicity (Lu et al., 2012) and acts as a multi-target inhibitor of key enzymes in pro-inflammatory lipid mediator biosynthesis (Gerstmeier et al., 2019). Compound **3.1** has also shown strong antibacterial activity against gram-positive bacteria (Hua et al., 2017). Very recently, it was demonstrated that compounds **3.1** and **3.2** exhibited strong SARS-CoV-2 3Clpro inhibition with IC_{50}'s of 3.45 ± 0.78 µM and 1.19 ± 0.15 µM, respectively (Xiong et al., 2021). Compound **3.1** also inhibited SARS-CoV-2 Papain-like protease and 3C-like protease with IC50s of ca. 16.3 µM and 1.79 µM, respectively, *in vitro* in a dose-dependent manner. The compound also blocked SARS-CoV-2 replication with an EC_{50} of ca. 8.3 µM in Vero E6 cells (Chen et al., 2021)

3.5. Conclusions

The present study aimed to assess the anthelmintic potential of different organs of *O. insignis*, a medicinal plant used in traditional folk medicine for the treatment of intestinal worms. The results obtained demonstrated for the first time the *in vitro* anthelmintic potential of the fruits extract, which had the best activity compared to stem bark, leaves, and roots. To recognize the active principles in the fruits, we isolated using bioassay-guided fractionation 3 known compounds and evaluated their anthelmintic properties against a panel of parasites mainly infecting humans and/or other mammals like rodents, dogs, cats, and baboons. The evaluation of the anthelmintic properties revealed strong antiparasitic activity of all the isolated compounds (**3.1** –**3.3**) against *S. mansoni* and could justify the use of *O. insignis* roots in the treatment of schistosomiasis. In addition, the cytotoxic and antiproliferative potential of the compounds was evaluated against PC-3 (human prostate cancer) and HT-29 (human colorectal adenocarcinoma) cell lines, respectively. Compound **3.3** was the most active, showing antiproliferative activity against PC-3 cell lines. In summary, the anthelmintic activities, cytotoxic and antiproliferative potential of compounds **3.1** –**3.3** are promising, and this study gives evidence that *O. insignis* fruit extracts deserve further studies in order to fully investigate the potential of discovering new anthelmintics and anticancer drugs. So far, the

pharmacodynamics as well as the pharmacokinetic parameters are unknown and should be investigated. Moreover, expanded toxicological investigations should be performed to validate the safety of the compounds.

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Supplementary material

The following are available online at https://www.mdpi.com/article/10.3390/biom11121893/s1, Figures S1–S9: HRMS, 1D and 2D NMR of compound **3.1**; Figures S10–S18: HRMS, 1D and 2D NMR of compound **3.2**; Figures S19–S27: HRMS 1D and 2D NMR of compound **3**; Figure S28: Important fragment ions on the TOF MS2 spectra of compounds **3.1**–**3.3**; Tables S1 and S2: Anthelmintic activity of extracts and fractions (testorganism *C. elegans*). Table S3: ^{1}H NMR data (400 MHz, $CDCl_3$, δ in ppm) of 6-[8(Z)-pentadecenyl] anacardic acid (**3.1**), 6-[10(Z)-heptadecenyl] anacardic acid (**3.2**) and 3-[7(Z)-pentadecenyl] phenol (**3.3**) including HMBC (H to C); Table S4: ^{13}C NMR data (100 MHz, $CDCl_3$, δ in ppm) of 6-[8(Z)-pentadecenyl] anacardic acid (**3.1**), 6-[10(Z)-heptadecenyl] anacardic acid (**3.2**) and 3-[7(Z)-pentadecenyl] phenol (**3.3**); Tables S5 and S6: Anthelmintic activity of **3.1**–**3.3** (testorganism *C. elegans*). Figure S29: Graph for LC_{50} of 6-[8(Z)-pentadecenyl] anacardic acid (**3.1**) and 6-[10(Z)-heptadecenyl] anacardic (**3.2**) (testorganism *C. elegans*).

4. *Albatrellus confluens* (Alb. & Schwein.) Kotl. & Pouz.: Natural fungal compounds and synthetic derivatives with *in vitro* anthelmintic activities and antiproliferative effects against two human cancer cell lines.

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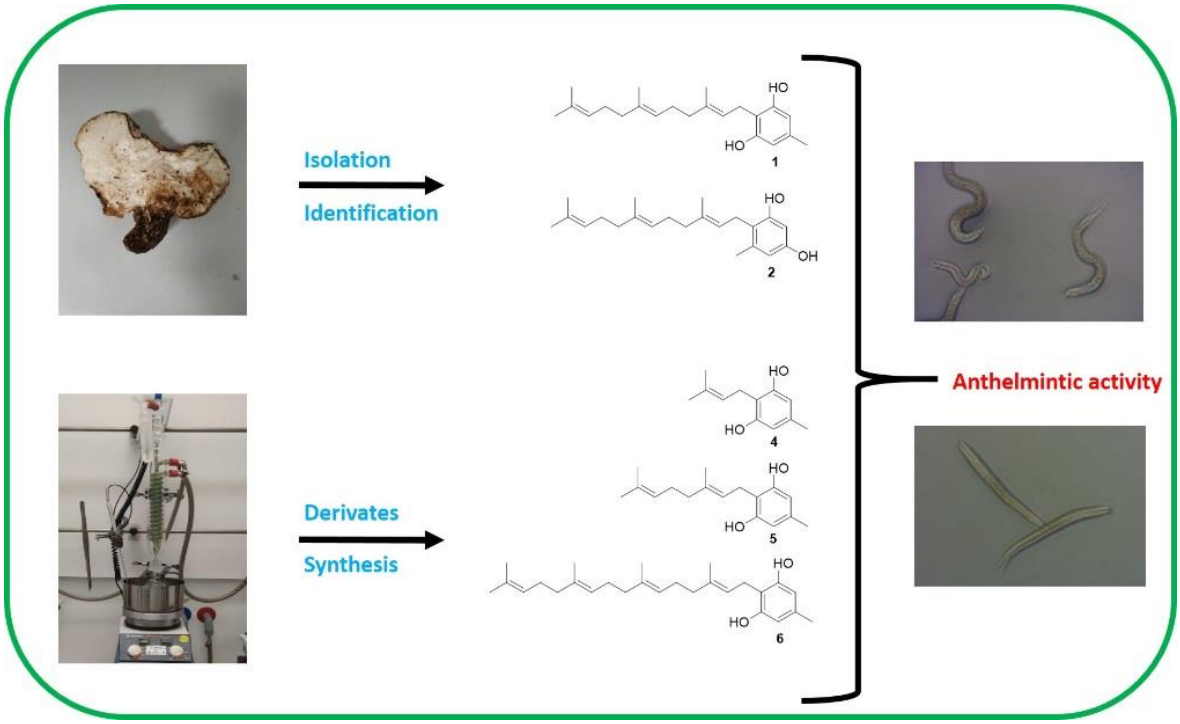


Abstract: Neglected Tropical Diseases affect the world's poorest populations with soil-transmitted helminthiasis and schistosomiasis being among the most prevalent ones. Mass drug administration is the currently most important control measure, but use of the few available drugs is giving rise to increased resistance of the parasites to the drugs. Different approaches are needed to come up with new therapeutic agents against these helminths. Fungi are a source of secondary metabolites but most fungi remain largely uninvestigated as anthelmintics. In this report the anthelmintic activity of *Albatrellus confluens* against *Caenorhabditis elegans* was investigated using bio-assay guided isolation. Grifolin (**4.1**) and neogrifolin (**4.2**) were identified as responsible for the anthelmintic activity. Derivatives **4.4**–**4.6** were synthesised to investigate the effect of varying the prenyl chain length on anthelmintic activity. The isolated compounds **4.1** and **4.2** and synthetic derivatives **4.4**–**4.6** as well as their educts **4.7**–**4.10** were tested against *Schistosoma mansoni* (adult and newly transformed schistosomula), *Strongyloides ratti*, *Heligmosomoides polygyrus*, *Necator americanus* and *Ancylostoma ceylanicum.* Prenyl-2-orcinol (**4.4**) and geranylgeranyl-2-orcinol (**4.6**) showed promising activity against newly transformed schistosomula. The compounds **4.1**, **4.2**, **4.4**, **4.5** and **4.6** were also screened for antiproliferative or cytotoxic activity against

two human cancer lines, viz. prostate adenocarcinoma cells (PC-3) and colorectal adenocarcinoma cells (HT-29). Compound **4.6** was determined to be the most effective against both cell lines with IC_{50} values of 16.1 µM in PC-3 prostate cells and 33.7 µM in HT-29 colorectal cells.

4.1 Introduction

Neglected Tropical Diseases (NTDs) affect more than 1.7 billion of the world's poorest populations. The two most common are the soil-transmitted helminthiases and schistosomiasis. Soil-transmitted helminths (STHs) are parasitic worm infections affecting mainly marginalized population groups in the tropics and subtropics. The STHs with the highest infection rates are the "large roundworm" *Ascaris lumbricoides*, the whipworm *Trichuris trichiura*, and the hookworms *Ancylostoma duodenale* (Old World hookworm) and *Necator americanus* (New World hookworm) which affect more than 1.5 billion people worldwide (Moser et al., 2019). Schistosomiasis (snail fever, bilharzia) is caused by parasitic flatworms of the genus *Schistosoma* with about 240 million infected people worldwide and more than 700 million people living in endemic areas (Spangenberg, 2021). The coronavirus disease 2019 (COVID-19 pandemic) has not only caused the death of millions, but has also severely disrupted health systems and economies throughout the world (Sarkodie and Owusu, 2021). This resulted in the suspension of many health services and programs, including those for neglected tropical diseases (NTDs) (Brooker et al., 2021). The prevalence of NTDs including STHs probably increased due to the suspension of Mass Drug Administration (MDA) programs which were the main control measure for STHs (Ehrenberg et al., 2021) and schistosomiasis was identified as one of the diseases that was most affected by disruption of control programs (Brooker et al., 2021). Approaches tapping into neglected resources might therefore even be more required for the control of STHs and schistosomiasis. Moreover, the current control measure, which is MDA, makes synthetic anthelmintic drugs potential environmental contaminants (Mooney et al., 2021). The chemical residues from anthelmintic drugs affect dung beetles, bacteria, fungi, mites and worms that live in the soil, through pollution of soil and water (Perez-Cogollo et al., 2018). Lastly, MDA programs are also responsible for creating a selective pressure on human parasites which will give rise to increasing levels of resistance (Wit et al., 2021). The extensive use of anthelmintic drugs in the control of animal parasites lead to widespread anthelmintic resistance. The resistance to anthelmintic drugs in veterinary species serves as an indicator of how anthelmintic resistance of human parasites may increase (Sharpton et al., 2020). Although in humans there is no standardized surveillance protocol for monitoring resistance to anthelmintic drugs in places where mass drug administration takes place (Tinkler, 2020), there is already evidence of anthelmintic drug resistance. In Uganda, the efficacy of praziquantel has been shown to be lower in schools that have a longer duration of mass drug administration (Crellen et al., 2016). Another example is in Pemba Island where poor efficacy of albendazole against *T. trichiura* and the hookworms has been reported. Pemba Island has a long history of MDA (Walker et al., 2022). Drug resistance is an emerging problem and there is therefore a need for new natural product based anthelmintics to help reduce the global prevalence of human nematode infections.

One approach could be to look at fungi as a possible source of anthelmintic compounds. There are 2.2–3.8 million species of fungi worldwide (Hawksworth and Lucking, 2017) and of these between 140,000–160,000 are mushrooms and only about 10% of the mushrooms have been investigated (Hawksworth, 2001; Wasser, 2014). Mushrooms have been used across the globe for their medicinal properties and they are sources of potent pharmaceuticals (Zeb and Lee,

2021). Edible mushrooms have shown anthelmintic activity against *Haemonchus contortus* which is also known as the barber's pole worm, and is a very common parasite and one of the most pathogenic nematodes of ruminants (Comans-Perez et al., 2020). Recently a cyclodepsipeptide PF1022A, isolated from fungal culture *Mycelia sterilia* showed anthelmintic activity against *Ascaridia galli* in chickens (Sasaki et al., 1992). Emodepside, a semisynthetic derivative of PF1022A, has shown efficacy against a variety of gastrointestinal nematodes in animals including but not limited to *H. contortus* (sheep), *Heterakis spumosa* (mice), *Cooperia oncophora* (cattle) and *Ascaris suum* (pigs) (Harder et al., 2005). Emodepside is currently under clinical development for the treatment of onchocerciasis (Assmus et al., 2022). A study has shown that ten species of gilled fungi including the oyster mushroom *Pleurotus ostreatus* attack and consume nematodes (Thorn and Barron, 1984). The ability of edible mushrooms to produce nematocidal compounds that immobilize nematodes has been confirmed (Thorn and Barron, 1984; Heydari et al., 2006). A fraction from the hydro-ethanolic extract of *Pleurotus djamor*, which is also an edible mushroom, showed ovicidal activity against *H. contortus* (Pineda-Alegria et al., 2017). The red pigment from *Aspergillus terreus* has also exhibited anthelmintic activity against *H. contortus* (Sreedevi and Pradeeb, 2016). The reason for the various biological activities of fungi may be due to the fact that fungal fruiting bodies are a highly demanded food and habitat source in an ecosystem. A high number of symbiotic relationships between fungi and invertebrates or vertebrates are known to exist. For example, while feeding on fungal fruiting bodies or feeding on fungi, organisms ingest spores and ensure their dissemination over a greater distance (Trappe and Claridge, 2005). In parallel, some fungi produce secondary metabolites which can protect the asco- and basidiomatas against different predators. These chemical constituents act either as constitutive defense compounds with toxic, bitter, or pungent properties or as wound-activated defense compounds which convert inactive precursors enzymatically to the active agent (Spiteller, 2008). Half of the fungal biomass in the soil is consumed by nematodes. Therefore fungi possess defense compounds as a means to protect themselves (Anke and Sterner, 1997). Fungi are therefore a good source of anthelmintic compounds and in some respect fungi are a better alternative to vascular plants as sources of naturally occurring antinematodal compounds due to the less complex anatomical structure of fungi and their ability to adapt to growth in large fermenters (Chitwood, 2002). Compounds isolated from plants may present some cost and sustainability issues, for example if the compound is found in a slow growing plant or found in tissues that cannot be sustainably harvested like root bark (Garcia-Bustos et al., 2019).

In this report the extract of the fruiting bodies of eleven fungal species were assayed for *in vitro* anthelmintic activity using *Caenorhabditis elegans.* The species were selected based on observations that their fruiting bodies were usually not attacked by insect larvae in the field which is why we assume that they may contain biologically active compounds. Fruiting bodies of one species, *Albatrellus confluens* (Alb. & Schwein.) Kotl. & Pouz. showed anthelmintic activity. Using bioassay guided isolation, we isolated grifolin (**4.1**) and neogrifolin (**4.2**) which were responsible for the anthelmintic activity. As anthelmintic compounds do not generate high economic returns, a low cost synthesis is crucial for commercial development (Garcia-Bustos et al., 2019). Therefore we synthesised analogues of grifolin (**4.1**), namely prenyl-2-orcinol (**4.4**), geranyl-2-orcinol (**4.5**), and geranylgeranyl-2-orcinol (**4.6**) in one step reactions. Synthesised compounds **4.4**–**4.6** together with the educts orcinol (**4.3**)**,** prenol (**4.7**), geraniol (**4.8**), farnesol (**4.9**), and geranylgeraniol (**4.10**) were evaluated against *C. elegans*, followed by assaying against the five human and animal parasites *Schistosoma mansoni* (adult and newly

transformed schistosomula), *Strongyloides ratti*, *Heligmosomoides polygyrus*, *Necator americanus* and *Ancylostoma ceylanicum* (all larval stages).

4.2 Materials and methods

4.2.1 General

Column chromatography for fractionations or purifications was performed on, silica gel (0.040–0.063 mm, Merck, Darmstadt, Germany). Analytical TLC was performed on pre-coated silica gel F_{254} aluminum sheets (Merck, Darmstadt, Germany) using the solvent system *n*-hexan-EtOAc (3:1) the solvent system and spots were detected by their color, their absorbance under UV-light (254 nm and 366 nm), or after spraying with vanillin and heating.

Alumina (activated, basic, Brockmann I) was purchased from Sigma-Aldrich (Darmstadt, Germany). Orcinol and prenyl alcohols prenol, geraniol, farnesol, and geranylgeraniol were purchased from Sigma-Aldrich (Darmstadt, Germany). Solvents were purchased from Roth (Karlsruhe, Germany), reagent grade, and used without further purification.

NMR spectra were recorded with an Agilent DD2 400 MHz NMR spectrometer (Varian, Palo Alto, CA, USA) operating at a proton NMR frequency of 400 MHz using a 5-mm inverse detection cryoprobe. 2D NMR spectra were recorded using standard CHEMPACK 8.1 pulse sequences (^{1}H,^{1}H zTOCSY, ^{1}H,^{13}C gHSQCAD, ^{1}H,^{13}C gHMBCAD) implemented in Varian VNMRJ 4.2 spectrometer software (Varian, Palo Alto, CA, USA). The mixing time for the TOCSY experiments was set to 80 msec. The HSQC experiment was optimized for ^{1}JCH = 146 Hz with DEPT-like editing and ^{13}C-decoupling during acquisition time. The HMBC experiment was optimized for a long-range coupling of 8 Hz; a two-step ^{1}JCH filter was used (130–165 Hz). ^{1}H chemical shifts are referenced to internal TMS (^{1}H δ = 0 ppm), while ^{13}C chemical shifts are referenced to $CDCl_3$ (^{13}C δ = 77.0 ppm).

The negative ion electron spray ionization high-resolution mass spectra (ESI-HRMS) were obtained from an API 3200 Triple Quadrupole System (Sciex, Framingham, MA, USA) equipped with a turbo ion spray source, which performs ionization with an ion spray voltage on 70 eV. Sample introduction was performed by direct injection through an Agilent-HPLC 1200 (Agilent, Santa Clara, CA, USA) syringe pump. During the measurement the mass/charge range from 5 to 1800 can be scanned.

4.2.2 Fungal material

The collected fungal fruiting bodies were were stored at −20 °C in a refrigerator: *Albatrellus confluens* (Alb. & Schwein.) Kotl. & Pouzar, under Pinus sp., Paintner Forst near Kelheim, Bavaria, Germany (8 October 2019, leg./det. N. Arnold, coll. 22/2019); *Albatrellus subrubescens* Kotl. & Pouzar, under Abies sp. and Picea sp., Paintner Forst near Kelheim, Bavaria, Germany (18 October 2019, leg./det. N. Arnold, coll. 15/2019); *Caloboletus calopus* Pers., under Fagus sp., near Gungolding, Bavaria, Germany (Oktober 2019, (leg./det. N. Arnold); *Rhodocollybia maculata* (Alb. & Schwein.) P. Kumm., under *Picea* sp., Reisberg near Ingolstadt, Bavaria, Germany (8 October 2019, leg./det. N. Arnold, coll. 10/2019); *Cortinarius alboviolaceus*, under *Picea* sp., near Hormersdorf, Bavaria, Germany (8 October 2019, leg./det. N. Arnold, coll. 12/2019); *Cortinarius infractus* (Pers.) Fr., under Picea sp., near Hormersdorf, Bavaria, Germany (8 October 2019, leg./det. N. Arnold, coll 3/2019); *Cortinarius vulpinus* (Velen.) R. Henry, under *Fagus* sp., Paintner Forst near Kelheim, Ba-varia, Germany (8 October 2019, leg./det. N. Arnold, coll. 16/2019); *Hygrophoropsis aurantiaca*, Pegnitz, under *Picea* sp.,

Pegnitz, Veldensteiner Forst near Wilpark Hufeisen, Ba-varia, Germany (28 November 2020, leg./det. N. Arnold, coll. 61/2020); *Paralepista flaccida* (Sowerby) Pat., under *Picea* sp. and *Fagus* sp., Pegnitz, Veldensteiner Forst near Wilpark Hufeisen, Bavaria, Germany (28 November 2020, leg./det. N. Arnold, coll. 60/2020); *Clitocybe nebularis* (Batsch) Harmaja, under *Pinus* sp., Pegnitz, Veldensteiner Forst near Wilpark Hufeisen, Bavaria, Germany (28 November 2020, leg./det. N. Arnold, coll. 59/2020); *Suillus tridentinus* (Bres.) Singer, under *Larix* sp., Reisberg near Ingolstadt, Bavaria, Germany (18 October 2019, leg./det. N. Arnold, coll. 23/2019). Voucher specimens are deposited at Leibniz-Institute of Plant Biochemistry.

4.2.3 Extract preparations and preliminary anthelmintic screening

Frozen fungal fruiting bodies (1 g) of each species were macerated and extracted by sonication three times for 15 min with 5 mL of 80% MeOH at room temperature. The resulting solutions were evaporated to dryness under reduced pressure using a rotary evaporator maintained at 40 °C to afford the crude extracts. From each crude extract a stock solution of 1 mg/mL in 4% DMSO was prepared. The samples were screened for anthemintic properties against *C. elegans* at the final concentration of 500 µg/mL as described previously (Dube et al., 2021). Ivermectin (10 µg/mL) was used as a positive control.

4.2.4 Isolation of compounds 4.1 and 4.2

Frozen fungal fruiting bodies of *Albatrellus confluens* (375 g) were macerated and ex-tracted three times by sonication for 15 mins with 80% MeOH (3 × 400 mL). The slight yellow solution was evaporated to dryness under reduced pressure using a rotary evapo-rator maintained at 40 °C to afford 11.6 g of crude extract. The crude extract was redissolved in 100 mL of water and partitioned between *n*-hexane (200 mL × 2), chloroform (200 mL × 2), EtOAc (200 mL × 2) and *n*-butanol (200 mL × 2). The resulting fractions were evaporated to dryness at 40 °C to yield 3.6 g of *n*-hexane, 1.6 g of chloroform, 0.3 g of EtOAc, 1.2 g of *n*-BuOH, and 4.7 g of the remaining aqueous fractions.

The *n*-hexane, chloroform and EtOAc fractions were combined based on their TLC profiles to give 5.5 g of combined fractions which was adsorbed on an equivalent mass of silica gel and chromatographed over a silica gel column (7 × 34 cm) using *n*-hexane-EtOAc and EtOAc-MeOH gradients as eluents. The column was monitored by UV lamp (254 and 366 nm). Fractions of 35 mL were collected as follows: [(1–6), *n*-hexane-EtOAc (90:10)] [(7–24), *n*-hexane-EtOAc (8:2)], [(25–29), *n*-hexane-EtOAc (7:3)], [(30), *n*-hexane-EtOAc (6:4)], [(31), *n*-hexane-EtOAc (1:1)], [(32), *n*-hexane-EtOAc (4:6)], [(33), *n*-hexane-EtOAc (3:7)], [(34), *n*-hexane-EtOAc (2:8)] [(35), *n*-hexane-EtOAc (1:9)], [(36), EtOAc-MeOH (9:1)] [(37), MeOH (100%)]. These fractions were pooled according to their TLC profiles into 10 subfractions F1 to F10 as follows: F1 (1–3; 0.05 g), F2 (4–7; 2.13 g), F3 (8–10; ,0.24 g), F4 (11–13; 0.04 g), F5 (14–18; 0.04 g), F6 (19–23; 1.96 g), F7 (24–28; 0.39 g), F8 (29–31; 0.12 g), F9 (32–35; 0.06 g), and F10 (36–37; 0.39 g). 1.5 g of F2 was separated by sil-ica gel column chromatography (7 × 34 cm) and using n-hexane-EtOAc 9:1 as eluent sys-tem. 10 fractions of 25 mL each were collected and fractions 4 to 7 were combined to yield compound **4.1** (767 mg). 1.5 g of F6 was separated by column chromatography using silica gel column chromatography (7 × 34 cm) and *n*-hexane-EtOAc 4:1 as eluent system. 14 fractions of 25 mL each were collected and fractions 5 to 9 were combined to give compound **4.2** (843 mg).

4.2.5 Synthesis of 4.1, 4.4–4.6

Compounds **4.1**, **4.4**–**4.6** were synthesized as described in literature (Jentsch et al., 2020). Biefly, in each reaction to a solution of orcinol (**4.3**, 2 g, 16 mmol) the corresponding prenyl alcohol (**4.7**, 0.55 mL, 5 mmol; **4.8**, 0.94, 5 mmol; **4.9**, 1.35 mL, 5 mmol; **4.10**, 1.75 mL, 5 mmol) in 20 mL dichlormethane acidic aluminium (4 g) was added. In each synthesis, the reaction was refluxed at 60 °C for one week and monitored by TLC plates using vanillin as a spray reagent. After one week the reaction was cooled down and the reaction mixture filtered through celite plug. The filter cake was rinsed with ethyl acetate (500 mL). The ethyl acetate phase was dried with brine and concentrated *in vacuo*. The crude extract from each reaction was separated by column chromatography on silica gel using *n*-hexane-EtOAc (10:1) as isocratic eluent system and afforded compounds **4.4** (R_f 0.75, 295 mg, 28.1%), **4.5** (R_f 0.80, 424 mg, 30.4%), **4.1** (R_f 0.83, 629 mg, 35.5%), and **4.6** (R_f 0.86, 819 mg, 38.2%).

4.2.6 *In vitro* anthelmintic bioassay

4.2.6.1. *Caenorhabditis elegans*

In the *in vitro* anthelmintic assay, the Bristol N2 wild-type strain of C. elegans was used. The nematodes were cultured on NGM (Nematode Growth Media) Petri plates using the uracil auxotroph *E. coli* strain OP50 as a food source according to the methods described by Stiernagle (2006). The bioassay using *C. elegans* was carried out following the method described earlier (Thomsen et al., 2012). Briefly, an NGM plate containing a 4 day old *C. elegans* culture was used to harvest worms by washing the plate twice with 2 mL of M9 buffer. The worm suspension was centrifuged at 800g and resuspended in 2 mL of M9 buffer. The number of worms was adjusted to between 30–40 worms per 20 µL. Using a 384 microtiter well plate 20 µL of the worm suspension was incubated with 20 µL of the test substance for 30 minutes after which the number of living and dead worms in each well were enumerated using a microscope (Olympus BX 41, Tokyo, Japan). *In vitro* anthelmintic activity was expressed as the percentage of dead worms. The solvent DMSO (2%) and the standard anthelmintic drug ivermectin (10 µg/mL) were used as negative and positive controls, respectively. All assays were carried out in triplicate and LC_{50} values were calculated using SigmaPlot 14.0.

4.2.6.2. Parasitic helminths

The drug sensitivity assays with *S. mansoni* [adult and newly transformed schistosomules (NTS)] and *S. ratti*, *H. polygyrus*, *N. americanus*, and *A. duodenale* (L3 larvae) to test compounds **4.1**, **4.2**, **4.4**–**4.10** were carried out in triplicates and conducted as described in previous publications (Dube et al., 2021; Lombardo et al., 2019; Keiser and Haeberli, 2021). All *in vitro* studies were carried out in accordance with Swiss national and cantonal regulations on animal welfare under permission number 2070 at the Swiss Tropical and Public Health Institute (Swiss TPH).

4.2.7. Cytotoxic effects on human cancer cell lines

The investigated cell lines, PC-3 (human prostate adenocarcinoma) and HT-29 (human colorectal adenocarcinoma), were purchased from ATCC (Manassas, VA, USA). The cell culture medium RPMI 1640, the supplements FCS and L-glutamine, as well as PBS and trypsin/EDTA were purchased from Capricorn Scientific GmbH (Ebsdorfergrund, Germany). Culture flasks, multi-well plates and further cell culture plastics were purchased from Greiner Bio-One GmbH

(Frickenhausen, Germany) and TPP (Trasadingen, Switzerland), respectively. Anti-proliferative and cytotoxic effects, respectively, of the compounds were investigated by performing colorimetric MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) and CV (crystal violet)-based cell viability assays (Sigma-Aldrich, Taufkirchen, Germany), respectively.

4.2.7.1. Cell culture

Two human cancer cell lines, PC-3 (prostate adenocarcinoma) and HT-29 (colorectal adenocarcinoma) were used to study cytotoxic and anti-proliferative effects, respectively, of the compounds **4.1**, **4.2**, **4.4**, **4.5** and **4.6**. Both cell lines were cultured in RPMI 1640 medium, supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine and 1% penicillin/streptomycin, in a humidified atmosphere with 5% CO_2 at 37 °C. Routinely, cells were cultured in T-75 flasks until reaching subconfluency (~80%). Subsequently, the adherent cells were harvested by washing once with PBS and detaching from plastics by using trypsin/EDTA (0.05% in PBS), prior to cell passaging and seeding for sub-culturing and assays in 96-well plates, respectively.

4.2.7.2. Cytoxicity assay

Cells handling and assay techniques were in accordance to the methods described by Khan et al., (2020). In brief, anti-proliferative and cytotoxic effects of the five compounds under investigation were examined by performing colorimetric MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) metabolic cell viability assays and CV (crystal violet)-based cytotoxicity assays, respectively. For this purpose, cells were seeded in low densities in 96-well plates (6000 cells/100 µL/well for PC-3 and 10,000 cells/100µL/well for HT-29) using the aforementioned cell culture medium. The cells were allowed to adhere for 24 h, followed by the 48 h compound treatment. Based on 20 mM DMSO stock solutions, the compounds **4.1**, **4.2**, **4.4**, **4.5** and **4.6** were serially diluted in standard growth medium to reach final concentrations of 100, 50, 25, 12.5, 6.25, 3.125, 1.56 and 0.78 µM for cell treatment. Furthermore, two controls were used for data normalization (i) negative control (0.5% DMSO) and (ii) positive control (100 µM digitonin) to determine the 100% and 0% viability, respectively. Each data point was determined in independent biological triplicates, each with technical quadruplicates. As soon as the 48 h incubation was finished, MTT and CV assays were conducted.

For the MTT assay, cells were washed once with PBS, followed by incubation with MTT working solution (0.5 mg/mL MTT in culture medium) for 1h under standard growth conditions. After discarding the MTT solution, DMSO was added in order to dissolve the formed formazan, followed by measuring formazan absorbance at 570 nm, and additionally at the reference/background wavelength of 670 nm, by using a SpectraMax M5 multi-well plate reader (Molecular Devices, San Jose, CA, USA).

For the CV assay, cells were washed once with PBS and fixed with 4% paraformaldehyde (PFA) for 20 min at room temperature (RT). After discarding the PFA solution, the cells were left to dry for 10 min and then stained with 1% crystal violet solution for 15 min at RT. The cells was washed with water and were dried overnight at RT. Afterwards, acetic acid (33% in aqua bidest.) was added to the stained cells and the absorbance was measured at 570 nm and 670 nm (reference wavelength) using a SpectraMax M5 multi-well plate reader (Molecular Devices, San Jose, CA, USA). For data analyses, GraphPad Prism version 8.0.2 was used.

4.3 Results

The crude extracts (80% aqueous methanol) from fruiting bodies of *Albatrellus confluens* (Alb. & Schwein.) Kotl. & Pouzar, *Albatrellus subrubescens* (Murr.) Pouz., *Caloboletus calopus* (Pers.) Vizzini, *Rhodocollybia maculata* (Alb. & Schwein.) Sing., *Cortinarius alboviolaceus* (Pers.) Fr., *Cortinarius infractus* (Pers.) Fr., *Cortinarius vulpinus* (Velen.) R. Hry., *Hygrophoropsis aurantiaca* (Wulfen) Maire, *Paralepista flaccida* (Sow.) Vizzini, *Clitocybe nebularis* (Batsch) P. Kumm. and *Suillus tridentinus* (Bres.) Sing. were tested for their anthelmintic properties against *C. elegans* as described before (Dube et al., 2021). Only *A. confluens* showed anthelmintic activity above 50% while all other species tested had anthelmintic activity below 10% (Fig. 4.1). The percentage of anthelmintic activity is the percentage of dead worms.

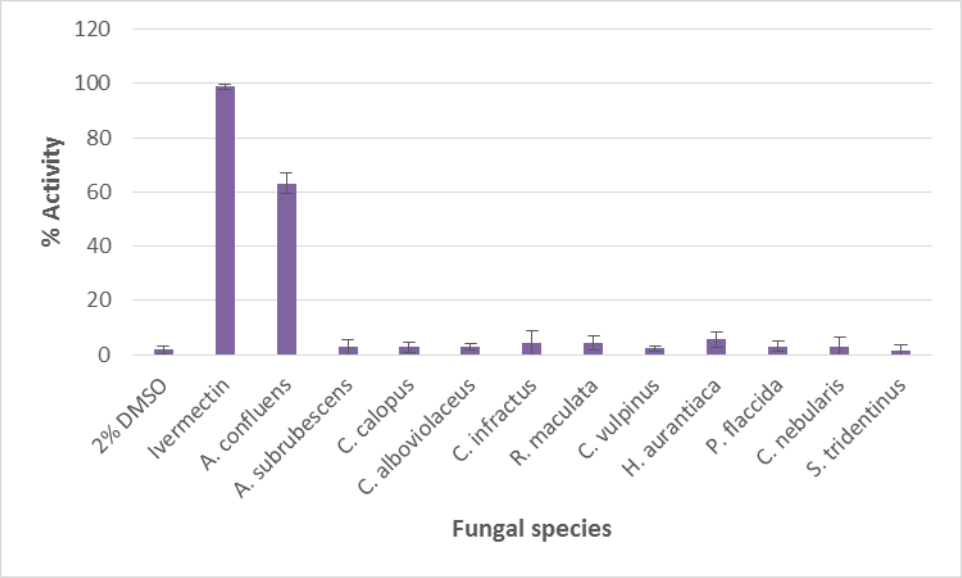


Fig. 4.1. *In vitro* anthelmintic activity of crude extracts (500 µg/mL) of fungal fruiting bodies against *C. elegans.*

A. confluens: Anthelmintic Activity of Crude Extract, Fractions and Metabolites

The mushroom showing activity, *A. confluens*, belongs to the family Albatrellaceae (Russulales) and is widely distributed across Europe, North America, and Asia. The mycorrhizal species grow in a variety of habitats but mainly occur in coniferous forests (*Pinus* spp., *Picea* spp.). The fruiting cap is pale peach-colored and circular with a diameter of 3–20 cm. The spores are cream-colored, the stipe up to 11 cm long and 3 cm thick (Ryvarden and Gilbertson, 1993).

The *in vitro* anthelmintic activity of 80% methanol crude extracts of fruiting bodies of *A. confluens* was evaluated using *C. elegans* as a model organism and revealed that the extract had good anthelmintic activity killing 63.2 ± 3.8% of the nematodes at a concentration of 500 µg/mL. After partitioning of the crude extract between water and different organic solvents, the anthelmintic activity of the organic fractions at a concentration of 500 µg/ml showed a killing rate against *C. elegans* of 72.3 ± 3.4% for *n*-hexane, 62.8 ± 4.5% for chloroform, and 75 ± 3.5% for ethyl acetate. The *n*-butanol and remaining water fraction had an activity of 17.1 ± 2.8% *(n*-butanol fraction) and 0.6 ± 0.9% (water fraction). The *n*-hexane, chloroform, and ethyl acetate fractions were combined and subjected to column chromatography on silica gel using

a mixture of *n*-hexane:ethyl acetate (increasing polarity) as eluents. Using bioassay-guided fractionation, compound **4.1** (Fig. 4.2) was isolated as an orange solid and identified as grifolin (**4.1**) (5-methyl-2-[(2*E*,6*E*)-3,7,11-trimethyldodeca-2,6,10-trienyl]benzene-1,3-diol) based on the spectral data, mainly ESI-HRMS, 1D, and 2D NMR (Fig. S1–S6, Table S1; Supplementary Material) and by comparison with detailed reported data (Koch and Steglich, 2007; Nakuta et al., 2002; Yaqoob et al., 2020). Compound **4.2** (Fig. 4.2) was isolated as a red oil and identified as neogrifolin (**4.2**) (5-methyl-4-[(2*E*,6*E*)-3,7,11-trimethyldodeca-2,6,10-trienyl]benzene-1,3-dio) by comparing its spectral data (Fig. S7–S13, Table S2; Supplementary Material) with the ones reported in the literature (Koch and Steglich, 2007; Nakuta et al., 2002; Yaqoob et al., 2020).

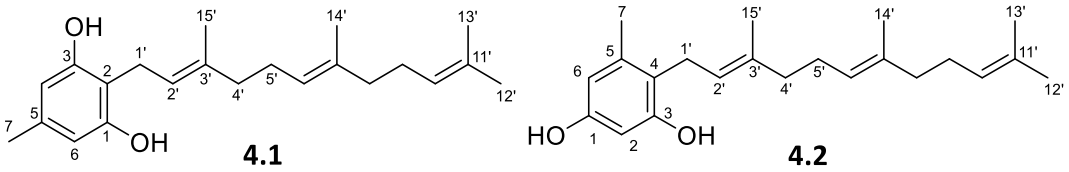
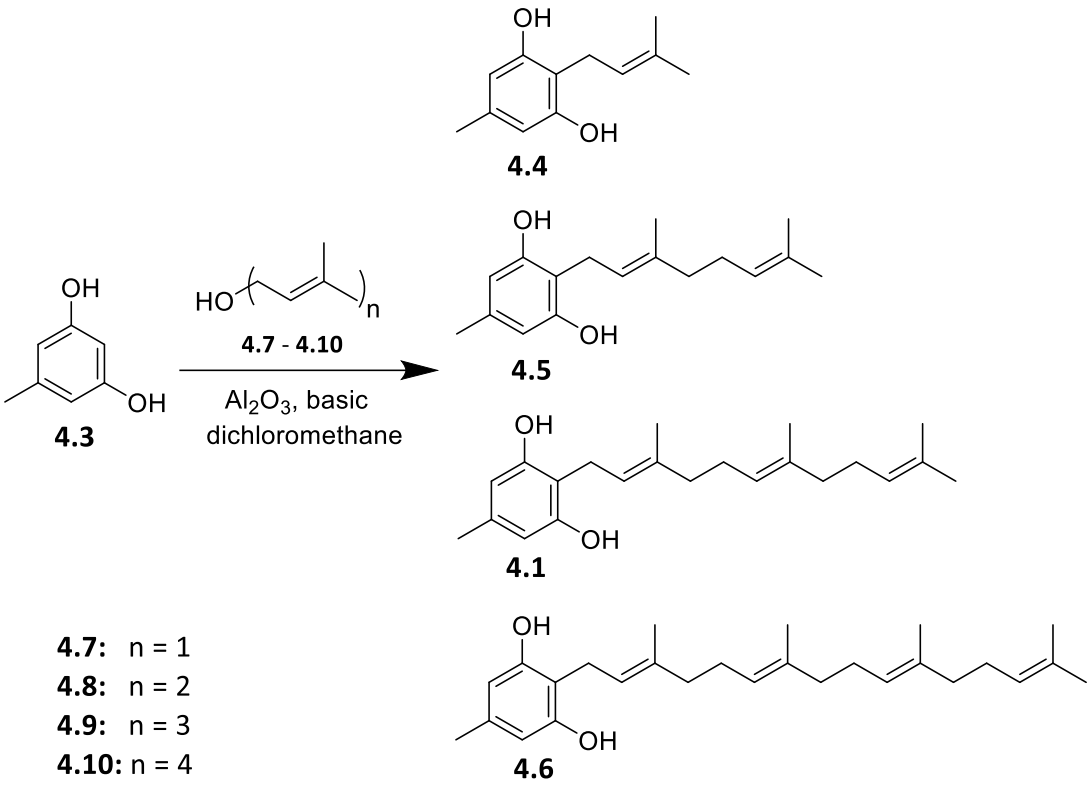


Fig. 4.2. Structures of the natural occurring compounds **4.1** and **4.2**.

