Anthelmintic activity in plant secondary metabolites from *Khaya senegalensis* (African mahogany)

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ABSTRACT

Gastrointestinal parasites, particularly the helminths, are a major constraint in livestock production systems, with serious worldwide socio-economic impact because they impose significant financial and production losses. This problem is exacerbated by the ever-increasing incidence of resistance to anthelmintic oral medication. Moreover, there is an increase in consumer awareness of drug residues in animal-sourced food, making it imperative to introduce a strategy for parasite control that is 'clean, green and ethical'(CGE). To address these issues, the general hypothesis tested in this thesis was that *Khaya senegalensis* produces bioactive secondary metabolites with anthelmintic activity. The hypothesis was tested in two experiments.

In the first experiment, leaf samples of *K. senegalensis* were subjected to methanolic extraction followed by bioassay-guided fractionation, with a bioassay based on *in-vitro* mortality of third stage larvae of the model helminth, *Ascaris suum*. This process revealed early-eluting fractions with anthelmintic activity, the most active of which was found to contain linoleic and oleic acids. To further confirm the identity of these anthelmintic molecules, synthetic linoleic and oleic acids were tested in the bioassay, and were found to have LD_{50} values of 496 µg/mL (linoleic acid) and 1562 µg/mL (oleic acid). This experiment thus confirmed the presence of anthelmintic activity in *K. senegalensis* and demonstrated the potential for the plant, or an extract of plant material, as a novel control for helminths. This experiment was also the first to demonstrate the anthelmintic activity of linoleic acid and oleic acid.

In the second experiment, we focussed on the later-eluting fractions of the *K. senegalensis* leaf extract in which we identified two major members of the limonoid group of compounds, mahonin (1) and methyl angolensate (2). Mahonin, the more active of the two, was able to kill a significant number of third stage larvae at concentrations as low as 60 µg/mL, with an LD_{50} value of 38 µg/mL. For methyl angolensate (2), the LD_{50} value was 152 µg/mL. Subsequently, it was found that, when combined, methyl angolensate (**2**) and mahonin (**1**) worked additively to cause larval mortality. Finally, analysis of additional, later fractions, revealed intermediate anthelmintic activity (LD_{50} 72 µg/mL) associated with a completely novel limonoid, assigned the trivial name 16-oxodelevoyin B (**3**).

With the work presented in this thesis we were able to demonstrate the anthelmintic activity of secondary metabolites produced by *Khaya senegalensis.* This plant could be utilized in

livestock management, either by feeding plant material directly to the animals or by isolating the identifying bioactive molecules. Among the interesting molecules are the limonoids, including 16-oxodelevoyin B (**3**), a compound that is new to science. In addition, linoleic acid might have a role to play in helminth control in livestock, in addition to its nutritional benefits for consumers of milk and meat. Importantly, African mahogany is harvested for its timber, so leaf material is a by-product that is both readily available and inexpensive. The next step should be to move beyond the *in vitro* larval bioassay towards *in-vivo* studies with livestock. These options must be explored further as we move towards a vision for clean, green and ethical animal management.

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Co-author signatures and dates:

Student signature: Date: 01/02/2022
I, Graeme B Martin, certify that the student's statements regarding their contribution to each of the works listed above are correct. As all co-authors' signatures could not be obtained, I hereby authorise inclusion of the co-authored work in the thesis. **Coordinating supervisor signature:** **Date**: 28 January, 2022

CHAPTER 1: GENERAL INTRODUCTION

The livestock sector is one of the most important players in the global food system and is integral to food security and agricultural development. It is estimated that the sector supports the livelihood and food security of around 1.3 billion people while contributing 40% to the global value of agricultural output. It also provides 34% of global food protein supply to the population (FAO, 2021). These roles of the sector are becoming increasingly critical as the world human population heads towards 10 billion by 2050 (UNDP, 2001). To feed that population, it has been suggested that the global numbers of cattle and small ruminants will need to double (Kiers et al., 2008; Thornton, 2010), putting additional pressure on a livestock sector that is already facing multiple animal health and production constraints.

One such constraint is gastrointestinal parasites, particularly the helminths, that cause diseases with a major worldwide socio-economic impact because they cause significant financial and production losses and in livestock industries. To better understand these losses in financial perspective, in Australia the cost of dealing with gastrointestinal parasites in sheep and cattle is around $1 billion annually (Sackett, Holmes, Abbott, Jephcott, & Barber, 2006). For Australian sheep farmers in Western Australia (WA), they cause an estimated loss of around $435.9 million annually (Lane, Jubb, Shephard, Webb-Ware, & Fordyce, 2015). The most common helminths in this region are *Trichostrongylus* and *Teladorsagia spp.*, two species that often come as mixed infections in sheep and are considered to be the main cause of diarrhoea ('scouring'; (Cotter, 2017a). These two species are most problematic in winter rainfall areas like south-western WA (Besier & Love, 2003).

Gastro intestinal parasites are also a major problem in non-ruminants and humans. In pigs nematodes like *Ascaris suum*, *Oesophagostomim spp.*, *Trichostrongylus axei*, *Trichuris suis* (Roepstorff et al., 1998) and cestodes like *Taenia solium* (Dorny et al., 2004; Sah et al., 2017) are widely prevalent. It should be noted that some helminths like *Trichuris suis* and *Taenia solium* can be transmitted to humans (zoonotic parasites) and possess a serious public health concern (Stephenson, Holland, & Cooper, 2000; Carabin et al., 2011). The risk is even more pronounced in pastoral communities and developing countries where humans share environmental resources and proximity with domestic and wild animals (Barnes, Davaasuren, Baasandagva, & Gray, 2017). *Ascaris suum* closely resembles the highly prevalent human nematode *Ascaris lumbricoides* (Brooker & Pullan, 2013) that have infected around 819 million humans worldwide (Pullan, Smith, Jasrasaria, & Brooker, 2014). They have also been

widely used as a model parasite for different human studies (Shao et al., 2014; Midha et al., 2018), especially as model system for human intestinal nematodes (Timm et al., 2016). They are fairly common in pigs world-wide (Nansen & Roepstorff, 1999) and cause economic loss mainly due to liver condemnation, altered carcass composition and reduced feed conversation rate (Thamsborg, Nejsum, & Mejer, 2013).

The massive problems caused by helminth infection are compounded by the ever-increasing incidence of resistance to anthelmintic oral medication, a worldwide problem in small ruminants (Fleming et al., 2006). The level of resistance to anthelmintics in WA is one of the highest in the world and, without proper intervention and management, it is expected to worsen rapidly. There are almost no farms in WA where sheep are not resistant to white benzimidazole and clear levamisole anthelmintics and even the combination of these two is only fully effective on less than 20% of properties. Moreover, resistance of *Ostertagia spp*. to macrocyclic lactone anthelmintics has been observed in more than 80% of properties (Cotter, 2017b). Finally, in parallel with these problems, there is an increase in consumer awareness of drug residues in animal-sourced food, making it imperative to introduce a strategy for parasite control that is 'clean, green and ethical' (CGE; (Martin et al., 2004).

One promising CGE strategy is based on the use of specific members of the vast array of organic compounds synthesised by plants. Primary compounds are directly involved in essential plant functions such as photosynthesis, respiration, growth and development. Secondary compounds, however, are engaged as chemical defences to protect the plant against herbivores and infectious agents. These secondary compounds are toxic in nature so are potential sources of natural antimicrobials, herbicides and insecticides (cited by (Crozier, 2006).

In this thesis, we are focussing on one plant in particular – *Khaya senegalensis*, commonly known as African mahogany, a member of the *Meliaceae* family. It is a multipurpose, deciduous tree, native to tropical and subtropical Africa, that is valued for its timber, fuelwood and medicinal purposes (CABI, 2013). The therapeutic properties of *K. senegalensis* have been exploited by many communities in the region. In humans, it is extensively used to treat fever and malaria; in cattle, it is used for the treatment of diarrhoea, ulcers, and gastrointestinal worms (Iwu, 2014).

Extracts from *K. senegalensis* have also been shown to have potential as an anthelminthic in small ruminants. (Ademola, Fagbemi, & Idowu, 2004) reported that an ethanolic extract from

K. senegalensis given to sheep as a drench at 500 mg/Kg, reduced the faecal egg count (FEC) by about 90%, and, for the nematode *Haemonchus contortus*, steam extracts of *K. senegalensis* were more potent than other plants tested (China, Attindehou, Gbngboche, & Salifou, 2016). Though these studies have addressed the anthelmintic properties of *K. senegalensis,* no efforts have been made to identify the active compounds or to quantify them, although it has been suggested that high concentrations of condensed tannins could be responsible. Against this backdrop, two main objectives were developed for the project described in this thesis:

1) To investigate the anthelmintic properties of *K. senegalensis*;
2) To narrow down the number of potential candidate compounds, and identify those responsible for the anthelmintic activity.

To attain these objectives, we followed a process called 'bioassay-guided fractionation' in which the complex mixture of compounds in an extract of plant material is separated into fractions that are then screened for anthelmintic activity using an *in vitro* larval mortality assay. The most active fractions are further separate and re-assayed. This process is repeated several times until the compound(s) responsible for the bioactivity are sufficiently pure to be identified. Ideally, the larval mortality assay would be based on the helminth species that infect the livestock species of interest. However, not all helminth species are suited to *in-vitro* management. We therefore turned to a well-established assay based on *Ascaris suum* (Bonde et al., 2021). Large numbers of eggs can be harvested from adult female of *A. suum* collected from the intestine of a slaughtered pig, stored for long periods at 4°C, and hatched when needed to provide fresh L3 larvae area for the assay. This system overcomes the need to maintain a herd of experimentally infected animals as a source of larvae.

The general hypothesis tested in this thesis is that *K. senegalensis* produces bioactive secondary metabolites with anthelmintic activity that could be applied in the livestock industries.

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CHAPTER 2: LITERATURE REVIEW

2.1. Livestock helminths of importance

The most problematic helminths, the gastrointestinal nematodes, affecting small ruminants in Australia are *Haemonchus contortus*, *Teladorsagia circumcincta* and *Trichostrongylus spp*. These strongylids have a similar life cycle with few exceptions (Figure 2.1). The adults are present in the digestive tract and are sexually dimorphic. After fertilization the female produces a large number of eggs that are passed out into the environment when the host animal defecates. The eggs are generally 70-150 µm wide and usually hatch in 1-2 days, after which the resulting larvae undergo two moults to develop into ensheathed third stage larvae (L3). The sheath is a cuticular layer that protects the larvae from environmental factors but also prevents feeding. Third stage larvae are ingested by the host and then travel through the intestinal tract during which the sheath is lost. The L3s have a brief histotrophic phase before transforming to the L4 and pre-adult stage (Cheng, 1981).

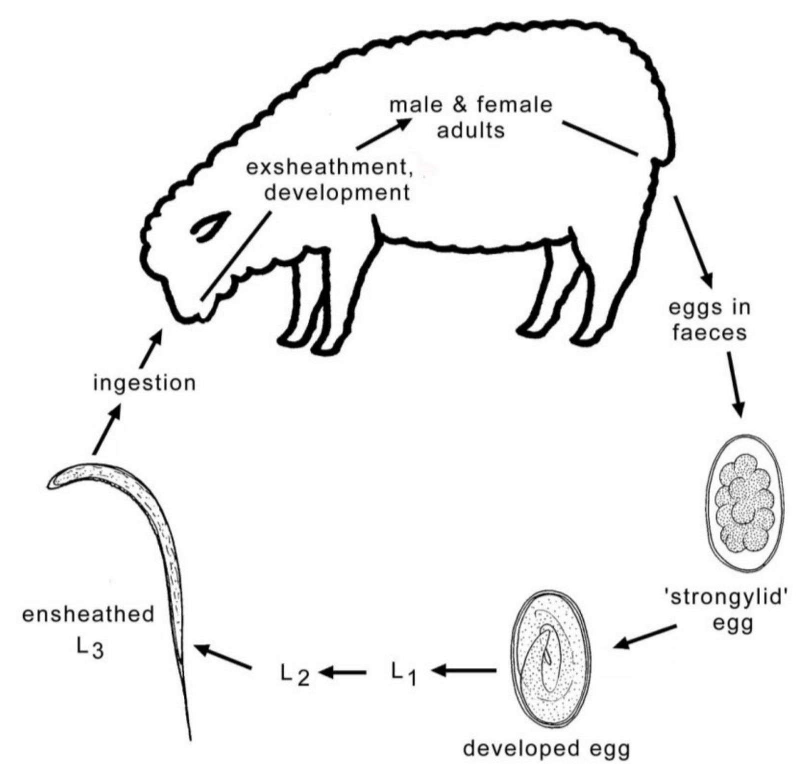

Figure 2.1. Representation of life cycle of gastrointestinal nematodes of small ruminant (M. A. Taylor, Coop, Wall, Coop, & Wall, 2013)

These helminths are known to have a shrewd survival adaptation known as 'hypobiosis'. With the arrival of unfavourable environmental conditions, the larvae undergo a period of arrested

development, the ‘hypobiotic stage’, and do not become active again until the environment is conducive. This is particularly typical for species of *Haemonchus* and *Teladorsagia*. For example, *Haemonchus* larvae generally undergo a hypobiotic stage after the end of the grazing season and resume their activity in the following spring (or autumn in the case of *Teladorsagia*). The re-activation may coincide with the start of lambing season and can result in high Faecal Egg Counts (FEC) during the peri-parturient stage in ewes (Salisbury & Arundel, 1970). This problem is exacerbated by a decrease in the immunity of the host during the peri-parturient stage, increasing the odds for survival and egg production by existing helminths. Further, peri-parturient loss of immunity can increase the susceptibility of the host to further infection when the pasture is already contaminated with L3s (Hungerford, 1990).

In field conditions, almost all livestock are infected with one or more species of these parasites, however, various factors determine the manifestation and severity of clinical symptoms (H. Gordon, 1983). The most important factors are the parasite species, the number of infective stage larvae being ingested, the number of parasites in the GI tract, the overall health and immunological status of the host, as well as various environmental factors such as the climatic condition, farm management, stocking rate, pasture type, stressors in the environment and diet (M. Taylor, 2000). Thus, in general, a heavy worm burden afflicts young non-immune, immuno-compromised adults and individuals ingesting a large number of L3 infective stage from the contaminated environment (Zajac, 2006).

2.1.1 Haemonchus contortus

Haemonchus contortus is commonly known as ‘barber’s pole worm’ because, when fresh, it has white ovaries winding spirally around the blood-filled intestine, resembling a barber’s pole. They are located specifically in the abomasum and are relatively long (2.0-3.0 cm). Males have an asymmetrical dorsal lobe and barbed spicules, while the females usually have a vulvar flap. Both sexes have cervical papillae and a tiny lancet inside the buccal capsule (M. A. Taylor, Coop, Wall, Coop, & Wall, 2007).

2.1.1.1 Life cycle

The lifecycle of *H. contortus* is direct and the pre-parasitic phase is typically trichostrongyloid (M. A. Taylor et al., 2007). They are one of the most fecund helminths, with the females producing thousands of eggs per day that can rapidly contaminate pasture (Cheng, 1981). The eggs hatch L1 and L2 stage larvae in the faeces and the infective L3 stages emerge from the faecal pellet. The L3 larva is encapsulated in a sheath that protects it

from environmental conditions. When ingested by a suitable host, the sheath sheds in the rumen (‘exsheathment’) a process that is very important for the successful establishment of the infection. The details are not perfectly clear, but carbon dioxide in the rumen is believed to induce exsheathment (Nikolaou & Gasser, 2006). After exsheathment, the larvae move further down into the abomasum where they develop into L4, L5 and adult blood-sucking helminths (Florian Roeber, Aaron R. Jex, & Robin B. Gasser, 2013a).The prepatent period (the period from when the host is first infected to the earliest time when they start shedding eggs or larvae in faeces) is 2-3 weeks in sheep and 4 weeks in cattle (M. A. Taylor et al., 2007).

2.1.1.2 Pathogenesis

Adult *H. contortus* are voracious, with each individual draining 0.05 mL of blood every day. With 5,000 adult worms, for example, a sheep can easily loose 250 mL of blood daily, leading to acute haemorrhagic anaemia – the hallmark symptom of ‘haemonchosis’. This acute anaemia becomes apparent after 2 weeks of infection, at which point there is a progressive and sharp fall in packed cell volume (PCV). In the following weeks, the blood marrow tries to compensate by increasing the erythropoiesis 2-3-fold but, along with blood, there is also the continuous loss of iron and protein in the gastrointestinal tract. The animal becomes anorexic and weak, and eventually the bone marrow becomes exhausted, further reducing the haematocrit. Death ensues. When there is a heavy infection of around 30,000 worms, animals can die within one day due to severe gastric haemorrhage. However, the more common and problematic form of haemonchosis is the chronic form, during which a small but persistent burden of several hundred worms leads to a continuous loss of blood that is enough to cause the clinical symptoms, including weight loss, weakness, anorexia, marked anaemia, and overall production loss. Other more physical clinical signs include submandibular oedema and ascites which can be attributed to the protein loss. Unlike most other gastrointestinal parasites, *H. contortus* does not primarily causes diarrhoea (M. A. Taylor et al., 2007).

2.1.2 Teladorsagia species (alternative genus name Ostertagia)

Teladorsagia circumcincta and *T. trifurcate* are the two most important species of this genus. *Teladorsagia* generally inhabit the abomasum and are small (7-12 mm long) with a brownish colour and a short buccal cavity. Females are 9-12 mm long and with vulva close to the posterior end. The females have a vulval flap while the males have slender trifurcated spicules. They are not as fecund as *H. contortus* and produce an average of 100-200 eggs per

day (Cole, 1986b). Unlike *H. contortus, Teladorsagia* do not feed on blood, and the pathological effects are caused by the physical presence of the larvae. The larvae develop in the gastric glands in the abomasum and form nodules that cause extensive damage to the parietal cells, leading to decreased production of hydrochloric acid (McKellar, 1993), and thus an increase the pH in the abomasum. This high pH will affect the conversion of pepsinogen into active pepsin, ultimately hampering protein digestion and causing an increased plasma level of pepsinogen. Factors that affect the severity of infection include concurrent infections, nutrition, and the ability of the host to develop a suitable immune response against the parasite (Stear, Bishop, Henderson, & Scott, 2003).

2.1.3 Trichostrongylus species

Species in this genus include an important group of helminths that reside in the anterior small intestine and generally affect pasture-fed small ruminants. *Trichostrongylus* infections are often asymptomatic but, when sheep are heavily infected (more than 10,000 worms) they present symptoms such as protracted watery diarrhoea. This is most apparent in lambs and weaners, but old stressed sheep in poor condition are also affected. The faeces become dark green and can stain the fleece in the rear of the animal ('black scours'; (Levine, 1968). The most common species in Australia are *T. colubriformis*, *T. vitrinus* and *T. rugatus* (I. Beveridge, A. L. Pullman, R. R. Martin, & A. Barelds, 1989). When infective *Trichostrongylus* L3 larvae penetrate between the enterocytes of the intestinal villi, they form a tunnel beneath the epithelium (I. Beveridge et al., 1989) where young larvae develop and, following rupture of the sub-epithelial layer, are released into the lumen 10 -12 days post infection. When these young larvae migrate, they cause extensive damage leading to generalized enteritis. The hallmarks of *Trichostrongylus* infection are haemorrhage, oedema and loss of protein into the lumen of intestine, leading to hypoalbuminaemia and hypoproteinaemia (Barker & Titchen, 1982). Clinical symptoms, seasonal occurrence and post-mortem findings, including larva counts, are the basis of diagnosis. During post-mortem examination of a heavily infected animal, the lining of the intestine is seen to be inflamed with bloody spots, with excess mucus and enlarged mesenteric lymph nodes (Levine, 1968).