Compounds **4.1** and **4.2** were tested at a concentration of 500 µg/mL against *C. elegans* and compound **4.1** showed only moderate anthelmintic activity killing 32 ± 4.8% while compound **4.2** showed strong anthelmintic activity killing 100% of the worms and had an LC_{50} 410.6 µg/mL (Fig. S34; Supplementary Material).

To assess the influence of the prenyl side chain in compounds **4.1** and **4.2** towards their anthelmintic activities, derivatives of compound **4.1** with different side chain lengths were synthesized in an alumina-promoted regioselective aromatic allylation one-step reaction adopted from Jentsch et al. (2020) (Scheme 3.1; Fig. 3.3).

The structures of the prenylated orcinol (**4.3**) derivatives, prenyl-2-orcinol (**4.4**) (5-methyl-2-(3-methylbut-2-enyl)benzene-1,3-diol), geranyl-2-orcinol (**4.5**) (2-[(2*E*)-3,7-dimethylocta-2,6-dienyl]-5-methylbenzene-1,3-diol), and geranylgeranyl-2-orcinol (**6**) (5-Methyl-2-(3,7,11,15-tetramethyl-2,6,10,14-hexadecatetraen-1-yl)-1,3-benzen) were confirmed by their ESI-HRMS, 1D and 2D NMR data (Supplementary Material: **4.4**: Fig. S14–S18, Table S3; **4.5** Fig. S23–S25, Table S4; **4.6:** Fig. S26–S33, Table S5). The educts of the synthesis of **4.1**, **4.3–4.6,** namely, orcinol (**4.3)** together with the prenyl alcohols prenol (**4.7**), geraniol (**4.8)**, farnesol (**4.9**), and geranylgeraniol (**4.10**), were also tested against *C. elegans* at a concentration of 500 µg/mL. Orcinol (**4.3**) showed no activity against *C. elegans* while synthesized compounds geranyl-2-orcinol (**4.5**) and geranylgeranyl-2-orcinol (**4.6**) exhibited similar activity to their corresponding prenyl alcohols geraniol (**4.8**) and geranylgeraniol **4.10** (see Table 4.1 below). Only compound **4.4** had a much higher anthelmintic activity compared to prenol (**4.7**).



Scheme 4.1. Synthesis of compounds **4.1**, **4.4**–**4.6** by regioselective alkylation of orcinol (**4.3**).

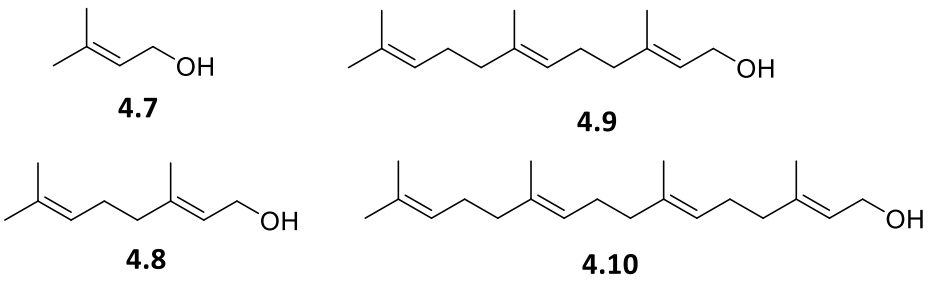


Fig. 4.3. The prenyl alcohols **4.7**–**4.10** used in the synthesis of the analogues.

Table 4.1. *In vitro* anthelmintic activity of compounds **4.1**–**4.10** at 500 µg/mL in % mortality.

Natural and Synthetic Compounds	**4.1**	**4.2**	**4.3**	**4.4**	**4.5**	**4.6**
Anthelmintic activity against *C. elegans*	32.0 ± 4.8	100 ± 0 *	0.7 ± 0.9	85.6 ± 5.1 *	95.3 ± 2.8 *	1.6 ± 2.3
Corresponding alcohols	**4.9**	**4.9**	-	**4.7**	**4.8**	**4.10**
Anthelmintic activity against *C. elegans*	32.1 ± 3.0	32.1 ± 3.0	-	17.8 ± 1.7	99.5 ± 0.7 *	0

* Compound activities determined to be significant versus control worms using Kruskal-Wallis non parametric test (Statsdirect software version 3.2.8) with $p < 0.05$.

All compounds were tested *in vitro* against parasitic helminths. Compounds **4.1**, **4.2**, and **4.4**–**4.6** were tested against *S. mansoni* (adult and newly transformed schistosomules (NTS)), and the larval stages of *N. americanus*, *S. ratti*, *H. polygyrus*, and *Ancylostoma ceylanicum* at a concentration of 10 µM. Compounds showing anthelmintic activity above the threshold of 60% against the parasitic organisms were also tested at 1 µM (Table 4.2).

Table 4.2. *In vitro* anthelmintic activity of compounds **4.1**–**4.6** against parasitic helminths.

	Activity % *					
Organism	**4.1**	**4.2**	**4.4**	**4.5**	**4.6**	**Reference**
NTS ** (10 µM)	36.0 ± 4	26.0 ± 2	93.3 ± 0 ***	55.0 ± 5.0 ***	75.0 ± 5.0 ***	Auranofin 100
NTS ** (1 µM)	nt	nt	38.9 ± 1.7	nt	55.0 ± 1.7 ***	nt
S. mansoni (10 µM)	27.3 ± 2	35.1 ± 2	29.2 ± 4.2	0	16.7 ± 0	Praziquantel 100
N. americanus (10 µM)	16.5 ± 12.5	13.4 ± 8.4	12.1 ± 8.9	29.6 ± 2.4	28.2 ± 7.4	Levamisole 100
S. ratti (10 µM)	18.6 ± 7.8	18 ± 2	1.9 ± 13.1	2.9 ± 5.6	0	Levamisole 100
H. polygyrus (10 µM)	16.4 ± 0.4	16 ± 4.3	23.4 ± 7.3	38.6 ± 4.8	36.6 ± 1.9	Levamisole 100
A. ceylanicum (10 µM)	2.0 ± 2.5	7.5 ± 0	24.4 ± 7.8	19 ± 14.1	17.5 ± 3.9	Abamectin 100

* Mortality % based on three replicates; ** NTS = newly transformed schistosomula; nt = not tested *** compound activities determined to be significant versus control worms using Kruskal-Wallis non parametric test (Statsdirect software version 3.2.8) with $p < 0.05$. Reference compounds were auranofin for NTS, praziquantel for adult *S. mansoni* and levamisole for *N. americanus*, *S. ratti*, *H. polygyrus* and *A. ceylanicum* (all at 10 µM).

Compounds **4.4** and **4.6** showed good activity against newly transformed schistosomula affecting 93.3% and 75.0% of the organisms at a concentration of 10 µM. At a concentration of 1 µM compound **4.4** and compound **4.6** had a reduced activity rate of 38.9% and 55.0% against NTS. All compounds showed moderate, weak to no activity with respect to the other four parasitic organisms. Against adult *S. mansoni*, compounds **4.1**, **4.2**, and **4.4** had activities between 27 to 35% while compounds **4.5** and **4.6** had less than 20% activity. Compounds **4.5** and **4.6** had similar activity against *N. americanus* killing about 30% of the worms while compounds **4.1**, **4.2** and **4.4** killed less than 20%. Compounds **4.5** and **4.6** showed moderate activity against *H. polygyrus* with almost 40% activity. Except for compound **4.4**, showing weak activity (24.4%), the compounds **4.1**, **4.2**, **4.5** and **4.6** were almost inactive against *A. ceylanicum.*

The prenyl alcohols **4.7**–**4.10** showed no significant activity against the parasites NTS, *N. americaus*, *S. ratti*, *H. polygyrus*, and *A. ceylanicum*. (Table 4.3). They were not tested against adult *S. mansoni* as the activity against NTS was less than 50%.

Table 4.3. *In vitro* anthelmintic activity of prenyl alcohols 7–10 against parasitic helminths.

	Activity %*				
Organism	**4.7**	**4.8**	**4.9**	**4.10**	**Reference**
NTS ** (10 μM)	33.3 ± 0	31.3 ± 2	25 ± 0	31.3 ± 2	Auranofin 100
S. mansoni (10 μM)	nt	nt	nt	nt	nt
N. americanus (10 μM)	11.6 ± 2	11.3 ± 10	21.3 ± 1	36.3 ± 1	Levamisole 100
S. ratti (10 μM)	13 ± 5	39.6 ± 0.4	0.2 ± 9	0	Levamisole 100
H. polygyrus (10 μM)	0	1.8 ± 5	34.6 ± 5.5	0	Levamisole 100
A. ceylanicum (10μM)	18.8 ± 2.9	13.7 ± 1.7	10 ± 6.4	23.6 ± 5.9	Abamectin 100

* Mortality % based on three replicates; ** NTS = newly transformed schistosomula; nt = not tested. Reference compounds were auranofin for NTS, praziquantel for adult *S. mansoni* and levamisole for *N. americanus*, *S. ratti*, *H. polygyrus* and *A. ceylanicum* (all at 10 μM).

Compounds **4.1**, **4.2**, **4.4**, **4.5** and **4.6** were screened for their potential antiproliferative and cytotoxic activity against two human cancer lines, namely prostate adenocarcinoma cells (PC-3) and colorectal adenocarcinoma cells (HT-29). The compounds' effect on the metabolic cancer cell viability was determined by conducting a MTT assay, general cytotoxic effects were determined by using a CV assay, both after 48 h cancer cell treatment with increasing concentrations up to 100 μM of the compounds. The MTT and CV assay read-outs were normalized to 100% cell viability (0.5% DMSO negative control) and 0% cell viability (digitonin positive control), and analyzed by non-linear curve regression and IC_{50} values calculation using GraphPad Prism 8 software.

As shown in Fig. 4.4, compound **4.4** lacked any substantial antiproliferative activity in both cell lines and both assays, as indicated by IC_{50} values > 100 μM. Whereas the compounds **4.1**, **4.2**, **4.5** and **4.6** were detected to permit antiproliferative, and ultimately cytotoxic activity, in PC-3 and HT-29 cells with IC_{50} values in the range of 15–95 μM.

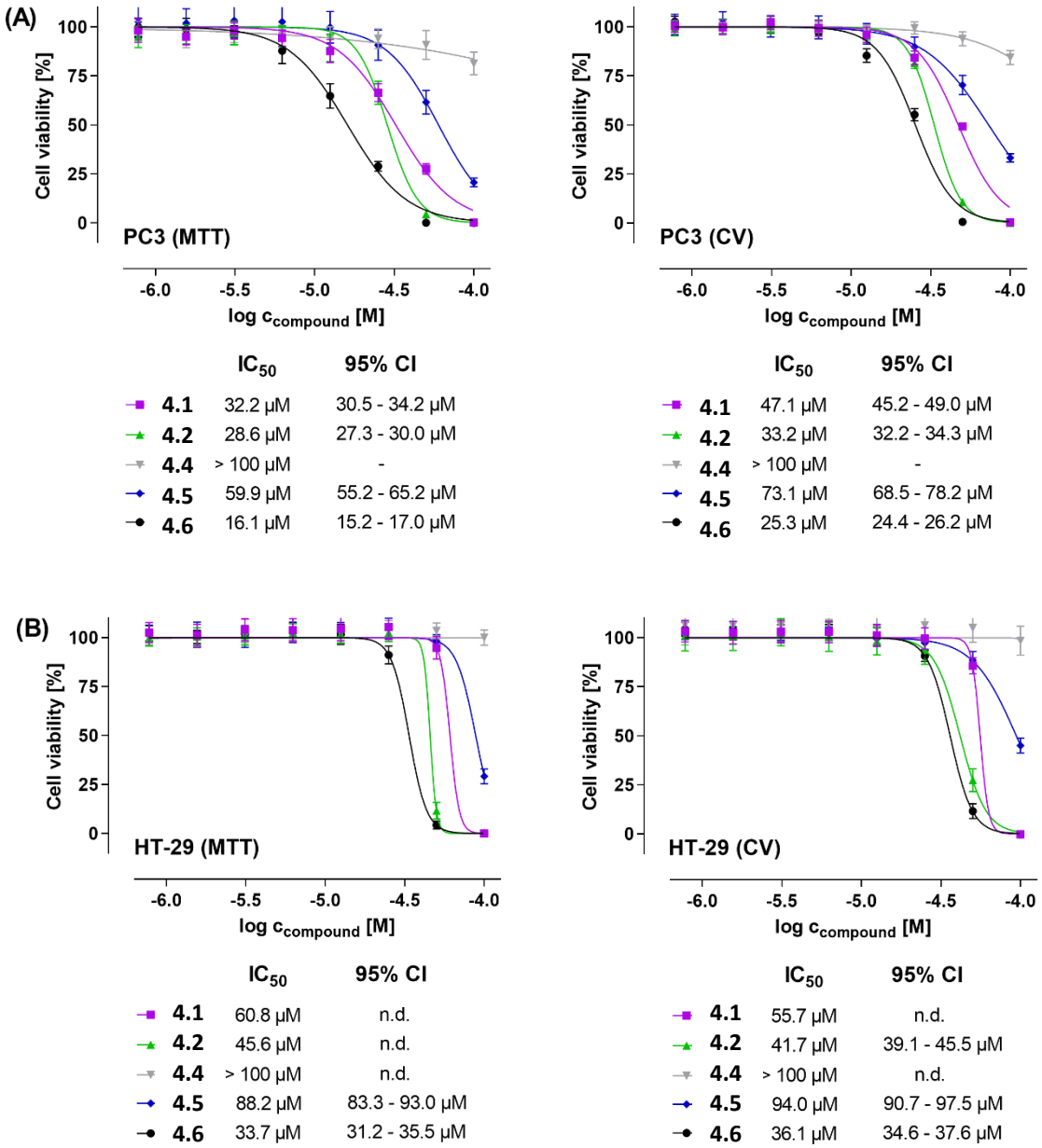


Fig. 4.4. Effect of the compounds **4.1**, **4.2**, **4.4**, **4.5** and **4.6** on the metabolic cell viability of (**A**) prostate PC-3 and (**B**) colorectal HT-29 cancer cells, respectively, as determined by MTT assay (**left**), and general cytotoxic and antiproliferative effect as determined by using crystal violet (CV) assay (**right**) after 48 hours cell treatment. Data represent biological triplicates, each comprising technical quadruplicates. IC_{50} curves were analyzed and drawn by using GraphPad Prism software. IC_{50} values are given with its calculated 95% confidence intervals (95% CI); n.d.: mathematically not definable based on the available data.

4.4. Discussion

Phytochemical investigations on fruiting bodies of *A. confluens* have been extensively reported (Besl et al., 1977; Zhi-Hui et al., 2001; Yang et al., 2008; Hashimoto et al., 2005) and also secondary metabolites of different compound classes are described from cultures of this mushroom (Guo et al., 2015; Hellwig et al., 2003; Wang et al., 2005). In general, mushrooms of the genus *Albatrellus* including *A. confluens* are well known for producing monomeric

farnesylphenols, such as grifolin (**4.1**) and neogrifolin (**4.2**). The main constituent **4.1** of *A. confluens* was first described from the misnamed mushroom *Grifola confluens* (Hirata and Nahanishi, 1950). Neogrifolin (**4.2**), which was first reported from the organic-chemical synthesis of grifolin (**4.1**) (Cardillo et al., 1969) was later recognized in different *Albatrellus* species (Besl et al., 1977; Quang et al., 2006).

The monomeric grifolin (**4.1**) and neogrifolin (**4.2**) are well known to possess diverse biological activities (Quang et al., 2006) such as anti-oxidative activity, anti-microbial effect, activity on human and rat vanilloid receptor 1, inhibition of tumor cell growth, promotion of melanin synthesis by B 16 melanin, inhibition of nitric oxide production in RAW 264.7 cells, anti-cholesteremic activity level in blood and liver and plant growth inhibition. For compound **4.1**, a significant *in vitro* activity against three *Leishmania* species and *Trypanosoma cruzi* was reported (Mahiou et al., 1995).

The isomeric compounds grifolin (**4.1**) and neogrifolin (**4.2**) showed activity against *C. elegans* with neogrifolin (**4.2**) exhibiting higher activity against the free-living nematode. The higher activity may be due to the shift of the hydroxyl group at C-3 in grifolin (**4.1**) to C-5 in neogrifolin (**4.2**) and the shift of the methyl substituent in **4.1** from C-5 to C-3 in **4.2**. In general, phenolic compounds possess biological activity based on the ability to form a phenoxy radical which can then scavenge free radicals (Ali et al., 2013). The anthelmintic activity observed against *C. elegans* was however probably not due to the antioxidant activity of the phenoxy anion as orcinol (**4.3**) showed no activity against *C. elegans* while the activity seemed to be due to the presence of the prenyl chain as we demonstrated through the tested prenyl alcohols **4.7**–**4.10** (Table 4.2). From the activity profile of synthesized derivatives **4.4**–**4.6** of the farnesylphenol grifolin (**4.1**) it can be concluded that increasing prenyl chain length n (Scheme 1) reduced the anthelmintic activity against *C. elegans*. Compounds **4.4** (n = 1) and **4.5** (n = 2) bear shorter prenyl side chains than compound **4.1** (n = 3) and are more active while compound **4.6** (n = 4) exhibited almost no activity against *C. elegans*. There was also similar activity between the prenyl alcohols **4.8**–**4.10** and their corresponding phenolic compounds **4.1**, **4.5**, and **4.6** except for compound **4.4** whereby the corresponding prenyl alcohol **4.7** had lower activity. Prenol (**4.7**) is known to elicit behavioural responses in *C. elegans* through the AWC neurons (Baiocchi et al., 2020). The observed anthelmintic activity may be due to the interaction of **4.4** with these neurons. The higher activity of **4.4** compared to the corresponding alcohol **4.7** (prenol) against *C. elegans* may also be explained by the fact that prenol (**4.7**) itself is a very small molecule, but as a substituent in **4.4** is part of a larger molecule that can bind more easily to the AWC receptor.

Previously, anthelmintic activity against *C. elegans* for geraniol (**4.8**) which is the main component in the essential oil of *Cymbopogon martini*, and other *Cymbopogon* species, has been reported (Kumaran et al., 2003; Otify et al., 2022). The essential oils of *C. martini* and *Thymus bovei* have also shown anthelmintic activity against adult Indian earthworms *Pheretima posthuma* and this was attributed to the high content of geraniol (**4.8**) in the oils of both species (Nirmal et al., 2007; Jaradat et al., 2016). Farnesol (**4.9**) has also been reported to have anthelmintic properties against *C. elegans*, but less potent than the monoterpenoid **4.8** (Abdel-Rahman et al., 2013) as also seen in our results. Furthermore, farnesol (**4.9**) exhibited nematicidal properties against the human parasitic *Anisakis* species which infects individuals who eat raw fish contaminated with the parasite (Navarro-Moll et al., 2011). Geranylgeraniol (**4.10**) isolated from *Pterodon pubescens* seed oil showed antitrypanosomal activity against blood-stream trypomastigotes of *Trypanosoma cruzi* (Menna-Barreto et al., 2008). In traditional medicine, terpenes play an important role as they are thought to be

responsible for the anthelmintic properties of some plants such as Asian wormwood (*Artemisia annua*) and American wormseed (*Dysphania anthelmintica*) (Mirza et al., 2020). The use of terpenes as anthelmintics is however challenging as they are rapidly absorbed in the stomach and proximal intestine and therefore large doses are required to reach the target site where the intestinal parasites reside, resulting in toxic side effects (Michiels et al., 2008). The prenyl alcohols showed weak *in vitro* anthelmintic activity against the parasitic helminths (Table 4.3) and no correlation could be made between the *in vitro* anthelmintic activity of compounds **4.1**, **4.4**, **4.5** and **4.6** and their corresponding prenyl alcohols.

The NTS against which compounds **4.4** and **4.6** showed good activity, is a target for drug and vaccine development as it is susceptible to the immune response (Gobert et al., 2010). Praziquantel kills adult worms and compounds that affect NTS and juvenile worms would offer a complementary approach in the fight against schistosomiasis. The activity shown by compounds **4.4** and **4.6** against NTS could therefore be a starting point for the development of similar compounds with modifications to enhance activity and bioavailability at the site of infection. As human schistosomiasis is only second to malaria in mortality (Sirak et al., 2021) the importance of finding new alternative therapeutic agents to the current drug used cannot be overemphasized.

Besides the *in vitro* anthelmintic screening, compounds **4.1**, **4.2**, **4.4**, **4.5** and **4.6** were tested *in vitro* for their effects on the viability and proliferation of human cancer cell lines. Since two of the compounds, namely **4.1** (grifolin) and **4.2** (neogrifolin), have been tested and described by others, for their impact on human HT-29 colorectal cancer cells (Yaqoob et al., 2020), we decided to use the same cell line for our screenings, as well as human PC-3 prostate cancer cells as one of our most frequently used human cancer cell lines. Both allowing comparison of the data with published data of others and with internal data of other natural products.

Compound **4.6** was detected *in vitro* to be the most active of these compounds affecting cancer cells' viability with IC_{50} values of 16.1 µM in PC-3 prostate cells and 33.7 µM in HT-29 colorectal cells (both in MTT assay), followed by the compounds **4.2**, **4.1** and **4.5**, in that order. In all cases, the respective IC_{50} value was lower by 1.5–2 in PC-3 prostate cancer cells compared to HT-29 colorectal cancer cells. However, the IC_{50} values determined in our study are in very good agreement with data very recently published, at least for the compounds **4.1** and **4.2** (Yaqoob et al., 2020). Furthermore, the calculated IC_{50} values based on our MTT and CV data are very similar, with slightly lower IC_{50} values in the metabolic MTT cell viability assay. This indicates that metabolic cell viability is affected by compounds **4.1**, **4.2**, **4.5** and **4.6**, triggering a cytotoxic effect that is ultimately leading to cancer cell death.

As mentioned above, cytotoxic activities towards human cancer cells of compound **4.1** (grifolin) and **4.2** (neogrifolin) have been described by others. Yaqoob and coworkers, for instance, tested these compounds for anti-cell viability activity against human colon cancer cells, and published IC_{50} values in a quite narrow range of ~25–35 µM for all cell lines they tested (Yaqoob et al., 2020), in very good accordance with the antiproliferative and cytotoxic activities, respectively, described here. The cancer cells' growth inhibiting properties of grifolin and neogrifolin motivated researchers to investigate the underlying mode of cell death in several cancer cell lines (Bouyahya et al., 2022). Ye et al. postulated that grifolin induces the cell death in naso-pharyngeal carcinoma cell line CNE1 through caspase-mediated apoptosis process (Ye et al., 2005). Furthermore, Che et al. investigated the role of autophagy in grifolin-treated human ovarian cancer cells (Che et al., 2016). The research of Che and coworkers suggested that autophagic cell death is induced by grifolin in human ovarian cancer cells by

inhibiting the Akt/mTOR/S6K pathway. However, many other grifolin derivatives are still underinvestigated. Especially the influence of adding or substracting one or more isoprene units to or from, respectively, the polyisoprene chain of grifolin (**4.1**)—resulting in compounds **4.4**, **4.5** and **4.6**—on the anticancer properties is not yet investigated. Therefore, further advanced studies should be conducted to investigate in more detail both the compounds' potential as anti-cancer treatments and the safety of these compounds in non-cancer treatments in humans. All the more, since the latter aspect, the impact of those compounds on normal, i.e. healthy cells was not investigated so far.

To the best of our knowledge, this is the first report of the anthelmintic activity of constituents from *Albatrellus confluens* against *C. elegans* and the first investigation of the isolated grifolin (**4.1**) and neogrifolin (**4.2**) against the parasitic organisms *S.mansoni* (adult and NTS, and the larval stages of *N. americanus*, *S. ratti*, *H. polygyrus*, and *A. ceylanicum*. It is also the first time the effect of prenyl chain length on the anticancer activity of grifolin derivatives, compounds **4.4**, **4.5** and **4.6**, is investigated.

4.5. Conclusions

The *in vitro* anthelmintic activity against *C. elegans* shown by compounds **4.1** and **4.2** was a clear demonstration of structure activity relationship showing that the position of functional groups has an effect on biological activity. There was also clear correlation between *in vitro* anthelmintic activity of the prenyl alcohols against *C. elegans* whereby reducing the length of the prenyl chain increased activity. Although no correlation could be made between the structure of the compounds and anthelmintic activity in the *in vitro* parasitic assays compounds **4.4** and **4.6** displayed promising antischistosomal activity. Compound **4.6** was also the most promising when the cytotoxic and anti-proliferative effects were investigated. Based on our promising bioactivity for compounds **4.1**–**4.2, 4.4**–**4.6**, synthesis of farnesyl phenols is still an area of interest and should be pursued (Grabovyi and Mohr, 2016). Even though prenyl compounds are said to have limited chemical developability they are still enticing scaffolds for the generation of derivatives with the ability to interfere with essential pathways in target organisms like disrupting the transfer or synthesis of essential aliphatic prenyl groups to protein (Zhang and Casey, 1996).

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Supplementary materials

The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/molecules27092950/s1, Figures S1–S6: ESI-HRMS, 1D and 2D NMR of compound **4.1**, Table S1. ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR data of compound **4.1** in $CDCl_3$; Figures S7–S13: ESI-HRMS, 1D and 2D NMR of compound **4.2**, Table S2. ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR data of compound **4.2** in $CDCl_3$; Figures S14–S22: HRMS, 1D and 2D NMR of compound **4.4**, Table S3. ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR data of compound **4.4** in $CDCl_3$; Figures S23–S25: HRMS, ^{1}H NMR spectrum and ^{13}C NMR of compound **4.5**, Table S4. ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR data of compound **4.5** in $CDCl_3$; Figures S26–S33: HRMS, 1D and 2D NMR of compound **4.6**, Table S5. ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR data of compound **4.6** in $CDCl_3$; Figure S34 LC50 curve for in vitro anthelmintic activity of compound **4.2** (neogrifolin) against *C. elegans*.

5. Medicinal plant preparations administered by Botswana traditional health practitioners for treatment of worm infections show anthelmintic activities.

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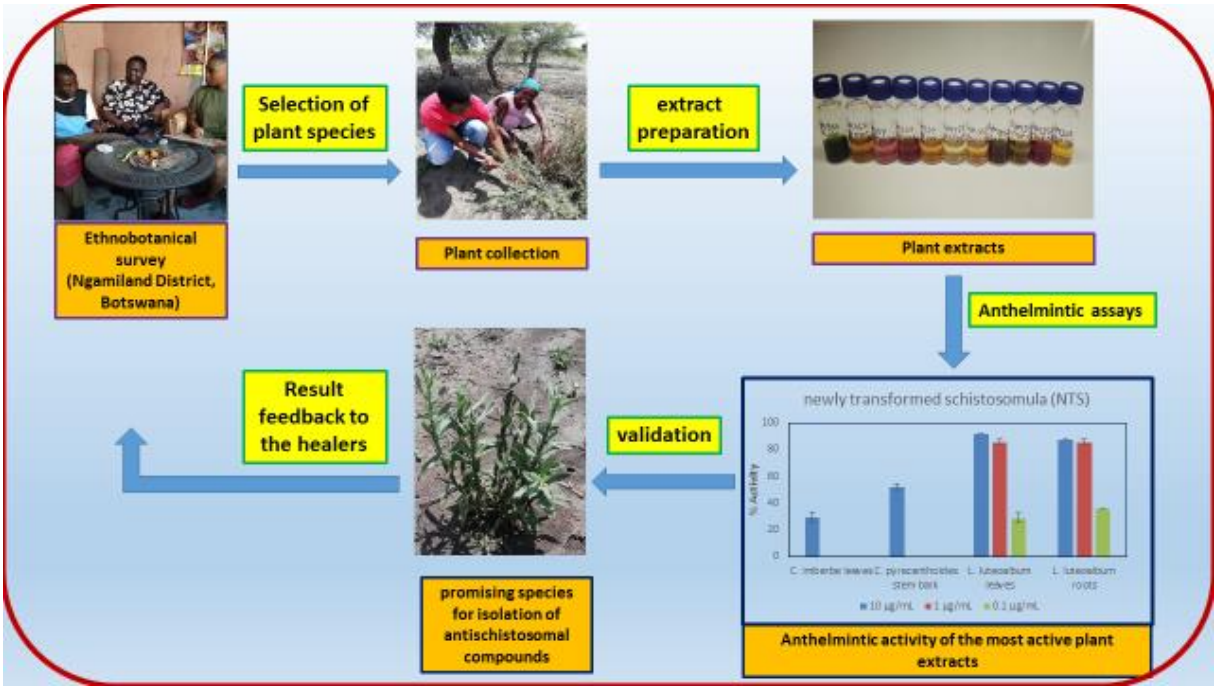

Abstract: Schistosomiasis and soil-transmitted helminths are some of the priority neglected tropical diseases (NTDs) targeted for elimination by the World Health Organisation (WHO). They are prevalent in Botswana and although Botswana has begun mass drug administration with the hope of eliminating soil transmitted helminths as a public health problem, the prevalence of schistosomiasis does not meet the threshold required to warrant large scale interventions. Although Botswana has a modern healthcare system, many people in Botswana rely on traditional medicine to treat worm infections and schistosomiasis. In this study, ten plant species used by traditional medicinal practitioners against worm infections were collected and tested against *Ancylostoma ceylanicum* (zoonotic hookworm), *Heligmosomoides polygyrus* (roundworm of rodents), *Necator americanus* (New World hookworm), *Schistosoma mansoni* (blood fluke) [adult and newly transformed schistosomules (NTS)], *Strongyloides ratti* (threadworm), and *Trichuris muris* (nematode parasite of mice) *in vitro*. Two plants, *Laphangium luteoalbum* and *Commiphora pyaracanthoides* displayed promising anthelmintic activity against NTS and adult *S. mansoni* respectively. *L. luteoalbum* displayed 85.4 % activity at 1 µg/mL against NTS while *C. pyracanthoides* displayed 78.5 % activity against adult *S. mansoni* at 10 µg/mL.

5.1 Introduction

Neglected tropical diseases (NTDs) are diverse groups of communicable diseases that globally infect or affect more than 2.7 billion of the most impoverished populations living in Low-to Middle-Income Countries of Africa, Asia, and Latin America (Herricks et al., 2017). In Africa 90% of the disease burden of NTDs is in Sub-Saharan Africa due to widespread poverty and the suitability of specific climates in Africa for some NTDs to thrive (Ochola et al., 2021). In Botswana schistosomiasis and soil-transmitted helminths are some of the priority NTDs targeted for elimination (WHO, 2019; WHO, 2021). Schistosomiasis and soil transmitted helminths are prevalent in Botswana particularly in the North-West Inland Wetland, the Okavango Delta, and along the Chobe River, both regions of major tourist attraction. Botswana has begun mass drug administration of albendazole in the hope of eliminating soil transmitted helminths (WHO, 2021). Although the prevalence of schistosomiasis infections does not meet the threshold required to warrant large scale interventions, the World Health Organization (WHO) recommends combining praziquantel (the drug used to treat schistosomiasis) with albendazole for the treatment of school age children and other high risk groups, as co-infections of *Schistosoma* spp. and soil-transmitted helminths (STH) are common in many endemic areas in Africa (Aula et al., 2021). There has been an increase in the prevalence of *Schistosoma* species in Botswana between 2010 and 2019 (Kokaliaris et al., 2022) and although there has not been an increase in the prevalence of schistosomiasis it was predicted that there may be future epidemics of schistosomiasis based on the correlation between the flow of the rivers in the Okavango Delta and prevalence patterns of snail populations and subsequently schistosomiasis (Chimbari et al., 2020; Appleton et al., 2008). Further, it was cautioned that the suspension of mass drug administration programs due to the COVID-19 pandemic may lead to future outbreaks of schistosomiasis (Aula et al., 2021). Mass drug administration may be an effective control measure against soil transmitted helminths and schistosomiasis, however the reliance on praziquantel for the treatment of schistosomiasis in mass drug administration control programs presents a constant threat for the development of drug resistance (Molehin et al., 2022). An example of this can be seen in Uganda, where the efficacy of praziquantel has been shown to be lower in schools that have a longer duration of mass drug administration (Crellen et al., 2016). The limited number of anthelmintic drugs and their prolonged use will inevitably lead to anthelmintic drug resistance. This is a well-documented issue in animal populations (Redman et al., 2015) and the resistance to anthelmintic drugs in veterinary species serves as a reference for how anthelmintic resistance may increase within the human population (Sharpton et al., 2020) and some mutations associated with benzimidazole resistance have been identified in eggs from human stools (Rashwan et al., 2016). There is therefore a need for new anthelmintic drugs and plants used in traditional medicine against parasitic helminths could provide promising leads.

The role of traditional medicine in health systems was re-affirmed when the WHO declared 2011-2020 as the Second Decade of African Traditional Medicine and extended by formulating the WHO traditional medicine strategy 2014-2023. Traditional medicine plays an important role in the diagnosis, prevention or elimination of physical, mental and social illnesses. In Africa and the Diaspora traditional medicine usage has been, and remains, a significant contributor to primary healthcare delivery (WHO, 2013; Andrae-Marobela et al., 2021).

In Botswana, the public healthcare system is made up primarily of modern biomedical formal structures. These include hospitals, clinics and outreach health posts and this ensures that every inhabitant lives no more than 15 km away from a health facility. In spite of their close proximity to modern biomedical structures many people in Botswana still seek healthcare

from traditional health practitioners. This is because it is familiar to them as it is ingrained in their culture and due to the fact that public health facilities are often overwhelmed and are not always well equipped. It can also be argued that access to traditional health practitioners is usually easier, faster and cheaper than finding a modern healthcare facility (Okatch et al., 2013). Seeking help from traditional health practitioners is also perceived as more personalized and confidential. An example of this is the fact that although there was an increase to accessibility of combination anti-retroviral therapy, individuals with moderate and advanced HIV infection continued to use traditional medicine (Tietjen et al., 2018).

Traditional medicine is widely used in Africa in the management of worm infections and schistosomiasis (Mhlongo and Van Wyk, 2019; Odiambo et al., 2016; Bah et al., 2006; Allan et al., 2014). Patient observations by traditional health practitioners therefore might lead us to botanicals useful for the treatment/management of worm infections and schistosomiasis. Previously the main screening approaches used for the discovery of new anthelmintics were animal based, target based and phenotypic methods (Geary et al., 2015). Animal based methods are low through put, time consuming and require a large amount of extract or compound and therefore *in vitro* screening methods (target based and phenotypic) are now used (Geary and Thompson, 2003). Target based methods screen extracts and compounds against one or more molecules with essential functions in the parasite metabolism (Zheng et al., 2013). These methods however do not consider the bioavailability of the compound to the parasite. Phenotypic screening employs whole parasites *in vitro* and quantitatively measures the phenotypic features after treatment of the parasites with extracts or compounds (Geary et al., 2015).As control these infections continues to rely on chemotherapy, the emergence of resistances remains a problem. Natural products from botanicals might increase the available pool of potential new anthelmintics with unique structures and/or unique modes of actions and hence delay or prevent resistance (Jayawardene et al., 2021; Liu et al., 2020).

In this study, therefore, we document traditional medical knowledge in relation to worm infections and schistosomiasis in one area of high prevalence, the Ngamiland-District in Botswana, and determined anthelmintic bioactivities of medicinal plants, administered by local traditional health practitioners, against a battery of hookworm, roundworm, nematode and *Schistosoma* species. To the best of our knowledge, this is the first comprehensive characterization of anthelmintic bioactivities of traditional medicinal plants in Botswana.

5.2. Materials and methods

5.2.1 Study site

The study was conducted in the Ngamiland District in the North-West of Botswana, which is the site of a unique ecosystem, the Okavango Delta, where over 95% of its inhabitants depend on wetland resources to sustain their livelihoods. It is further a hotspot for biodiversity in Botswana, which attracts traditional health practitioners to procure and use medicinal plants from the area. Historically *Schistosoma mansoni* transmission is known to occur in the Okavango delta due to the abundance of *Biomphalaria pfeifferi*, the snail intermediate host for the parasite (Appleton et al., 2008). Many Okavango Delta inhabitants from poorer sections of the society and in rural villages around the Okavango Delta are subsistence farmers who are engaging in flood recession farming ('Molapo-Farming'). This farming practice utilizes wetland flooding patterns for planting. Unfortunately, the risk of exposure of farmers to *Schistosoma* is high as flood waters bring with it the snails serving as vectors. Usually, whole families are involved in Molapo-Farming, which has led in the past to schistosomiasis in school

students, which seriously affected their performance. Two sites in the Okavango served as sampling origins, the Ngamiland District capital Maun and the Village of Sehithwa, 80 km from Maun (Fig. 5.1). Both villages are home to the traditional health practitioners who collaborated with us in this study.