2.1.4 Ascaris suum

The helminth species described above commonly infect grazing ruminants. For monogastric livestock, particularly pigs, *Ascaris suum* is an important helminth. The female of the species are larger (up to 40 cm) than the male (up to 25 cm) and resides in the small intestine of pigs.

Females have high fecundity, producing around 200,000 to a million eggs per day. The eggs are highly resistant and can survive for 5 to 11 years under optimal conditions (Ballweber, 2015). When ingested by a suitable host, these eggs hatches into larvae in the intestine. The larvae then penetrates the intestinal wall and through portal circulation reaches the liver and then the lungs (Roepstorff, Eriksen, Slotved, & Nansen, 1997). After around 10 days of infection the larvae travels up the bronchial tree and are swallowed back while coughing. These larvae matures into adult in the intestine and the host animal would start shedding the parasite eggs approximately 6 to 7 week post infection (Ballweber, 2015).

The clinical signs are generally a manifestation of the physical damage done by the migrating larvae. They causes distinct granulomatous to fibrous lesions in the liver tissue commonly referred to as milk spots (Roepstorff et al., 1997) and acute pneumonia (Lassen et al., 2019). The economic losses are from condemnation of the liver during slaughter and reduced growth in heavily infected pigs (Hale, Stewart, & Marti, 1985; Roepstorff et al., 1997).

2.1.5 Epidemiology

The type and the severity of gastrointestinal helminth infection depends on several factors that can be mainly categorized as:

- Host-related: age, sex, species, immunity, genetic resistance;
- Parasite-related: life history, duration of the histotrophic phase, survival of larvae in the environment, site in the host;
- Environmental: climate, weather, season, type of vegetation, microclimate.

The relationships among the host, parasite and environment factors are illustrated in Figure 2.2.

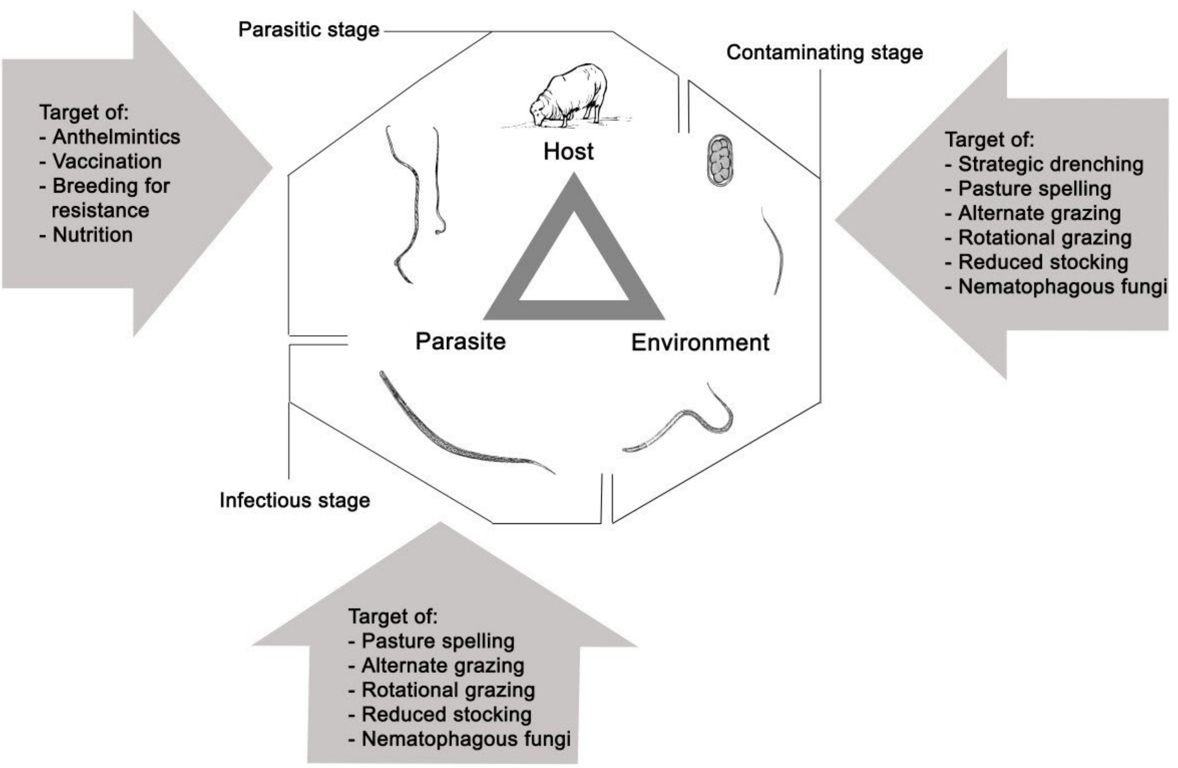

Figure 2.2. Relationships among host, parasite and environment, and factors affecting the parasite control strategy (H. M. Gordon, 1948; Levine, 1968; Donald, Southcott, & Dineen, 1978).

The probability and the pattern of disease occurrence are primarily determined by the interaction between host and parasite, whereas disease transmission is primarily influenced by host-environment and parasite-environment interactions (Levine, 1968). Differences in climate, even within the same region, can play a major role in the epidemiology of helminth infection because every parasite has its own unique ecological needs for the successful development of their free-living stages, particularly temperature and humidity (Cole, 1986a; I. Beveridge, A. Pullman, R. Martin, & A. Barelds, 1989). Although climate plays a major role in the distribution of the parasite, management practices such as anthelmintic treatment and host movement can also influence their prevalence and distribution (H. M. Gordon, 1948). There have been many studies and insights regarding the epidemiology and ecology of nematodes, but this subject is so complex and diverse that there are still considerable gaps in the knowledge in this field. This can be mainly attributed to the lack of accurate diagnostic tools in the past to study the individual parasites (Florian Roeber, Aaron R. Jex, & Robin B. Gasser, 2013b).

2.1.6 Seasonal distribution of helminth infection in Australia

Autumn is commonly the best time for the survival and development of the eggs of most helminth species in Australia because, at that time of year, the temperatures and evaporation rates are generally moderate to low. However, once spring arrives, the rising temperatures increase the development and migration of larvae. In summer, the level of contamination is highly dependent on rainfall, with low rainfall keeping contamination low.

The development of *H. contortus* is mainly restricted to spring, summer (if this season is not too dry) and early autumn. As it cannot tolerate low temperature and desiccation, it is not very common in winter rainfall regions that have long dry summers. On the other hand, *T. colubriformis* is prevalent during summer in summer-rainfall areas and *T. circumcincta* is widespread on pasture in late winter and early spring (H. Gordon, 1983).

2.1.7 Epidemiology of helminths in winter rainfall areas

The southern part of Australia, including Tasmania, most of Victoria, and the southern part of Western Australia, South Australia and New South Wales, is a winter rainfall zone. Accordingly, the major problematic helminths in this zone are *T. circumcincta* and *T. axei*, both of which reside in the abomasum, and the more pathogenic *T. vitrinus* that resides in the small intestine (I Beveridge, AL Pullman, PH Phillips, et al., 1989) and can cause production losses in adult animals and death in weaners. *Chabertina ovina* and *Nematodirus spp*. have also been frequently reported, but they are not considered important because they are not associated with decreased production or death.

The load of infective L3 larvae in the field is correlated to the pattern of rainfall, which peaks in late winter, decreases in spring, and is at the lowest in summer (Donald et al., 1978). Generally, in lambs born in spring and weaned between December and January, the worm burden is low in summer and then increases during autumn because, after the onset of rain, the pasture is generally contaminated with large numbers of infective L3 larvae. However, lambs born in autumn and weaned in August are often heavily infected in late winter and spring, and can carry high worm burdens during their first summer than spring-born lambs (Parnell, 1962). After these sheep have grown older, to around a year, their worm burdens starts to reduce as they develop resistance. Consequently, the worm burden is relatively constant throughout the year in adult animals, although peri-parturient ewes can have a significantly greater burden because their immune system is down-regulated (Donald et al., 1978).

When sheep ingest L3 larvae of *T. circumcincta* during winter, spring and early summer, those larvae enter the hypobiotic stage within the host, and then becomes active in the following summer and early autumn. This phenomenon is particularly important because it can exacerbate the pathogenicity of the parasite at times of nutritional stress, and can restock the adult worm population even during the season when pasture contamination would normally be low (Florian Roeber, Aaron R Jex, & Robin B Gasser, 2013). There is generally a marked increase in FECs during summer and early autumn in sheep in these winter rainfall zones. Similar observations were made by (N Anderson, 1973) who reported a negative correlation between the intake of infective L3 larvae and FEC, with the highest FEC values during summer when larval intake was low. On the other hand, during autumn when pasture L3 contamination was high, low FEC values were recorded.

In conclusion, perhaps, the most important tool in livestock helminths control is our knowledge on the epidemiology of the parasite in the given area. A successful and sustainable parasite control program can only be devised by taking into account the host, parasite and environmental factors. In the following section I will be discussing more on our existing parasite control strategy, its downside and why a better strategy has become so necessary. I will also present our idea and through the experimental section, try to verify them.

2.2. Parasite control

2.2.1 Commonly used anthelmintics

To control gastrointestinal parasitic infection in livestock, the most widely accepted practice until now has been the use of chemotherapeutic anthelmintic medication, commonly known as ‘drenches’ or ‘dewormers’. The first commercially used broad spectrum anthelmintic was Thiabendazole, discovered in 1961, that belongs to a group known as ‘benzimidazoles’. It quickly became popular and widely used because it was more efficient and less toxic than previously available dewormers. In the following years, a variety of substituted benzimidazoles were gradually introduced. This group of drugs attaches to ß-tubulin, thus preventing the polymerisation of microtubules, eventually leading to the disassembly of the existing cytoplasmic microtubule structure in the helminth cells (Lacey, 1990).

Another popular anthelmintic, Ivermectin, came to the market back in 1981. In contrast to the benzimidazoles, this molecule belongs to the group known as macrocyclic lactones which is a highly potent against a broad spectrum of endo- and ecto-parasites. Though the exact

mechanism of action of Ivermectin is less understood, it acts on glutamate-gated chloride channels to increase chloride ion permeability, thus hampering the parasite's motility, feeding and reproduction (Vassilatis et al., 1994). Ivermectin and other macrocyclic lactones have strong markets, not just in the livestock sector, but also in small animal topical applications. They have also been licenced to control canine heartworm (*Dirofilaria immitis*) and are often used in combination with pyrantel to control gastrointestinal roundworm in dogs.