Fig. 5.1. Map of Botswana showing areas where plants where collected (adapted from https://austria-forum.org/af/Geography/Africa/Botswana/Maps/Botswana).

5.2.2 Study design, data collection methods and ethical considerations

The study design follows an exploratory, mixed-methods approach (Creswell and Clarke, 2011; Creswell, 2003) to solicit traditional medical knowledge and therefore is of qualitative nature. Three traditional health practitioners collaborated with us in this study, the late Mrs. Tshwanelo Seputhe, Mr. Nkaelang Seputhe, both from Maun, and Mrs. Tsholofelo Tiroyakgosi based in Sehitwa. All three health practitioners have collaborated with one of us (K. Andrae-Marobela) for over fifteen years and have demonstrated reliable, in-depth traditional medical knowledge. Both female traditional health practitioners were/are involved in Molapo-Farming contributing to their livelihood, which is representative of many Okavango Delta inhabitants (Ngwenya et al., 2017). Initial traditional medical knowledge about worm infections were shared by the health practitioners during an ethno-survey undertaken between 2008 and 2010 (Andrae-Marobela et al., 2010), which included in-depth interviews and informal discussions conducted after obtaining community consent and individual prior informed consent. We also used informal conversations to discover categories of meaning (Fetterman, 1989) during the celebrations of the African Traditional Medicine Day in 2013 in Toteng, Ngamiland District Botswana, which was organized by the Ministry of Health, Botswana. These

data were supplemented by recent (January 2019), subsequent semi-structured and unstructured conversations with the three traditional health practitioners to obtain more detail knowledge and to confirm previously generated data to enhance credibility. Though conversations were unstructured, the interviewer had a guideline in mind to focus on knowledge of worm infections and schistosomiasis, but the idea is to let respondents express themselves freely on their own terms (Bernard, 1995). Data were also collected through informal participant observations of traditional health practitioners outside of their homes while accompanying them during medicinal plant collection. These participant observations provided nuances of subjective meaning and valuable narratives of health practitioner's experiences. Ethical approval and research permit was granted by the Ministry of Infrastructure, Science & technology, Botswana (Permit no.: ETH 5 (1), and the Ministry of Health (Permit no.: PPME: 13/18/1 Vol VIII (354); HPDME 13/18/1).

5.2.3 Plant collection and extract preparation

The traditional medicinal plants investigated in this study were collected together with the traditional health practitioners to avoid misidentification. The plant species were taxonomically identified using the dichotomous key in Coates Palgrave and Ellery & Ellery (Coates Palgrave, 1983; Ellery and Ellery, 1997) and authenticated species were deposited in the University of Botswana Herbarium. Correct botanical names were counterchecked using the WFO plantlist (www.wfoplantlist.org). The plant samples were air dried indoors at room temperature and ground to a fine powder using a blender. 1 g of each powdered sample was extracted by sonication for 15 minutes with 10 mL of 80% MeOH (methanol and water have proved to be the solvents with the highest extraction efficiency, Borges et al., 2020) at room temperature. The resulting solutions were evaporated to dryness under reduced pressure using a rotary evaporator maintained at 40 °C to afford crude extracts. 10 mg of each crude extract was tested at the Swiss Tropical and Public Health Institute (Swiss TPH) to investigate their anthelmintic activities.

5.2.4 Anthelmintic assays

In vitro studies using parasitic helminths were carried out in accordance with Swiss national and cantonal regulations on animal welfare under the permission number 2070. The anthelmintic assays to test the activity of the plant extracts against *Ancylostoma ceylanicum* (zoonotic hookworm), *Heligmosomoides polygyrus* (roundworm of rodents), *Necator americanus* (New World hookworm), *S. mansoni* (blood fluke) [adult and newly transformed schistosomula (NTS)], *Strongyloides ratti* (threadworm), and *Trichuris muris* (nematode parasite of mice) were carried out as described previously (Lombardo et al., 2019, Keiser et al., 2021, Dube et al., 2021). Three-week-old female NMRI mice were obtained from Charles River (Sulzfeld, Germany). Three week-old female C57BL/6NRj mice and three-week-old male Syrian golden hamsters were purchased from Janvier Laboratories (Le Genest-Saint-Isle, France). Rodents were kept in types 3 and 4 macrolon cages under environmentally controlled conditions (temperature: 25 °C, humidity: 70%, light/dark cycle 12 h/12 h) and had free access to water (municipal tap water supply) and rodent food. Rodents were allowed to acclimatize for 1 week before infection. Statistical analysis (one way ANOVA, P= <0.001) was performed using SigmaPlot 14.0.

5.2.4.1 *In vitro* tests on *A. ceylanicum*, *H. polygyrus, N. americanus*, *S. ratti* and *T. muris*

The life cycles of the assayed nematodes are maintained at the Swiss TPH. Hamsters were infected per os with 140 *A. ceylanicum* L3 or subcutaneously with 150 *N. americanus* L3. The feces of infected hamsters were filtered to obtain *A. ceylanicum* and *N. americanus* eggs which were then cultivated on an agar plate for 8-10 days in the dark at 24°C to obtain larvae (L3) while mice were used to obtain *H. polygyrus* larvae (L3) following the same procedure. *S. ratti* L3 were acquired as summarized by Garcia and Bruckner (1997). For the drug assay, 30-40 L3 were placed in each well of a 96-well plate for each extract. Larvae were incubated in 198 µl culture medium with the test samples at a concentration of 100 µg/mL. RPMI 1640 (Gibco, Waltham MA, USA) medium supplemented with 5% amphotericin B (250 µg/mL, Sigma-Aldrich, Buchs, Switzerland) and 1% penicillin 10,000 U/mL, and streptomycin 10 mg/mL solution (Sigma-Aldrich) was used for the assays with *H. polygyrus* L3. Phosphate-buffered saline (PBS, Sigma-Aldrich) supplemented with 1% penicillin (10,000 U/mL) and streptomycin (10 mg/mL) solution was used to incubate *S. ratti* L3. *A. ceylanicum* and *N. americanus* L3 stages were incubated in Hanks' balanced salt solution (HBSS; Gibco, Waltham MA, USA) supplemented with 10% amphotericin B and 1% penicillin (10,000 U/mL) and streptomycin (10 mg/mL) solution. Larvae were kept in the dark at room temperature for 72 hours, except *A. ceylanicum*, which were incubated at 37 °C and 5% CO_2, after which the effect of the extract was evaluated. For this the total number of L3 per well was determined. Then, 50-80 µl of hot water (≈80°C) was added to each well and the larvae that responded (the moving worms) were counted. The proportion of larval death was determined and the percentage of survival was determined by the ratio of moving larvae to the total number of larvae present in the well. The *N. americanus* L3 assay was an exception as the wells were stimulated by vigorous up and down pipetting. The *in vitro* tests on adult *H. polygyrus* were carried out by first infecting female NMRI mice with 88 *H. polygyrus* L3. Mice were dissected two weeks post infection and three hookworm adult pairs were placed in each well of a 24-well plate at a volume 1980 µl and exposed to the test extracts at a concentration of 100 µg/mL. For the *in vitro* assay with *T. muris* adult worms, female C57BL/6NRj mice were infected with 200 embryonated *T. muris* eggs. Seven weeks post infection *T. muris* adult worms were collected from the intestines. Three *T. muris* adult worms were placed in each well of a 24-well plate containing 1980 µl culture medium and the test extracts at a concentration of 100 µg/mL. The adult worms of *H. polygyrus* and *T. muris* were scored microscopically based on their phenotype, using a viability scale ranging from 3 to 0 (3: good motility and no morphological changes; 2: low motility and light changes in morphology; 1: very low motility and morphologically impaired; and 0: death). In case the adult worms did not move enough for a clear scoring, they were stimulated with hot water at the last evaluation time-point. The reference compounds were abamectin 10 µM (for *A. ceylanicum* L3), levamisole 10 µM (for *N. americanus* L3, *H. polygyrus* L3, *S. ratti* L3) and tribendimidine 10 µM (for adult *H. polygyrus* and adult *T. muris*). The negative control was 1% DMSO.

5.2.4.2 *In vitro* tests using *S. mansoni*

To obtain NTS, cercariae were collected from infected *Biomphalaria glabrata* snails (maintained at Swiss TPH) and were mechanically transformed. Briefly, 5-6 weeks post infection, infected snails were each placed in a single well of a 24 well plate. The snails were left under a neon lamp for 3-4 hours. After removal of the snails the plate was examined for cercariae. The cercariae were collected using a Pasteur pipette and the cercarial suspension was poured through a 100 µm filter into a 50 mL tube. The cercariae were transformed to NTS

by placing 7 mL of cercarial suspension in a 10 mL syringes and connecting each syringe to a Luer Lok. After connecting another empty syringe to the opposite side of the Luer Loks, the liquid was pushed back and forth three to four times vigorously. The suspensions were then poured into 15 mL tubes and placed on ice in the dark for 7 minutes. The supernatants were removed and discarded by slowly pipetting leaving the sedimented NTS.. The NTS were kept in the incubator (37 °C and 5% CO2) in medium M199, supplemented with 5% FCS and 1% penicillin/streptomycin and 1% (v/v) antibacterial/antifungal solution39 until usage. In order to obtain adult *S. mansoni*, cercariae were collected following the same steps mentioned above. The cercarial concentration was adjusted to 100 cercariae/ 100 µL and 100 µL aliquots were aspirated with a 1 mL syringe ensuring there were no air bubbles in the aspirates. The cercarial suspensions were then each injected subcutaneously into the neck of mice After infection the mice were kept at 25 °C with a 12 hour day/ night cycle. After 7 weeks post infection mice were euthanized with CO_2 for 5 minutes. The mesenteric veins of infected mice were dissected at day 49 post-infection in order to collect adult *S. mansoni* worms. For adult *S. mansoni* and NTS, transparent flat-bottom 96- and 24-well plates were used, respectively (Sarstedt, Switzerland). 30-40 NTS were incubated with the test extract (0.1, 1, 10, 50 and 100 µg/mL) in 198 - 199.8 µL of M199 medium (Gibco, USA) supplemented with 5% (v/v) FCS (Bioconcept AG, Switzerland), 1% (v/v) penicillin/streptomycin solution (Sigma–Aldrich, Switzerland), 1% (v/v) antibacterial/antifungal solution for up to 72 h at 37 °C and 5% CO_2. The experiment was conducted in triplicate. For the adult *S. mansoni* assay at least three worms (both sexes) were incubated in a final volume of 1980 µl - 1998 µl RPMI 1640 supplemented with 5% (v/v) FCS and 1% (v/v) penicillin/streptomycin at 37 °C and 5% CO_2 for 72 h and the test extract at 1, 10 and 50 µg/mL. The experiment was conducted in duplicate. Adult worms and NTS were judged via microscopic readout 72 h after incubation; they were scored according to motility, morphology, and granularity (scores from 0 to 3) (Lombardo et al., 2019). The reference compounds were auronofin 10 µM (for NTS) and praziquantel 10 µM (for adult *S. mansoni*). The negative control was 1% DMSO.

5.3. Results

5.3.1 Traditional medicinal plants for treatment/management of worm infections

During our interaction with three traditional health practitioners, medicinal plant uses of ten plant species were documented (Table 5.1).

Table 5.1. Traditional medicinal anthelmintic plants used in North West District, Botswana.

Traditional Health Practitioner	Plant species	Plant Family	Local name	Plant part	Location	Preparation and dosage	Supporting literature
Sephuthe	*Aloe ferox* Mill.	Xanthorrhoeaceae	-	Leaves	Maun	Pierce the leaves to extract the juice and infuse in cold or warm water. Take one cup twice a day after food	*In vitro* anthelmintic activity against *Haemonchus contortus* and *in vivo* anthelmintic activity against *Heterakis gallinarum* (Ahmed et al., 2013; Maphosa et al., 2010; Mwale and Masika, 2015)
Sephuthe	*Aloe zebrina* Baker	Xanthorrhoeaceae	Ghopha	Leaves	Maun	Cut the leaves and infuse in water. Drink one cup two or three times a day.	The leaf extract is used against parasites and the root extract is used to treat bilharziosis (schistosomiasis) (Bossard, 1996)
Sephuthe	*Terminalia sericea* Burch. ex DC.	Combretaceae	Mogonono	Roots	Maun	Boil the roots and drink the water extract 3 times a day	Roots are used to treat bilharzia (schistosomiasis) (Mongalo et al., 2016)

Table 5.1. continued

Traditional Health Practitioner	Plant species	Plant Family	Local name	Plant part	Location	Preparation and dosage	Supporting literature
Sephuthe	*Colophospermum mopane* (Benth.) Leonard	Leguminosae	Mophane	Seeds	Maun	Grind to a powder and put one tablespoon in a cup of hot water. Drink one cup three times a day	Used in traditional medicine for the treatment of tapeworms (Mashabane et al., 2001)
Sephuthe	*Boscia albitrunca* (Burch.) Gilg & Benedict	Capparaceae	Motopi	Leaves	Maun	Infuse one tablespoon of leaf powder in a warm cup of milk. Drink three times a day	No reports-
Sephuthe	*Combretum imberbe* Wawra	Combretaceae	Motswere	Leaves	Maun	Boil the leaves and drink one cup of warm water extract three times a day	Has shown *in vitro* biological activity against *Schistosoma haematobium* (Aremu et al., 2012)
Sephuthe	*Commiphora pyracanthoides* Engl.	Burseraceae	Moroka	Stem bark	Maun	Boil the bark and drink one cup two times a day	No reports
Sephuthe	*Solanum panduriforme* E. Mey.	Solanaceae	Tholwatholwane	Fruit	Maun	Mix the inner pulp of the fruit with milk. Three fruit pulps are mixed with one cup of milk. Drink the infused milk once a day	Used in the treatment of tapeworm in conjunction with the roots of *Pseudeminia benguellensis* (Bossard, 1996)

Table 5.1. continued

Traditional Health Practitioner	Plant species	Plant Family	Local name	Plant part	Location	Preparation and dosage	Supporting literature
Tiroyakgosi	*Laphangium luteoalbum* (L.) Tzvelev	Compositae	Mookojane	Leaves	Sehithwa	Boil the roots and drink the water extract three times a day. After drinking the root extract drink one cup of the boiled leaf extract at the end of the day.	No report
Tiroyakgosi	*Laphangium luteoalbum* (L.) Tzvelev	Compositae	Mookojane	Roots	Sehithwa		No reports
Tiroyakgosi	*Moringa oleifera* Lam.	Moringaceae	Moringa	Leaves	Sehithwa	Boil the leaves and drink two cups of the water extract twice a day	Anthelmintic activity against *H. contortus*, *Trichostrongylus colubriforms, Oesophagastum columbianum, Trichuris* sp. and *Ostertagia* sp (Moyo et al., 2013; Pedraza-Hernandez et al., 2021)

With the exception of the *A. ferox* and *A. zebrina* species, preparations were made mostly from dried plant parts. The majority of the samples were leaves (55%) followed by roots (18%). Seeds, fruit and stem bark were each 9%. Roots are normally used in traditional medicine in Botswana since secondary metabolites are usually stored there so it is interesting to note the majority of samples are leaves. Most of the samples are boiled (55%) and some are infused with cold, warm or hot water. Two samples are mixed with milk and this may be in order to reduce the amount of bioavailable compounds from the plant sample as milk is known to reduce the bioavailability of certain compounds.

Milk however may also increase or decrease the excretion of certain metabolites in the extract (Reis and Joaquim, 2015). The dosage for the majority of the samples was three times a day (60%) and only one sample (*Solanum panduriforme*) is taken once a day. To the best of our knowledge there is no supporting literature on the use of *Boscia albitrunca, Commiphora pyracanthoides* and *Laphangium luteoalbum* as anthelminticsmaking this investigation the first to report their use to treat helminth infectionsasis. This is also the first *in vitro* bioactivity report on anthelmintic activity for *Aloe zebrina, Terminalia sericea, Colophospermum mopane, Boscia albitrunca, Commiphora pyracanthoides*, *Solanum panduriforme* and *Laphangium luteoalbum.*

5.3.2 Bioactivities of medicinal plant extracts against parasites

Crude extracts prepared from the ten plant species were tested at the starting concentration of 100 µg/mL against the larvae (L3) of *A. ceylanicum*, *N. americanus* and *S. ratti, H. polygyrus* (adult and L3 larvae) *T. muris* (adult worms) and NTS (Fig. 5.2; see Table S1 and S2 in Supplementary Material for numerical data). Only the most active extracts against NTS (threshold 70% at 50 µg/mL) (Fig. 5.3) were further tested against adult *S. mansoni* beginning at 50 µg/mL (Fig. 5.4). Activity above 50% at a concentration of 100 µg/ml for a plant extract is considered relevant (Cos et al., 2006; Butterweck and Nahrstedt, 2012). Therefore activity at a concentration of 100 µg/ml above 65% was considered 'good', between 30-65% 'moderate' and below 30% 'weak'.

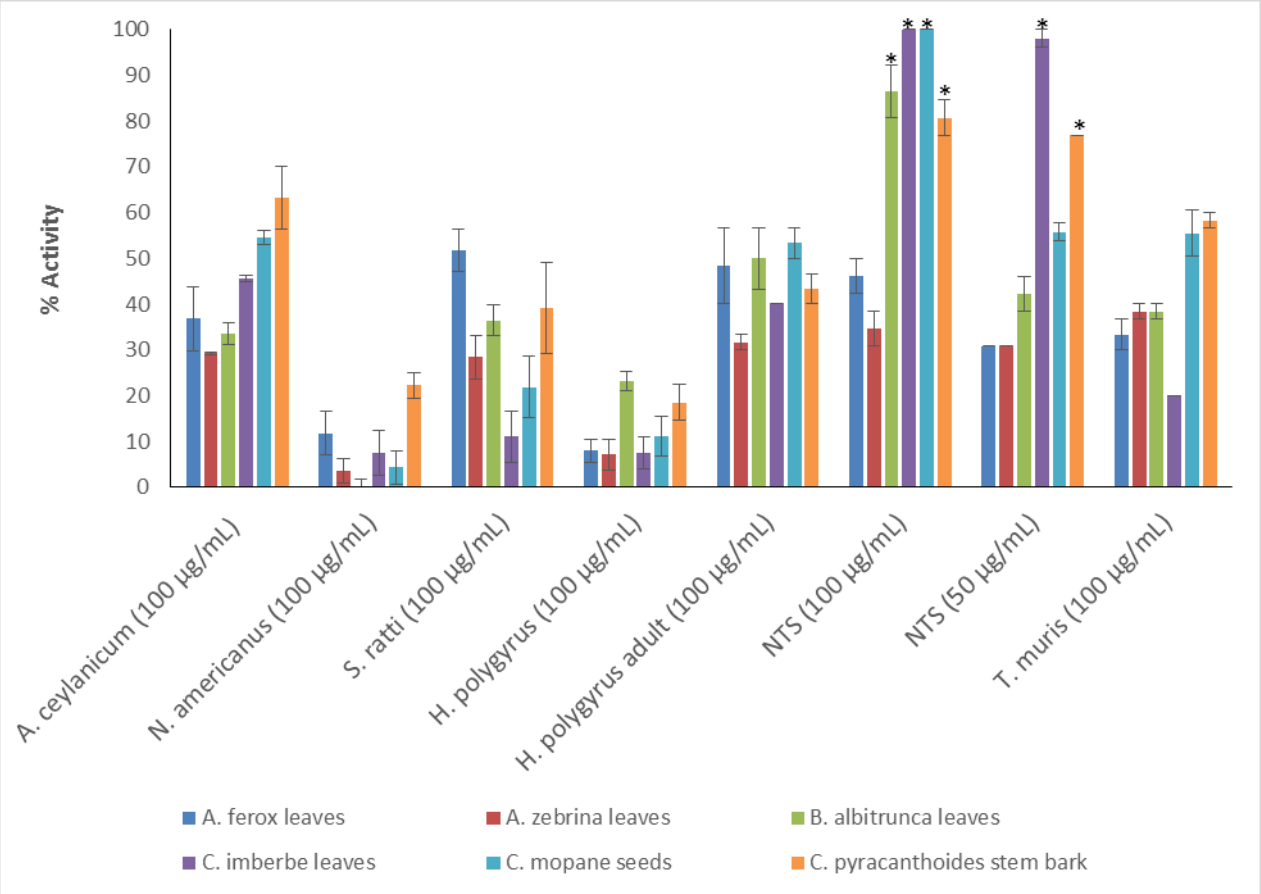

Fig. 5.2. Anthelmintic activity of the plant extracts against the parasitic organisms.

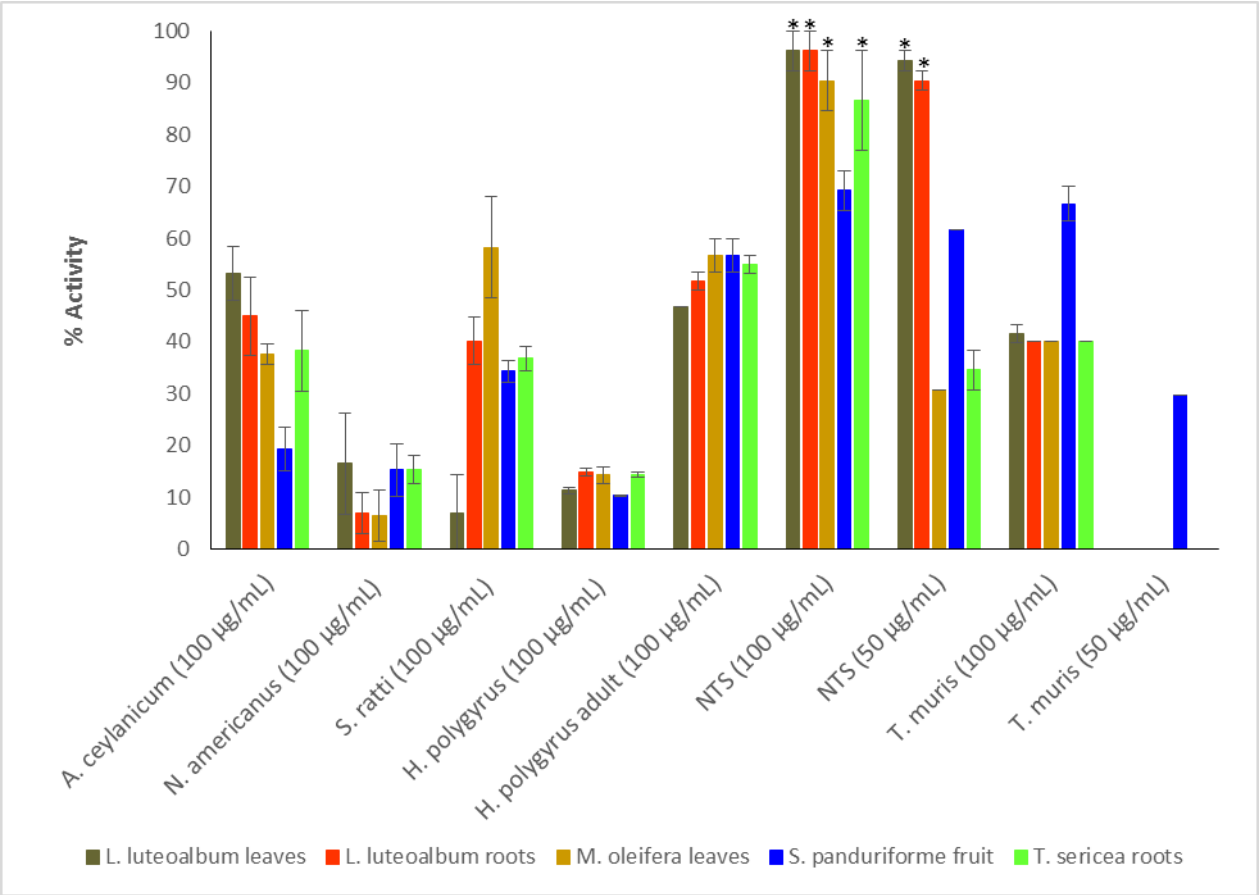

Fig. 5.2 continued.

Anthelmintic activity of the plant extracts against the parasitic organisms. Activity based on three replicates. Statistical analysis (one way ANOVA, P= <0.001 and an all pairwise multiple comparison procedure (Tukey Test) * extract activities determined to be significant) was performed using SigmaPlot 14.0 (Supplementary Materials, Table S3).

C. pyracanthoides stem bark (63.3 ± 6.9%) had the highest activity against *A. ceylanicum* followed by *C. mopane* seeds (54.5 ± 1.9%) and *L. luteoalbum* leaves (53.1 ± 5.2%). The other plant extracts exhibited activity between 19.4 ± 4.2% and 45.6 ± 0.8%. *M. oleifera* leaves (58.2 ± 9.8%) exhibited the highest activity against *S. ratti* larvae. All plant extracts showed low activity against *N. americanus* larvae with activity between 0.3 ± 1.5% and 22.2 ± 2.7%. The plant extracts displayed activity between 31.7 ± 1.7% and 56.7 ± 3.3% against adult *H. polygyrus* worms but showed lower activity against the larvae (between 7.1 ± 3.3% and 23.2 ± 2%). *M. oleifera* leaves and *S. panduriforme* fruit had the highest activity (both at 56.7 ± 3.3%) against adult *H. polygyrus* worms while *B. albitrunca* leaves (23.2 ± 2%) had the highest activity against *H. polygyrus* larvae. *S. panduriforme* fruit (66.7 ± 3.3%) was the only extract that showed activity above 65% against adult *T. muris* and was therefore tested at a lower concentration of 50 µg/mL at which it displayed reduced activity (29.6 ± 0%). The plant extracts generally showed the highest activity against NTS (between 69.2 ± 3.8% and 100 ± 0%) (except for *A. ferox* leaves 46.1 ± 3.8%, and *A zebrina* leaves 34.6 ± 3.8%) and were tested at the reduced concentration of 50 µg/mL were four plant extracts (*C. imberbe* seeds (98.1 ±

1.9%), *C. pyracanthoides* stem bark (76.9 ± 0%) and *L. luteoalbum* leaves (94.3 ± 1.9%) and roots (90.4 ± 1.9%)) displayed the highest activity. These four plant extracts were then tested at further reduced concentrations against NTS (Fig. 5.3).

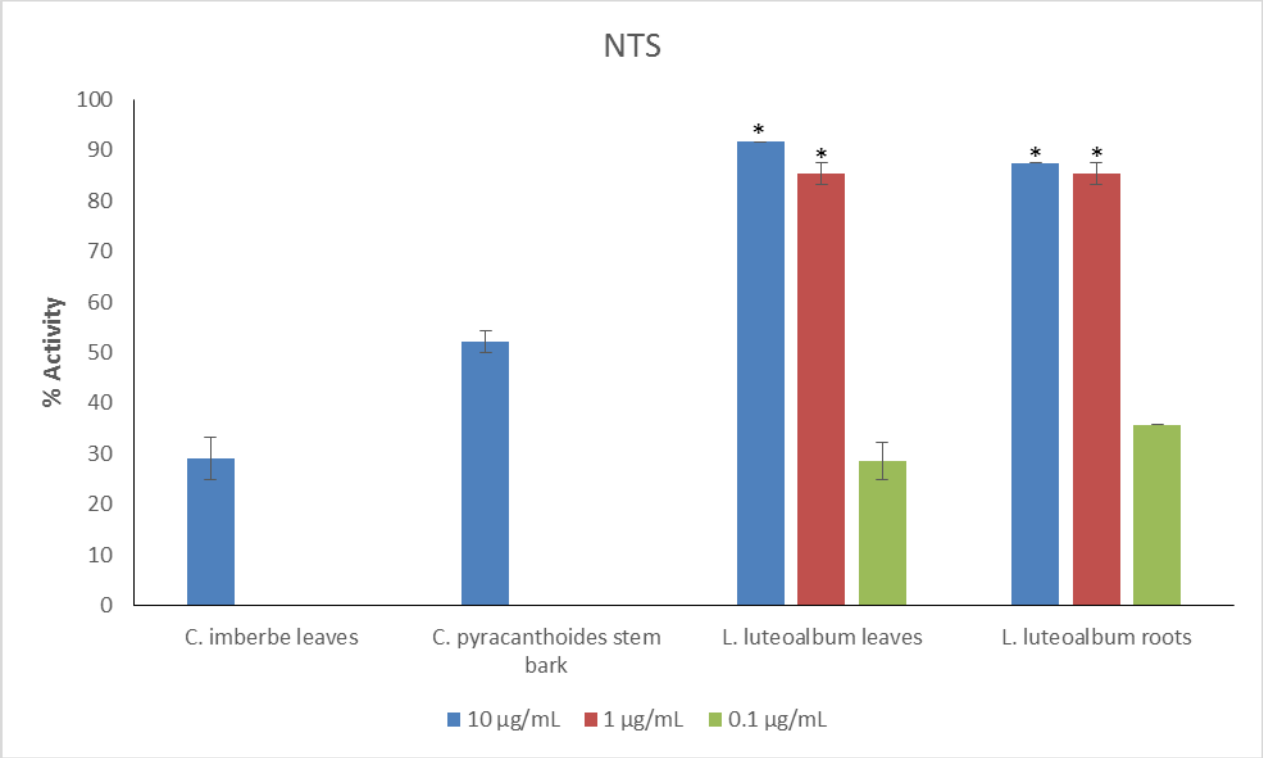

Fig. 5.3. Anthelmintic activity of the most active plant extracts at reduced concentrations against newly transformed schistosomula (NTS).

Activity based on three replicates. Statistical analysis (one way ANOVA, P= <0.001, and an all pairwise multiple comparison procedure (Tukey Test), * extract activities determined to be significant) was performed using SigmaPlot 14.0 (Supplementary Materials, Table S4).

The leaves and roots of *L. luteoalbum* showed the highest activity against NTS with activity above 90% at 1 µg/mL. At 0.1 µg/mL the activity was 28.6 ± 3.6% for *L. luteoalbum* leaves and 35.7 ± 0% for *L. luteoalbum* roots. *C. imberbe* leaves had 29.2 ± 4.2% activity while *C. pyracanthoides* stem bark displayed 52.1 ± 2.1% activity both plant extracts were tested at the test concentration of 10 µg/mL (Fig. 5.3).

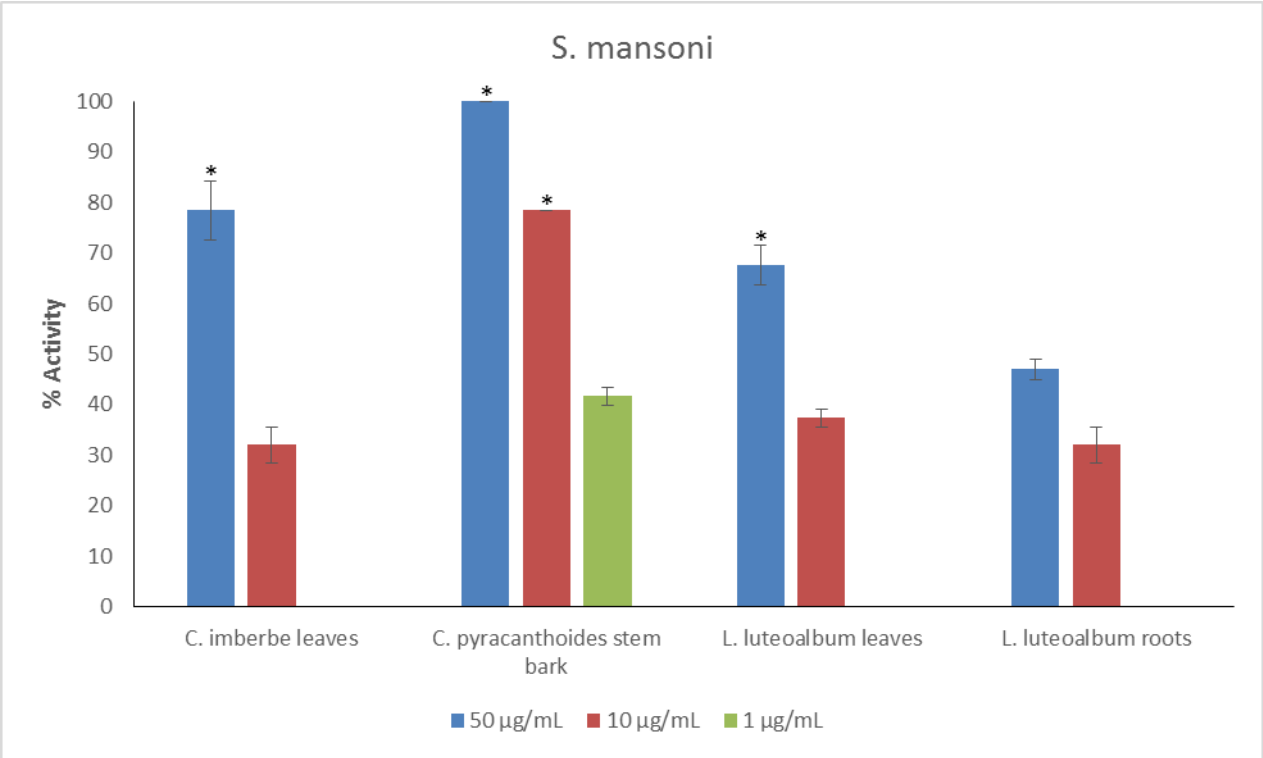

Fig. 5.4. Anthelmintic activity of the plant extracts against *S. mansoni.* Statistical analysis (one way ANOVA, P= <0.001, and an all pairwise multiple comparison procedure (Tukey Test) * extract activities determined to be significant) was performed using SigmaPlot 14.0 (Supplementary Materials, Table S5).

C. pyracanthoides exhibited the highest activity against adult *S. mansoni*, displaying 78.5 ± 0% activity at 10 µg/mL and 41.7 ± 1.7% activity at the lower concentration of 1 µg/mL. *C. imberbe* leaves displayed 78.4 ± 5.9% activity at 50 µg/mL while *L. luteoalbum* leaves displayed 67.7 ± 3.9% activity also at 50 µg/mL (Fig. 5.4).

5.4. Discussion

In this investigation extracts from medicinal plants that are used as anthelmintics in traditional medicine by the traditional health practitioners from North-Western Botswana were tested for their *in vitro* anthelmintic activity against various helminth parasites. Conventional drug screening usually uses compound libraries to test for various biological activities *in vitro* and the active compounds are then further tested *in vivo*. However, there is a very high failure rate with this approach as often compounds that gave good *in vitro* activity can be inactive when tested in animal models (Butterweck and Nahrstedt, 2012) or can be toxic. About 60% of failures for potential new therapeutic drugs are due to lack of efficacy and toxicity making these the two main causes for lack of success in drug development (Chen et al., 2009). The approach of testing herbal extracts which are used in traditional medicine is referred to as reverse pharmacology (Takenaka 2001). The idea is that if a plant is already widely used in traditional medicine without reported toxicity then the likelihood of the plant based remedy being safe and effective is high (Butterweck and Nahrstedt, 2012). Heinrich (2010) stated

however that working with a plant extract brings the major challenge that it is a mixture of active, partially active and inactive compounds whose activity is often not on a single target. Some of the compounds in the mixture can also be prodrugs, meaning that they need to be converted to an active form usually by the gut microflora. Flavonoids are a group of compounds whereby there is increasing evidence that they could possibly act as prodrugs. For example flavonols are metabolized by the intestinal microflora to their corresponding hydroxyphenylacetic acids (Aura et al., 2002; Blaut et al., 2003; Griffiths and Smith, 1972; Scalbert, 2002). The presence of prodrugs may lead to lack of activity in *in vitro* assays as the conditions required for the prodrug to become active are not available. The biological activity of plant extracts can result from the overall effect of compounds with synergistic, additive or antagonistic activity and this can lead to loss of activity when fractionation is done in an effort to isolate the active principle (Caesar and Cech, 2019). Studies have also shown that disease resistance is less likely to occur when an extract with many compounds is used rather than a single active compound (van Vuuren and Viljoen, 2011; Burfield and Reekie, 2005). Plenty of plants used in traditional medicine are now registered and marketed as botanical drugs in their crude form without having the active ingredients isolated (Wagner, 2011). A remarkable example is the phytopharmaceutical preparation Iberogast®, sold in Germany which consists of nine different plant extracts. The preparation is used in the treatment of dyspepsia and showed therapeutic equivalence when it was compared with the synthetic drugs cisapride and metoclopromide but it had fewer side effects than the synthetic drugs (Wagner and Allescher, 2006). The plant extracts in our study are used by traditional health practitioners against helminths as extracts and were therefore tested *in vitro* as extracts. The plant extracts all showed anthelmintic activity to varying degrees.

The overall best anthelmintic activity was exhibited by *C. pyracanthoides* stem bark extract against *S. mansoni* adult worms, *A. ceylanicum* L3 and *N. americanus* L3. Only *S. panduriforme* fruit extracts had better activity against *T. muris* than *C. pyracanthoides* stem bark extract, while *B. albitrunca* leave extract was the only plant extract with better anthelmintic activity against *H. polygyrus* L3 than *C. pyracanthoides* extract. Plant extracts and compounds with broad spectrum anthelmintic activity are desirable as multiple parasitic helminths are often endemic in the same regions. A plant extract showing anthelmintic activity against several target species across the Nematoda and Platyhelminthes phyla could be helpful in the isolation of compounds with potential to be broad spectrum therapeutic agents (Patridge et al., 2021). *Commiphora* (Burseraceae) species are often used in traditional medicine in Southern Africa for various ailments including malaria where the stem is used and stomach aches where the bark, resin and leaf are used. Investigations have shown that *C. pyracanthoides* essential oil extract has various biological properties including anti-inflammatory (5-LOX enzyme inhibition), anti-cancer (against HT-29, MCF-7 and SF-268 cell lines), antimicrobial (*Bacillus cereus*) and antioxidant activity (ABTS and DPPH asays) (Paraskeva et al., 2008). *C. pyracanthoides* was not cytotoxic against kidney epithelial cells indicating that it has selective activity against cancer cells.