2.2.2 Resistance to anthelmintic medication

In basic terms, resistance means the ability of the parasite to survive a specific dose of a drug that would normally kill that parasite (Besier & Love, 2003). Current control strategies for helminths in livestock rely heavily on anthelmintic drenches drawn from a limited number of chemical groups, and it is inevitable that the parasites will continue to evolve and thus develop resistance to these drugs. In Australia, the first case of helminth resistance to benzimidazole was reported in 1966, only after 5 years of the release of the medication (West, Pomroy, & Leathwick, 2004) and, by 2011, it was evident in almost 90% of the properties across Australia. All three important sheep nematodes, *T. circumcincta*, *T. colubriformis* and *H. contortus*, are now showing varying degrees of resistance to most of the widely used broad-spectrum anthelmintics. Analysis by state has shown that the greatest levels of resistance to macrocyclic lactones in *Trichostrongylus spp* are found in Tasmania and WA (Playford et al., 2014).

With increasing resistance, anthelmintic effectiveness declines, leading to decreases in productivity and increases in mortality, leading to higher doses and more frequent anthelmintic treatments. In the short term, this strategy can minimize clinical signs and production losses (Johnstone et al., 1979) but more frequent and higher doses will expedite the development of resistance (Besier & Love, 2003). Indeed, the most significant factors associated with anthelmintic resistance are excessive frequency, along with under-dosing (Besier & Love, 2003).

The role of excessively frequent treatment in the development of resistance has been recognised ever since recommendations on how to deal with resistance were first proposed (Prichard, Hall, Kelly, Martin, & Donald, 1980) and subsequently backed up by field experimentation (Martin, Anderson, Jarrett, Brown, & Ford, 1982). Frequent anthelmintic treatments close to the time that larvae develop into fertile adults will produce a generation that is more resistant to that treatment. Before resistance had been recognised, researchers

encouraged the suppression of worm burdens by recommending frequent, short-interval, anthelmintic treatments to maximize health and production from sheep (N. Anderson, 1972; Brown, Ford, Miller, & Beveridge, 1985). This might explain the rapid increase in resistance to the benzimidazoles and the macrocyclic lactones during the 1970s and early 1980s (Besier & Love, 2003).

Another significant factor in the development of anthelmintic resistance is the survival of worms subjected to a dose that discriminates between those that are resistant and those that are susceptible. This issue is more critical when the dose used is enough to kill most non-resistant helminths while allowing the survival of some resistant helminths (Smith, Grenfell, Isham, & Cornell, 1999). This principle has been proven through laboratory testing, in which worms treated with low doses of anthelmintics produced highly resistant populations (Ranjan, Wang, Hirschlein, & Simkins, 2002). It is difficult to identify the specific cause for under-dosing in Australia (Besier & Hopkins, 1988) but one plausible reason is underestimation by farmers of the weight of their sheep, compounded by the inaccuracy older drenching guns (Besier & Love, 2003).

2.2.3 Growing voices for alternative strategies

It is now apparent that helminth control in livestock based solely on chemotherapy is not sustainable, and there is increasing interest in alternative control strategies. In addition, the traditional aim of maximal worm control is being challenged (Van Wyk, Stenson, Van der Merwe, Vorster, & Viljoen, 1999) and complete elimination may no longer be feasible if we want to preserve the efficacy of our remaining anthelmintics. One tactic is to focus more on minimising the impact of parasites on animal health and production, rather than a "kill all" strategy. In doing so, we can expect a minor loss of production but that outcome is better than a world full of resistant parasite populations (Besier & Love, 2003).

Within the life cycle of a helminth infection, there are 3 steps where we can disrupt the continuity: i) kill the parasite present in the host; ii) improve the resistance and resilience of the host, and iii) reduce contact between the infective L3 larvae and the host (Torres-Acosta & Hoste, 2008). Alternative control strategies should target all three stages. Some of the non-chemical-based approaches, such as breeding sheep for worm resistance, use of nematophagous fungi, grazing management, vaccines or bioactive plant secondary metabolites, have shown promise, but no individual approach can deliver results comparable to the highly potent chemical anthelmintics. However, long-term sustainable helminth control

can be obtained through an integrated approach, with additive effects combining all of the non-chemical approaches. Even more effective would be an integrated helminth management program specially tailored for a specific production system for a given climatic condition. Success in this endeavour would allow anthelmintics to be reserved for emergencies.

2.3. Bioactive plants to control GI parasites

Humans started domesticating wild species of livestock at least 10,000 years ago, and early on discovered how animal disease can reduce production. Early therapeutics for these animals most probably had humble beginnings with preparations derived from plants. Though unscientific and without any sense of aetiology and pathogenesis, some of these treatments were effective and were passed down through generations as anecdotes in many cultures (Bracegirdle, 1992). With the advent of modern chemotherapy, many of these practices have virtually disappeared. However, with drug resistance and growing public demand for ‘organic’ food, there has been a resurrection in interest on plant compounds (Ingebrigtsen et al., 2001) and a growing need to validate their efficacy by measurement of bioactivity and identification of the active molecules in phytochemical studies.

There are two ways of evaluating the anthelmintic properties of a plant and plant compounds: *in vitro* and *in vivo*. The *in vitro* approach is used in the laboratory for a primary screening of candidates because it is relatively simple, inexpensive, reproducible and rapid (Hoste et al., 2008). *In-vitro* screening has been used successfully to validate the anthelmintic properties of many African and East Asian traditional medicinal plants (Ademola, Fagbemi, & Idowu, 2004; Iqbal, Lateef, Ashraf, & Jabbar, 2004; Kamaraj & Rahuman, 2011), as well as Australian plants (Kotze et al., 2009; Payne, Kotze, Durmic, & Vercoe, 2013). Several factors affect the bioactivity of the plant extract, with the stage of helminth lifecycle being among the most an important. Some plants extracts can affect all the three stages – egg hatching, larval migration, and adult survival. Others affect a specific stage – for example, extracts of *Carica papaya* are toxic to all three stages whereas extracts of *Zanthoxylum zanthoxyloides* are most effective against egg hatching (Hounzangbe-Adote, Paolini, Fouraste, Moutairou, & Hoste, 2005). Another important factor is the type of solvent used for the extraction – for some plants, tannins were better extracted by ethanol than by water; in other species, anthelmintic activity was limited to a water extract (Kotze et al., 2009). Thus, in planning *in vitro* experiments on bioactive plants, it is essential to consider both the stage of lifecycle of the parasite of interest and the type of solvent.

The *in vivo* approach to evaluating candidate plants for anthelmintic activity involves the use of host animals. There are two major alternative types of *in vivo* study. First, experimental animals can be treated orally with a known amount and concentration of an extract – for instance, (Ademola et al., 2004) tested whether an oral dose of an ethanolic extract of *K. senegalensis* affected FEC in sheep; similarly, (Gogoi & Yadav, 2016) used the same approach to test the effect of leaf extract of *Caesalpinia bonducella* on pinworm *Syphacia obvelata* which was experimentally infected in albino mice. In the second type of *in-vivo study*, host animals are allowed to graze pasture containing the anthelmintic candidate plant – for example, using this approach, (Marley, Cook, Keatinge, Barrett, & Lampkin, 2003) found the birdsfoot trefoil (*Lotus corniculatus*) has anti-parasitic activity in sheep. One of the difficulties with a grazing experiment is variation – feeding preference will vary among individual animals, affecting intake, and individual plants will vary in chemical composition. Consequently the results can be inconsistent because the dose of anthelmintic compound is not well controlled. This issue probably explains why *Lotus corniculatus* was anthelmintic for grazing sheep (Marley et al., 2003) but not in the study by (Niezen, Robertson, Waghorn, & Charleston, 1998).

2.4. Plant secondary metabolites (PSM)

Plants synthesise a vast array of organic compounds and they are generally classified as primary compounds and secondary compounds (or metabolites), a distinction probably developed by Albrecht Kössel (1891). Primary compounds are directly involved in essential plant functions such as photosynthesis, respiration, growth and development. Secondary compounds refers to the numerous compounds which do not have a direct role in essential plant biochemical processes (Hartmann, 2007). More than 200,000 secondary compounds, of great diversity, have been identified and they fulfil a wide range of eco-physiological roles (Wink, 2016). Some secondary metabolites produce attractive colours in the petals (e.g. carotenoids, anthocyanins), or fragrances (e.g., terpenes, amines) to attract pollinators. Others confer certain colours, fragrances or tastes to fruits to attract animals to eat and disperse the seeds (Geilfus, 2019).

Some of secondary metabolites are always present in the plant whereas others are only produced in response to an abiotic stress (*e.g*., drought), when their production and concentration increase. Plants can also produce when they are physically damaged, for example by an attack by invertebrate pests or on grazing or pruning (Harbone, 1991).

Substantial resources are needed to produce these secondary compounds (Wink, 2011) so they are usually found in small concentrations – well below 1% of the dry weight of plant material (Akula & Ravishankar, 2011). Most of the time, several secondary metabolites work synergistically against the stressor, making it difficult for the pathogens to develop a chemical resistance against them (Wink, 2015).

We can classify secondary compounds based on their biosynthetic origin into three major groups; phenolics, terpenoids and nitrogen containing alkaloids (Goyal, Joy, & Rasul Suleria, 2019) as illustrated in figure 2.3.

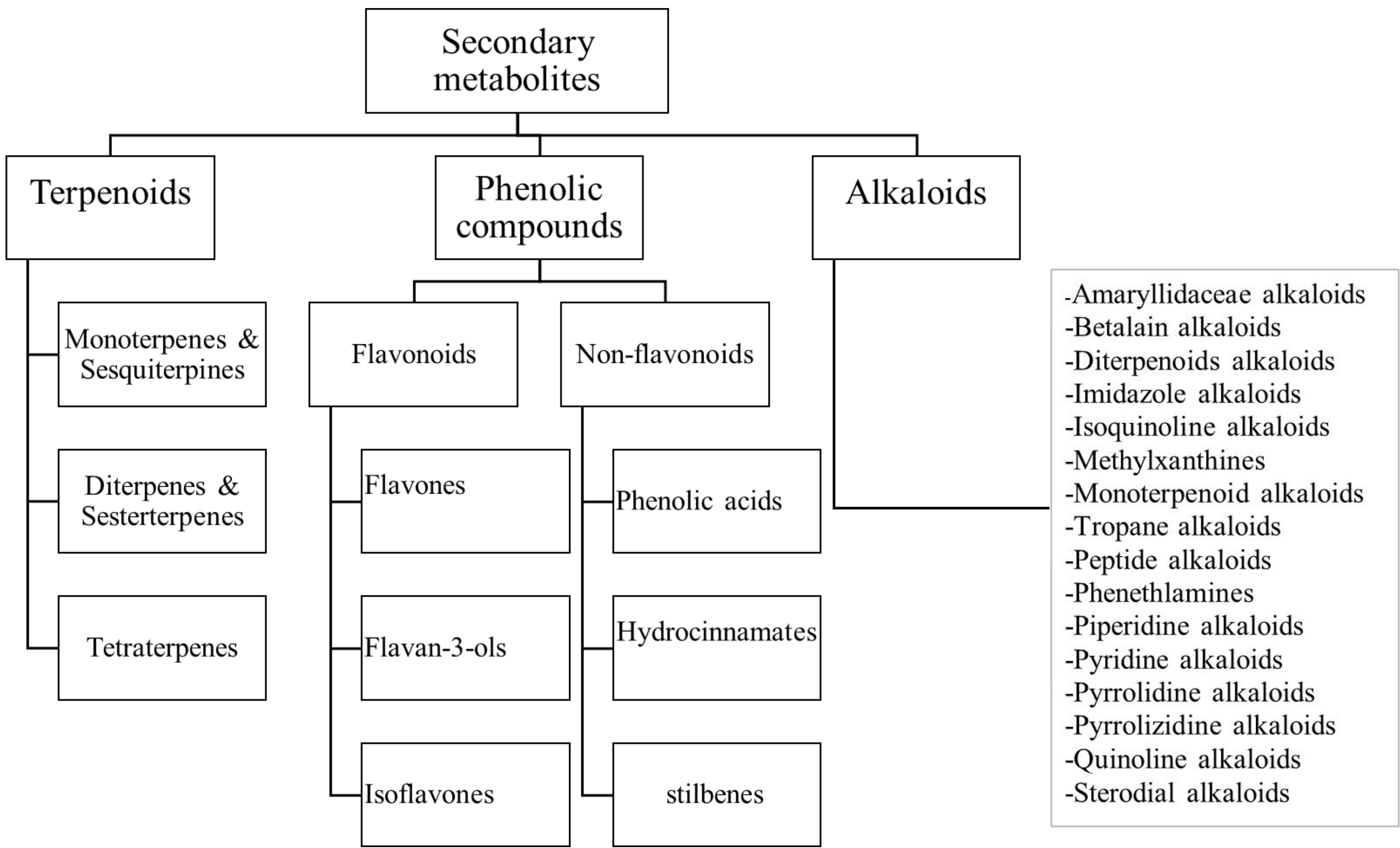

Figure 2.3. Classification of plant based secondary metabolites (Goyal et al., 2019).

2.4.1 Phenolics

Phenolics are one of the largest groups of secondary compounds and all of them have at least one aromatic ring with one or more hydroxyl groups attached. They can be single, low molecular weight compounds, or larger and complex molecules such as the tannins. They are usually classified on the basis of the numbers and arrangement of the carbon atoms (Table 2.1), and are commonly found conjugated to sugars and organic acids (Crozier, 2003). Phenolics are often further classified into subclasses – the flavonoids, coumarins, quinones, isoflavonoids, stilbenoids and tannins (Harborne, 1999).

Table 2.1. Structures of phenolic and polyphenolic compounds (Crozier, 2003).

Carbon No.	**Skeleton**	**Classification**	**Example**	**Basic structure**
7	C_6-C_1	Phenolic acid	Gallic acid	COOH
8	C6-C2	Acetophenones	Xanthoxylin	O O CH$_3$
8	C6-C2	Phenylacetic acid	p-Hydroxyphenyl acetic acid	COOH
9	C_6-C_3	Hydroxycinnamic acids	Caffeic acid	COOH
9	C_6-C_3	Coumarins	Esculetin	O O
10	C_6-C_4	Naphthoquinones	Juglone	O O
13	C_6-C_1-C_6	Xanthoes	Gentisin	O O

14	C_6-C_2-C_6	Stilbenes	Resveratrol	
15	C_6-C_2-C_6	Flavonoids	Quercetin	

Amongst all phenolics, tannins and flavonoids are the most important and widely studied, so they will be the focus of further discussion.

2.4.2 Tannins

Tannins are a diverse group and one of the most abundant polyphenolic compounds in plants. They can form complexes with proteins and precipitate them (Hagerman & Butler, 1978). They have received huge attention and are probably the most studied compounds in relation to animal production and nutrition (Waghorn, 2008).

Tannins can be broadly divided into:

a) Hydrolysable tannins (HT) that generally consist of a polyol core, mostly a D glucose, which is esterified with mainly gallic acid (Haslam, 1989); they can be easily hydrolysed by acids or bases, and degraded and absorbed in the gut, potentially causing toxicity in ruminants (McLeod, 1974);
b) Condensed tannins, or proanthocyanidins, are made of oligomers or polymers of flavin-3-ol subunits (Haslam, 1989) and are the most commonly found tannins in forage legumes and plants; they are not as easily hydrolysed as HT, and require a strong oxidative and acidic hydrolysis; they are not susceptible to anaerobic enzyme degradation (McSweeney, Palmer, McNeill, & Krause, 2001) so they can stay intact in the gut of ruminant livestock; due to their ability to bind protein, they can protect dietary protein from ruminal degradation, thus increasing the availability of protein in the lower digestive tract and offering nutritional benefit to ruminants; however, when concentrations of CT exceeds 7% of dry matter (DM), there can be a severe decrease in feed intake and an overall decrease in production (Hoste, Jackson, Athanasiadou, Thamsborg, & Hoskin, 2006).

2.4.3 Flavonoids

Flavonoids are the first class of polyphenols and are water soluble pigments generally found in the vacuoles of plant cells. They are widely distributed in plants and have many functions, with the most important being attraction of pollinators by provision of various colour pigments in flowers and petals (Justin et al., 2014). In recent times, flavonoids have become notorious for offering benefits to human health, especially after being associated with red wine. Red wine contains high levels of flavonoids (mostly quercetin and rutin) that are known to reduce the development of atherosclerosis by preventing the oxidation of low-density lipoprotein (Mink et al., 2007). Some authors suggest that this might be the reason for longevity and low instance of coronary heart diseases in French people (who consume more wine) compared to other Europeans (Justin et al., 2014). In the livestock sector, flavonoids had a bad reputation, being associated with decreased growth and a reduction in fertility because many of them have oestrogenic properties (Dakora, 1995). On the other hand, their antioxidant, antimicrobial and other bioactivities can improve livestock performance, and the quality of milk and meat (North, Dalle Zotte, & Hoffman, 2019; Li et al., 2020). Some are thought to reduce methane production in ruminants (Oskoueian, Abdullah, & Oskoueian, 2013). Flavonoids are also known to have anthelmintic properties against major livestock parasites – for examples, extracts rich in phenolics and flavonoids from *Solanum nigrum* L. (Saddiqe, Maimoona, & Khalid, 2013) and *Onobrychis viciifolia* (Barrau, Fabre, Fourasté, & Hoste, 2005) showed significant activity against *H. contortus* in sheep.

2.4.4. Terpenes and terpenoids

Terpenes and terpenoids are among the most abundant plant secondary products and have diverse structures. Terpenes are simple hydrocarbons and are made up of five carbon isoprene units, assembled in numerous ways. Terpenoids are mostly modified forms of terpenes with different functional groups and an oxidized methyl group that is in various locations, or completely removed. They can be further classified on the basis of the number of carbon units (presented in figure 4) into monoterpenes, sesquiterpenes, diterpenes, sesterpenes and triterpenes (Shagufta, 2018). Terpenes and terpenoids generally serve roles in a variety of specialized chemical interactions that protect plants from biotic and abiotic factors. Commercially, terpenoids are widely used as flavours, fragrances, and spices, as well as in cosmetics (Roba, 2021). They are known to have antitumor (Andrade et al., 2015), anti-inflammatory (Bi et al., 2017), antibacterial (Banerjee, Parai, Chattopadhyay, & Mukherjee,

2017), antiviral (Armaka, Papanikolaou, Sivropoulou, & Arsenakis, 1999), antimalarial (Creek et al., 2008) and many other biological activities (Yang et al., 2020).

C_{10} Monoterpenes (2 isoprene units)

C_{15} Sesquiterpenes (3 isoprene units)

C_{20} Diterpene (4 isoprene units)

C_{25} Sesterpenes (5 isoprene units)

C_{30} Triterpenes (6 isoprene units)

C_{40} Tetraterpene (8 isoprene units)

Figure 2.4. Classification of terpenes (Shagufta, 2018).

2.4.5. Khaya senegalensis

Khaya senegalensis, commonly known as African mahogany, is a member of the *Meliaceae* family that comprises 51 genera and around 1400 species. The *Khaya* family, the most popular and commercially important genus, is valued for a hard timber that is resistant to insect and fungal infection (Adesida, Adesogan, Okorie, Taylor, & Styles, 1971). African mahogany was probably introduced into Australia by colonists for planting around streets and parklands. In the 1950s, The Commonwealth Scientific and Industrial Research Organisation (CSIRO) decided to conduct a timber trial and established a plantation near Darwin. The trees were also included in a mine rehabilitation program in Cape York during early 1970s. More recently, there has been lot of interest from farm forestry groups and state government organizations, and Managed Investment Schemes (MIS) has even proposed a large scale plantation (Armstrong, 2007).