L. luteoalbum leave and root extract exhibited excellent activity against NTS with both the roots and leaves showing similar activity against the parasite. The plant originates from Europe and was introduced to Southern Africa by early settlers and it is now widespread and known as a winter weed of maizelands (Bromilow, 2010). The plant has shown antifungal activity and

the compounds responsible for the antifungal activity did not show cytotoxic activity when tested against the Vero cell line (Aderogba et al., 2014). Although no cytotoxic effects were observed against the Vero cell line further toxicological studies need to be done to ensure the safety of the plant. This is because plants sometimes produce toxic secondary metabolites to act as defence compounds against pathogens and herbivores (Wittstock and Gershenzen, 2002). The promising activity against schistosomes makes *L. luteoalbum* an ideal candidate for further investigations as it is a weed meaning it grows easily even in unfavourable conditions and obtaining adequate biomass would not be a challenge (Mdee et al., 2009).).

M. oleifera leaves exhibited the best anthelmintic activity against adult *H. polygyrus* and *S. ratti* L3 larvae. *M. oleifera* is a fast growing woody plant whose seeds, leaves and flowers have broad spectrum therapeutic applications (Mahfuz and Piao, 2019). Among the biological properties investigated, the leaves have shown anthelmintic activity reducing the worm burdens of *Haemonchus contortus*, *Trichostrongylus colubriforms* and *Oesophagastum columbianum* in goats (Moyo et al., 2013). The *M. oleifera* leaf extract has also exhibited anthelmintic properties against *Trichuris* sp. and *Ostertagia* sp. The bioactive compounds thought to be responsible for the anthelmintic activity are heneicosane, di(2-ethylhexyl)phthalate (as 1,2-benzenedicarboxylic acid in Pedraza-Hernandez et al., 2021), heptacosane pentatriacontane and hexadecanoic acid ethyl ester (Pedraza-Hernandez et al., 2021).

S. panduriforme fruits had the highest activity against adult *T. muris* and also shared the best activity against adult *H. polygyrus* with *M. oleifera* leaves. *T. muris* has low cure rates using benzimidazole drugs and therefore new approaches are needed to eliminate morbidity from trichuriasis (Patridge et al., 2021). Further investigations could be carried out on the fruits of *S. panduriforme* in order to isolate the active principle. *S. panduriforme*, *C. imberbe* and *T. sericea* are some of the plants used by traditional health practitioners in the treatment/management of HIV related opportunistic infections in Ngamiland District in Northern Botswana (Okatch et al., 2012). *S. panduriforme* and *T. sericea* are also used in South Africa for the treatment of tuberculosis and a study carried out by Green et al. (2010) showed that *T. sericea* bark extract (MIC 25 µg/mL) had better activity against *Mycobacterium tuberculosis* than *S. panduriforme* leaves although *S. panduriforme* leaves showed activity at a concentration higher than 100 µg/mL. The roots of *S. panduriforme* are also used for the treatment of oral diseases (More et al., 2008) and the plant is also used for skin infections, wounds and ulcers (Prozesky et al., 2001). The leaves of *S. panduriforme* have antiplasmodial activity against *Plasmodium falciparum*, although *Solanum nigrum* not *S. panduriforme* is reported to be used against malaria in traditional medicine (Prozesky et al., 2001).

The roots leaves and bark of *T. sericea* are widely used in South Africa to prepare remedies used in ethnoveterinary medicine for the treatment of wounds, ticks and diarrhea (Selogatwe et al., 2021). *T. sericea* is a multipurpose medicinal plant used to treat many ailments and the plant contains various biological activities including anti-HIV, antifungal, antibacterial, antiparasitic, anticancer, wound healing, antioxidant and anti-inflammatory activity (Mongalo et al., 2016).

A. ferox is used in traditional medicine in South Africa to treat intestinal worm infections (Wintola and Afolayan, 2015) and several studies have shown the *in vitro* and *in vivo*

anthelmintic activity of the plant against *H. contortus* and *Heterakis gallinarum* (Ahmed et al., 2013; Maphosa et al., 2010; Mwale and Masika, 2015). *A. zebrina* is used in southern Africa for the treatment of myiasis and *in vitro* studies have shown that the leaf extract reduces pupation rate and pupal mass of *Lucilia cuprina* and *Chrysomya marginalis* (Mukandiwa et al., 2012). *B. albitrunca* is widely used as a medicinal plant in Southern Africa. Its uses include the treatment of constipation, diarrhea, and epilepsy. In Botswana it is used for the treatment of skin diseases, haemorrhoids and in ethnoveterinary medicine (Maroyi, 2019). Antibacterial and antifungal activities have been reported from leaf and fruit extracts of *B. albitrunca* (Pendota et al., 2015; Tshikalange et al., 2017). *C. mopane* is a dominant tree occurring in the dry regions of southern Africa, it is used in traditional medicine for the treatment of tapeworms (Mashabane et al., 2001), syphilis, dysentery, diarrhea, inflamed eyes (Du et al., 2015) and is also used in ethnoveterinary medicine (Syakalima et al., 2018). *C. imberbe* is widely used in Africa to treat bacterial infections (Angeh et al., 2007), sexually transmitted infections (Chinsembu, 2016) and also in ethnoveterinary medicine (Chinsembu et al., 2014). The leaf extract of the plant has shown *in vitro* biological activity against *S. haematobium* (Aremu et al., 2012), and anti-inflammatory activity (McGaw et al., 2001). Isolated compounds were active against *Mycobacterium fortuitum* and *Staphylococcus aureus* (Katere et al., 2003).

Overall the plant extracts showed the best activity against NTS and only the best four were tested against adult *S. mansoni*. The plant extracts also showed moderate activity against adult *H. polygyrus* and adult *T. muris*. Activity of the plant extracts against *A. ceylanicum* L3 and *S. ratti* L3 was reduced. *N. americanus* and *H. polygyrus* L3 were the worms whereby the plant extracts had the least effect with activity ranging between 0.3 and 23.2%. The anthelmintic activity shown by the plant samples shows the importance of traditional medicine as traditional health practitioners have knowledge about which plants to use against various ailments. Further investigations need to be carried out to in order to identify the active principles in the plant extracts and validate the safety of the plant extracts through toxicological studies. Once the active principles are identified then crude extracts can be standardized with identified marker molecules.

5.5 Conclusions

In this study we report for the first time the use as anthelminctics and *in vitro* anthelmintic activity of *B. albitrunca, C. pyracanthoides* and *L. luteoalbum.* We also report for the first time the *in vitro* anthelmintic activity of *A. zebrine, T. sericea, C. mopane* and *S. panduriforme.* The promising antischistosomal activity exhibited by the leaves and roots of *L. luteoalbum* warrant further investigation of the plant as a potential source of compounds with antischistosomal properties. The overall anthelmintic activity exhibited by the different plant species especially *C. pyracanthoides* require further investigations to identify the active anthelmintic principles and also to perform cytotoxicity studies to give evidence to the safety of the plants. Our investigation confirms the importance of indigenous knowledge and further interviews should be held with traditional health practitioners in order to tap into the vast knowledge of medicinal plants which they have. These plants could be promising leads for the discovery of much needed new therapeutic agents against soil transmitted helminth infections and

schistosomiasis. Additionally, the possibility of using the plants for veterinary applications may also be studied.

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Supplementary materials

The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/plants11212945/s1, Table S1: Anthelmintic activity against parasitic helminths; Table S2: Most active plants against S. mansoni and NTS; Table S3: Statistical table for one way ANOVA using Sigma Plot 14.0. ANOVA table for data in Figure 1; Table S4: Statistical table for one way ANOVA using Sigma Plot 14.0. ANOVA table for data in Figure 2; Table S5: Statistical table for one way ANOVA using Sigma Plot 14.0. ANOVA table for data in Figure 3.

6. Anthelmintic activity of selected Ethiopian plant and fungi species: Could spices be an alternative to MDA?

This Chapter is a cooperative work (see author declaration for details) and is a manuscript in preparation which is to be published in a peer reviewed journal as Dube, Mthandazo; Ware, Ismail; Lam, Thi Hai Yen; Martinez-Aldino, Ingrid Y.; Kahsay, Birhanu Nigusse; Mariam, Tsige-Gebre; Moeller, Lucie; Franke, Katrin; Imming, Peter; Arnold, Norbert.

Abstract: The global prevalence of soil transmitted helminths (STH) is an ongoing problem and mass drug administration programs which were the main control measure were recently hindered by the Covid 19 pandemic and this may have lead to an increase in the prevalence of STH. Mass drug administration (MDA) programs, though effective are not sustainable long term and there is also the threat of development of drug resistance as well as undesired effects on the environment. In this study we tested the anthelmintic activity of Ethiopian plant and fungal extracts against *C. elegans* and found that some samples including spices exhibited anthelmintic activity. Using HRMS we annotated the compounds showing the major peaks in the most active extracts by comparing the MS^n data with databases such as Metfrag. We also used bioassay guided isolation to identify the anthelmintic compounds in *Echinops kebericho*. This investigation shows the potential spices have either as anthelmintics or as a source of anthelmintic compounds.

6.1 Introduction

Soil transmitted helminths (STHs) are the most prevalent neglected tropical disease affecting about 24 % of the world's population (WHO, 2022). The most affected individuals are children, due to their poor hygiene practices and the fact that they sometimes consume soil (Brown et al., 2013). Currently the most effective control measure is mass drug administration (MDA) but this is not sustainable long term and drug resistance is also an emerging problem. Mass drug administration also has negative effects on the environment as the drug residues may affect invertebrates as well as pose the threat of contaminating the water table. An example is seen with ivermectin, a drug widely used in MDA programs, which disrupts dung beetle diversity, soil properties and ecosystem functions (Verdu et al., 2018). There is therefore a need for new anthelmintic therapeutic agents to help fight the prevalence of STHs without the dangers of negatively affecting the environment. Plants and their constituents, are a good alternative because they have bioactive compounds that they have developed over the course of evolution to defend themselves against invading parasites, bacteria, fungi and even predators (Guerriero et al., 2018). The world health organization (WHO) reports that about 80 % of the world's population uses herbal medicines as their first-line primary health-care (Kahsay et al., 2021). So medicinal plants are especially a potentially good source of anthelmintics as through indigenous knowledge they have already been preselected and the chances of isolating bioactive compounds is high. An example of this is the isolation of anthelmintic compounds from the fruits of *Ozoroa insignis*, a plant widely used in traditional medicine in Southern Africa (Dube et al., 2021). There are concerns about the conservation of medicinal plants as usually the roots are used for the treatment of ailments as they are traditionally considered to be more effective than other parts of the plant (Mbuni et al., 2020)

and this may lead to over harvesting of plants that have medicinal properties. According to the International Union for Conservation of nature and the World Wildlife Fund between 50 000 and 80 000 plant species are used for medicinal purposes around the world and 15 000 among them are threatened with extinction from overharvesting and destruction of their habitat (Chen et al., 2016). Also other so far neglected organism like fungi can act as a potential source of anthelmintic compounds and our recent studies have demonstrated that the mushroom *Albatrellus confluens* contains compounds which are active against *Caenorhabditis elegans* (Dube et al., 2022).

Other possible under investigated sources of anthelmintic agents are herbs and spices. They have been used since around 5 000 BC for human consumption (Marc et al., 2022). Herbs are defined as plants whose leaves are used in cooking for the purpose of giving flavor to particular prepared meals, while a spice is any of the various aromatic products obtained from plants in the form of powder or seeds or other plant parts that is used to add taste to food (Motti, 2021). Spices are reported to possess a number of pharmacological effects and are frequently used as an active ingredient in medicines to treat different human ailments (Manju et al., 2011). A survey conducted in the United States showed that 54 % of adult participants were using spices on a daily basis for the promotion of good health and wellness and ginger, garlic and cinnamon were the three most used. The survey also showed that more than 10 % of infants and children were given spices to remedy stomach issues, coughs and colds (Jiang, 2019). Spices and herbs contain bioactive compounds with various activities such as antioxidant, antimicrobial, antiparasitic, anti-diabetics, anticancerous, and many more that help to maintain health and have no detrimental effects (Jamil et al., 2022). Spices have been used for centuries for preservation as well as for making food tastier. They are easy to grow as most of them are small plants which are quite robust. Their safety profile is known as they have been consumed for years and there would therefore be no toxicity concerns. Their fast growing nature makes it easy for them to be cultivated and therefore there would be no conservation issues which normally arise when medicinal plants are over harvested. They could also be easily made available to communities affected by STHs and would be a more sustainable alternative to MDA. They already are known to have various health benefits and offer the added advantage of not only dealing with the STHs but also improving the general well-being of individuals who consume them. A second alternative which would be even better from a conservation point of view would be waste products from the food industry. Two examples are papaya seeds which are usually thrown away and also pumpkin seeds. Both have shown anthelmintic activity (Ameen et al., 2018; Grzybeck et al., 2016) and could be potential solutions for dealing with the prevalence of STHs. Avocado seeds have also shown anthelmintic activity and might also be a source of anthelmintic compounds (Soldera-Silva et al., 2019).

In this study we investigated the anthelmintic properties of some Ethiopian plants and fungal species including spice samples as well as other food stuffs using *C. elegans* as a model organism for parasitic worms. While pumpkin seeds, papaya fruit, strawberries and carrots showed little to no anthelmintic activity, some spice samples and avocado fruit showed varying degrees of anthelmintic activity and the most active samples were *Cinnamomum verum* bark (37.7% activity), *Echinops kebericho* roots (36.9% activity), *Piper nigrum* seeds (92% activity) and *Trachyspermum ammi* seeds (41.6% activity). Although bioassay guided isolation is the best method to isolate anthelmintic compounds sometimes this is not always possible when there is inadequate plant material. Therefore mass spectrometry has become a useful analytical tool that can be used for the analysis of the chemical composition of natural

products when isolation is not possible. Mass spectrometry has the advantage of sensitivity and time as samples can be processed in a relatively short time compared to the time comsuming process in isolation. Using HRMS we were able to annotate the major compounds present in the samples showing the highest anthelmintic activity. We were also able to isolate the anthelmintic compounds from *E. kebericho* as there was adequate plant material available whereby we observed that thiophenes were responsible for the observed anthelmintic activity.

6.2 Materials and method

Column chromatography for fractionations or purifications was performed either on silica gel 60 (0.040–0.063 mm, Merck, Darmstadt, Germany) or Sephadex LH 20 (Fluka, Germany). Analytical TLCs were performed on pre-coated silica gel F_{254} aluminum sheets (Merck, Darmstadt, Germany) and spots were detected by their color, their absorbance under UV-light (254 nm and 366 nm), or after spraying with vanillin and heating. For ^{1}H NMR, ^{13}C NMR, and HRMS see Supplementary Material.

NMR spectra were recorded with an Agilent DD2 400 MHz NMR spectrometer (Varian, Palo Alto, CA, USA) operating at a proton NMR frequency of 400 MHz using a 5-mm inverse detection cryoprobe. 2D NMR spectra were recorded using standard CHEMPACK 8.1 pulse sequences (^{1}H,^{1}H zTOCSY, ^{1}H,^{13}C gHSQCAD, ^{1}H,^{13}C gHMBCAD) implemented in Varian VNMRJ 4.2 spectrometer software (Varian, Palo Alto, CA, USA). The mixing time for the TOCSY experiments was set to 80 msec. The HSQC experiment was optimized for ^{1}JCH = 146 Hz with DEPT-like editing and ^{13}C-decoupling during acquisition time. The HMBC experiment was optimized for a long-range coupling of 8 Hz; a two-step ^{1}JCH filter was used (130-165 Hz). ^{1}H chemical shifts are referenced to internal TMS (^{1}H δ = 0 ppm), while ^{13}C chemical shifts are referenced to $CDCl_3$ (^{13}C δ = 77.0 ppm).

The negative ion electron spray ionization high-resolution mass spectra (ESI-HRMS) were obtained from an API 3200 Triple Quadrupole System (Sciex, Framingham, MA, USA) equipped with a turbo ion spray source, which performs ionization with an ion spray voltage on 70 eV. Sample introduction was performed by direct injection through an Agilent-HPLC 1200 (Agilent, Santa Clara, CA, USA) syringe pump. During the measurement the mass/charge range from 5 to 1800 can be scanned.

The semi-preparative HPLC was performed on a Shimadzu prominence system which consists of a CBM-20A communications bus module, a SPD-M20A diode array detector, a FRC-10A fraction collector, a DGU-20A5R degassing unit, a LC-20AT liquid chromatograph, and a SIL-20A HT auto sampler, using a YMC Pack Pro C18 column (5µm, 120 Å, 150 × 10mm I.D, YMC, USA) with H_2O (A) and CH_3CN (B) at a flow rate of 2.5 ml/min.

6.2.1. Plant material

A total of 90 plant and fungi species comprising 40 families were collected from Gullele Botanical Garden and local supermarkets in Addis Ababa, Ethiopia. The families with the highest numbers of samples were Compositae (12), Xanthorrhoeaceae (10), Lamiaceae (7) and Fabaceae (6). The rest of the families had between 1 and 5 samples (See Appendix 1 for the entire list). All the plant and mushroom species were authenticated by the Ethiopian Biodiversity Institute, Addis Ababa, Ethiopia. The collection included plant varieties such as

cereals, leguminous plants, vegetables, fruits, spices and tea plants. In addition to the commonly used food items, attention was given to some indigenous *Aloe* species. The samples consisted of various plant parts with the majority being leaves, seeds and fruits (Fig. 6.1). One of the most common mushrooms found in Ethiopia, the *Pleurotus ostreatus*, was also included in the study. The fungi has shown anthelmintic activity against *Haemonchus contortus* (Comans-Perez et al., 2021). Except for *Daucus carota, Zingiber officinale* and *Echinops kebericho* all other plant samples were only the above ground parts. This was to ensure that the selected species can be cultivated and harvested at a rate that ensures the plants are conserved in a sustainable manner.

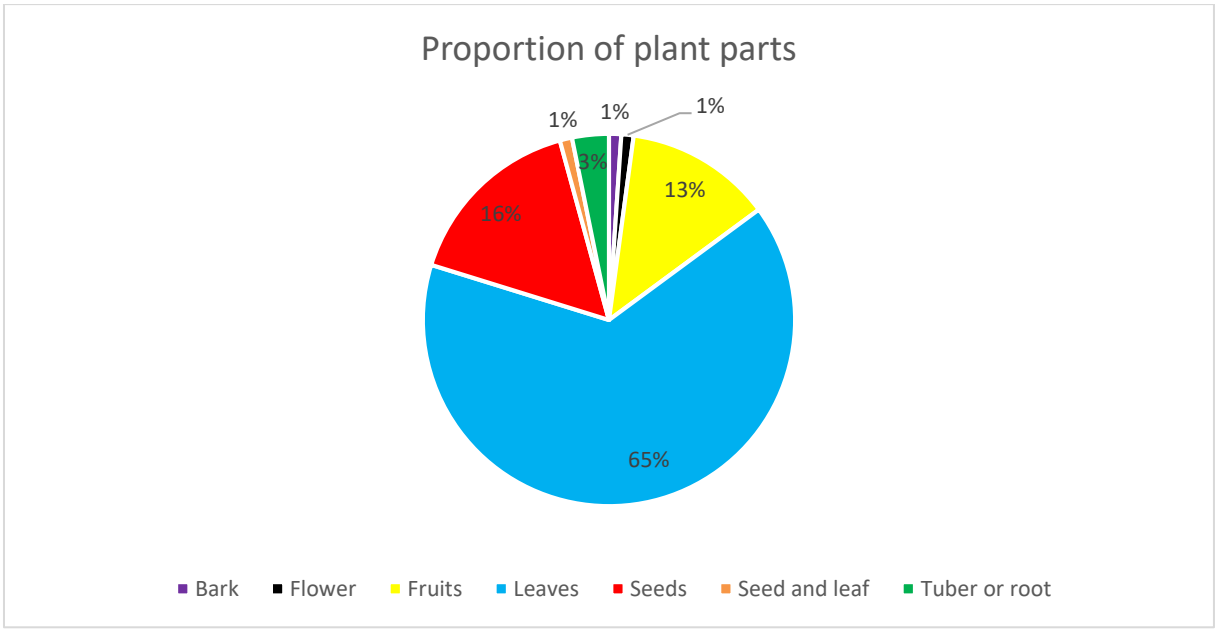


Fig. 6.1. Proportion of different plant parts

6.2.2 Extracts preparation and anthelmintic screening

Dried plant material (100 mg) was extracted by sonication for 15 minutes with 2 mL of 80 % MeOH at room temperature. The resulting solution were evaporated to dryness under nitrogen to afford a crude extract. A stock solution of 1 mg/mL in 4% DMSO was prepared. The sample was screened at the final concentration of 500 µg/mL.

The Bristol N2 wild type strain of *Caenorhabditis elegans* was used in the anthelmintic assay. The nematodes were cultured on NGM (Nematode Growth Media) petri plates using the uracil auxotroph *E. coli* strain OP50 as food source according to the methods described by Stiernagle (2006). The anthelmintic bioassay was carried out following the method developed by Thomsen et al. (2012). In all the assays, the solvent DMSO (2%) and the standard anthelmintic drug ivermectin (10 µg/mL) were used as negative and positive controls, respectively. All the assays were carried out in triplicate.

6.2.3 Bioassay guided isolation of *Echinops kebericho* roots

206.41 g of dried roots of *Echinops kebericho* was extracted two times by sonication for 30 mins with (2 x 900 mL) of 80% MeOH. Filtrates were obtained by using filter papers (Whatman no.1) and were evaporated to dryness under reduced pressure using a rotary evaporator maintained at 40 °C to afford 10.13 g of crude extract.

This extract was dissolved in 100 mL of water and partitioned between *n*-hexane (200 mL x 4), chloroform (200 mL x 4), EtOAc (200 mL x 3) and *n*-butanol (200 mL x 2). The resulting fractions were evaporated to dryness at 40 °C to yield 5.61 g of *n*-hexane, 0.27g of chloroform, 0.15 g of EtOAc, 1.64 g of *n*-BuOH and 1.71 g of the remaining aqueous fraction.

The *n*-hexane fraction was adsorbed on an equivalent mass of silica gel and chromatographed over a silica gel column chromatography (40-63 µm, 2.5 x 48 cm) using *n*-hexane-EtOAc and EtOAc-MeOH gradients as eluents. The column was monitored by UV lamp (254 and 366 nm). Fractions of 100-300 mL were collected as follows: [(1), *n*-hexane - EtOAc (97.5:2.5)], [(2-4), *n*-hexane - EtOAc (95:5)], [(5-6), *n*-hexane - EtOAc (92.5:7.5)], [(7-9), *n*-hexane - EtOAc (90:10)], [(10-11), *n*-hexane - EtOAc (87.5:12.5)], [(12-14), *n*-hexane - EtOAc (85:15)], [(15), *n*-hexane - EtOAc (82.5:17.5)], [(16), *n*-hexane - EtOAc (8:2)], [(17-18), *n*-hexane - EtOAc (77.5:22.5)], [(19), *n*-hexane - EtOAc (75:25)], [(20), *n*-hexane - EtOAc (72.5:27.5)], [(21), *n*-hexane - EtOAc (70:30)], [(22), *n*-hexane - EtOAc (67.5:32.5)], [(23), *n*-hexane - EtOAc (65:35)], [(24-25), *n*-hexane - EtOAc (60:40)], [(26), *n*-hexane - EtOAc (50:50)], [(27), *n*-hexane - EtOAc (40:60)], [(28), *n*-hexane - EtOAc (30:70)], [(29), *n*-hexane - EtOAc (20:80)], [(30), *n*-hexane - EtOAc (10:90)], [(31), EtOAc (100)] [(32), EtOAc - MeOH (97.5:2.5)] [(33), EtOAc - MeOH (95:5)] [(34), EtOAc - MeOH (92.5:7.5)] [(35), EtOAc - MeOH (9:1)] [(36), MeOH (100%)]. These fractions were pooled according to their TLC profiles into 11 subfractions F1 to F11 as follows: 0.13 g of F1 (1), 0.03 g of F2 (2),3.59 g of F3 (3-7), 0.23 g of F4 (8), 0.38 g of F5 (9-11), 0.35 g of F6 (12-14), 0.22 g of F7 (15-17), 0.14 g of F8 (18-20), 0.10 g of F9 (21-24), 0.09 g of F10 (25-31) and 0.05 g of F11 (32-35). F3 was further separated using silica gel chromatography with *n*-hexane - EtOAc (95:5) to give 2.3 g of compound **6.7**. F6 was further purified using HPLC to give 11.4 mg of compound **6.8**. 0.22 g of F7 was separated by column chromatography using sephadex LH20 column chromatography (1.7 x 40 cm) and DCM - MeOH 7:3 as eluent. 10 fractions were collected and fractions 7 to 9 were combined to give 107 mg of C2F1. C2F1 was separated using HPLC to give four subfractions C2F1-S1 to S4. 9.4 mg of C2F1-S2 was further purified by HPLC to afford 1.3 mg of compound **6.9** and 0.9 mg of **6.10** and **6.11,** which were in a 1.4:1 mixture.

6.2.4 High resolution mass spectra

The high resolution mass spectra in positive and negative modes were obtained from an Orbitrap Elite mass spectrometer (Thermofisher Scientific, Bremen, Germany) equipped with an ESI electrospray ion source (spray voltage 4.0 kV; capillary temperature 275 °C, source heater temperature 40 °C; FTMS resolution 60.000). Nitrogen was used as sheath gas. The sample solutions were introduced continuously via a 500 µl Hamilton syringe pump with a flow rate of 5 µl/min. The instrument was externally calibrated by the Pierce® LTQ Velos ESI positive ion calibration solution (product number 88323) and Pierce® ESI negative ion calibration solution (product number 88324) from Thermofisher Scientific, Rockford, IL, 61105 USA). The data were evaluated by the Xcalibur software 2.7 SP1. The collision induced dissociation (CID) MS^n measurements were performed using the relative collision energies given in the Supplemental material.

6.2.4.1 Metabolite annotation

Metabolites were annotated using characteristics based on their corresponding accurate high-resolution *m/z*, and fragmentation patterns with those reported in the literature and databases such as MetFrag and MassBank. Briefly, a compound was deemed identified if the mass error of MS1 was below or equal to 5 ppm. Then, the fragmentation patterns of MS/MS spectra were analyzed to identify the metabolites.

6.3 Results

Out of the 90 samples tested, ten showed anthelmintic activity above 8 %: *Cinnamomum verum* bark; *Cuminum cyminum* seeds; *Echinops kebericho* roots; *Ocimum basilicum* leaves; *Persea americana* fruit; *Phytolacca dodecandra* seeds; *Piper nigrum* seeds; *Sesamum indicum* seeds; *Trachyspermum ammi* seeds; *Zingiber officinale* tuber (Fig. 6.2). For the anthelmintic results of all 90 samples see Appendix 1.

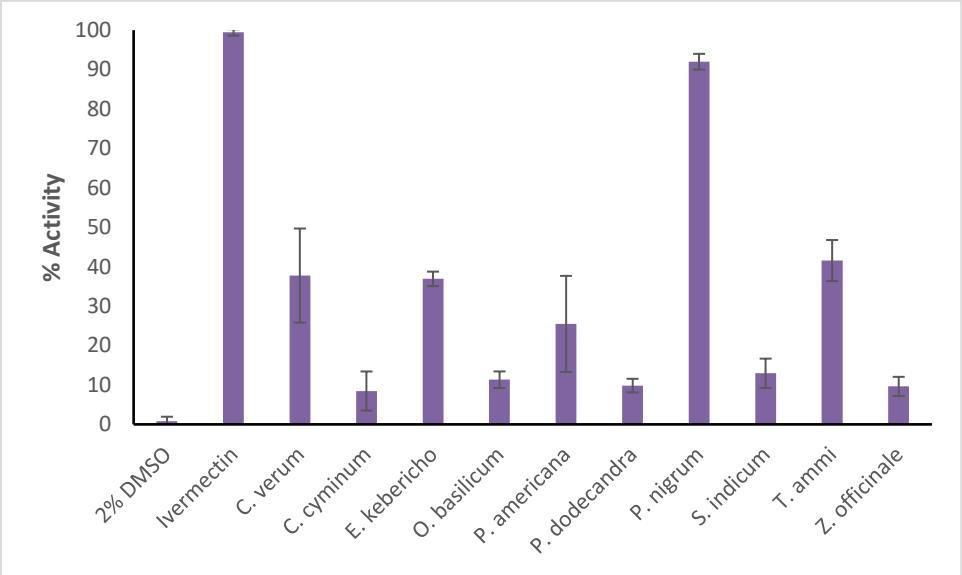


Fig. 6.2. Anthelmintic activity of plant extracts > 8%.

Available compounds which were known to be present in the active samples from literature and thought to be responsible for the anthelmintic activity, where then tested against *C. elegans*. The compounds tested where piperine (**6.1**), cinnamaldehyde (**6.2**), cinnamic acid (**6.3**), eugenol (**6.4**) (known to be present in *C. verum*, thymol (**6.5**) (known to be present in *T. ammi*) and zingerone (**6.6**) (known to be present in *Z. officinale*). The results are shown in Fig. 6.3 below.

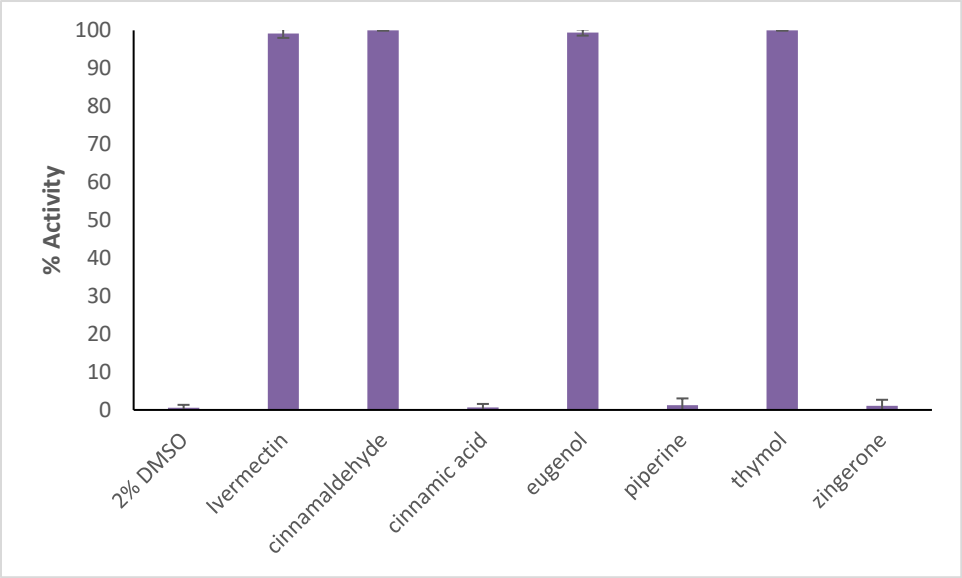
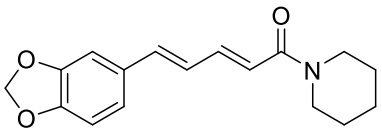
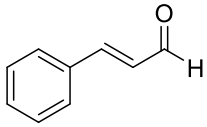


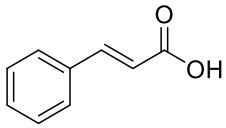
Fig. 6.3. Anthelmintic activity of selected compounds tested at 500 µg/ml against *C. elegans*



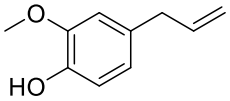
6.1



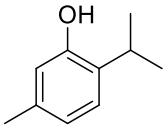
6.2



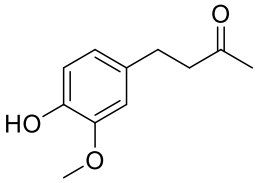
6.3



6.4



6.5



6.6

Cinnamaldehyde (**6.2**), eugenol (**6.4**) and thymol (**6.5**) showed excellent anthelmintic activity against *C. elegans* at 500 µg/mL and therefore the LC_{50}s were determined. Cinnamaldehyde had the best activity with an LC_{50} of 76.86 µg/mL followed by thymol (**6.5**) with an LC_{50} of 104.15 µg/mL and Eugenol with an LC_{50} of 145.80 µg/mL.

To check if the compounds thought to be active were present in the samples, the samples were sent for high resolution mass spectrometry. This was done also to try and identify the major compounds present. The results of the analysis are shown in table 6.1 below.

Table 6.1: Metabolites identified in spice samples by UPLC-ESI-MS in positive ionization modes

Sample	Rt	Compound name	Compound class	$[M+H]^+$	Molecular formula	Error (ppm)	MS/MS fragments (rel. intensity, %)
Piper nigrum	7.79	Piperylin	Piperamides	272.1280	$C_{16}H_{18}NO_3^+$	-0.4	201.0546 (**100**), 173.0559 (3), 135.0442 (8), 98.0600 (1)
	8.44	Piperine	Piperamides	286.1441	$C_{17}H_{20}NO_3^+$	1.1	201.0548 (**100**), 135.0442 (4), 112.0754 (1)
	9.11	Piperettine	Piperamides	312.1598	$C_{19}H_{22}NO_3^+$	1.3	227.0704 (**100**), 199.0756 (2), 164.1071 (3), 112.0757 (10)
	9.27	Piperettine (isomers)	Piperamides	312.1598	$C_{19}H_{22}NO_3^+$	1.3	227.0703 (**100**), 199.0753 (2), 164.1071 (3), 112.0757 (11)
	10.02	1-Piperidin-1-yl-deca-2,4-dien-1-one	Piperamides	236.2011	$C_{15}H_{26}NO^+$	0.9	151.1119 (**100**), 133.1012 (31), 112.0757 (28), 109.0648 (14), 95.0491 (49), 86.0964 (33)
	10.02	Chabamide	Piperamides	571.2803	$C_{34}H_{39}N_2O_6^+$	0.0	486.1908 (**100**)
	10.36	Piperolein B	Piperamides	344.2220	$C_{21}H_{30}NO_3^+$	0.0	314.2111 (60), 259.1326 (23), 222.1853 (95), 175.0753 (8), 161.0599 (4), 135.0441 (**100**), 112.0757 (6)
	12.81	*N*-isobutyl-octadeca-2,4,12- trienamide	Piperamides	334.3102	$C_{22}H_{40}NO^+$	-0.6	261.2215 (**100**), 233.2267 (20), 149.1328 (12), 135.1169 (24), 121.1014 (23), 109.1012 (23)
Cinnamomum verum	4.07	Procyanidin B2	Flavonoids	579.1498	$C_{30}H_{27}O_{12}^+$	0.2	427.1024 (**100**), 409.0920 (52), 301.0708 (19) 291.0865 (45), 247.0604 (18)
	4.72	Corydine	Aporphines	342.1702	$C_{20}H_{24}NO_4^+$	0.6	311.1279 (16), 299.1280 (19), 297.1123 (**100**), 265.0861 (16)
	4.78	Procyanidin C1	Flavonoids	867.2132	$C_{45}H_{39}O_{18}^+$	0.1	715.1664 (47), 697.1543 (40), 579.1500 (**100**), 559.1230 (20), 451.1023 (16), 427.1028 (24), 409.0919 (38), 289.0707 (11)
	4.96	Reticuline	Isoquinolines	330.1704	$C_{19}H_{24}NO_4^+$	1.2	299.1280 (6), 267.1028 (1), 192.1022 (**100**), 175.0757 (1), 137.0599 (1)
	9.73	Anabellamide	Dipeptides	507.2278	$C_{32}H_{31}N_2O_4^+$	0.0	256.1333 (**100**), 238.1227 (65)
Cuminum cyminum	5.04	Luteolin 7-*O*-glucopyranosiduronic acid-glucopyranoside	Flavonoids	625.1396	$C_{27}H_{29}O_{17}^+$	-0.5	449.1082 (8), 287.0554 (**100**)
	5.30	Apigenin 7-*O*-glucuronopyranosyl-*O*-glucopyranoside	Flavonoids	609.1453	$C_{27}H_{29}O_{16}^+$	0.5	433.1132 (9), 271.0605 (**100**)
	5.40	Luteolin-7-*O*-glucoside	Flavonoids	449.1082	$C_{21}H_{21}O_{11}^+$	0.9	287.0553 (**100**)
	5.72	Apigenin-7-*O*-glucoside	Flavonoids	433.1133	$C_{21}H_{21}O_{10}^+$	0.9	271.0604 (**100**)
	6.09	Apigenin 7-*O*-6''-malonylglucoside	Flavonoids	519.1130	$C_{24}H_{23}O_{13}^+$	-0.6	501.1021 (4), 475.1228 (6), 433.1124 (32), 271.0600 (**100**)
	6.38	Luteolin	Flavonoids	287.0551	$C_{15}H_{11}O_6^+$	0.4	287.0548 (**100**), 201.0548 (13), 153.0179 (8), 135.0440 (2)
	6.81	Apigenin	Flavonoids	271.0602	$C_{15}H_{11}O_5^+$	0.4	271.0600 (**100**), 153.0182 (14)
Trachyspermum ammi	2.71	Chlorogenic acid	Phenylpropanoids	355.1028	$C_{16}H_{19}O_9^+$	1.1	163.0392 (**100**), 145.0287 (3)
	5.26	Rutin	Flavonoids	611.1606	$C_{27}H_{31}O_{16}^+$	-0.2	465.1026 (30), 303.0501 (**100**)
	5.51	Scolymoside	Flavonoids	595.1663	$C_{27}H_{31}O_{15}^+$	1.0	449.1075 (30), 287.0551 (**100**)
	5.55	Monardin D	Monoterpene	432.1870 $[M+NH_4]^+$	$C_{19}H_{30}NO_{10}^+$	1.3	415.1612 (1), 397.1497 (12), 379.1391 (45), 361.1286 (29), 233.1176 (39),

							191.1070 (**100**), 149.0600 (33), 127.0393 (12)
	5.71	Monardin F	Monoterpene	436.2180 $[M+NH_4]^+$	$C_{19}H_{34}NO_{10}^+$	0.7	419.1912 (5), 401.1801 (27), 383.1703 (54), 249.0607 (**100**), 231.0501 (80), 213.0402 (3), 153.1281 (3)
	5.82	Luteolin 7-*O*-6″-malonylglucoside	Flavonoids	535.1079	$C_{24}H_{23}O_{14}^+$	-0.6	517.0975 (2), 329.0659 (2), 287.0550 (**100**)
Sesamum indicum	5.46	Acteoside	Triterpenoids	642.2394 $[M+NH_4]^+$	$C_{29}H_{40}NO_{15}^+$	0.3	625.2118 (33), 479.1538 (17), 471.1494 (70), 325.0919 (**100**)
	5.94	Sesaminol triglucoside	Lignans	874.2994 $[M+NH_4]^+$	$C_{382}H_{52}NO_{22}^+$	2.2	857.2660 (**100**), 695.2015 (48), 353.1006 (50), 325.1132 (43)
	6.20	Sesaminol diglucoside	Lignans	695.2190	$C_{32}H_{39}O_{17}^+$	1.2	533.1656 (70), 371.1129 (**100**), 353.1023 (68)
	6.46	Sesamolinol diglucoside	Lignans	714.2614 $[M+NH_4]^+$	$C_{32}H_{44}NO_{17}^+$	1.4	697.2325 (10), 535.1795 (5), 355.1178 (51), 233.0812 (**100**)
Ocimum basilicum	6.46	Phenylpropanoid derivatives	Phenylpropanoid	430.1707 $[M+NH_4]^+$	$C_{19}H_{28}NO_{10}^+$	-0.2	413.1445 (2), 395.1335 (15), 377.1231 (**100**), 359.1125 (38), 291.1223 (7), 273.1122 (79), 231.1014 (80), 207.1017 (29), 189.0912 (93), 127.0391 (23)
	8.84	Salvigenin	Flavonoids	329.1025	$C_{18}H_{17}O_6^+$	-1.3	315.0821 (18), 314.0786 (**100**), 297.0715 (17), 296.0680 (94)
	10.04	Fatty acid derivative	Fatty acids	532.3482 $[M+NH_4]^+$	$C_{27}H50_{28}NO_9^+$	-0.4	515.3212 (15), 497.3110 (77), 423.2742 (86), 405.2636 (**100**), 387.2531 (53), 353.2688 (83), 261.2216 (17)
	12.33	Unknown	Unknown	609.2717			591.2602 (**100**), 559.2340 (9), 531.2391 (15)
	12.53	Unknown	Unknown	593.2762			534.2577 (34), 533.2545 (**100**), 461.2319 (2)
	13.34	Unknown	Unknown	607.2916			575.2648 (37), 547.2700 (**100**), 473.2334 (2)
Persea americana	9.58	Fatty acid amide derivative	Fatty acid amides	382.2955	$C_{22}H_{40}NO_4^+$	0.5	364.2849 (**100**), 297.2430 (4), 279.2321 (13), 196.1699 (34)
Zingiber officinale	7.26	Steroid derivative	Steroids	450.2128 $[M+NH_4]^+$	$C_{23}H_{32}NO_8^+$	1.3	433.1863 (7), 373.1650 (**100**), 313.1439 (6)
	7.78	Steroid derivative	Steroids	494.2391 $[M+NH_4]^+$	$C_{25}H_{36}NO_9^+$	1.2	477.2118 (7), 417.1907 (**100**), 357.1696 (25)
	8.31	Steroid derivative	Steroids	508.2543 $[M+NH_4]^+$	$C_{26}H_{38}NO_9^+$	0.4	491.2272 (4), 431.2061 (**100**), 371.1851 (30), 193.0860 (8)
Phytolacca dodecandra	6.25	Esculentoside H	Saponins	1006.5236 $[M+NH_4]^+$	$C_{48}H_{80}NO_{21}^+$	1.9	725.0555 (16), 515.3350 (**100**), 497.323 (39), 479.3102 (45), 451.3189 (27), 327.1607 (14)
	7.26	Esculentoside A	Saponins	844.4698 $[M+NH_4]^+$	$C_{42}H_{70}NO_{16}^+$	1.1	827.4498 (80), 781.5104 (46), 748.4963 (62), 693.8446 (50), 665.3870 (75), 515.3347 (**100**)
	8.63	Saponin derivative	Saponins	960.5538 $[M+NH_4]^+$	$C_{48}H_{82}NO_{18}^+$	1.2	943.5152 (20), 781.4738 (40), 498.5652 (5), 439.3573 (**100**), 325.1136 (21)
	8.67	Saponin derivative	Saponins	814.4952 $[M+NH_4]^+$	$C_{42}H_{72}NO_{14}^+$		804.1304 (57), 781.5263 (**100**), 668.4880 (62), 529.4171 (53), 435.6560 (54), 301.5780 (51), 212.7496 (51)

E. kebericho roots were available in a large enough quantity to perform bioassay guided isolation and therefore this was done to identify the anthelmintic compounds.