In Africa, where *K. senegalensis* is endemic, its therapeutic properties have been exploited by many communities, especially for the treatment of fever and malaria, and, in cattle, the treatment of diarrhoea, ulcers, and gastrointestinal helminths (Iwu, 2014). An extract of the plant has been shown to have anti-bacterial, anti-inflammatory and anti-cancer effects (Lompo, Nikièma, Guissou, Moës, & Fontaine, 1998; Androulakis et al., 2006). Other studies have shown that extracts from *K. senegalensis* also have anthelmintic potential in small ruminants with an oral dose of 500 mg/kg reducing faecal egg count (FEC) by about 90% (Ademola et al., 2004). For *Haemonchus contortus*, steam extracts of *K. senegalensis* were more potent than other plants tested (China, Attindehou, Gbngboche, & Salifou, 2016).

Several studies have investigated the chemical composition of *K. senegalensis* – for example Wakirwa et al. (2013) demonstrated the presence of saponins, flavonoids, tannins and alkaloid glycosides. A summary of known secondary metabolites and potential therapeutic used is presented in Table 2.2.

Table 2.2. Therapeutic uses of Khaya senegalensis and detected bioactive molecules. Compiled by (Rabadeaux, Vallette, Sirdaarta, Davis, & Cock, 2017).

Therapeutic uses	**Plant product**	**Secondary compounds**	**References**
Antioxidant	Stem bark extract	Not determined	(Androulakis et al., 2006)
Antifungal	Fruit extract	Limnoids including seneganolides	(Androulakis et al., 2006)
Antiseptic	Root, stem, bark extract	Phenolics, Flavonoids, saponin, terpenoids	(Kubmarawa, Khan, Punah, & Hassan, 2008; Makut, Gyar, Pennap, & Anthony, 2008)
Anti-inflammatory	Stem bark extract	Not determined	(Androulakis et al., 2006)
Anti-cancerous	Stem bark extract	Limonoids	(Zhang, Wang, Chen, Androulakis, & Wargovich, 2007)
Anti-malarial	Leaf, seed and stem bark extract	Terpenoids	(Umar, Ibrahim, Fari, Isah, & Balogun, 2010)
Anti-*Trypanosoma brucii* (sleeping sickness)	Stem bark extract	Not determined	(Ibrahim, Njoku, & Sallau, 2008)
Anti-Leishmaniosis	-	Dimeric proanthocyanidins	(Kayser & Abreu, 2001)
Against Diabetes	Aqueous stem bark	Glibenclamide	(Kolawole, Kolawole, Ayankunle, & Olaniran, 2012)
Immunomodulatory		Dimeric proanthocyanidins	(Kayser & Abreu, 2001)

2.5. Research objectives

The overall objective of this research was to isolate and identify bioactive secondary compounds in *Khaya senegalensis* that have potential for managing livestock helminths. The specific aims were:

- Isolate and identify compounds in *K. senegalensis* with maximum anthelmintic activity;
- Measure the anthelmintic efficacy of these active compounds.

The general hypothesis tested in this thesis was that *K. senegalensis* contains bioactive compounds which have anthelmintic properties against livestock gastrointestinal parasites, with the specific hypotheses detailed in the introduction of each experimental chapter.

To attain these objectives, we followed a process called 'bioassay-guided fractionation' in which the complex mixture of compounds in an extract of plant material is separated into fractions that are then screened for anthelmintic activity using an *in vitro* larval mortality assay. The most active fractions are further separated and re-assayed. This process is repeated several times until the compound(s) responsible for the bioactivity are sufficiently pure to be identified.

A flowchart of the planned experimental process is depicted in Figure 2.5.

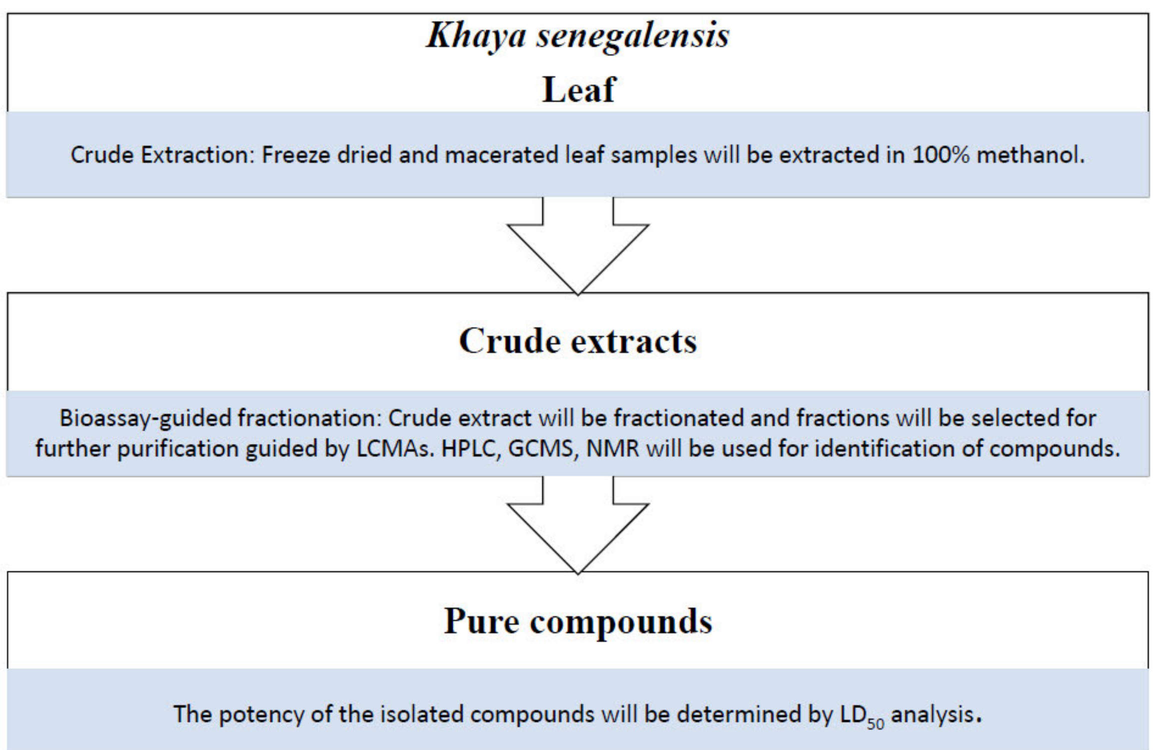

Figure 2.5. Flow chart of the experimental design (LMAs: Larval Mortality Assays, HPLC: High Pressure Liquid Chromatography, GCMS: Gas Chromatography Mass Spectrometry, NMR: Nuclear Magnetic Resonance and LD50: Lethal Dose 50).

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CHAPTER 3: GENERAL MATERIALS AND METHODS

3.1 Experimental plan

The experiments described in this thesis were designed to test the general hypothesis that *Khaya senegalensis* produces bioactive secondary metabolites with anthelmintic activity that could be applied in the livestock industries.

We conducted a stepwise experimental process, beginning with extracts from the leaf of *K. senegalensis* that were fractionated, with the fractions subsequently tested for anthelmintic activity by measuring the *in vitro* mortality of the third stage larvae of the common pig parasite, *Ascaris suum*. The active fractions were then further separated and purified, with progress at each stage monitored using the *in vitro* bioassay. After repeating this process, we were able to refine the extract down to a single compound, or a group of similar compounds, that could be isolated and subjected to structural elucidation. Finally, the pure compounds, individually and in combination (to detect synergistic activity), were tested over a range of concentrations to determine their anthelmintic efficacy.

The materials and methods that are common to the experiments are provided below, whereas more specific details are provided in the experimental chapters.

3.2 Plant materials

The plant material was collected from a plantation owned by Bioactive Solutions Pty Ltd (Floreat, WA) in the dry, tropical far-north-western region of Australia (Fig 3.1). From trees that had been planted in 1998, leaves were harvested with extreme care, sealed in an airtight plastic bag and transported to University of Western Australia. The material was immediately lyophilized using a VirTis BenchTop Pro with Omnitronics 9L (SP Scientific, Warminster, PA) and milled to make a course powder using a cyclone grinder (CYCLOTECH 1093 sample mill; Tecator, Hoganas, Sweden) fitted with a screen of 4 mm. It was milled again to a finer powder using a 1 mm screen. The powdered plant products were stored in air-tight containers at –20 °C until further use.

Figure 3.1. The region of Western Australia where the sample was collected.

3.3 Crude extraction

Crude extraction is the first and one of the most important steps in fractionation, with the main objective of obtaining soluble plant metabolites while leaving behind insoluble residues (Nn, 2015). Many factors determine the solvent to be used, and we chose 100% methanol because it had been used in previous studies that successfully detected anthelmintic activity in *K. senegalensis* (Ademola, Fagbemi, & Idowu, 2004). The resulting extract was then filtered through Whatman No 1 filter paper and the filtrate was dried under reduced pressure in a water bath using a rotary evaporator (Buchi, R-210) at a temperature not exceeding 40 °C.

3.4 Chromatography

3.4.1 Rapid silica filtration (RSF)

The dried extracts were reconstituted using a minimal amount of methanol and put through a vacuum assisted silica column of internal diameter 4 cm and height 8 cm (Davisil chromatographic silica media LC60A 40-63 µm, Grace Discovery Sciences). The column was loaded with 200 mL of hexanes initially and then eluted with 200 mL volumes of increasing concentrations of ethyl acetate (in hexane, v/v) at 20%, 40%, 60%, 80% and 100%. This was followed by increasing the concentration of methanol in ethyl acetate from 10% to 100%. Thus, 10 fractions were obtained from the leaf extract. They were dried under reduced pressure in a water bath at a temperature not exceeding 40 °C and stored at 4 °C in glass vials with the headspace flushed with nitrogen gas.

3.4.2 Scaled up flash silica chromatography

Scaled-up flash silica chromatography was done using the Reveleris X2 chromatography system, with silica gel (Silica 40µm, 80 g, p/n 145146133, Reveleris, BUCHI) used as the stationary phase.