6.3.1 Anthelmintic activity of crude extract, fractions and metabolites from *E. kebericho*

The anthelmintic activity of 80% methanol crude extract of the roots of *E. kebericho* was evaluated using *Caenorhabditis elegans* as a model organism and revealed that the extract had moderate althemintic activity killing 36.9 ± 1.85 % of the worms at a concentration of 500 µg/ml. After partitioning of the crude extract between water and different organic solvents, the anthelmintic activity was observed in the *n*-hexane fraction with 78.67 ± 10.71 % mortality at a concentration of 500 µg/ml. The chloroform, EtOAc, n-butanol and aqueous fractions had activity below 10% as follows: chloroform 5,29 ± 4,1%, EtOAc and *n*-butanol had no activity at all with 0% and the aqueous fraction had 0.63 ± 0.89% anthelmintic activity. Using bioassay guided isolation, the *n*-hexane fraction was subjected to column chromatography on silica gel using mixtures of *n*-hexane / ethyl acetate (increasing polarity) as eluents, as well as sephadex LH20 (eluent DCM : methanol 7:3), sephadex LH60 (solvent methanol) and HPLC on RP-18 material (solvent Acetonitrile : Water). Compound **6.7** (Fig. 6.4) was isolated as a clear liquid and identified as dehydrocostus lactone (**6.7**) based on its spectral data, mainly HRMS, 1D and 2D NMR, and by comparison with detailed reported data (Abegaz et al., 1991; Ito et al., 1984). Compound **6.8** (Fig. 6.4) was isolated as an amorphorous powder and identified as diaspanolide B by comparing its spectral data with the ones reported in literature (Zeng et al., 2017; Adegawa et al., 1987; Wu et al., 2011). Compound **6.9** (Fig. 6.4)was isolated as a yellow wax and identified as 2-(4-hydroxybut-1-ynyl)-5-(penta-1,3-diynyl) thiophene by comparing its spectral data with the ones reported in literature (Bohlmann et al., 1968; Abegaz et al., 1991; Chang et al., 2015). Compound **6.10** and **6.11** (Fig. 6.4) were isolated as a yellow wax which was a 1.4:1 mixture of both *trans*-and *cis*-2-[pent-3-en-1-ynyl]-5-[4-hydroxybut-1-ynyl]-thiophenes and were identified by comparing the spectral data of the mixture as reported previously (Abegaz, 1991).

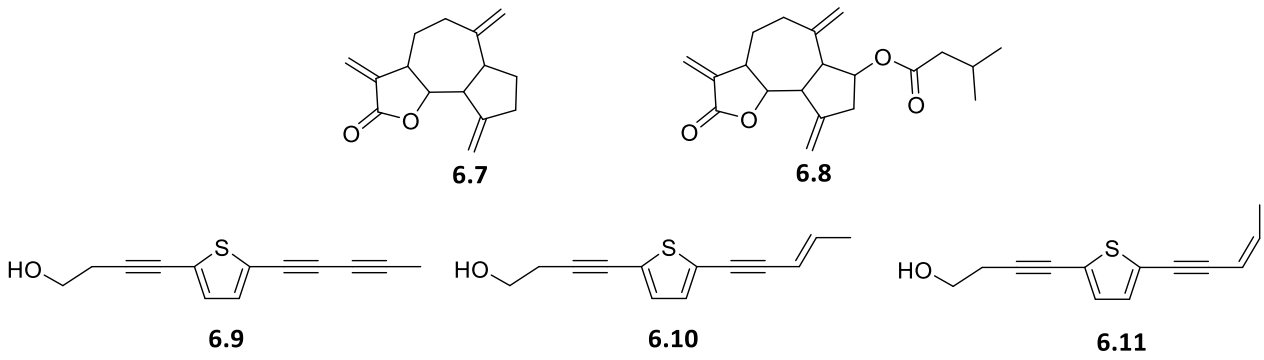


Fig. 6.4: Structures of the natural occurring compounds **6.7**- **6.11.**

Compounds **6.7** to **6.11** were tested against *C. elegans* at a concentration of 500 µg/mL. Compound **6.7** showed moderate activity killing 40.00 %, compound **6.8** killed 89.5 % while compound **6.9** showed strong anthelmintic activity killing 100% of the worms at 500 µg/ml. Compound **6.9** had an LC_{50} of 80.09 µg/ml. Compound **6.10** and **6.11** which were isolated as 1.4:1 mixture had an LC_{50} of 192.76 µg/ml. To the best of our knowledge this is the first reported isolation of compounds **6.8**, **6.9**, **6.10** and **6.11** from *E. kebericho*.

6.4. Discussion

The fruit extract of *Piper nigrum* had the highest anthelmintic activity at 91.99 % and previous studies have shown that piperine (**6.1**) isolated from the fruits of *Piper nigrum* displayed anthelmintic activity against the Indian adult earthworm *Pheretima posthuma* (Simham et al., 2013). Piperine (**6.1**) was shown to be present in our sample of *Piper nigrum* (table 6.1) but it is not the compound responsible for the anthelmintic activity as shown in our results (Fig. 6.3). *P. nigrum* is also known to contain alkamides, piptigrine, wisanine, and dipiperamide (Jiang, 2019) and the essential oil of black pepper is known to contain α-pinene, sabinene, β-pinene, δ-3-carene (Dosoky et al., 2019), *trans*-caryophyllene and limonene (Nikolić et al., 2015). These or other compounds may be responsible for the anthelmintic activity. The extract of the bark of *Cinnamomum verum* showed moderate anthelmintic activity (38%) against *C. elegans*. Other studies have shown that the bark of *C. verum* has anthelmintic activity against the Indian earthworm *Pheritima posthuma* (Lakshmi et al., 2015) and essential oils from *C. verum* have shown activity against the pine wood nematode *Bursaphelenchus xylophilus* (Park et al., 2005). The bark of *Cinnamomum verum* is known to contain cinnameladehyde (**6.2**) as one of the major components. Other compounds known to be present in cinnamon include eugenol (**6.4**), cinnamic acid (**6.3**) and cinnamate (Rao and Gan, 2014). We however did not manage to annotate cinnameladehyde (**6.2**) from the MS-MS data we obtained. We tested commercially available *trans*-cinnameladehyde (**6.2**), cinnamic acid (**6.3**) and eugenol (**6.4**) against *C. elegans*. While cinnamic acid (**6.3**) displayed no notable activity against *C. elegans,* cinnamaldehyde (**6.2**) and eugenol (**6.4**) showed excellent *in vitro* anthelmintic activity with LC_{50} of 76.86 µg/mL and 145.80 µg/mL respectively. The anthelmintic activity of cinnamaldehyde (**6.2**) is also supported by Williams et al., (2015) who demonstrated *in vitro* anthelmintic activity of *trans*-cinnamaldehyde (**6.2**) against the swine nematode *Ascaris suum*. Eugenol (**6.4**) has previously displayed anthelmintic activity against *C. elegans* (Hernando et al., 2019; Abdel-Rahman et al., 2013). The seed extract of *T. ammi* in our study displayed 41% anthelmintic activity against *C. elegans* and a previous study has shown that the aqueous extract and dry powder of *T. ammi* seeds have shown *in vivo* anthelmintic activity against equine helminths (Imani-Baran et al., 2020). Khan et al (2019) investigated the components of the volatile oil of *T. ammi* and found that the major constituents were thymol (**6.5**), *γ*-terpinene, *ρ*-cymene, *β*-pinene and carvacrol. Thymol (**6.5**) was used for the treatment of ascarids and hookworms in the early 1900s and has also displayed anthelmintic activity against *C. elegans* (Hernando et al., 2019; Rahman et al., 2013). It may be the active principle in *T. ammi* as demonstrated by the anthelmintic activity displayed by the commercial thymol (**6.5**) tested (LC_{50} 104.15 µg/mL). A previous study by Mirza et al. (2020) showed that thymol (**6.5**), eugenol (**6.4**) and cinnamaldehyde (**6.2**) displayed fast acting anthelmintic activity against adult *Ancylostoma ceylanicum in vitro*. Thymol (**6.5**) and cinnamaldehyde (**6.2**) also showed similar activity against adult *Trichuris muris in vitro* while (**6.4)** eugenol was less effective. When tested against *C. elegans* thymol (**6.5**) and cinnamaldehyde (**6.2**) proved equally effective against wild type and albendazole (**2.1**) resistant *C. elegans* with an LC_{50} around 100 µg/ml (Mirza et al., 2020) which is similar to the values obtained in our study.

Echinops kebericho has been used for the relief of various diseases including cough, diarrhea, fever, headache, malaria, stomach ache, typhus and is also used as a treatment for tapeworms (Ameya et al., 2016). Ethnosurveys have shown that *E. kebericho* is commonly used in traditional medicine in Ethiopia for the treatment of not only tapeworms but other intestinal worm infections (Alemu et al., 2018). Powdered tubers of the plant mixed with water are used in veterinary medicine for the treatment of black leg disease, liver disease, respiratory manifestations, and skin infections (Yigezu et al., 2014). The plant is frequently reported for

its antinociceptive and anti-inflammatory potentials (Abera, 2014; Getnet et al., 2016) and extracts and essential oils from the roots of *E. kebericho* have been assessed for their anthelminthic, antimicrobial, and molluscicidal activities (Beressa et al., 2020). The essential oils have shown inhibitory effects against egg hatching of *Haemonchus contortus* (Hussien et al., 2011). Chemical constituents known to be present in *E. kebericho* include alkaloids, carotenoids, essential oils, lignans, polyphenols, phytosterols, saponins, sesquiterpene alcohols, acetylenic and thiophene compounds (Hymete et al., 2007). Members of the genus primarily contain thiophenes and terpenes. Thiophenes which are the main bioactive constituent are biosynthetically derived from fatty acids and reduced sulphur (Arroo et al., 1997) while sesquiterpene lactones are the most prevalent secondary metabolites (Chadwick et al., 2013). Our study revealed that the thiophenes (compounds **6.9**-**6.11**) had higher anthelmintic activity than the sesquiterpene lactones (**6.7**-**6.8**).

Thiophenes have exhibited various biological effects including nematicidal, anti-inflammatory, anti-leishmanial, antimicrobial, antioxidant, cytotoxic, chemo-preventive, herbicidal, insecticidal, larvicidal and phototoxic activities (Ibrahim et al., 2016; Ibrahim et al., 2022). The reason for the biological effects of thiophenes is because they are produced as defense compounds and are toxic to various pathogens, such as nematodes, bacteria, fungi and insects (Gill et al., 2002; Champagne et al., 1984). Dehydrocostus lactone (**6.7**) which is known to be the major sesquiterpene in *E. kebericho* has also shown various biological activities including anthelmintic, anticancer, antimicrobial, antifungal, antiviral, anti-inflammatory, antidiabetic, antiulcer and antioxidant (Lin et al., 2015).

The anthelmintic activity displayed by the fruits of *Persea americana* were unexpected as the avocado fruit is popular for its use in the food and cosmetic industry. The seed of *P. americana* has shown anthelmintic activity against *H. contortus* and the compounds thought to be responsible for this activity are epicatechin (EC_{50} = 10 µg/mL) and rutin (EC_{50} = 30 µg/mL) (Soldera-Silva et al., 2019). The ethanolic extract of *S. indicum* seeds have shown *in vitro* anthelmintic activity against the earthworm *Pheretima posthuma* (Bhandare, 2018) supporting the anthelmintic activity shown in our study against *C. elegans*. In our study the leaves of *O. basilicum* displayed low anthelmintic activity against *C. elegans*, in previous studies the fruit extracts of *O. basilicum* has displayed anthelmintic activity against the earthworm *Eudrilus eugeniae* (Akoto et al., 2020). The methanolic extract of *Zingiber officinale* (ginger) has shown *in vitro* ovicidal effects against the eggs of the liver fluke *Fasciola hepatica* (Moazeni and Khademolhoseini, 2014). Some of the bioactive compounds found in ginger are gingerols, shogaols, paradols and zingerone (**6.6**) (Jiang, 2019). We tested commercially available zingerone (**6.6**) against *C. elegans* and observed no notable effect. *Cuminum cyminum* (seeds) displayed low anthelmintic activity against *C. elegans* but the hot water seed extract of *C. cyminum* has shown anthelmintic activity against the adult earthworm *Pheretima posthuma* (Madhavan and Thakaran, 2017). *Phytolacca dodecandra* seed extract displayed higher anthelmintic activity than the leaf extract, although the leaf extract has been shown to have anthelmintic effect against *Fasciola*, *Strongyles*, and *Moniezia* species (Tumwesigye et al., 2015). The leaf extract has also shown *in vitro* egg hatching inhibition against *H. contortus* (Tsehayneh and Melaku 2019; Mohammed et al., 2013). The berries of *P. dodecandra* have been extensively studied as a plant molluscicide and it has been suggested that the molluscicidal activity could be due to the hemolytic activity of saponins known to be present (Beressa et al., 2020). We were not able to identify specific saponins from the annotation of the MS-MS data of the *P. dodecandra* seed extract but the high molecular weight of the compounds suggests that they may be saponins. The results show that anthelmintic activity

can vary depending on the plant part investigated. An example of this is seen with *Hagenia abyssinica* (leaf) which only had 2.36% anthelmintic activity whereas the flowers have shown much higher anthelmintic activity at a tens time lower concentration also against *C. elegans* (Thomsen et al., 2012). The results also show that anthelmintic activity may vary depending on the organism, this was seen with *Matricaria chamomilla* (flower) which showed only 2.67 % anthelmintic activity against *C. elegans* but essential oil of *M. chamomilla* has shown promising anthelmintic activity against the human parasitic *Anisakis* species which affects individuals after they eat fish infected with the parasite (Romero et al., 2012). The extract of *Nigella sativa* displayed *in vivo* anthelmintic activity in Aseel chickens (*Gallus gallus domesticus*) infected with *Ascaridia galli*, *Heterakis gallinarum* and *Syngamus trachea* (Angel et al., 2019). In our results the extract of *N. sativa* seeds displayed low activity (5.36%) against *C. elegans* even though other researchers have reported anthelmintic activity of *N. sativa* seed extract against *Fasciola hepatica* (the common liver fluke) in buffaloes (Kailani et al., 1995; Maqbool et al., 2004) and the essential oils of *N. sativa* has shown anthelmintic activity against earthworms, tapeworms and nodular worms (Agarwal et al., 1979). The fruiting body of *P. ostreatus* showed low anthelmintic activity against *C. elegans* even though fruiting bodies of the mushroom and other species in the genus have shown anthelmintic activity against *H. contortus* (Comans-Perez et al., 2021).

While searching for anthelmintic compounds with the potential of becoming drug candidates is a sensible and logical approach to dealing with the challenge of the prevalence of STHs, this does not usually lead to a product. One of the reasons could be that there is no financial incentive for pharmaceutical companies to invest in drugs for human intestinal parasites and therefore compounds showing good anthelmintic activity are not followed up and the pipeline ends at publication of data. The result of the lack of interest of pharmaceutical companies in researching new antiparasitic drugs is that most clinical trials focus on malaria while ignoring other parasitic diseases which are widespread (Dziduch et al., 2022). It is practical therefore to have an inexpensive easily implementable solution to help reduce the prevalence of STHs. Even though there may be concerns about anthelmintic drug resistance, anthelmintic drugs like ivermectin (**2.4**; **2.5**), albendazole (**2.1**) and mebendazole (**2.2**) are still effective enough to be used in MDA programs and the fact that they are donated by the pharmaceutical industry means that there is little financial incentive for investment into a new drug.

Further investigation needs to be carried out to determine the *in vivo* anthelmintic effect of spices against parasitic worms. Researchers have already shown that some of the plant samples in this report like *Artemisia afra*, *P. nigrum*, *N. sativa* and *C. verum* have displayed *in vivo* antiparasitic activity against various parasites affecting chickens (Pop et al., 2019; Lee et al., 2012; Lan et al., 2016; Orengo et al., 2012; Angel et al., 2019). This serves as a model for the potential of the plant samples as anthelmintics in humans. The different plant parts of each species need to be investigated as previous studies have shown that anthelmintic activity may differ depending on the plant part used. A good example is *Ozoroa insignis* which displayed differing anthelmintic activity depending on the plant part (Dube et al., 2021). From the information obtained through personal communication, it was established that some species of chillies are used to treat stomach ailments. The chillies are used whole and in some countries are encapsulated in a ball of thick porridge and swallowed whole. This mechanism helps in delivering the active ingredients as this would slow down absorbtion in the stomach. A possible *in vivo* experiment could involve encapsulating spice samples which show *in vitro* anthelmintic activity against parasitic organism. This may help show proof of concept that indeed the strategy that is said to be used by some communities does indeed work. The

anthelmintic compounds isolated from *E. kebericho* need to be further tested against parasitic worms to gain knowledge of their potential use as anthelmintics.

Previous investigations have shown that there are plant species that display anthelmintic activity both *in vitro* and *in vivo*. However developing an anthelmintic drug would only be possible with a compound showing very high broad spectrum activity. This in itself still takes time due to the regulations that have to be met and it is quite an expensive process. It is still very necessary to keep searching for natural products in order to find a compound which has broad spectrum activity and may be the next big drug. The anthelmintic drug industry is still commercially viable especially when it comes to parasites affecting animals and crops. The process of finding new drugs will take time due to the challenges faced in drug development.

Traditional medicine is becoming more widely used and more products containing extracts are being developed due to the not so stringent regulations as compared to drugs. There are concerns though about conservation and sustainability. In this respect spices and food waste products offer a potentially safe, easy to implement alternative to MDA. The belief that roots have a higher bioactivity than other plants is clearly not always true. Dube et al (2021) showed that the fruits of *O. insignis* have a higher anthelmintic activity than the roots and Wang et al (2009) discovered that extracts from ginseng leaf stems had similar pharmacological activities to the roots.

6.5 Conclusions

Herbs and spices have the potential to be used as anthelmintics and our study has shown that *C. elegans* has shown susceptibility to some spice extracts and based on previous studies, extracts and compounds that have shown activity against *C. elegans* have been also active against one or more parasitic helminths but at a lower concentration. Annotation of the HRMS data gave a hint at which compounds may be responsible for the anthelmintic activity but these have to be determined by bioassay guided fractionation as was done with E*. kebericho*. Once known they can be used as marker molecules to standardize extracts. The broad spectrum potential of the spice extracts needs to be determined by testing the extracts against various parasitic helminths. This would help to identify the spices that may be used against the various human parasitic helminths. The ease at which spices can be grown coupled with their known health benefits would make spices an excellent alternative to mass drug administration and there would be no worries about drug residues affecting the environment.

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Supplementary materials

The following supporting information is available in Appendix 1 and 2. Table A1**:** List of Ethiopian plant samples and anthelmintic assay results. Table A2:^{1}H and ^{13}C NMR data of compound **6.7** dehydrocostus lactone (400/100 MHz, $CDCl_3$, δ in ppm). Table A3: ^{1}H and ^{13}C NMR data of compound **6.8** Diaspanolide B (400/100 MHz, $CDCl_3$, δ in ppm). Table A4: ^{1}H and ^{13}C NMR data of compound **6.9** 5-(4-hydroxybut-1-ynyl)-2-(pent-1,3-diynyl)-thiophene. Table A5: ^{1}H and ^{13}C NMR data of compound **6.10** and **6.11**. Figure A1 – A6 HRMS and 2D NMR data for compound **6.7**. Figure A7 – A14 HRMS and 2D NMR data for compound **6.8.** Figure A15 – A21 HRMS and 2D NMR data for compound **6.9.** Figure A22 – A27 HRMS and 2D NMR data for compound **6.10** and **6.11.**

General discussion and conclusion

The therapeutic need

Human nematode infections are still a major global health problem and with the effects of Covid 19 leading to the neglected tropical diseases being more neglected as focus shifted to dealing with the global pandemic, there is a high probability that the global prevalence of human nematode infections has increased. Neglected tropical diseases are built around the concept of low socioeconomic status and poverty as the most important social determinants (Hotez et al., 2009). However conflicts not only facilitate the relationship between poverty and poor health but also exacerbate poor health outcomes independent of poverty (Hotez, 2016). This is because conflicts can lead to situations where people who previously were not subjected to poverty may end up without access to safe drinking water and ablution facilities due to displacement and destruction of infrastructure. Cross sectional studies in Sierra Leone, Sri Lanka and Palestine found a high prevalence of intestinal parasites among people displaced due to conflict (Du et al., 2018). As a substantial portion of the globe is still engulfed in some form of conflict, combined with the effects of Covid 19 there is a high probability that the prevalence of STH, schistosomiasis and other NTDs is increasing. Apart from socioeconomic and political parameters, resistance to current anthelmintics continues to be a growing concern as well as the effect of anthelmintic drugs administered in mass drug administration on the environment. This thesis was therefore focused on using natural products as a source of anthelmintic compounds in order to investigate the possibility of finding active compounds that may provide much needed locally available arsenal in the global fight against STH and schistosomiasis.

The strategy for finding plant-derived anthelmintics

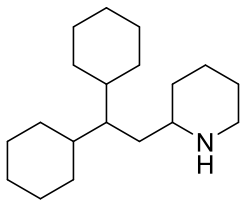
The approach was to use *C. elegans* as a model organism as it is easy to cultivate and maintain, and has similarity to parasitic nematodes (Buckingham et al., 2014). Although *C. elegans* is a good model organism, there is the possibility that it may give some false negative results, as there are plant extracts and compounds that are effective against parasitic nematodes but show no effect against *C. elegans*. This may result from the ability of *C. elegans* to detect and detoxify xenobiotics efficiently in its natural habitat, the soil (Bargmann and Mori, 1997). Parasitic organisms have the luxury of living inside a host and therefore are not as efficient when it comes to dealing with xenobiotics. An example of this phenomenon is compound **3.3** isolated from the fruits of *O. insignis* which showed little activity against *C. elegans* but was active against NTS and *S. mansoni* (Chapter 3). Therefore natural products that showed no or low activity against *C. elegans* cannot be excluded as potential anthelmintics, but it can only be stated that they are not active against *C. elegans*, but no final statement can be made about the activity against other parasitic organisms. Hoewever, quite a few natural products showed activity against *C. elegans* and were active against one or more parasitic organisms, proving that *C. elegans* is a good screening organism. It is also noteworthy that the initial concentration for the test against the parasitic organisms (100 µg/mL for extracts, 100 µM or 10 µM for compounds) was lower than the initial concentration for the test against *C. elegans* (500 µg/mL) and yet the extracts and compounds were active against the parasites. This shows that the compounds that were active against *C. elegans* found the free living nematode more difficult to kill than the parasitic organisms.

Structure-activity hypotheses

Compounds isolated from *O. insignis* fruit extract (chapter 3) allowed the best structure activity relationships insights. They showed which functional groups were necessary for the activity of the compounds against *C. elegans*.

The mushroom *Albatrellus confluens* was the only mushroom out of 12 tested that showed activity against *C. elegans* (chapter 4). The mushroom species were selected based on the observation that no insect larvae were found in basidiomata. The compounds grifolin (**4.1**) and neogrifolin (**4.2**) demonstrated how a change in the position of one functional group changed activity significantly. The synthetic analogues (**4.4**-**4.6)** were prepared to evaluate the effect of the prenyl chain length on activity. What was observed was that reducing the prenyl chain length seemed to increase anthelmintic activity. Testing the prenyl alcohols **4.7**-**4.10** on their own revealed that they may actually be responsible for the anthelmintic activity against *C. elegans* as the anthelmintic activity of each prenyl alcohol was similar to the anthelmintic activity of the prenyl alcohol after addition of orcinol (**4.3**) in three of the four compounds synthesised (**4.1**, **4.5** and **4.6**). Orcinol (**4.3**) on its own displayed no noteable activity. Only the shortest prenyl alcohol, compound **4.7**, had different activity from the compound **4.4** with orcinol added. Probably **4.7** on its own was not able to have an effect due to it lacking structural moieties needed for penetration into the target cells or needed for binding to a protein or other site and interfere with the worm's metabolism, but once orcinol was added, the molecule was able to have anthelmintic activity. Compounds containing thiophene rings (**6.9**-**6.11**) also showed anthelmintic activity against *C. elegans* (Chapter 6). The thiophene skeleton is essential for nematicidal activity, while an increased number of acetylene moieties improves activity (Liu et al., 2019).

There was no quantitative correlation between activity against *C. elegans* and activity against the parasitic organisms. The compounds most active against *C. elegans* were not the most active against the parasitic organisms. This may be due to the fact that the compounds were acting on different targets in *C. elegans* than in the parasitic worms. However, this does not mean that *C. elegans* is not a good model organism, as it shares drug targets with parasitic worms. An example is the perhexiline (**7.1**), which was originally developed as an anti-anginal drug. Perhexiline (**7.1**) is a putative inhibitor of carnitinepalmitoyl transferase (CPT), exhibiting anthelmintic activity against *C. elegans*, *H. contortus*, *A. ceylanicum* and *Onchocerca lienalis* (Tyagi et al., 2018). This is just one example that the free living nematode *C. elegans* and the parasitic worms share common drug targets.



7.1

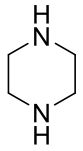
Since the anthelmintic assays were not mechanism-based, one can only postulate preliminary hypotheses on the possible molecular mechanism of action by referring to related drug substances with known mechanism of action. However, the isolated compounds do not have notable similarities to known anthelmintic drugs. This is of course good because they represent new chemical space, no matter what the target is.

For the two compounds that were isolated from *O. insignis* (compound **3.1** and compound **3.2**, chapter 3) and showed good activity against *C. elegans*, we hypothesise that they act as

γ-amino butyric acid (GABA) receptor agonists, inhibiting this ligand gated chloride channel. The basis for this suggestion is that like the anthelmintic drug piperazine (**7.2**), the compounds have a ring structure and also have a carboxyl group like the neurotransmitter GABA. The absence of the carboxyl group in compound **3.3** would explain its inactivity against *C. elegans*. Further proof would consist in preparing a chemical chimaera of piperazine and structural elements of **3.1** and **3.2**. The aliphatic chain in compounds **3.1**, **3.2** and **3.3** most probably facilitates membrane permeability and this could be the reason why salicylic acid (**7.4**) showed no activity against *C. elegans* although it also has the carboxyl group. It is also possible that the compounds directly interfere with the membrane as a proton ionophore like the anthelmintic drug nitroxinil (**7.3**) which has a ring structure and like compounds **3.1** and **3.2** has a hydroxyl group, but instead of carboxyl it has a nitro group. The similarities are not sufficient of course to prove a mode of action, but can guide the direction of mechanistic studies. Terpenoids are known to elicit their anthelmintic effects on *C. elegans* by paralysis through their effect on the levamisole-sensitive acetylcholine receptor (L-AChRs) and γ-aminobutyric acid (GABA) type A (UNC-49) receptors (Hernando et al., 2019). The anthelmintic effects of compounds **4.1**, **4.2**, **4.4** and **4.5** (Chapter 4) against *C. elegans* may be due to their effect on the L-AChRs and UNC-49 receptors. The basis for this hypothesis is the similarity in their structures to the monocyclic phenolic compounds, caravacrol (**7.5**), eugenol (**6.4**) and thymol (**6.5**) as these where the ones tested when determining the effect on the LAChR and UNC-49 receptors.

Overall bioassay-guided isolation using *C. elegan*s is a viable method for finding new anthelmintic compounds. Generally from the active compounds we isolated - both from *O. insignis* and *A. confluens* - a pattern emerged: phenolic groups with aliphatic side chains lead to anhelminticactivity. The aliphatic side chains probably facilitate penetration into the cell membrane of *C. elegans*. This may explain why there were no active compounds isolated from the polar fractions (*n*-butanol and water) as the compounds may have been unable to penetrate the *C. elegans* membrane.

The isolated anthelmintic compounds from *E. kebericho* containing a thiophene ring (chapter 6) were also very non-polar and this also may have enabled them to penetrate the *C. elegans* membrane. Compounds containing thiophene rings have the potential to be used as anthelmintics as they are known to be used by plants as defence compounds against nematodes, insects, bacteria and fungi (Ibrahim et al., 2022). They are found in *Tagetes* species (marigold), common ornamental plants, where they accumulate mainly in the roots. It has been established that root extracts of *Tagetes* species are effective in controlling a variety of nematodes in the soil (Marotti et al., 2010). Marigolds are one of the most popular flowers globally (Salachna, 2022; Kaur et al., 2022) and the roots could therefore provide a large biomass which could be used as a source of anthelmintic compounds.



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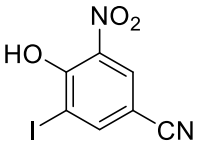

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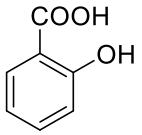

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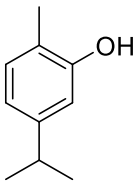

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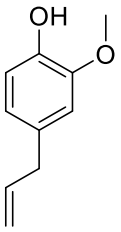

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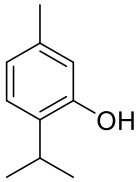

6.5

Other pharmacological effects of the compounds isolated

The anticancer activity of the anthelmintic compounds **3.1** – **3.3** isolated from *O. insignis* and *A. confluens* (chapter 3 and chapter 4) is also interesting as anthelmintic drugs are being repurposed as anticancer drugs. This is because of the ability of some anthelmintic drugs to interfere with crucial oncogenic pathways (Laudisi et al., 2020). An example is the anthelmintic drug ivermectin (**2.4** and **2.5**) which was used as a positive control in the *C. elegans* anthelmintic assay. Ivermectin (**2.4** and **2.5**) has shown *in vitro* and *in vivo* anticancer activity against gastric cancer cells and has also been shown to inhibit various metabolic pathways in colorectal cancer cells and blocked colon cancer stem cell self-renewal in a clonogenic assay (Nambara et al., 2017; Melotti et al., 2014). Repurposing anthelmintic drugs with known safety profiles for anti-cancer activity is a much quicker and less expensive way to find anti-cancer drugs than using new compounds with unknown safety profiles.

There was controversy regarding ivermectin (**2.4** and **2.5**) and Covid 19 when numerous claims were made that the anthelmintic drug was helping patients recover from the SARS-CoV-2 virus. Although ivermectin (**2.4** and **2.5**) has shown a relatively homogeneous *in vitro* activity against SARS-CoV-2 regardless of the strains or variants except for one omicron strain (Delandre et al., 2022), there is no substantive evidence that proves its activity in patients (Reardon, 2021). However, the *in vitro* activity displayed by ivermectin (**2.4** and **2.5**) does warrant the investigation of the antiviral activity of other anthelmintic compounds. We therefore tested compounds **3.1**, **3.2**, **3.3**, **4.1**, **4.2**, **4.4**, **4.5** and **4.6** in an assay to determine if the compounds had any inhibitory effect on the enzyme SARS-CoV-2 Main protease (Mpro) which plays a leading role in the replication cycle of all coronaviruses (Xue et al., 2008). The assay was conducted following a protocol similar to Chen et al. (2005). Three compounds showed good inhibitory activity against SARS-CoV-2 Mpro, namely compound **3.3** IC_{50} 19.2 µM, compound **4.1** IC_{50} 56 µM and compound **4.2** IC_{50} 11.3 µM (ongoing studies, unpublished data). These results are a hint that anthelmintic compounds have the potential of being broad spectrum drugs not only against parasitic worms, but also against cancer and SARS-CoV-2.

The strengths of investigating traditional medicine

Even though the use of herbal and traditional medicine is gaining popularity worldwide, there is still controversy regarding traditional healers as some people still believe they do not know

what they are doing. One of the reasons why there are debates concerning how knowledgeable traditional medicinal practitioners are is the lack of evidence to prove the efficacy of some of the plants they use. There are also perceptions that healers give inappropriate dosages to patients (Marobela et al., 2021). In our study we proved the *in vitro* anthelmintic activity of plants administered by traditional health practitioners from Botswana (Chapter 5). Seven out of ten plant species tested showed activity above the 70% threshold against NTS while one plant species had activity above 70% against *T. muris*. The high number of plant species showing activity against NTS is an indication that the traditional health practitioners are knowledgeable about plants effective against diseases that are prevalent in their habitat as schistosomiasis is prevalent in the areas where the healers reside.

Although this does not put an end to the debate regarding traditional health practitioners, it does show that there is ample justification to further investigate plants used by healers against various ailments.

The most promising result obtained from this study is the anthelmintic activity of the plant extracts used by traditional health practitioners in Botswana (chapter 5) and plant extracts from Ethiopia (chapter 6) most of which were herbs and spices. The medicinal plant extracts which displayed antischistosomal activity (Chapter 5) do present a challenge when it comes to isolating the active compounds as working with parasitic organisms is not as simple as working with *C. elegans* which is easy to culture. Obtaining the parasites takes more time as they have to be grown in a host which then has to be sacrificed to obtain the parasites. Medicinal plants have been used since ancient times for pharmaceutical purposes but their use was always kept in the customs of a confined population and were never fully explored. An example is seen with the oil from *Chenopodium ambrosioides* which was used as an anthelmintic against *Strongylus*, *Parascaris* and *Ascaris* spp. in the United Kingdom (Githiori et al., 2006).