3.4.3 High Performance Liquid Chromatography (HPLC)

Semi-preparative and analytical HPLC was performed using either an Agilent 1200 HPLC system with a diode array detector (DAD) and fraction collector, or using a Hewlett-Packard 1050 equipped with a DAD and Pharmacia Biotech RediFrac fraction collector. Analytical work was conducted using an Apollo C18 reversed-phase column (250 mm × 4.6 mm, 5 µm, Grace Discovery Sciences) with a flow rate of 1.0 mL/min, and semi-preparative HPLC was undertaken with an Apollo C18 reversed-phase column (250 mm × 10 mm, 5 µm, Grace Discovery Sciences) with a flow rate of 4.0 mL/min.

3.5. Parasitological techniques

3.5.1 Source of parasite eggs

Gravid *Ascaris suum* females were collected from the intestine of pigs at a slaughter house (Danish Crown, Ringsted, Denmark). An incision was made on the ventral part of the helminth to expose the 5-7 cm-long uterus that was then excised at the terminal, allowing the eggs to be obtained by gentle massaging. The eggs were then placed in a petri dish containing a sodium hypochlorite (1-15%) and water, diluted at the ratio of 1:29. To remove the outer proteinaceous shell, the eggs were suspended in a 0.5N NaOH solution, stirred and left to sit

for 15 min. After the eggs had settled, the supernatant was discarded and replaced with fresh NaOH. This treatment was repeated twice or thrice until the 'cotton ball'-like appearance of the egg had disappeared. The de-coated eggs were then washed with deionized water and centrifuged at 1500 rpm for 10 min; this process was repeated 3 times. The de-coated eggs were then suspended in 0.1N H_2SO_4 and diluted to a concentration of around 25 eggs/µL. The egg suspension was transferred to a flask and incubated at 25 °C in an incubator (INCU-LINE 68R:390-0728, VWR) for 6 weeks. During incubation, every 2 or 3 days, the flasks were removed from the incubator, opened and allowed to sit at room temperature for 3 min. After 6 weeks, the eggs were checked under a microscope and, when most of them were embryonated, they were store in refrigerator at 5°C until further use.

3.5.2 Egg hatching

Egg hatching was initiated using the procedure described by Bonde et al. (2021). Briefly, 10 mL of egg suspension was placed into a 15 mL falcon tube and washed thoroughly in Hank's Balanced Salt Solution (HBSS; Thermofisher Scientific, Denmark), followed by centrifugation at 500 G for 2 min. The supernatant was discarded and the process was repeated 4 times to ensure removal of all H_2SO_4. The egg pellet was then pipetted into a conical flask containing a magnetic spinning bar. Glass beads (2 mm diameter) were added to cover the highest point of the magnet, and the flask was covered with aluminium foil and placed on a magnetic stirrer inside a CO_2 incubator (10% CO_2) at 37°C. The contents were slowly agitated for 40 min to mimic the churning and grinding motion of the pig intestines and thus promote egg hatching. The contents of the flask were then checked using an inverted microscope to verify that most eggs had been hatched. A Baermann funnel was set up under a 20 µm sieve and the contents of the flask were poured into the sieve. The funnel was flooded with HBSS containing penicillin, streptomycin and amphotericin B to inhibit bacterial and fungal growth. The top of the Baermann funnel was sealed with plastic wrap and the funnel was then placed inside the CO_2 incubator overnight to allow the larvae to migrate to the bottom of Baermann funnel.

3.5.3 Larval collection

Larvae were harvested using a 5 mL pipette and placed in a Falcon tube containing Roswell Park Memorial Institute (RPMI) 1640 Medium with 2 mM L-glutamine (Sigma, Denmark) and infused with 100 U/mL penicillin and streptomycin. The suspension was centrifuged at 500 G for 2 min and the supernatant discarded. The process was repeated three times to wash

the larvae thoroughly. The required concentration of 100 larvae per 150 µL was achieved by determining the average number of larvae in 5 µL drops under a microscope and then diluting the solution with RPMI medium as needed.

3.5.4 Larval mortality assay (LMA)

The assay was conducted in 96-well (flat bottom) plates with each well containing 150 µL of adjusted larval solution and 1.5 µL of the fraction/compound to be tested. For negative controls, 1.5 µL dimethyl sulfoxide (DMSO) was analysed in duplicate. The plates were sealed and incubated at 37 °C in a CO_2 (10%) incubator. After 24 h, live and dead larvae in each well were counted. Larvae that were motile and curled were considered alive, whereas straight and stationary larvae were considered dead. Counting was limited to 15 min periods because the larvae die if left too long outside the incubator, so plates were replaced inside the CO_2 incubator for at least 15 min before the counting resumed. If enough activity was not observed during 24 h, the plates were counted again after 48 h. Percentage survival for each well was calculated as alive/(alive+dead) ×100 and the result corrected according to the negative controls to acquire the mortality rate:

$$Mortality\ rate = \left(1 - \frac{survival\%\ of\ larvae\ exposed\ to\ extract}{survival\ \%\ of\ negative\ control}\right)$$

The LD_{50} of the isolated compounds were determined using 2-fold serial dilutions, performed in triplicates.

3.6 Statistical methods

Prism 9 statistical software (GraphPad Software, CA, USA) was used to calculate the mean and standard deviation, and for calculating the LD_{50} of isolated and test compounds, using non-linear regression.

3.6.1 Synergistic effects

Synergy between the two isolated compounds was also tested in the LMA. The least potent compound was tested at a series of 2-fold serial dilutions with the second compound being added at a concentration equivalent to its LD_{20}. The LD_{20} was calculated based on the LD_{50} curve using the equation:

$$LD_{20} = \left(\frac{20}{100 - 20}\right)^{1/_{Hill\ Slope}} \times LD_{50}$$

A predicted additive effect curve was calculated using an equation as described below. The two compounds were assumed to have a synergistic effect if the mortality of the larvae in the LMA was above the predictive additive curve.

$$Predicted\ additive\ effect = \left(1 - \frac{mortality\ of\ test\ larvae\ exposed\ to\ compound\ x}{100}\right) \times \left(1 - \frac{LD_{20}\ of\ compound\ Y}{100}\right) \times 100$$

3.7. Preliminary work

Initially leaf and bark samples were collected from eight different trees. These materials were separately lyophilized and milled to make course powders using a cyclone grinder fitted with a screen of 4 mm. They were milled again to finer powders using a 1 mm screen. The powders were stored in air-tight containers at –20 °C until further use.

To study the effects of the extracts on helminths, I needed a parasitological bioassay based on viable eggs from two specific helminths that infect sheep. These eggs must be collected directly from the rectum. The problem is that Western Australia is a winter rainfall area so winter is the season of helminth infection, and the parasite load is therefore low during summer. I would therefore only be able to perform parasitological experiments during winters, an impractical limitation, especially for a PhD project.

I therefore began my project by working on the antimicrobial activity of the plant material using field strains of *Salmonella typhimurium* and *Escherichia coli* that were sourced from Department of Primary Industries and Regional Development (DPIRD) and maintained in culture at CSIRO microbiology laboratory in Floreat.

Methanol extracts of the leaf and bark from all 8 trees were subjected to agar-well diffusion assays using *S. typhimurium* and *E. coli*. Unfortunately, no zone of inhibition was observed. We then fractionated the crude extracts, hoping that this process would purify and concentrate any active molecules and thus present a strong zone of inhibition. We also used thin-layer chromatography to identify the best combination of solvents for the fractionation process. Using a rapid silica filtration technique, we obtained 10 unique fractions from each crude extract of bark and leaves. The dozens of resulting fractions were also subjected to HPLC to obtain a chemical profile, and they were all subjected to agar well diffusion tests.

Again, no zone of inhibition was observed even with repeated runs. We tried fractions from some other trees, but there was no inhibition.
By this stage, we had already performed antibacterial bioassays for 10 fractions each from leaf and bark of a single tree, against two strains of bacteria, a total of 120 treatments, in triplicate. Each bioassay also included control and standard wells, so management of the testing process had become massively challenging. We therefore decided to run all the fractions from the other seven trees through HPLC and compare the individual chemical profiles with those from the tree one that had been bioassayed and shown to have no antimicrobial activity. The HPLC profiles did not differ significantly among the trees.
We thus concluded that none of our plant samples would show any significant antimicrobial activity. I also managed to obtain some samples from the same plant from the same plantation that had been collected years earlier by another researcher, so I was able to test whether there was a change in activity with time. Interestingly, those samples showed a significant zone of inhibition whereas my samples showed none. Moreover, HPLC analysis revealed differences in chemical composition between the older samples and my own.
Many explanations come to mind – for example, perhaps the trees were going through some kind of stress during the earlier sampling period and thus produced bioactive molecules. However, with no solid information on which to base pursuit of the phenomenon, and with insufficient older plant material to support further investigation, we decided to abandon our investigation of potential antimicrobial activity. It was clear that that *Khaya senegalensis* can have antimicrobial activity but, despite a massive amount of work, we had insufficient data for publication and the time constraints of a PhD project were beginning to weigh heavily.
All was not lost because the HPLC profiles of the fractions showed that the eight trees were effectively identical, allowing us to use the material from a single tree in our subsequent parasitological work.

3.8. Contributions from collaborators

The nature of project was such that a significant proportion of the work required deep knowledge and skills in analytical chemistry. I had some background in chemistry but clearly needed high-quality collaborators so we teamed up with Dr Gavin Flematti and Samuele Sala at the School of Molecular Sciences at UWA. As the project progressed, they trained me in the basics and, by the end of my PhD work, I was confident enough to perform all of the fractionation procedures and to run the HPLC and GCMS. Thus, I performed all of the

preparatory ground work, from sample preparation through to thin-layer chromatography and rapid silica filtration, and I did much of the HPLC and GCMS (with some help on the analysis and interpretation of the outputs). Importantly, procedures such as LCMS and NMR required deep knowledge and skills in analytical chemistry, and were performed by our collaborating chemists who are, of course, going to be co-authors on the publications.