The scientific community is re-exploring ethnopharmacological traditions in order to discover alternative anthelmintic drugs and remedies (phytomedicines) due to the shortage of new anthelmintics and the widespread phenomena of reported drug resistance (Castagna et al., 2022). Phytomedicines have less quality and declaration requirements than conventional drugs and it is therefore easier to register a phytomedicine than a synthetic drug (Balekundri and Mannur, 2020). If a product is registered as a food supplement, then the development and production costs are even lower. Herbs and spices with anthelmintic properties could therefore offer a solution to the prevalence of STH that is easy to implement. One other aspect of herbs and spices is that they are known to have antibacterial properties and it has been hypothesized that the host gut microbiome plays a role in the host parasite relationship (Kupritz et al., 2021). This hypothesis stemmed from the observation that gastrointestinal helminth infections are associated with alterations in the host gut microbiota composition and function in both humans and animals (Rooney et al., 2022). While it is not clear what role the bacteria play it is postulated that they may prevent parasite expulsion, modulate the host immune response to the parasites or play a role in the establishment of parasite infection (Zaiss and Harris, 2016). Herbs and spices may therefore have the advantage of not only affecting the parasites but aslo influencing the host microbiome leading to easier parasite expulsion. The anthelmintic activity against *C. elegans* exhibited by 10 of the Ethiopian plant extracts, the majority of which were herbs and spices, therefore warrants further investigation. *Piper nigrum*, *Cinnamomum verum* and *Trachyspermum ammi* were the three species showing the best activity against *C. elegans*. Further investigations into the anthelmintic activity of herbs and spices against parasitic organisms could be conducted to determine the potential they have as phytomedicines used against STH. This would involve

determining the effective dosage required against the various stages of different parasites through *in vitro* and *in vivo* studies.

In conclusion, STH and schistosomiasis continue to be a global problem due to effects of Covid 19 and political and economic conflicts. There is therefore a need for continued research into new therapeutic agents as the anthelmintic drugs used in MDA have the potential to cause pollution of ground water and also harm non target organisms in the environment. The end goal would be to find a potent anthelmintic compound or phytomedicine with broad spectrum activity against both human and animal parasites. This is because commercialization of an anthelmintic drug that works only against human parasites may be challenging as the drugs used in MDA programs are donated since the people who need them cannot afford them.

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Appendix

Appendix 1

Table A1: List of Ethiopian plant samples and anthelmintic assay results

Species	Family	Part	Anthelmintic activity (%)
Acacia abyssinica Benth.	Leguminosae	Leaves	4,92 ± 3,98
Acanthus senni Chiov.	Acanthaceae	Leaves	4,53 ± 3,38
Aframomum corrorima (A.Braun) P.C.M.Jansen (Cardamom)	Zingiberaceae	Seeds	0,46 ± 0,65
Aloe ankoberensis M.G.Gilbert & Sebsebe	Xanthorrhoeaceae	Leaves	1,17 ± 0,82
Aloe benishangulana Sebsebe & Tesfaye	Xanthorrhoeaceae	Leaves	5,00 ± 3,83
Aloe debrana Christian	Xanthorrhoeaceae	Leaves	3,84 ± 2,22
Aloe percrassa Tod.	Xanthorrhoeaceae	Leaves	3,00 ± 2,29
Aloe pirottae A.Berger	Xanthorrhoeaceae	Leaves	0,60 ± 0,84
Aloe sinana Reynolds	Xanthorrhoeaceae	Leaves	0 ± 0
Aloe tewoldei M.G.Gilbert & Sebsebe	Xanthorrhoeaceae	Leaves	0,56 ± 0,79
Aloysia citriodora Palau	Verbenaceae	Leaves	5,97 ± 3,06
Ananas comosus (L.) Merr (Pineapple)	Bromeliaceae	Fruit	0 ± 0
Arachis hypogaea L. (peanut)	Fabaceae	Seeds	3,26 ± 2,95
Araucaria heterophylla (Salisb.) Franco	Araucariaceae	Leaves	0 ± 0
Artemesia afra Jacq. ex Willd.	Compositae	Leaves	2,52 ± 1,86
Asparagus africanus Lam.	Asparagaceae	Leaves	0,69 ± 0,98
Brassica carinata A.Braun (Ethiopian mustard)	Brassicaceae	Seeds	3,03 ± 2,65
Brassica nigra (L.) K.Koch	Brassicaceae	Leaves	0,52 ± 0,74
Cannabis sativa L.	Cannabaceae	Leaves	7,36 ± 1,77
Capsicum annuum L. (hot peppers)	Solanaceae	Fruit	1,08 ± 0,78
Capsicum frutescens L. (Ethiopian chili)	Solanaceae	Fruit	2,6 ± 1,84
Carica papaya L. (papaya)	Caricaceae	Fruit	0 ± 0
Chiliocephalum schimperi Benth.	Compositae	Leaves	5,80 ± 4,24
Cinnamomum verum J.Presl (cinnamon)	Lauraceae	Bark	37,74 ± 11,94
Citrullus lanatus (Thunb.) Matsum. & Nakai (watermelon)	Cucurbitaceae	Fruit	1,04 ± 1,48
Coriandrum sativum L. (coriander)	Apiaceae	Leaves	0 ± 0
Crotalaria exaltata Polhill	Fabaceae	Leaves	4,27 ± 6,04
Croton macrostachyus Hochst. ex Delile	Euphorbiaceae	Leaves	3,27 ± 3,24
Cucumis sativus L. (cucumber)	Cucurbitaceae	Fruit	0 ± 0
Cucurbita maxima Duchesne (Pumpkin)	Cucurbitaceae	Seeds	7,49 ± 6,47
Cucurbita pepo subsp. pepo convar. Giromontiina (zucchini)	Cucurbitaceae	Fruit	0 ± 0
Cuminum cyminum L. (cumin)	Apiaceae	Seeds	8,46 ± 4,96
Cymbopogon citratus (DC.) Stapf (lemon Grass	Poaceae	Leaves	4,88 ± 3,98
Daucus carota subsp. Sativus (Hoffm.) Arcang. (carrot)	Apiaceae	Tuber	1,17 ± 1,65
Echinops kebericho Mesfin	Compositae	Roots	36,90 ± 1,85
Erythrina brucei Schweinf.	Fabaceae	Leaves	6,36 ± 4,34

Foeniculum vulgare Mill.	Apiaceae	Leaves	2,24 ± 0,82
Fragaria × *ananassa* (Duchesne ex Weston) Duchesne ex Rozier (strawberry)	Rosaceae	Fruit	1,04 ± 1,48
Guizotia abyssinica (L. f.) Cass.	Compositae	Seeds	1,84 ± 1,41
Hagenia abyssinica (Bruce) J.F.Gmel	Rosaceae	Leaves	2,36 ± 2,23
Helianthus annuus L.	Compositae	Seeds	1,31 ± 1,85
Hypericum revolutum Vahl	Hypericaceae	Leaves	4,55 ± 4,03
Impatiens rothii Hook.f.	Balsaminaceae	Leaves	0,61 ± 0,86
Juniperus procera Hochst. ex Endl.	Cupressaceae	Leaves	4,21 ± 3,02
Kniphofia foliosa Hochst.	Xanthorrhoeaceae	Leaves	1,17 ± 0,84
Kniphofia hildebrandtii Cufod.	Xanthorrhoeaceae	Leaves	2,55 ± 1,81
Kniphofia insignis Rendle	Xanthorrhoeaceae	Leaves	0 ± 0
Lactuca sativa L. (lettuce)	Compositae	Leaves	0,82 ± 0,60
Laggera tomentosa (Sch.Bip. ex A.Rich.) Oliv. & Hiern	Compositae	Leaves	1,41 ± 1,00
Linum usitatissimum L. (flax)	Linaceae	Seeds	0,40 ± 0,56
Lippia abyssinica (Otto & A.Dietr.) Cufod.	Verbenaceae	Leaves	0 ± 0
Lippia adoensis Hochst. ex Walp. Apparently synonym of *Lippia abyssinica* (Otto & A.Dietr.) Cufod.	Verbenaceae	Leaves	0,71 ± 1,00
Lobelia rhynchopetalum Hemsl	Campanulaceae	Leaves	1,75 ± 2,48
Maesa lanceolata Forssk.	Primulaceae	Leaves	0,50 ± 0,70
Matricaria chamomilla L. (chamomile)	Compositae	Flowers	2,67 ± 2,28
Maytenus addat (Loes.) Sebsebe	Celastraceae	Leaves	4,94 ± 1,92
Melia azedarach L.	Meliaceae	Leaves	0,57 ± 0,81
Mentha × *piperita* L. (peppermint)	Lamiaceae	Leaves	2,76 ± 2,20
Millettia ferruginea subsp. darassana (Cufod.) J.B.Gillett	Fabaceae	Leaves	3,44 ± 2,50
Myrtus communis L.	Myrtaceae	Leaves	3,85 ± 1,19
Nigella sativa L.	Ranunculaceae	Seeds	5,36 ± 1,43
Ocimum basilicum L. (Mexican spice basil)	Lamiaceae	Leaves	11,32 ± 2,11
Ocimum basilicum L. (Mexican spice basil)	Lamiaceae	Seeds	0 ± 0
Ocimum lamiifolium Hochst. ex Benth.	Lamiaceae	Leaves	0 ± 0
Olea europaea subsp. cuspidata (Wall. & G.Don) Cif	Oleaceae	Leaves	1,46 ± 1,05
Persea americana Mill. (avocado)	Lauraceae	Fruit	25,49 ± 12,18
Phaseolus vulgaris L.	Fabaceae	Seeds	1,72 ± 1,22
Phytolacca dodecandra L'Hér	Phytolaccaceae	Leaves	1,32 ± 0,94
Phytolacca dodecandra L'Hér	Phytolaccaceae	Seeds	9,83 ± 1,73
Piper nigrum L. (black pepper)	Piperaceae	Seeds	91,99 ± 2,00
Plantago lanceolata L.	Plantaginaceae	Leaves	1,01 ± 1,43
Pleurotus ostreatus (Jacq. ex Fr.) P.Kumm.	Pleurotaceae	Fruiting body	3,85 ± 2,82
Otostegia fruticosa (Forssk.) Schweinf. ex Penzig	Lamiaceae	Leaves	4,32 ± 2,01
Plectocephalus varians (A.Rich.) C.Jeffrey in Cufod.	Compositae	Leaves	3,64 ± 3,69
Rhamnus prinoides L'Hér.	Rhamnaceae	Leaves	4,66 ± 3,93
Rhus glutinosa Hochst. ex A.Rich.	Anacardiaceae	Leaves	3,32 ± 3,23
Ricinus communis L.	Euphorbiaceae	Leaves	1,17 ± 1,65
Rumex abyssinicus Jacq. (mekmako)	Polygonaceae	Leaves	3,41 ± 2,73

Salvia Rosmarinus Schleid. (rosemary) apparently synonym of *Rosmarinus officinalis* L.	Lamiaceae	Leaves	0,9 ± 1,27
Sesamum indicum L. (sesame)	Pedaliaceae	Seeds	12,96 ± 3,72
Solanecio gigas (Vatke) C.Jeffrey	Compositae	Leaves	2,18 ± 1,74
Syzygium aromaticum (L.) Merr. & L.M.Perry (clove)	Myrtaceae	Flower	5,00 ± 2,08
Thymus schimperi Ronniger	Lamiaceae	Leaves	0,93 ± 1,31
Trachyspermum ammi (L.) Sprague	Apiaceae	Seeds	41,55 ± 5,21
Trigonella Foenum-graecum L. (fenugreek)	Fabaceae	Seeds	2,46 ± 1,94
Solanum lycopersicum L. (tomato)	Solanaceae	Fruit	3,41 ± 2,73
Vernonia amygdalina Delile	Compositae	Leaves	3,78 ± 2,88
Vernonia leopoldi (Sch.Bip. ex Walp.) Vatke	Compositae	Leaves	4,52 ± 1,82
Zehneria scabra Sond.	Cucurbitaceae	Leaves	3,06 ± 2,75
Zingiber officinale Roscoe (ginger)	Zingiberaceae	Tuber	9,62 ± 2,44

Appendix 2

Compound **6.7** QED014C1F7 (QED014-P1NH) dehydrocostus lactone

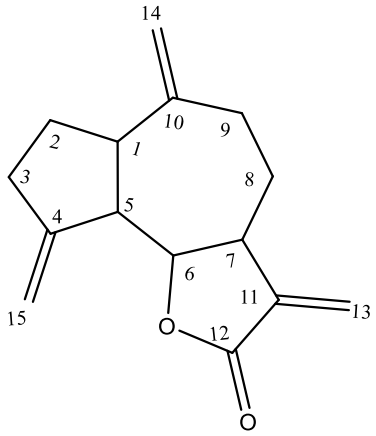

Table A2: ^{1}H and ^{13}C NMR data of compound **6.7** dehydrocostus lactone (400/100 MHz, $CDCl_3$, δ in ppm)

No	δ_H (Multiplicity, *J* in Hz)	δ_C
1	2.86 [a]	52.0
2	1.90 [a]	30.3
3	2.53 [a]	32.6
4		151.2
5	2.92 [a]	47.6
6	3.97 t 9.2	85.2
7	2.88 [a]	45.1
8'	1.43 [a]	30.9
8''	2.24 [a]	
9'	2.16 [a]	36.2
9''	2.50 [a]	
10		149.2
11		139.7
12		170.3
13'	6.22 d 3.3	120.2
13''	5.49 d 3.3	
14'	4.82 br s	112.6
14''	4.90 br s	
15'	5.07 d 2.4	109.6
15''	5.27 d 2.4	

[a]Overlapping signals, chemical shifts were determined from ^{1}H and ^{13}C HSQC correlation peaks.

Reference: Ito K, Iida T. and Kobayashi T., *Phytochemistry* **1984**, 23 (1), 188-190.

Compound **6.8** QED014C1F14HPF2, Diaspanolide B

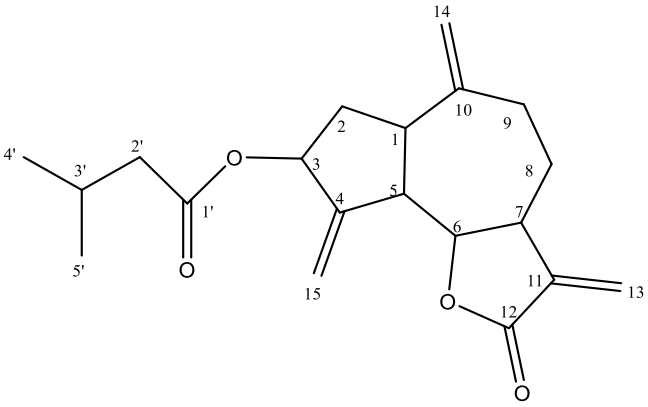

Table A3: ^{1}H and ^{13}C NMR data of compound **6.8** Diaspanolide B (400/100 MHz, $CDCl_3$, δ in ppm)

No	δ_H (Multiplicity, *J* in Hz)	δ_C
15A	5.48 t 2.2	113.5
15B	5.29 t 2.2	
14AB	4.96[a]	114.4
13A	5.50 d 3.3	120.3
13B	6.23 d 3.3	120.3
2	-	36.6
3	5.59[a]	74.3
4	-	148.21
12	-	170.0
11	-	139.56
8	-	30.65
9	-	34.57
10	-	147.82
1	-	44.62
5	-	50.28
6	4.07 t 9.4	83.80
7	-	45.21
1‘	-	172.8
2'AB	2.23[a]	43.6
3‘	2.11[a]	25.76
5‘-Me	0.98 (d, 6.6 Hz, 3H)	22.4
4‘-Me	0.98 (d, 6.6 Hz, 3H)	22.4

[a]Overlapping signals, chemical shifts were determined from ^{1}H and ^{13}C HSQC correlation peaks.

Reference: Adegawa S, Miyase T. and Ueno A., *Chem Pharm Bull* **1987**, 35 (4), 1479-1485.

Compound **6.9** QED014C1F18HPF6, 5-(4-hydroxybut-1-ynyl)-2-(pent-1,3-diynyl)-thiophene

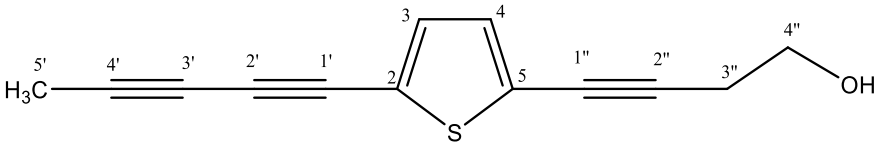

Table A4: ^{1}H and ^{13}C NMR data of compound **6.9** 5-(4-hydroxybut-1-ynyl)-2-(pent-1,3-diynyl)-thiophene

(600/150 MHz, $CDCl_3$, δ in ppm)

No	δ_H (Multiplicity, *J*, nH)	δ_C
2	-	125.5
3	7.09 d 3.7	133.6
4	6.97 d 3.7	131.3
5	-	122.8
1‘	-	66.7
2‘	-	79.0
3‘	-	64.2
4‘	-	83.2
5‘-Me	2.04 s	4.8
1“	-	75.1
2“	-	91.9
3“	2.71 td 6.2/1.9	24.1
4“A	3.82 t-like 5.9	60.9
4“B	3.81 t-like 5.9	

Reference: Abegaz B.M., *Phytochemistry* **1991**, 30 (3), 879-881.

Compound **6.10** and **6.11** 1.4/1 Isomeric Mixture of cis- and trans-

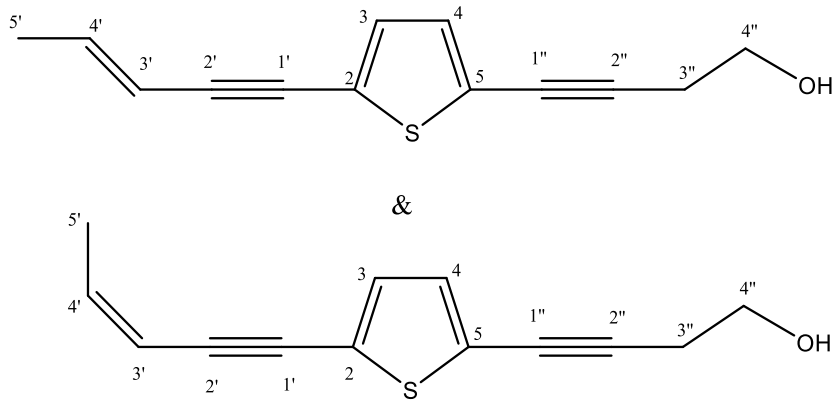

Table A5: ^{1}H and ^{13}C NMR data of Compound **6.10** and **6.11** (600/100 MHz, $CDCl_3$, δ in ppm)

	trans-		**Cis-**	
No	**δ_H (Multiplicity, *J* in Hz)**	**δ_C**	**δ_H (Multiplicity, *J* in Hz)**	**δ_C**
2	-	n.d.	-	n.d.
3	6.97 d 3.6	131.1	6.97 d 3.6	131.1
4	6.98 d 3.6	131.4	6.98 d 3.6	131.4
5	-	124.3	-	124.3
1‘	-	80.3	-	74.9
2‘	-	92.6	-	90.6
3‘	6.26 dq 15.8/6.9	140.9	6.07 dq 10.8/6.9	139.6
4‘	5.70 dq 15.8/1.8	110.3	5.69 dq 10.8/1.8	109.7
7‘-Me	1.85 dd 6.9/1.8	18.9	1.94 dd 6.9/1.8	16.3
1“	-	75.2	-	75.2
2“	-	91.5	-	91.5
3“	2.71 td 6.2/3.3	24.2	2.71 td 6.2/3.3	24.2
4“	3.81 br s	60.9	3.81 br s	60.9

Reference: Abegaz B.M., *Phytochemistry* **1991**, 30 (3), 879-881.

HRMS data for compound **6.7**

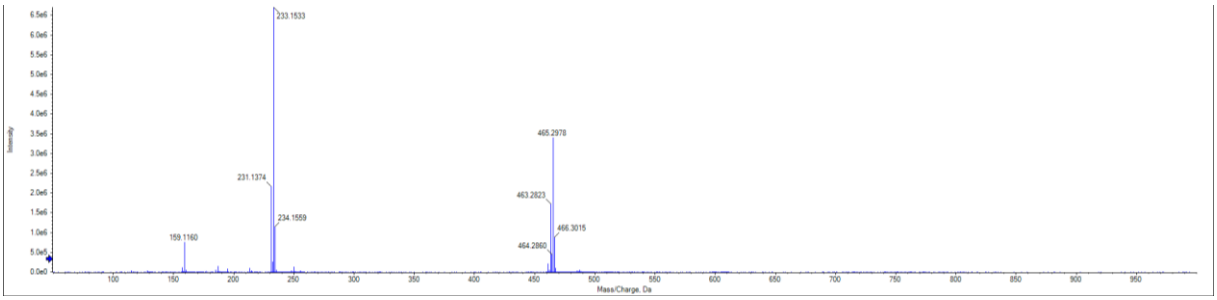

Figure A1: HRMS data for compound **6.7**

NMR data for compound **6.7**

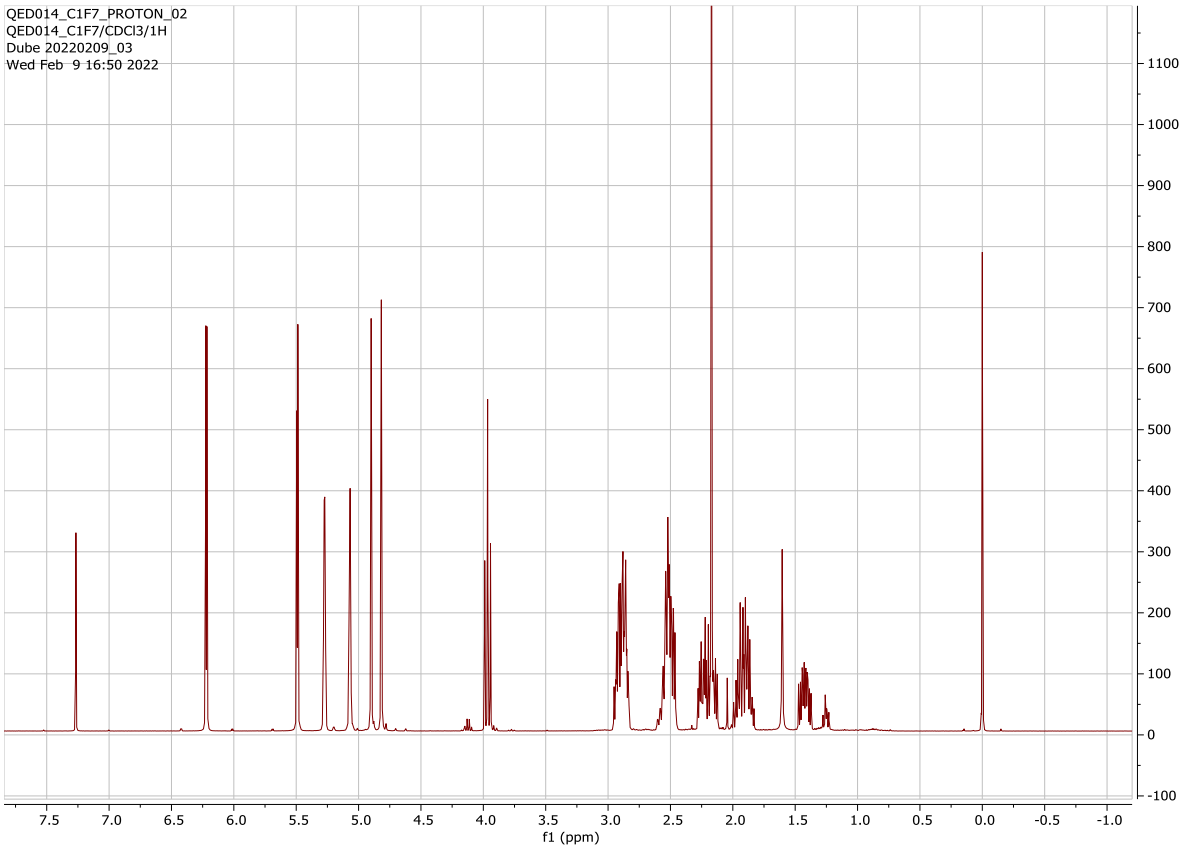

Figure A2: ^{1}H NMR spectrum of dehdydrocostus lactone (**6.7**) in $CDCl_3$ (400 MHz, δ in ppm).

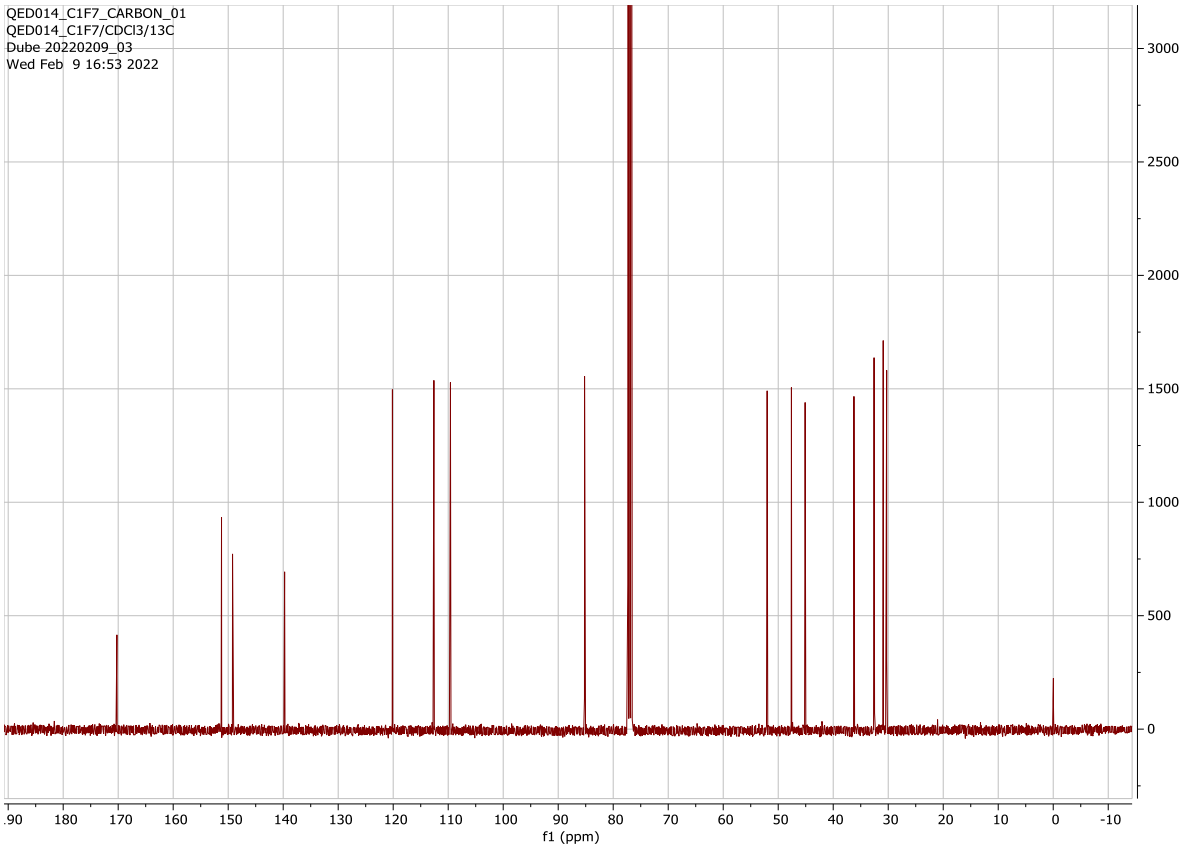

Figure A3: ^{13}C NMR spectrum of dehydrocostus lactone (**6.7**) in $CDCl_3$ (100 MHz, δ in ppm).

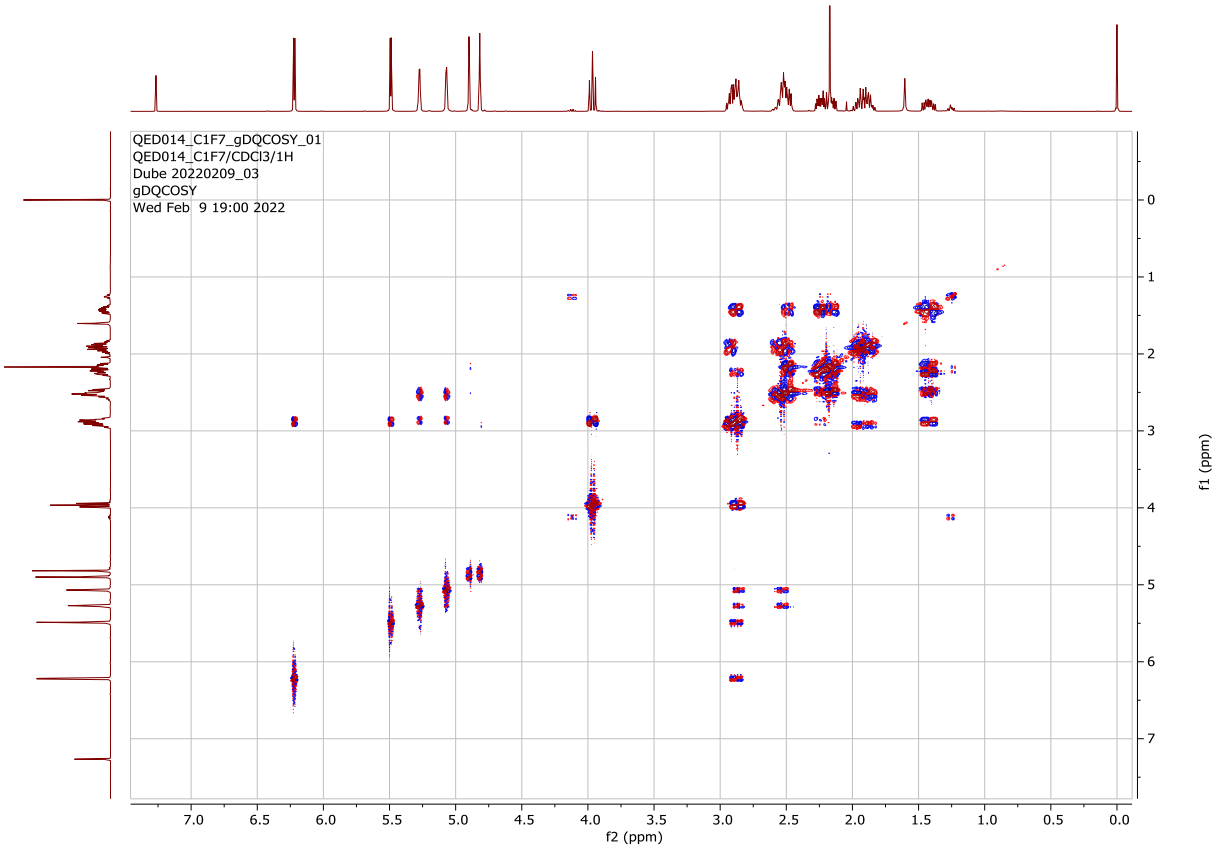

Figure A4: gDQCOSY spectrum of dehydrocostus lactone (**6.7**) in $CDCl_3$ (400 MHz, δ in ppm).

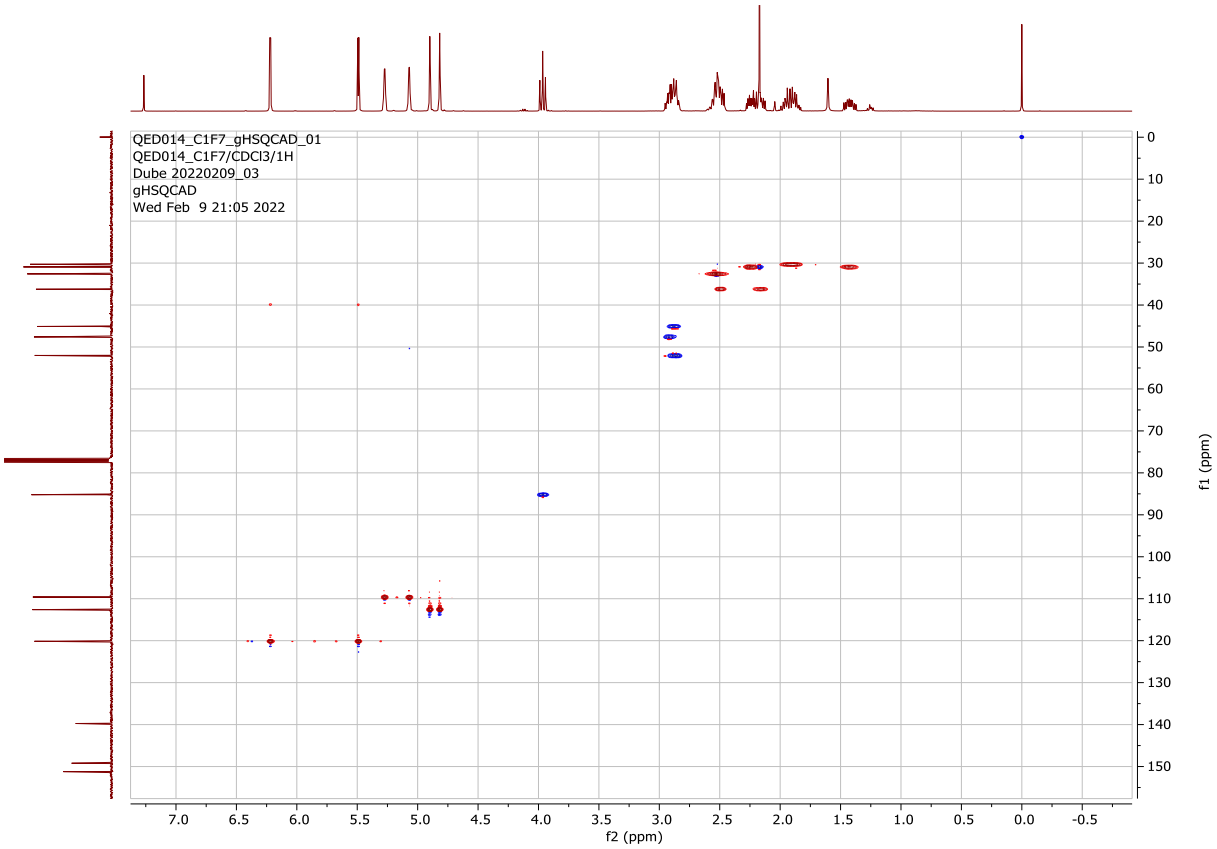

Figure A5: gHSQC spectrum of dehydrocostus lactone (**6.7**) in $CDCl_3$ (400 MHz, δ in ppm).

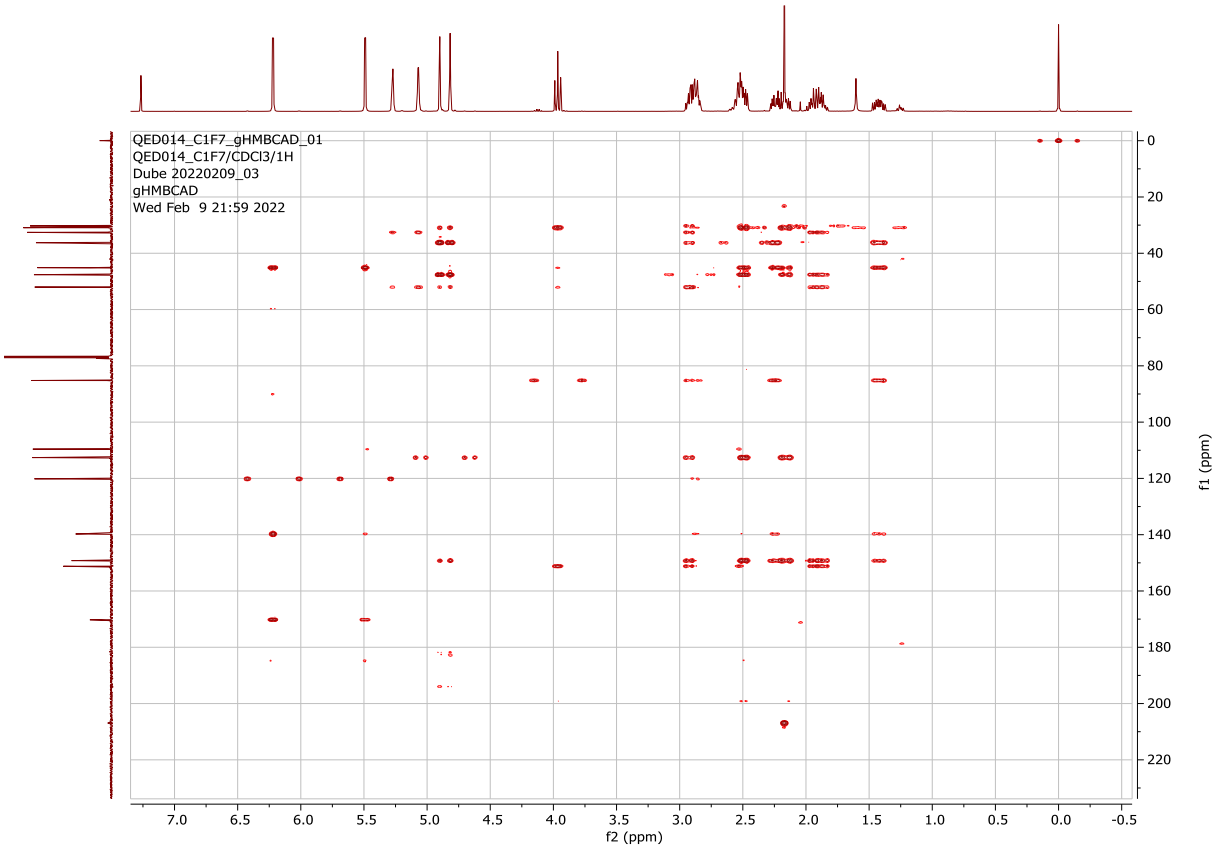

Figure A6: gHMBC spectrum of dehydrocostus lactone (**6.7**) in $CDCl_3$ (400 MHz, δ in ppm).

MS Data for compound **6.8**

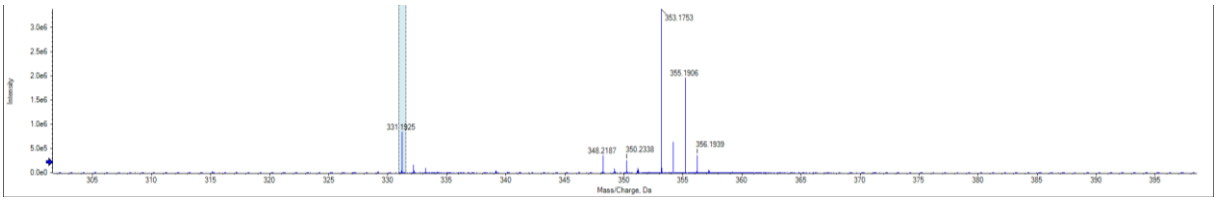

Figure A7: HRMS data for compound **6.8**

NMR data for compound **6.8**

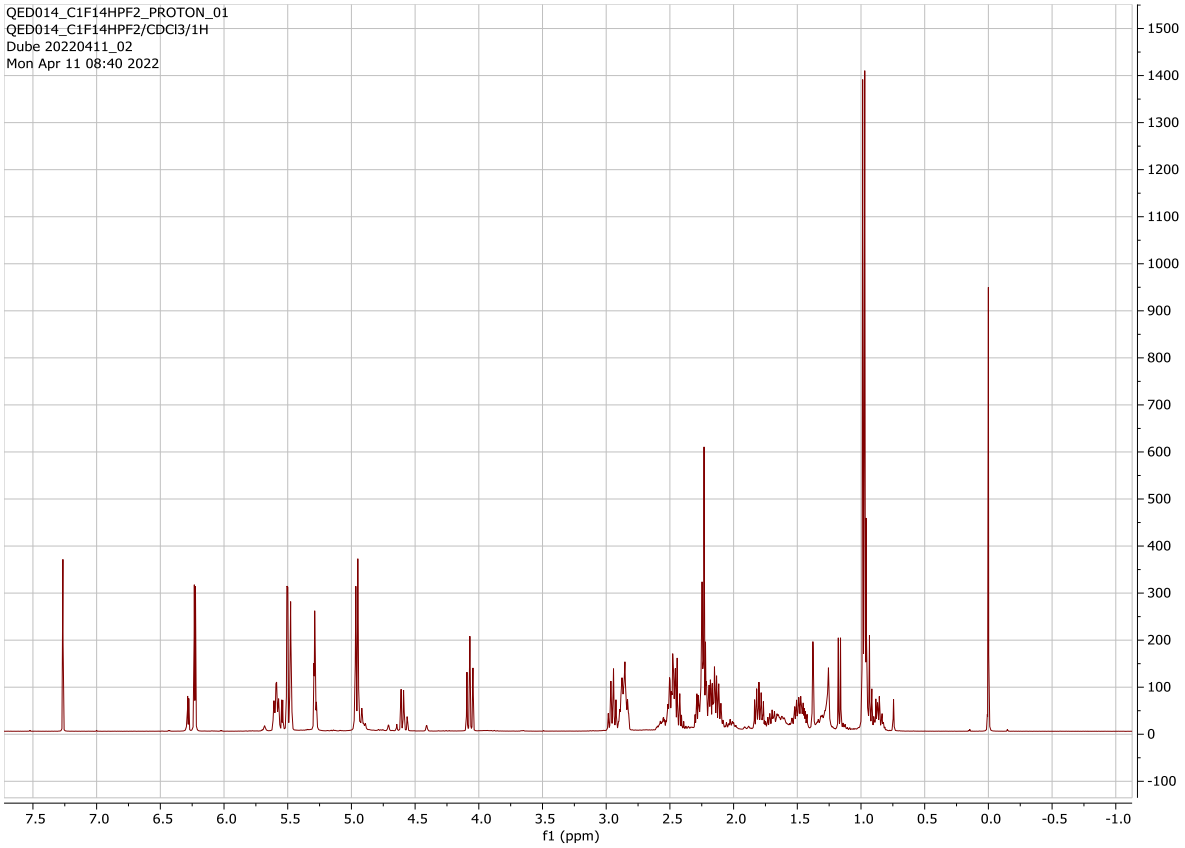

Figure A8: ^{1}H NMR spectrum of (**6.8**) in $CDCl_3$ (400 MHz, δ in ppm).

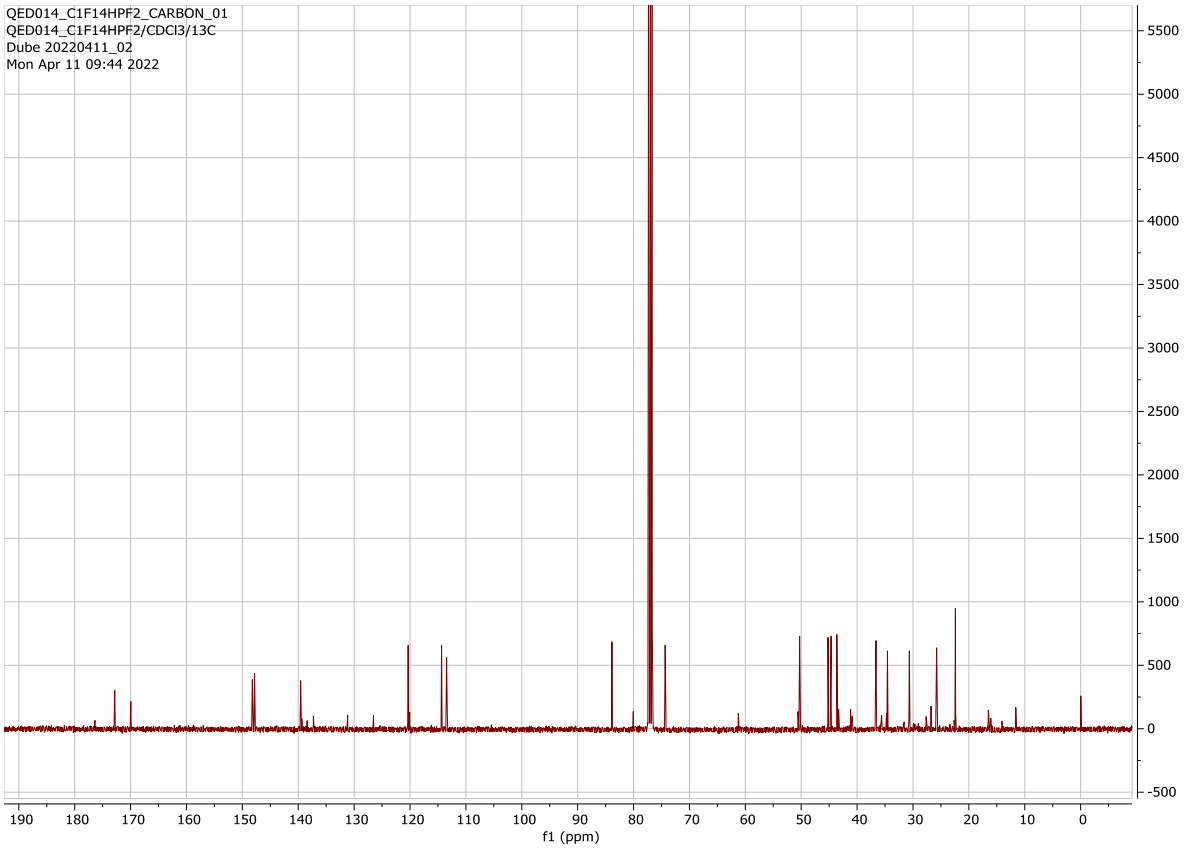

Figure A9: ^{13}C NMR spectrum of (**6.8**) in $CDCl_3$ (100 MHz, δ in ppm).

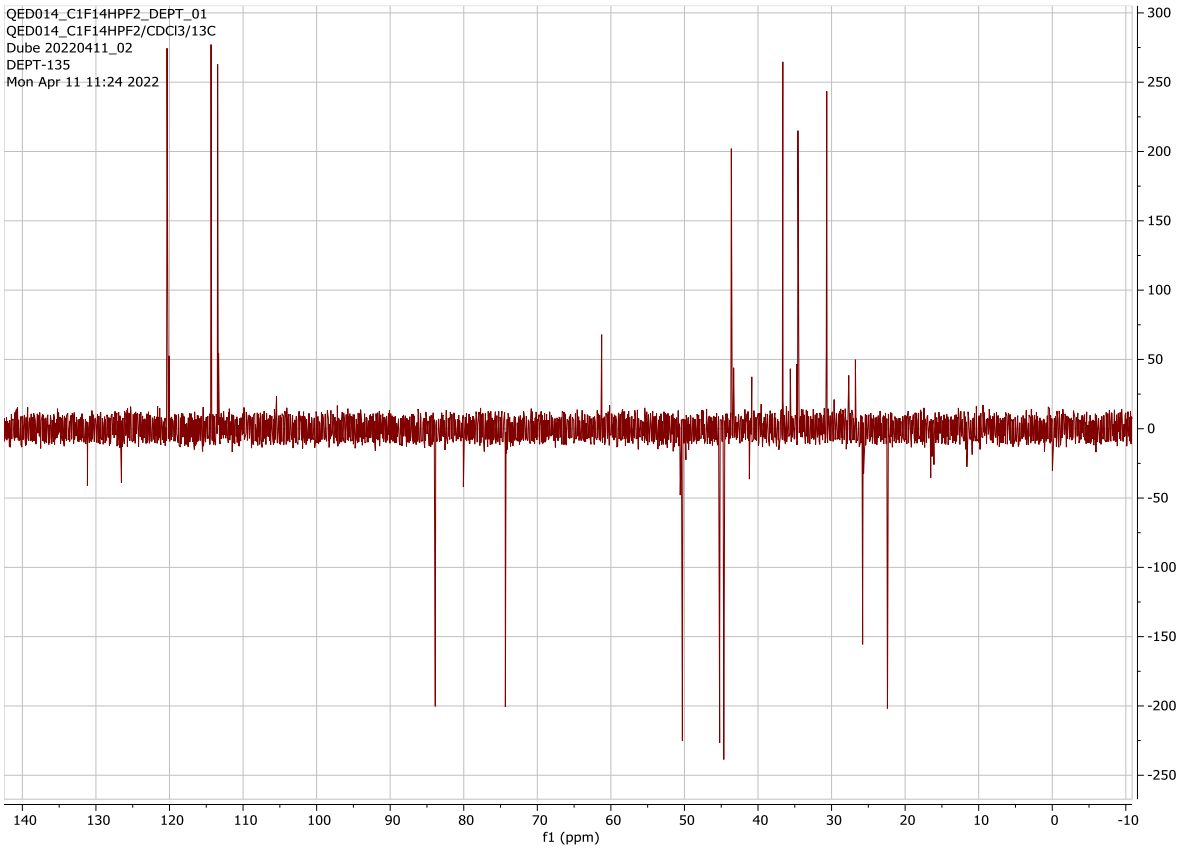

Figure A10: DEPT 135 spectrum of (**6.8**) in $CDCl_3$ (100 MHz, δ in ppm).

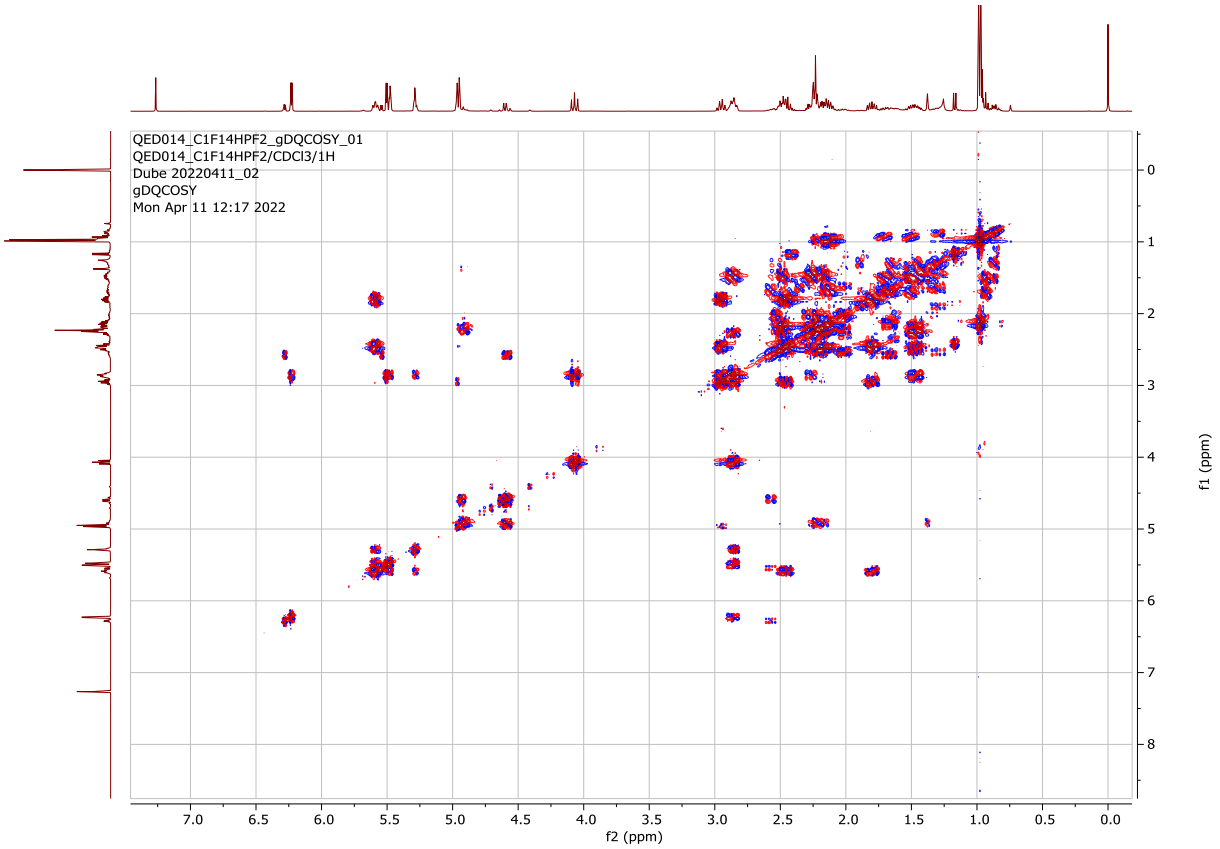

Figure A11: gDQCOSY spectrum of (**6.8**) in $CDCl_3$ (400 MHz, δ in ppm).

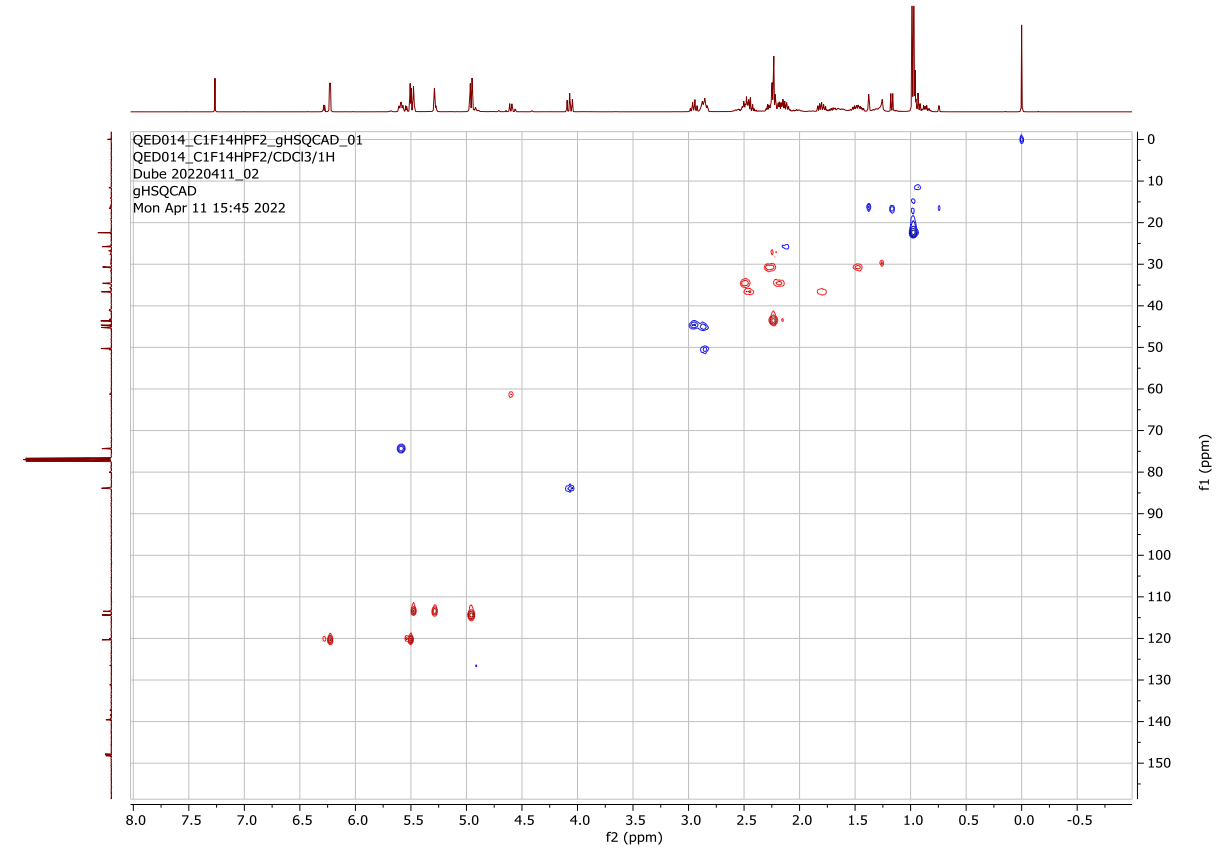

Figure A12: gHSQC spectrum of (**6.8**) in $CDCl_3$ (400 MHz, δ in ppm).

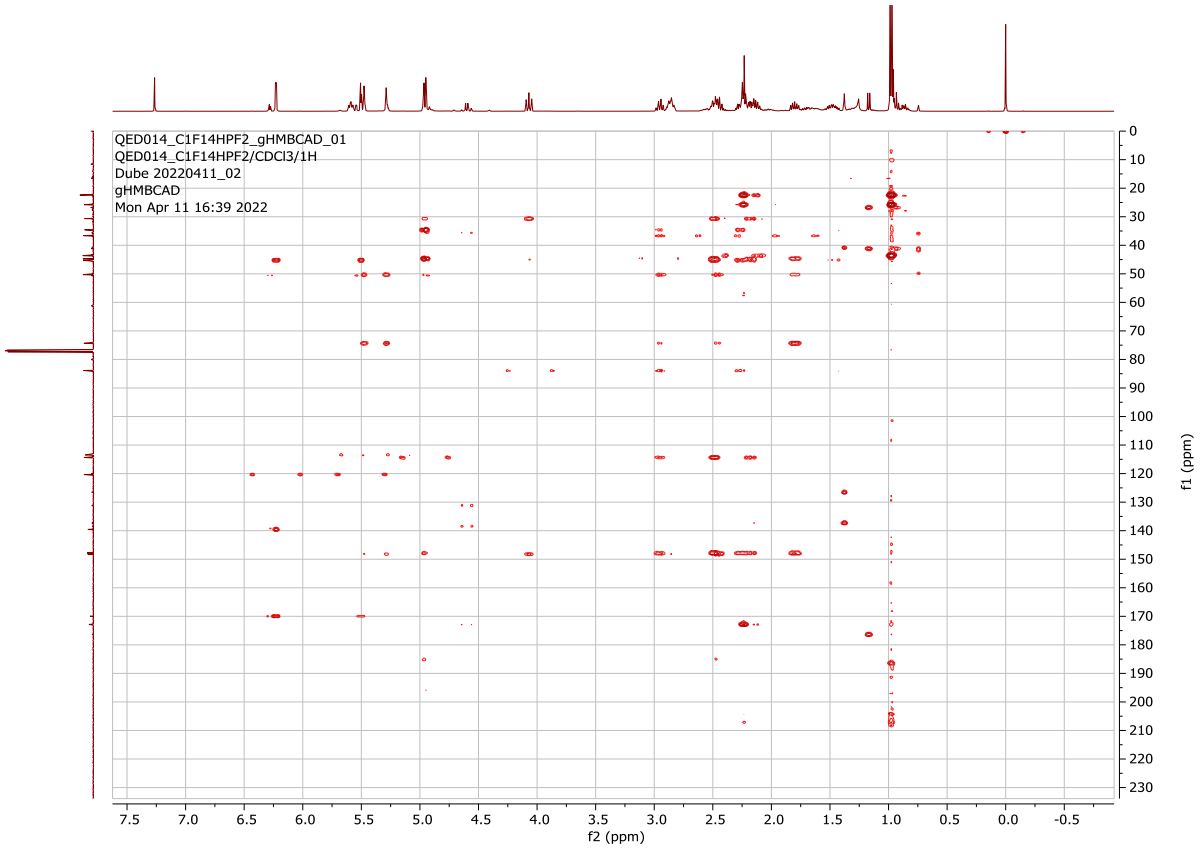

Figure A13: gHMBC spectrum of (**6.8**) in $CDCl_3$ (400 MHz, δ in ppm).

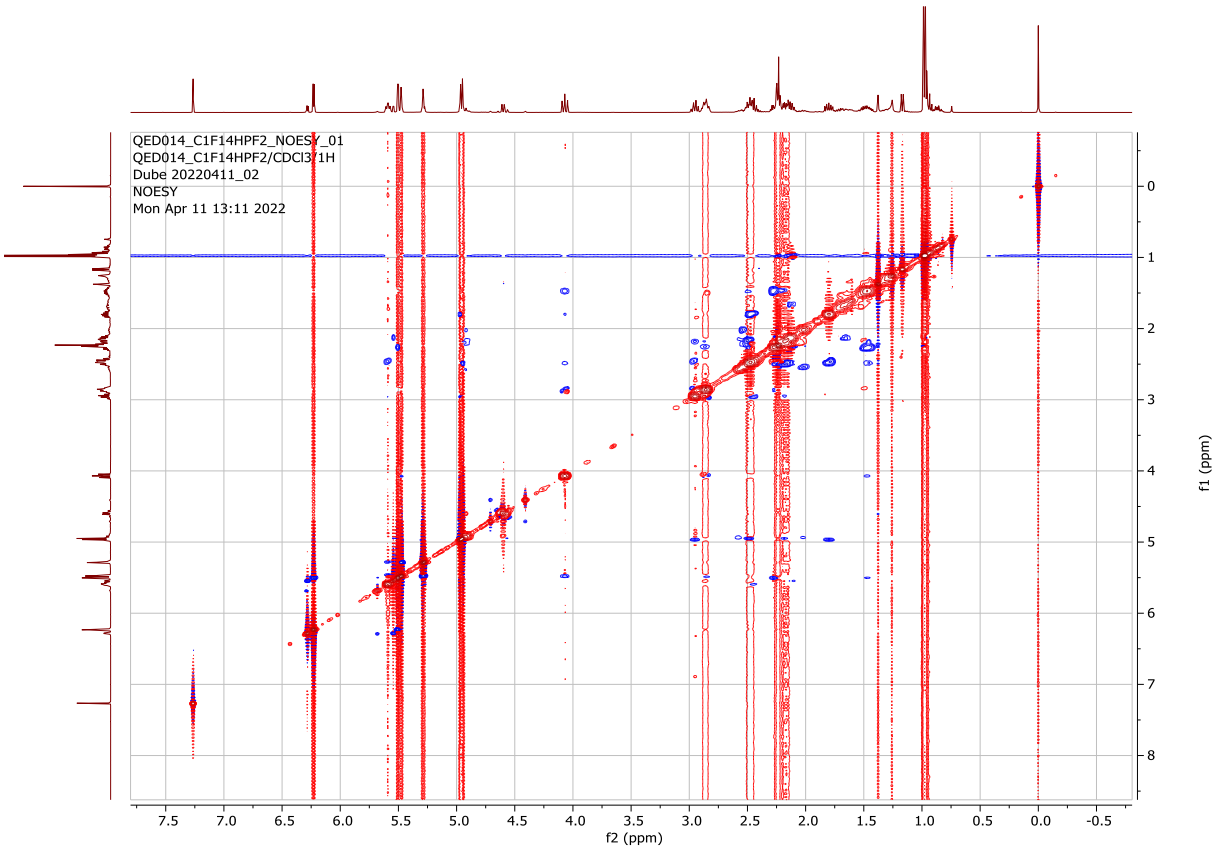

Figure A14: NOESY spectrum of (**6.8**) in $CDCl_3$ (400 MHz, δ in ppm).

MS Data for compound **6.9**

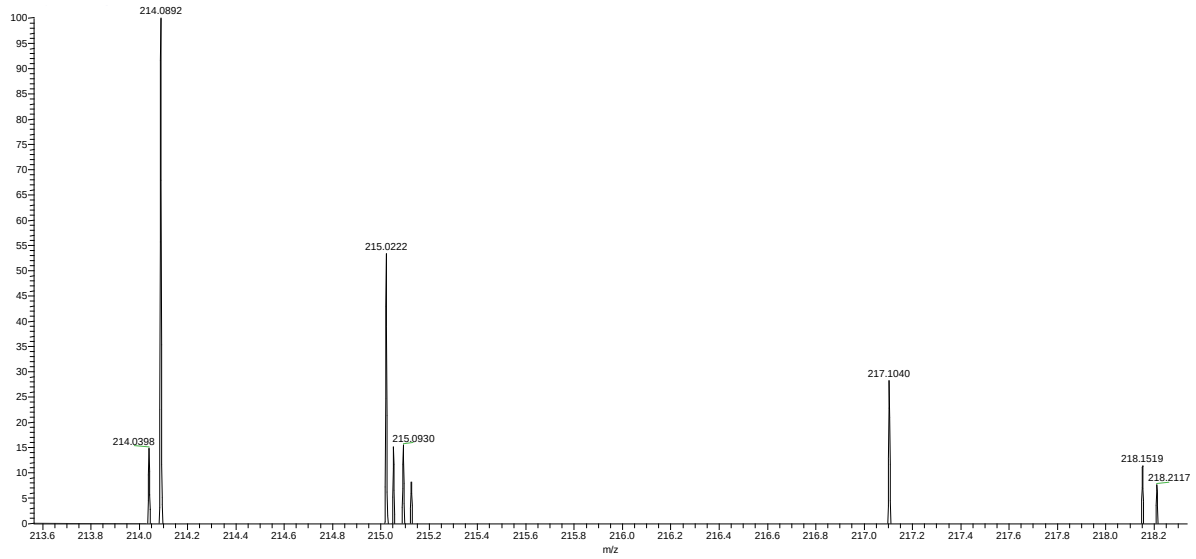

Figure A15: HRMS data for compound **6.9**

NMR data for compound **6.9**

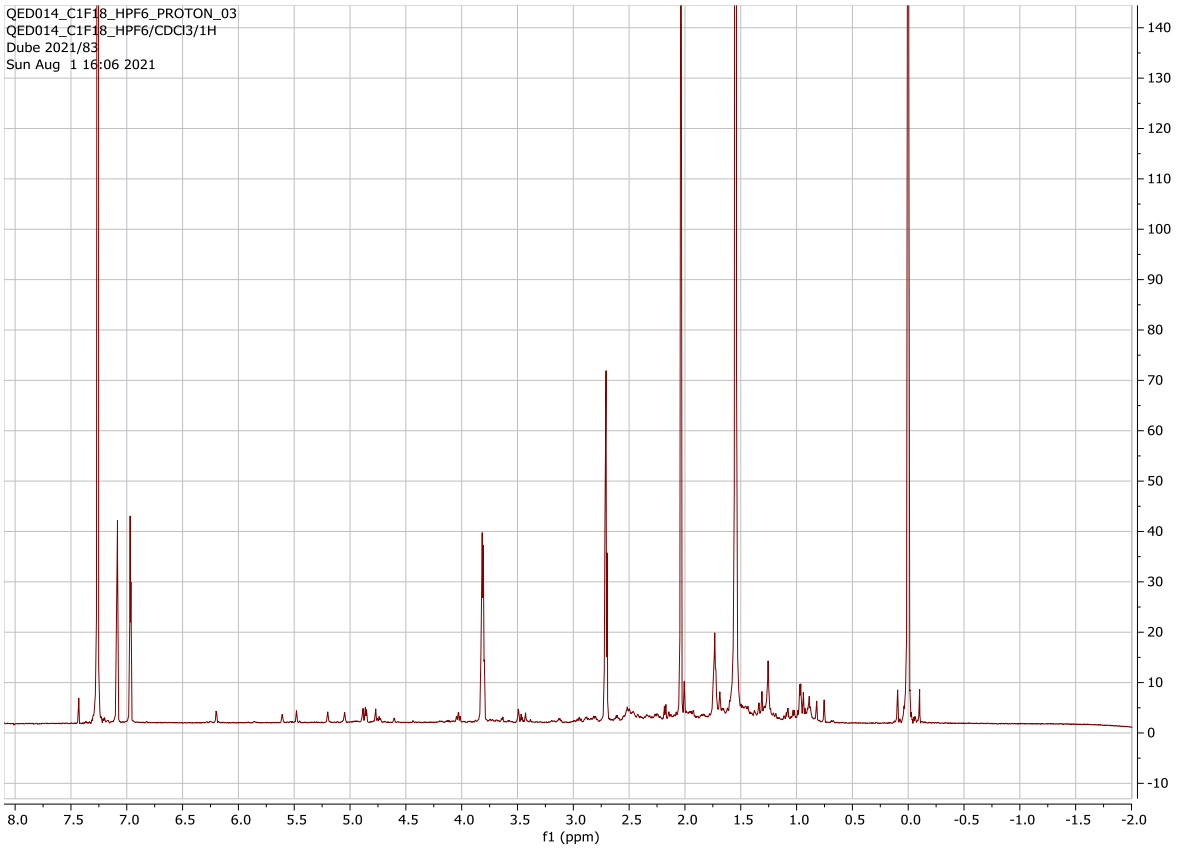

Figure A16: ^{1}H NMR spectrum of 4-(5-(penta-1,3-diyn-1-yl)thiophen-2-yl)but-3-yn-1-ol (**6.9**) in $CDCl_3$ (600 MHz, δ in ppm).

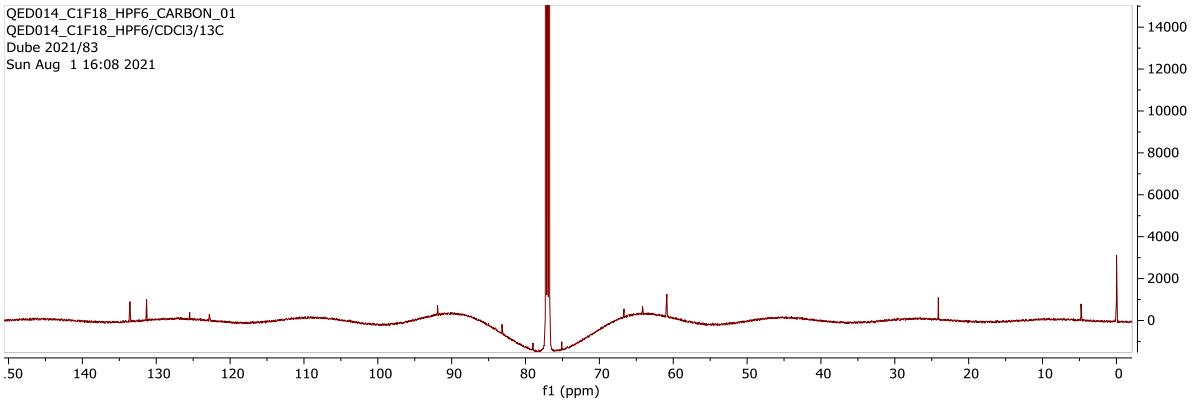

Figure A17: ^{13}C NMR spectrum of 4-(5-(penta-1,3-diyn-1-yl)thiophen-2-yl)but-3-yn-1-ol (**6.9**) in $CDCl_3$ (150 MHz, δ in ppm).

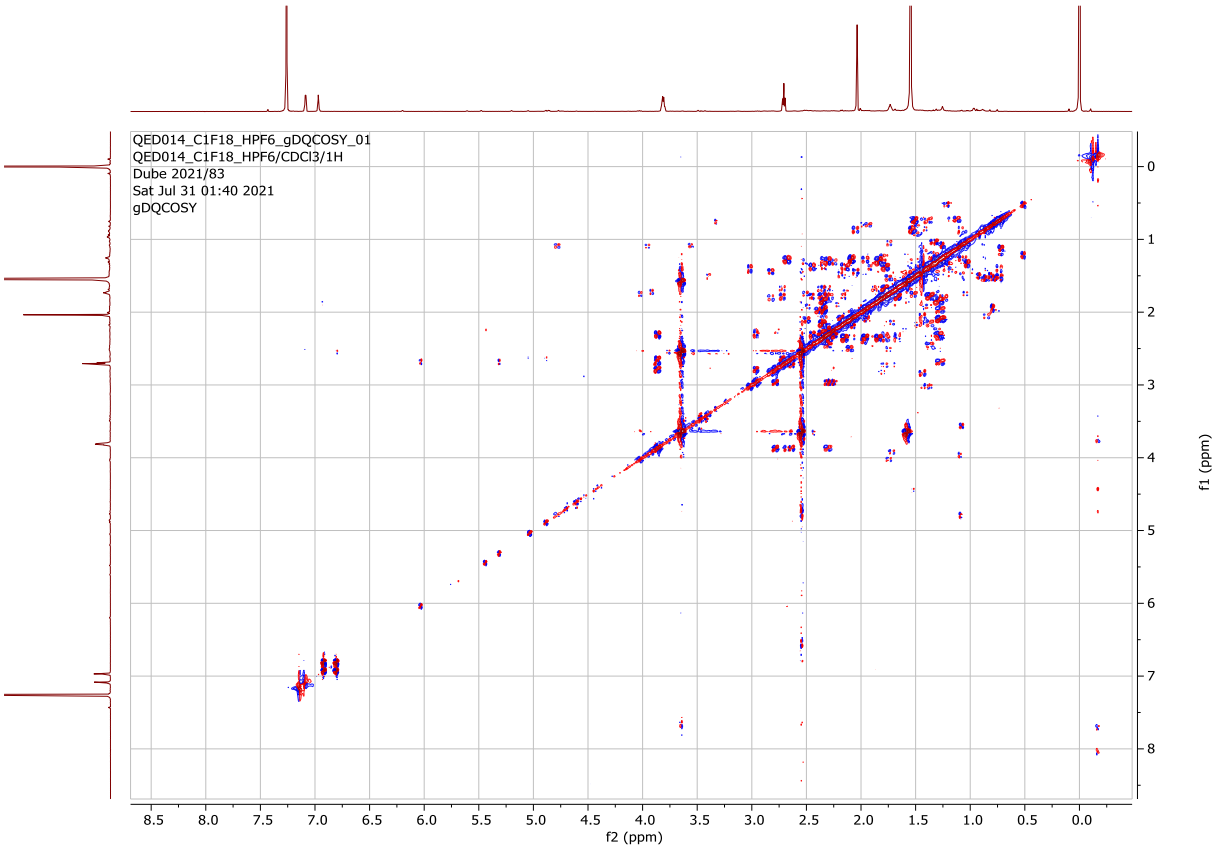

Figure A18: gDQCOSY spectrum of 4-(5-(penta-1,3-diyn-1-yl)thiophen-2-yl)but-3-yn-1-ol (**6.9**) in $CDCl_3$ (600 MHz, δ in ppm).

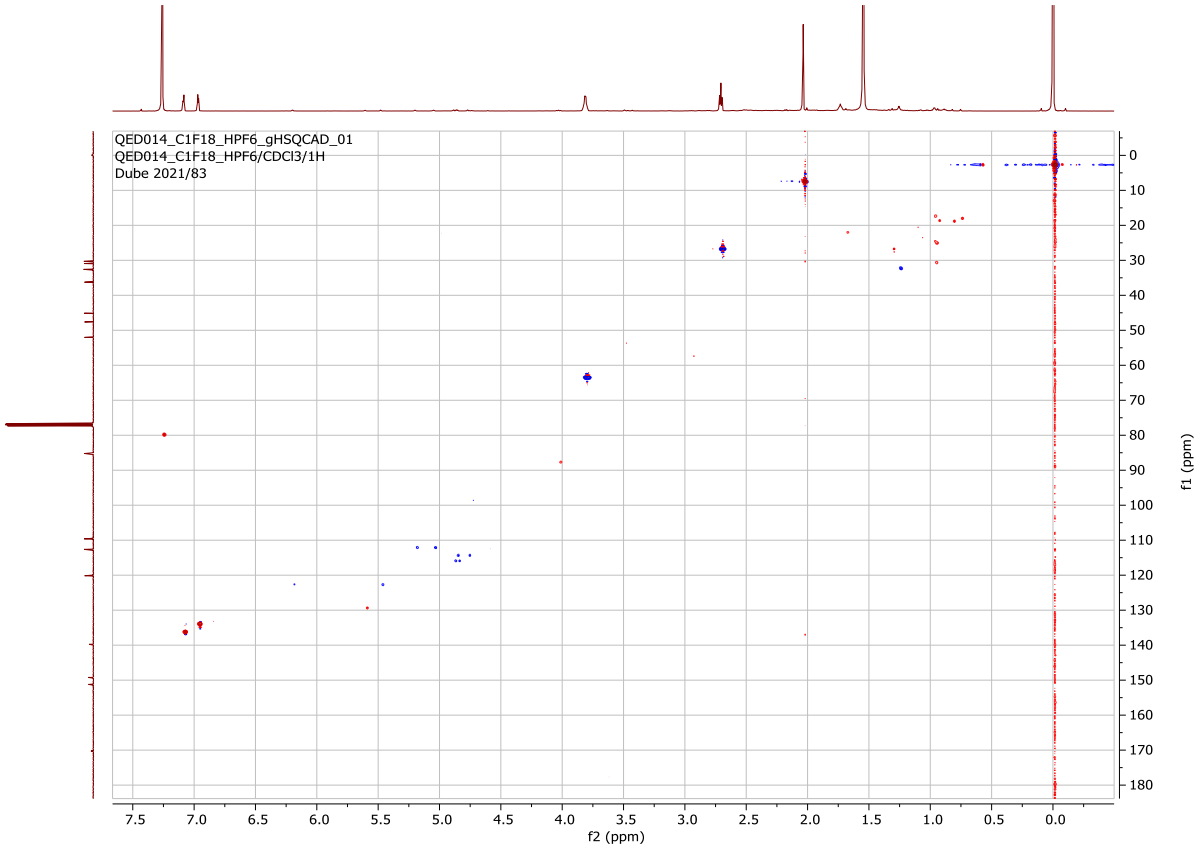

Figure A19: gHSQC spectrum of 4-(5-(penta-1,3-diyn-1-yl)thiophen-2-yl)but-3-yn-1-ol (**6.9**) in $CDCl_3$ (600 MHz, δ in ppm).

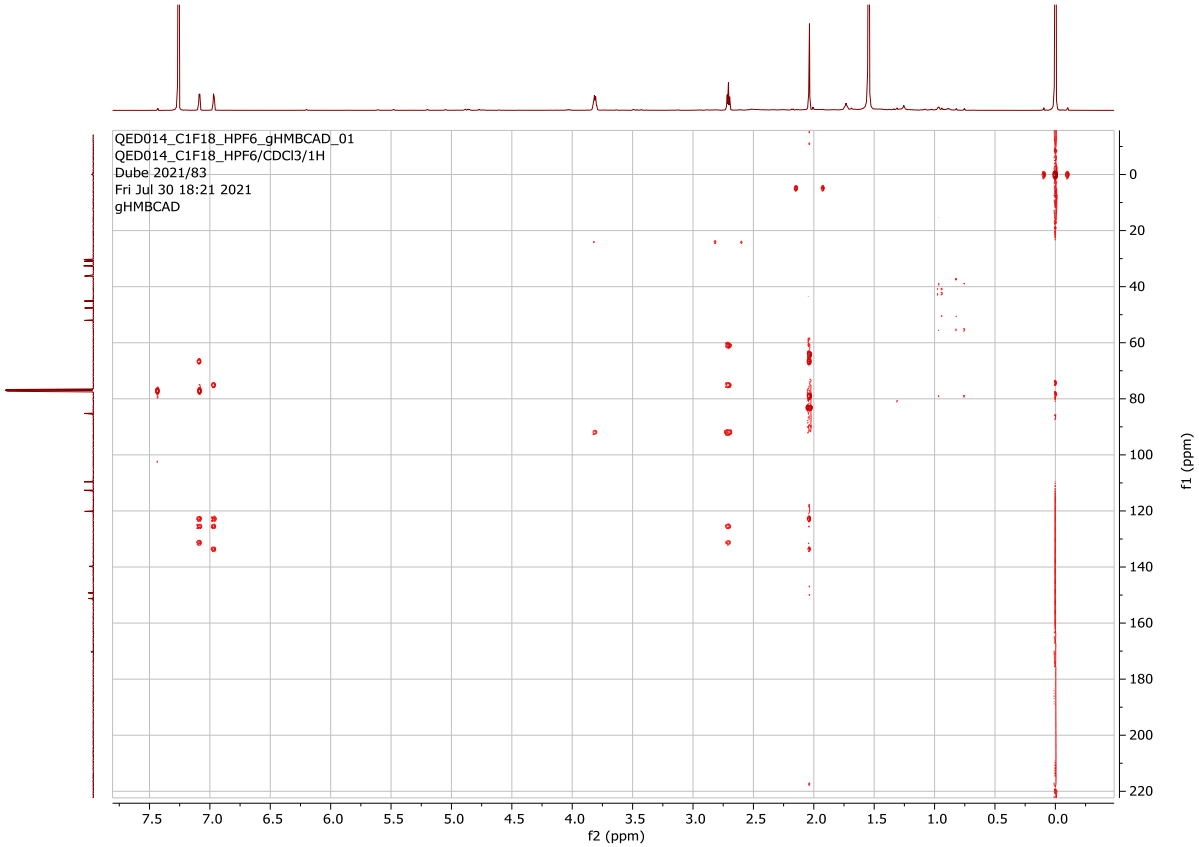

Figure A20: gHMBC spectrum of 4-(5-(penta-1,3-diyn-1-yl)thiophen-2-yl)but-3-yn-1-ol (**6.9**) in $CDCl_3$ (600 MHz, δ in ppm).

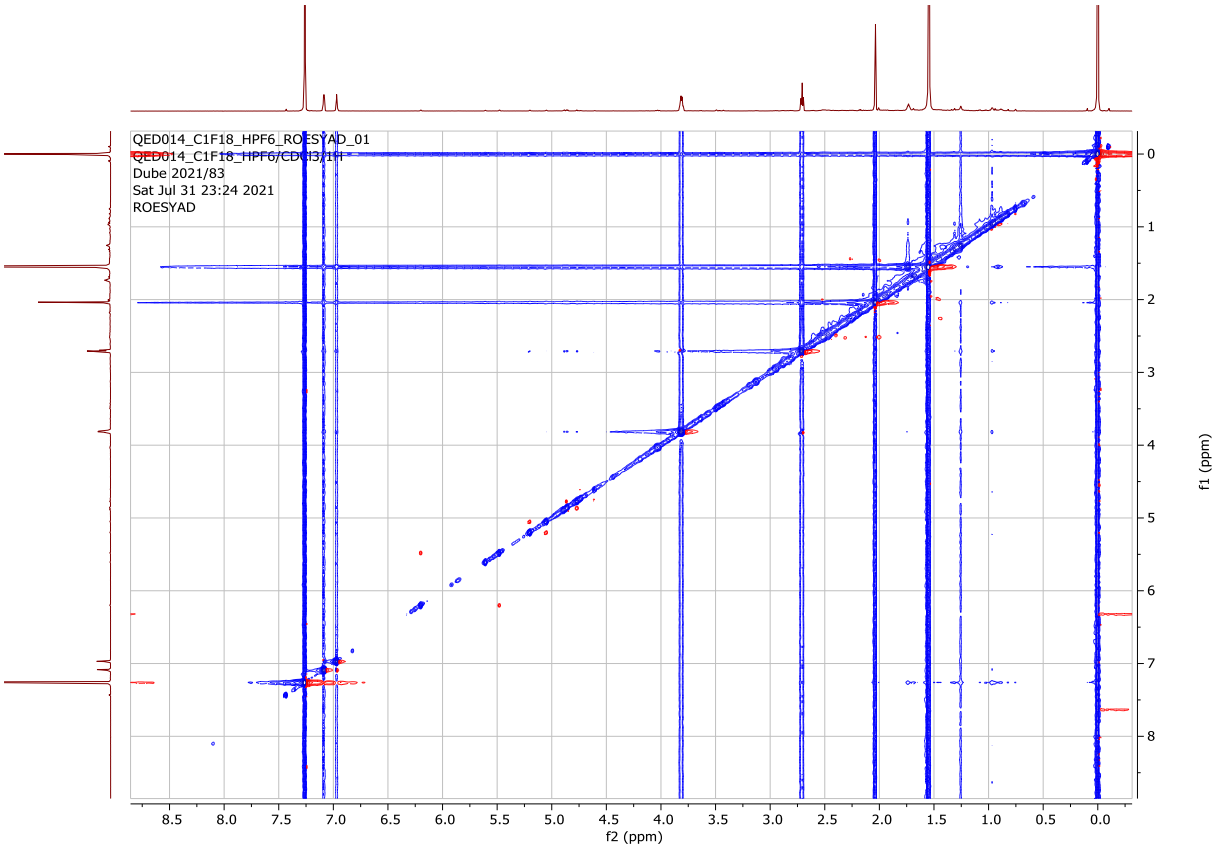

Figure A21: ROESY spectrum of 4-(5-(penta-1,3-diyn-1-yl)thiophen-2-yl)but-3-yn-1-ol (**6.9**) in $CDCl_3$ (600 MHz, δ in ppm).

HRMS data for compounds **6.10** and **6.11**

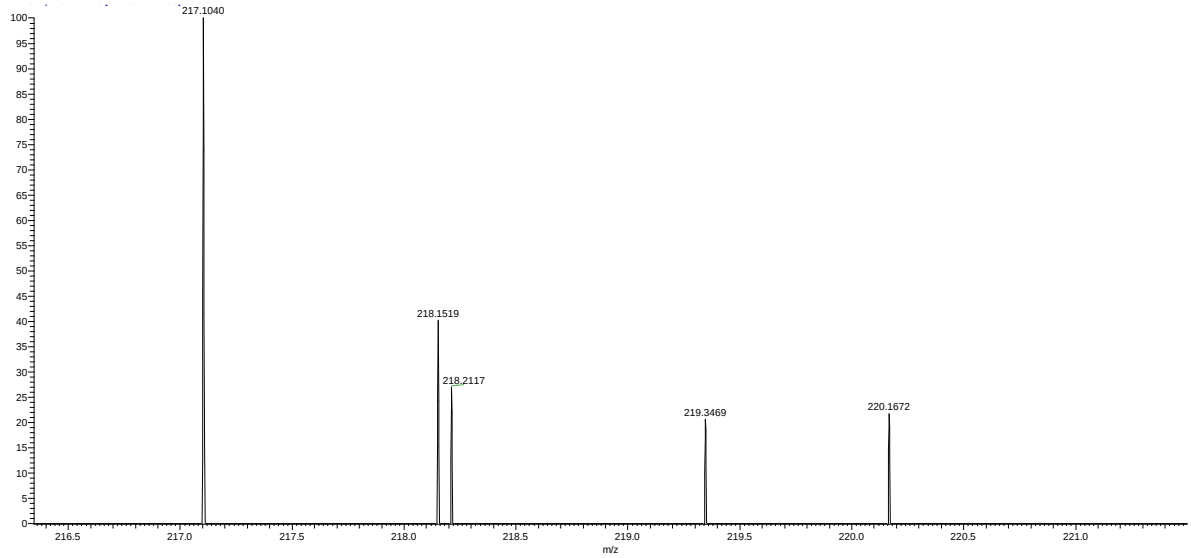

Figure A22: HRMS data for compounds **6.10** and **6.11**

NMR data for compound **6.10** and **6.11**

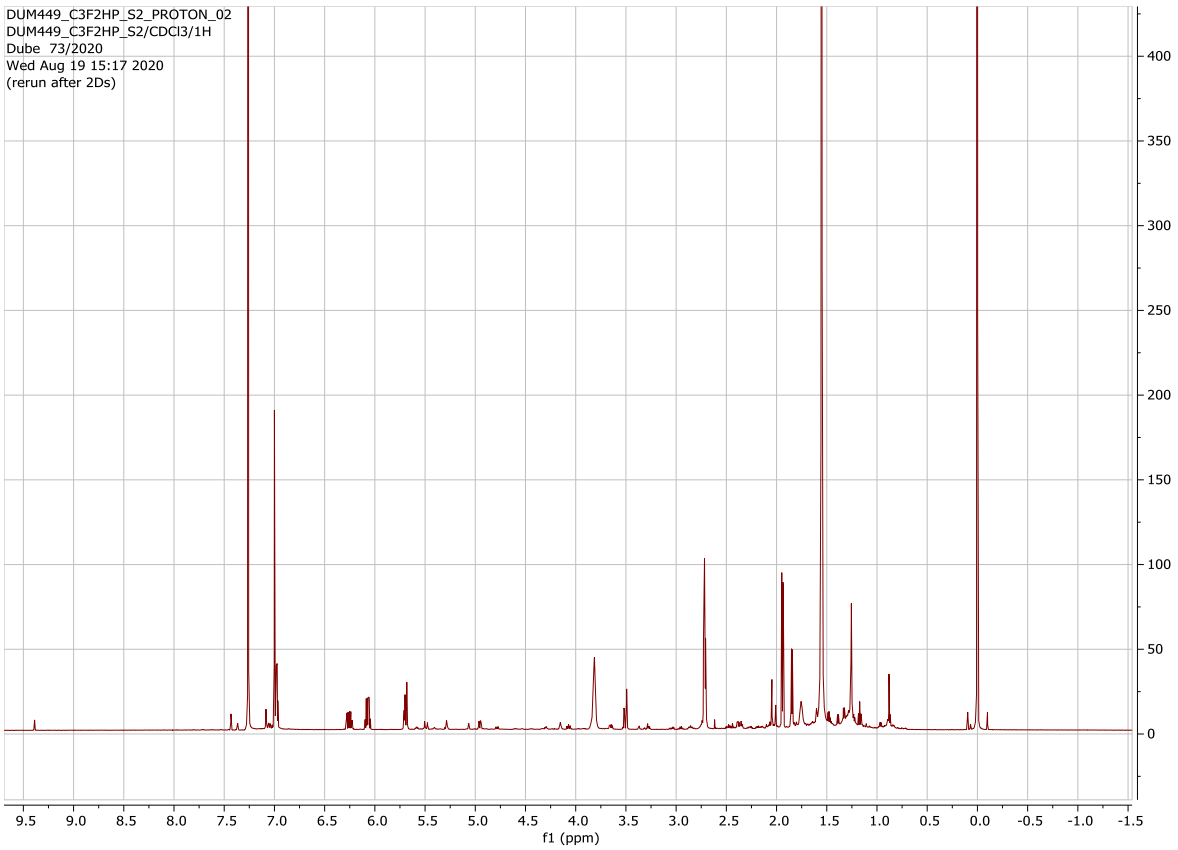

Figure A23: ^{1}H NMR spectrum of (**6.10** and **6.11**) in $CDCl_3$ (600 MHz, δ in ppm).

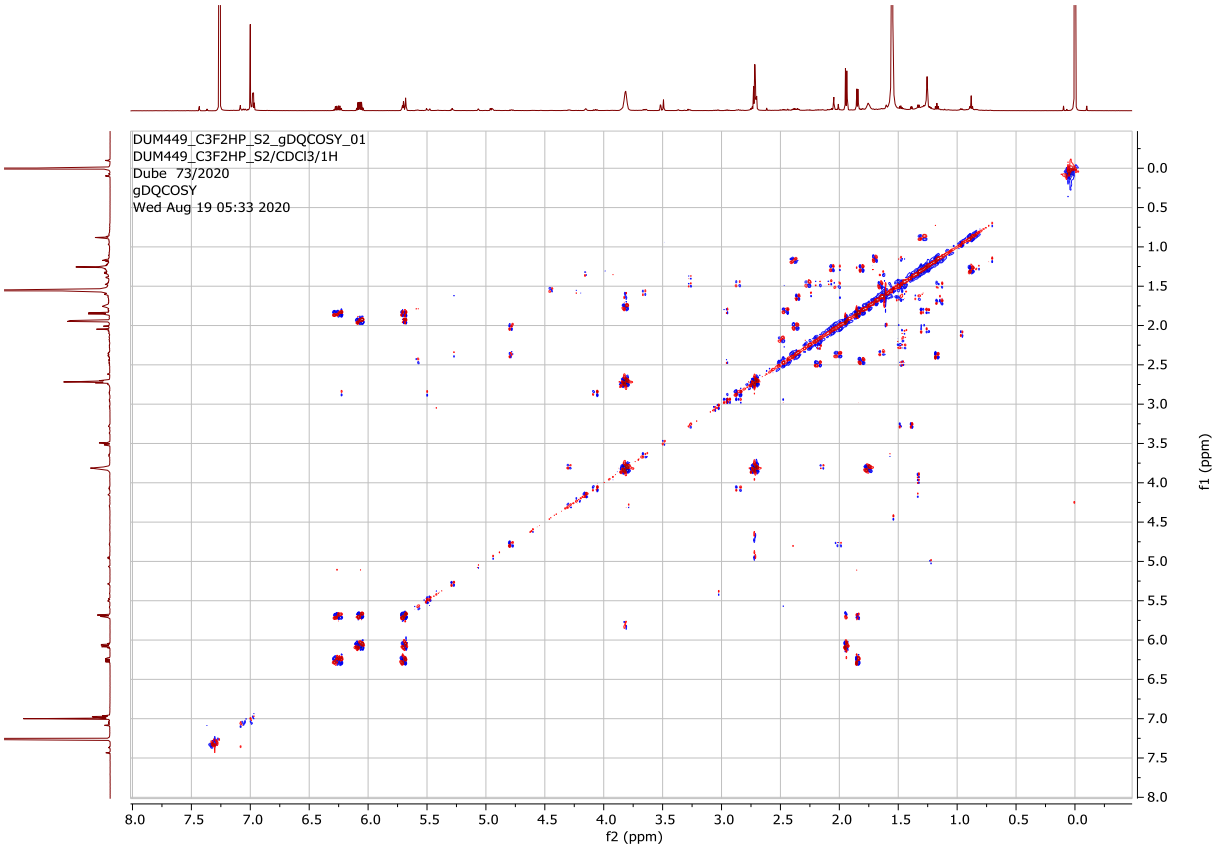

Figure A24: gDQCOSY spectrum of (**6.10** and **6.11**) in $CDCl_3$ (600 MHz, δ in ppm).

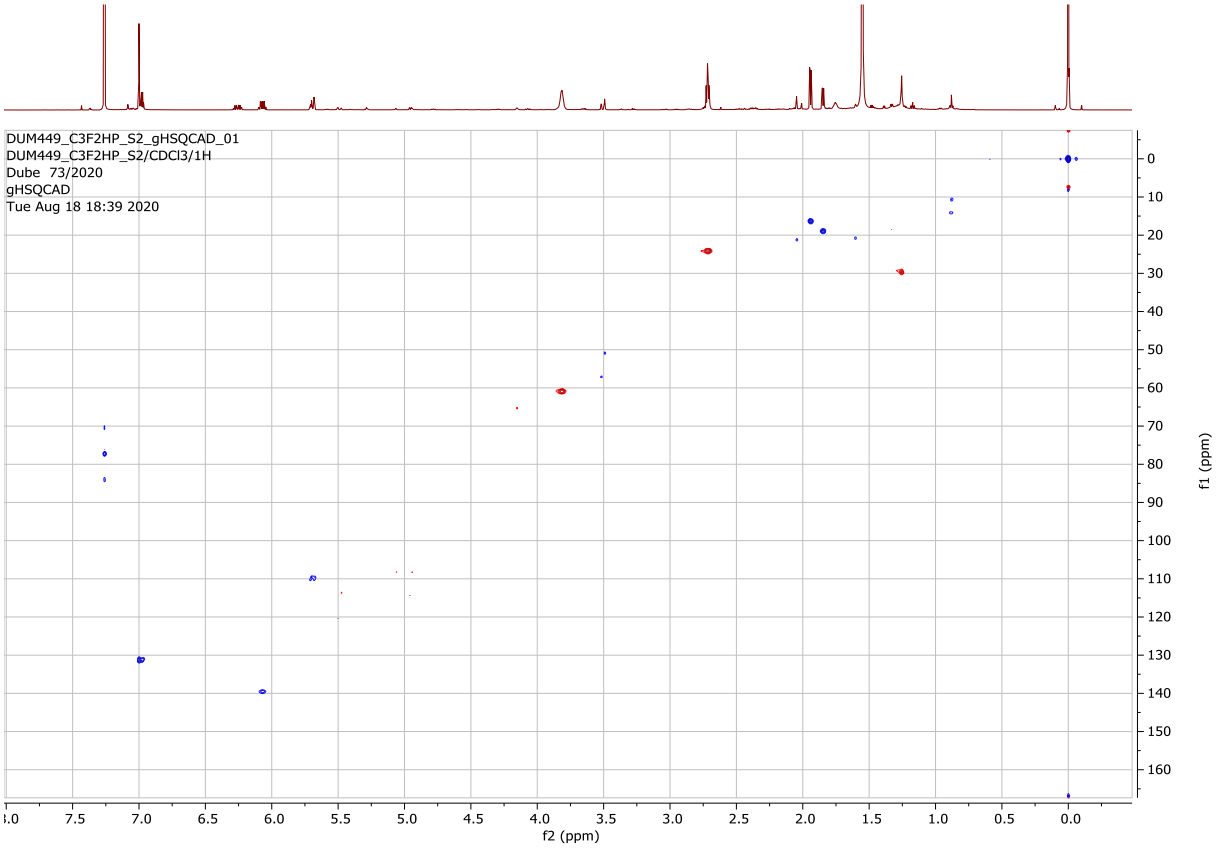

Figure A25: gHSQC spectrum of (**6.10** and **6.11**) in $CDCl_3$ (600 MHz, δ in ppm).

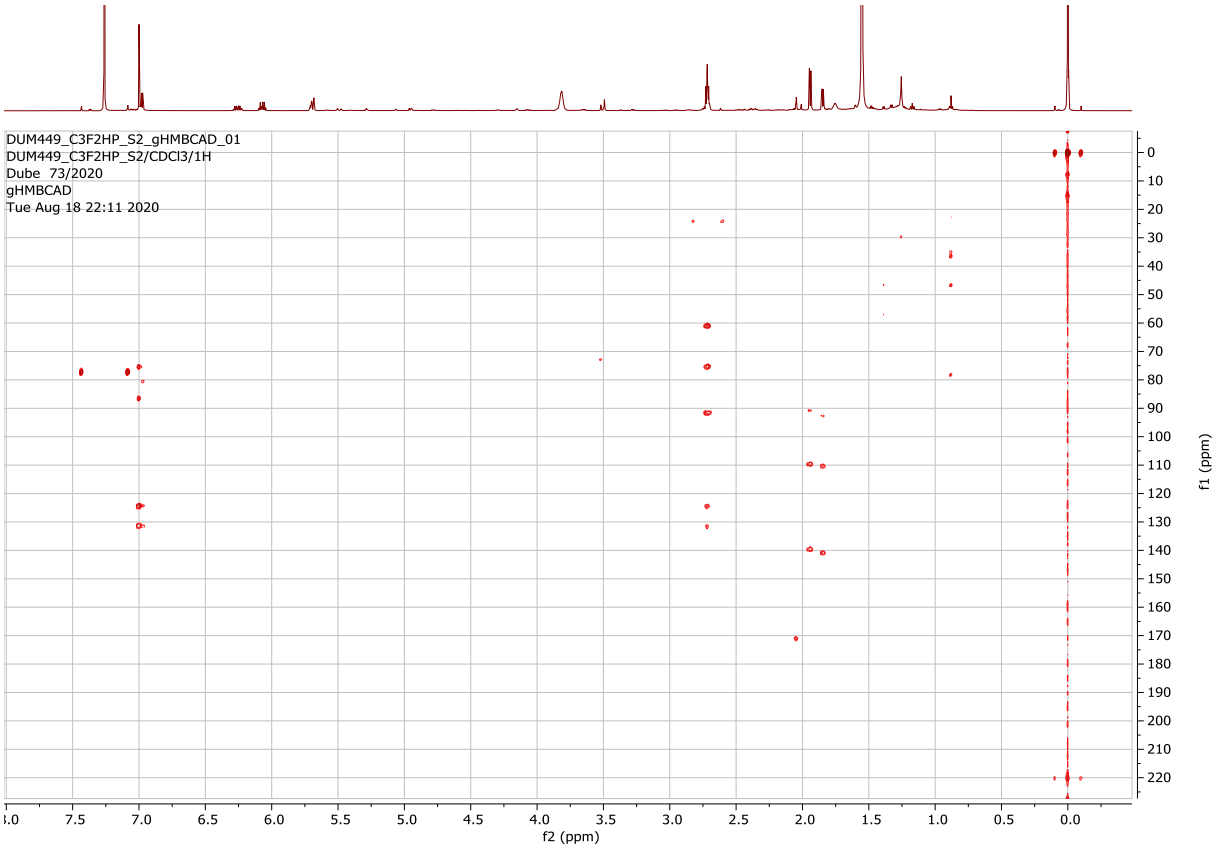

Figure A26: gHMBC spectrum of (**6.10** and **6.11**) in $CDCl_3$ (600 MHz, δ in ppm).

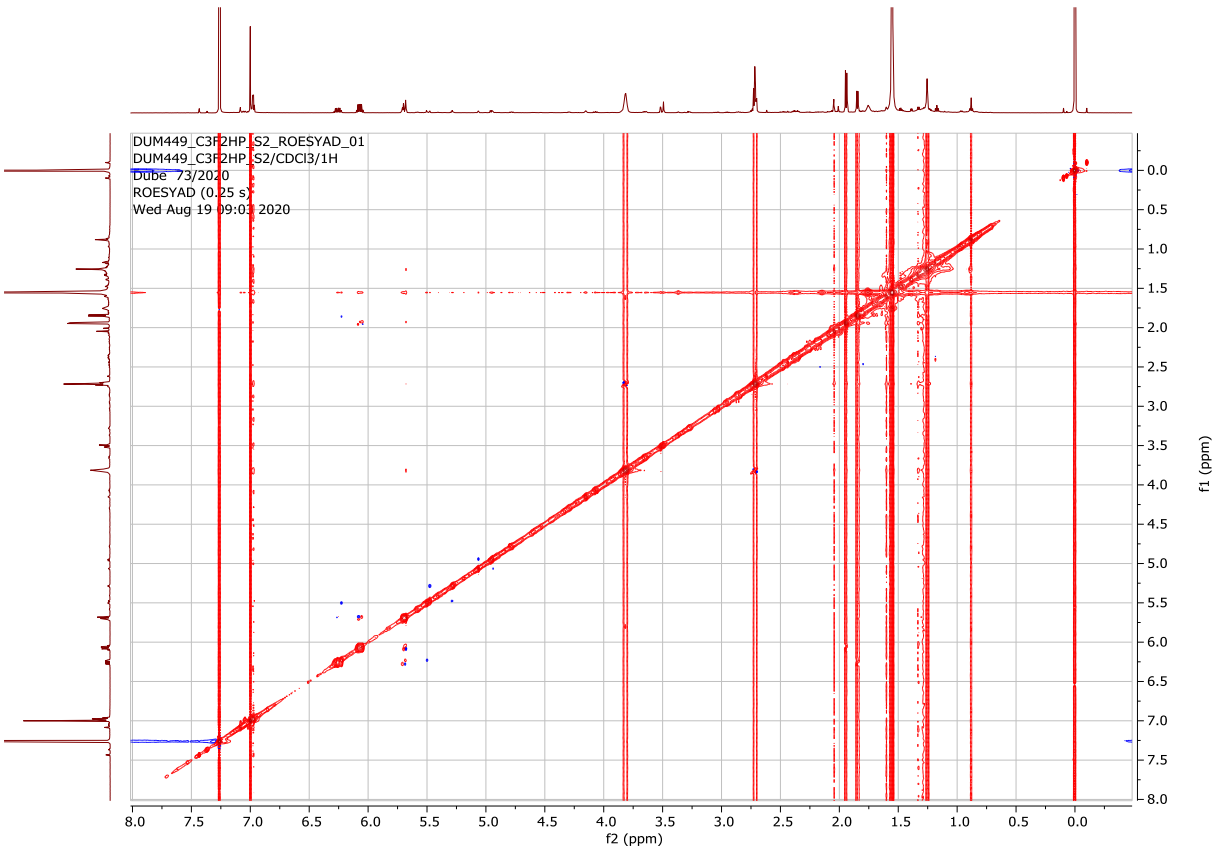

Figure A27: ROESY spectrum of (**6.10** and **6.11**) in $CDCl_3$ (600 MHz, δ in ppm).

Appendix 3

Compound code assignement

Thesis compound number	Compound name	Experiment code (3LC)
3.1	6-[8(Z)-pentadecenyl] anacardic	DUM336C1F26
3.2	6-[10(Z)-heptadecenyl] anacardic acid	DUM336C1F7_C2F10
3.3	3-[7(*Z*)-pentadecenyl] phenol	DUM336CF2_C2F6
4.1	Grifolin	DUM439C1F5
4.2	Neogrifolin	DUM439C1F20
4.3	Orcinol	DUM498
4.4	Prenyl-2-orcinol	DUM518
4.5	Geranyl-2-orcinol	DUM515
4.6	Geranylgeraniol-2-orcinol	DUM516
4.7	Prenol	
4.8	Geraniol	
4.9	Farnesol	
4.10	Geranylgeraniol	
6.7	Dehydrocostus lactone	QED014C1F7
6.8	Diaspanolide B	QED014C1F14HPF2
6.9	2-(4-hydroxybut-1-ynyl)-5-(penta-1,3-diynyl) thiophene	QED014C1F19HPF6
6.10	trans-2-[pent-3-en-1-ynyl]-5-[4-hydroxybut-1-ynyl]-thiophenes	DUM449C3F2HPS2
6.11	cis-2-[pent-3-en-1-ynyl]-5-[4-hydroxybut-1-ynyl]-thiophenes	DUM449C3F2HPS2

Declaration on the author contributions

Chapter 3 Dube, M., Saoud, M., Rennert, R., Fotso, G.W., Andrae-Marobela, K., Imming, P., Häberli, C., Keiser, J., Arnold, N., 2021**.** Anthelmintic activity and cytotoxic effects of compounds isolated from the fruits of *Ozoroa insignis* Del. (Anacardiaceae). *Biomolecules* 11 (12), 1893.

In this study Mthandazo Dube carried out the isolation of all compounds and perfomed the anthelmintic assay using *C. elegans*. Mohamad Saoud and Robert Rennert carried out the anticancer assays. Ghislain Wabo Fotso helped with the structural elucidation. Kerstin Andrae-Marobela helped with sourcing the plant material. Cecile Häberli and Jennifer Keiser performed the anthelmintic assays using parasitic organisms. Mthandazo Dube wrote the draft manuscript. Kerstin Andrae-Marobela, Peter Imming, Jennifer Keiser and Norbert Arnold edited the manuscript. The work was planned and supervised by Peter Imming and Norbert Arnold.

Chapter 4 Dube, M., Llanes, D., Saoud, M., Rennert, R., Imming, P., Häberli, C., Keiser, J., Arnold, N., 2022. *Albatrellus confluens* (Alb. & Schwein.) Kotl. & Pouz.: Natural fungal compounds and synthetic derivatives with *in vitro* anthelmintic activities and antiproliferative effects against two human cancer cell lines. *Molecules* 27 (9), 2950.

In this study Mthandazo Dube carried out the isolation and structure elucidation of compounds and perfomed the anthelmintic assay using *C. elegans*. Dayma Llanes carried out the synthesis of derivatives. Mohamad Saoud and Robert Rennert carried out the antiproliferative assays. Cecile Häberli and Jennifer Keiser performed the anthelmintic assays using parasitic organisms. Norbert Arnold collected and identified the fungal material. Mthandazo Dube wrote the draft manuscript. Peter Imming, Jennifer Keiser and Norbert Arnold edited the manuscript. The work was planned and supervised by Peter Imming and Norbert Arnold.

Chapter 5 Dube, M., Raphane, B., Sethebe, B., Seputhe, N., Tiroyakgosi, T., Imming, P., Häberli, C., Keiser, J., Arnold, N., Andrae-Marobela, K., 2022. Medicinal plant preparations administered by Botswana traditional health practitioners for treatment of worm infections show anthelmintic activities. *Plants* 11 (21), 2945.

In this study Mthandazo Dube collected the plant material and prepared the methanol extracts. Boingotlo Raphane collected the plant material and carried out the interviews with the traditional healers. Nkaelang Sephuthe and Tsholofelo Tiroyakgosi provided the ethnobotanical knowledge and also assisted with plant collection. Bongani Sethebe identified the plants. Cecile Häberli and Jennifer Keiser performed the anthelmintic assays using parasitic organisms. Mthandazo Dube wrote the draft manuscript. Kerstin Andrae-Marobela, Peter Imming, Jennifer Keiser and Norbert Arnold edited the manuscript. The work was planned by Mthandazo Dube, Norbert Arnold and Kerstin Andrae- Marobela and supervised by Peter Imming and Norbert Arnold.

Chapter 6 Dube, M., Ware, I., Lam T.H.Y., Martinez-Aldino, I.Y., Kahsay, B.N., Mariam, T.-G., Moeller, L., Franke, K., Imming, P., Arnold, N., 2022. Anthelmintic activity of selected Ethiopian plants and fungi species: Could spices be an alternative to MDA.

In this study Mthandazo Dube did the anthelmintic testing and isolation. Ismail Ware and Ingrid Martinez-Aldino did the metabolite annotation. Thi Hai Yen Lam carried out the structure elucidation. Birhanu Nigusse Kahsay and Tsige-Gebre Mariam collected the plant material. Lucie Moeller macerated the plant material. Mthandazo Dube wrote the draft manuscript. Katrin Franke, Peter Imming, and Norbert Arnold edited the manuscript. The work was planned by Mthandazo Dube, Lucie Moeller and Norbert Arnold and supervised by Peter Imming, Katrin Franke and Norbert Arnold.

Publications

Publications in peer-reviewed journals

Andrae-Marobela, K., Ntumy, A. N., Mokobela, M., **Dube, M**., Sosome, A., Muzila, M., Sethebe, B., Monyatsi, K. N., & Ngwenya, B. N., 2012. "Now I heal with pride"-The application of screens-to-nature technology to indigenous knowledge systems research in Botswana: Implications for drug discovery. In: *Drug Discovery in Africa: Impacts of Genomics, Natural Products, Traditional Medicines, Insights into Medicinal Chemistry, and Technology Platforms in Pursuit of New Drugs* (Vol. 9783642281754, pp. 239-264). Springer-Verlag Berlin Heidelberg.

Fotso, G.W., Ntumy, A.N., Ngachussi, E., **Dube, M**., Mapitse, R., Kapche, G.D.W.F., Andrae-Marobela, K., Ngadjui, B.T., Abegaz, B.M., 2014. Epunctanone, a new benzophenone, and further secondary metabolites from *Garcinia epunctata* Stapf (Guttiferae). *Helv. Chim. Acta* 97(7), 957-964.

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Agzamova, M.A., Mamadalieva, N.Z., Porzel, A., Hussain, H., **Dube, M**., Franke, K., Janibekov, A., Wessjohann, L.A., 2021. Lehmanniaside, a new cycloartane triterpene glycoside from *Astragalus lehmannianus*. *Nat. Prod. Res*. 27, 1-6.

Sirak, B., Asres, K., Hailu, A., **Dube, M**., Arnold, N., Häberli, C., Keiser, J., Imming, P., 2021. *In vitro* antileishmanial and antischistosomal activities of anemonin isolated from the fresh leaves of *Ranunculus multifidus* Forsk. *Molecules* 26, 7473.

Dube, M., Saoud, M., Rennert, R., Fotso, G.W., Andrae-Marobela, K., Imming, P., Häberli, C., Keiser, J., Arnold, N., 2021. Anthelmintic activity and cytotoxic effects of compounds isolated from the fruits of *Ozoroa insignis* Del. (Anacardiaceae). *Biomolecules* 11 (12), 1893.

Dube, M., Llanes, D., Saoud, M., Rennert, R., Imming, P., Häberli, C., Keiser, J., Arnold, N., 2022. *Albatrellus confluens* (Alb. & Schwein.) Kotl. & Pouz.: Natural fungal compounds and synthetic derivatives with in vitro anthelmintic activities and antiproliferative effects against two human cancer cell lines. *Molecules* 27, 2950.

Dube, M., Raphane, B., Sethebe, B., Seputhe, N., Tiroyakgosi, T., Imming, P., Häberli, C., Keiser, J., Arnold, N., Andrae-Marobela, K., 2022. Medicinal plant preparations administered by

Botswana traditional health practitioners for treatment of worm infections show anthelmintic activities *Plants* 11 (21), 2945.

Oral presentation

Dube, M. Bioassay guided isolation of anthelmintic compounds from natural products. Phytochemical society of Europe meeting on natural products in drug discovery and development – advances and perspectives 2022, Iasi, Romania, 19th – 22nd September 2022.

Poster presentations

Dube, M., Arnold, N., Andrae-Marobela, K., Imming, P. Screening of African traditional medicinal plants for anthelmintic activity. 15th Plant science student conference 2019, Halle (Saale), 18th – 21st June 2019.

Dube, M., Arnold, N., Andrae-Marobela, K., Imming, P. Screening of African traditional medicinal plants for anthelmintic activity. 1st Pharma Research Day, Halle (Saale), 26th June 2019.

Dube, M., Llanes, D., Saoud, M., Rennert, R., Imming, P., Häberli, C., Keiser, J., Arnold, N. *Albatrellus confluens*: Natural fungal compounds and synthetic derivatives with in vitro anthelmintic activities and antiproliferative effects. Plant science student conference 2022, Halle (Saale), 14th – 17th June 2022.

Curriculum vitae

Personal details

Name:	Mthandazo Dube
Date/place of birth:	13th July 1979 in Gwanda
Nationality:	Zimbabwean

School education

01/1985-12/1991	Tennyson Primary School, Bulawayo
01/1992-12/1997	Milton High School, Bulawayo

Academic studies

08/1998-08/2002	Bachelor of Science (Hons) in Applied Biology and Biochemistry, National University of Science and Technology Title of Project: 'Occurrence of Clostridium botulinum spores in honey'
01/2016-10/2018	Master of Philosophy in Biological Sciences, University of Botswana Title: 'Screening of natural products for anti-parasitic and insecticidal activities using whole organism systems and G-protein coupled receptor based screens'
11/2018-12/2022	PhD candidate, Leibniz Institute of Plant Biochemistry, Department of Bioorganic Chemistry, Halle (Saale) Title: 'Bioassay guided fractionation and isolation of anthelmintic compounds from natural products' (Supervisor: Prof. Dr. Peter Imming)

Work experience

09/2002-01/2004	Biology teacher, Mzingwane High School, Esigodini
04/2004-02/2008	Section Head Quality Assurance, Mitchell and Mitchell fresh exports, Marondera
08/2008-05/2017	Teaching Assistant, University of Botswana, Department of Biological Sciences, Gaborone
06/2018-07/2022	Research Assistant, Martin Luther Univeristy Halle-Wittenburg, Department of Pharmacy, Halle (Saale)

Eidesstattliche Erklärung

Ich erkläre an Eides statt, dass ich die vorliegende Arbeit selbständig und nur unter Verwendung der angegebenen Hilfsmittel und Quellen angefertigt habe.

Alle Stellen, die wörtlich oder sinngemäß aus Veröffentlichungen entnommen sind, habe ich als solche kenntlich gemacht. Des Weiteren versichere ich, dass ich diese Arbeit an keiner anderen Institution eingereicht habe.

Mir ist bekannt, dass bei Angabe falscher Aussagen die Prüfung als nicht bestanden gilt. Mit meiner Unterschrift versichere ich die Richtigkeit dieser Angaben und erkenne die rechtlichen Grundlagen an.

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Datum (Mthandazo Dube)