With respect to bioassays, I performed all of the microbiological work that, unfortunately, is not included on the thesis. For parasitological bioassays, we chose to collaborate with the veterinary parasitology laboratory at the University of Copenhagen in Denmark because they had a long history with a well-established, high-quality assay based on *Ascaris suum*. I travelled to Copenhagen with my samples to learn the technique and do the assays. *A suum* is an ideal helminth for the bioassay because large numbers of eggs can be harvested from adult females collected from the intestine of pigs at the slaughterhouse, stored for long periods at 4°C, and then hatched when needed to provide fresh L3 larvae for the assay. This system overcomes the need to maintain a herd of experimentally infected animals as a source of larvae.

I screened the initial fractions I carried to Copenhagen myself using this larval mortality assay and mastered the technique. However, the pandemic intervened and the later fractions and purified compounds were shipped to the University of Copenhagen to be assayed by Associate Professor Andrew R Williams and his team. On the other hand, all of the parasitological data were analysed, interpreted and written up by me with some help from my supervisors and collaborators.

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CHAPTER 4: EXPERIMENTAL CHAPTER

This chapter has been formatted for submission to *Veterinary Parasitology* [Elsevier].

Fatty acids isolated from leaf extract of *Khaya senegalensis* have anthelmintic activity against the gastrointestinal parasite of livestock *Ascaris suum*.

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Abstract

This study aimed to isolate and identify the bioactive compound(s) responsible for the anthelmintic properties of the plant, Khaya senegalensis. *Crude methanolic extracts of leaf material were separated through a process of bioassay-guided fractionation, using a bioassay based on mortality of* Ascaris suum *larvae* in vitro. *The outcome was isolation of fractions that had good anthelmintic activity and the identification of linoleic acid (LD_{50} 496 µg/mL) and oleic acid (LD_{50} 1562 µg/mL) as the major anthelmintic compounds using gas chromatography-mass spectrometry. This therapeutic potential, combined with the health benefits of unsaturated fatty acids, suggests that* Khaya senegalensis *could be useful supplement in livestock production.* In vivo *work should now proceed to confirm the value of these compounds in a production system.*

4.1. Introduction

An increase in the demand for high quality animal protein has placed immense pressure on livestock production and, according to some commentators, is expected to double by 2050 (Rojas-Downing, Nejadhashemi, Harrigan, & Woznicki, 2017; Thornton, 2010) as we attempt to feed the projected population of around 10 billion humans (UNDP, 2019). To reach this target, the industry will have to develop smart solutions to existing animal health and production constraints. One of the major constraints is gastrointestinal (GI) parasites (helminths) because, worldwide, they can cause heavy production and economic losses. In Australia, for example, it is estimated that the cost of dealing with parasitic diseases in sheep and cattle is in excess of Au1 billion dollars annually (McLeod, 1995).

Livestock helminths have been controlled for several decades using synthetic anthelmintic drugs that were initially very effective but are now becoming susceptible to extensive and indiscriminate resistance (Kaplan, 2004). Even with strategic management of treatments, they are only partially effective, yet they still attract significant costs to producers. Moreover, drug

residues in animal products intended for human consumption are a growing issue for consumers. All of these issues have led to a need to find novel ways to combat livestock helminths whilst avoiding synthetic chemicals, providing industry solutions that are clean, green and ethical.

A sustainable alternative is using plants with anthelmintic properties, several of which have been recognized and used by various communities around the world, as documented in many studies (Athanasiadou & Kyriazakis, 2004; Ingebrigtsen et al., 2001; Payne et al., 2018). One such plant is *Khaya senegalensis*, also known as African mahogany. When an ethanolic extract of *Khaya senegalensis* was administered orally to sheep, it reduced faecal egg count (FEC) by 90% (Ademola, Fagbemi, & Idowu, 2004). In another study with sheep, a methanolic extract was found to be a potent anthelmintic against *Haemonchus contortus* (China, Attindehou, Gbngboche, & Salifou, 2016). Anthelmintic bioactivity in plants is linked to the presence of plant primary or secondary metabolites (PSM), some of which (eg, the tannins) have been isolated and identified (Payne et al., 2018), but little is known of the PSMs that play such roles in *K. senegalensis.*

To investigate the anthelmintic properties, plant material can be analysed by bioassay-guided fractionation in which crude extracts are separated into fractions using chromatography techniques followed by assessment of the resulting fractions using an *in-vitro* bioassay based on helminth mortality. One established bioassay uses *Ascaris suum*, a helminth that causes world-wide economic loss in pigs due to liver condemnation, altered carcass composition, and reductions in feed conversation rate (Nansen & Roepstorff, 1999) (Thamsborg, Nejsum, & Mejer, 2013). *A. suum* also closely resembles a highly prevalent human helminth, *Ascaris lumbricoides* (Brooker & Pullan, 2013), for which it is widely used as a model (Midha et al., 2018; Shao et al., 2014; Timm et al., 2016). Although it would be ideal for a larval mortality assay to be based on a helminth that infects the livestock species of interest, not all helminth species are suited to *in-vitro* management. *A. suum* offers the major advantage that large numbers of eggs can be collected from adult helminths at abattoirs, stored for long periods at 4°C, and then hatched when needed to provide fresh L3 larvae or the assay. This system overcomes the need to maintain a herd of experimentally infected animals as a source of larvae.

Therefore, the present study uses bioassay-guided fractionation, with bioassay based on *A. suum* larval mortality, to test whether i) an extract of *K. senegalensis* leaf has anthelmintic

activity and ii) the compound(s) responsible for that activity can be purified, isolated and identified.

4.2. Materials and Methods

4.2.1 Experimental design

Bioassay-guided fractionation was used to process a crude extract of *K. senegalensis* leaf. Rapid silica filtration (RSF), Reveleris flash chromatography (RSC) and semi-preparative High Performance Liquid Chromatography (HPLC) were used to separate compounds and Gas chromatography-Mass spectrometry (GC-MS) was used for identification of individual compounds. All of the chemistry was performed at the University of Western Australia whereas the parasite bioassay was performed at the University of Copenhagen.

4.2.2 Plant material

Leaves of *K. senegalensis* were collected from a plantation in tropical north-Western Australia (Bioactive Solutions Pty Ltd). They were harvested from an individual, 19-year-old tree on 13 July 2017, packed in airtight plastic bags and transported to The University of Western Australia. They were freeze-dried and ground to pass 1 mm screen using a cyclone grinder (CYCLOTECH 1093 Sample Mill; Tecator, Hoganas, Sweden). The powder was immediately stored in airtight plastic containers at –20°C until processing.

4.2.3 Fractionation and isolation of compounds

4.2.3.1 Solvent extracts

Leaf powder (20 g) was extracted with methanol (400 mL) by stirring overnight at room temperature. The mixture was filtered (Whatman No. 1) and the resulting extract was evaporated under reduced pressure in a water bath not exceeding 40°C to give a greenish oily residue.

4.2.3.2 Rapid Silica Filtration (RSF)

The dried extracts were reconstituted using a minimal amount of methanol (ca. 2 mL) and added to a vacuum assisted silica column (Davisil chromatographic silica media LC60A 40-63 µm, Grace Discovery Sciences, internal diameter 4 cm; height 8 cm). The column was loaded with 200 mL of 100% hexanes initially and then eluted with increasing concentrations of ethyl acetate (in hexane, v/v) at 20%, 40%, 60%, 80% and 100% with 200 mL volumes. This was followed by increasing the concentration of methanol in ethyl acetate from 10% to 100%. Each solvent system was collected as a unique fraction to give 10 fractions overall. A

subsample (ca. 5 mL) from each fraction was taken, dried under N_2 gas and submitted for the bioassay.

4.2.3.3 Reveleris Silica Chromatography (RSC)

The most bioactive fractions from RSF were pooled and adsorbed onto celite and separated through a flash silica chromatography column (Silica 40 µm, 80 g, p/n 145146133, Reveleris, BUCHI) using a Reveleris flash chromatography instrument. The fractions were separated using a gradient solvent system, from 20 % ethyl acetate in hexanes to 60% ethyl acetate in hexanes, for 30 min using a flow rate of 60 mL/min. A total of 60 fractions each of around 25 mL were obtained, from which 2 mL was collected as a subsample for bioassay.

4.2.3.4 High Performance Liquid Chromatography (HPLC)

Semi-preparative and analytical HPLC were performed using either an Agilent 1200 HPLC system with a diode array detector (DAD) and fraction collector, or a Hewlett-Packard 1050 equipped with a DAD and Pharmacia Biotech RediFrac fraction collector. Analytical work was conducted using an Apollo C18 reversed-phase column (250 mm × 4.6 mm, 5 µm, Grace Discovery Sciences) with a flow rate of 1.0 mL/min, and semi-preparative HPLC was undertaken with an Apollo C18 reversed-phase column (250 mm × 10 mm, 5 µm, Grace Discovery Sciences) with a flow rate of 4.0 mL/min. Separations were optimised and started with 80% (v/v) acetonitrile/water which increased to 90% acetonitrile/water over 30 mins and finally 100% acetonitrile for 10 mins. A total of 40 fractions (one fraction every minute) 4 mL each, were collected from the semi-preparative HPLC separations. Consecutive sub fractions (2 mL×2 = 4 mL) were pooled and the resulting 20 mixed samples were dried under N_2 gas and used for the bioassay.

4.2.3.5 GC-MS analysis

The chemical profile of the most bioactive sub-fractions from the semi-preparative HPLC separation were dried and dissolved in dichloromethane and analysed by gas chromatography-mass spectrometry (GC-MS) using a Shimadzu GCMS-QP2010 (Kyoto, Japan) fitted with a Rtx-5 column (5% diphenyl-dimethyl-polysiloxane, 30m × 0.25 mm × 0.1 µm film thickness, Restek, Bellefonte, PA, USA). Helium was used as a carrier gas, maintained at a constant flow rate of 1.0 mL/ min. The oven temperature was programed to 40°C, where it was held for 1 min, and then the temperature was increased at a rate of 7°C/min to 250°C, where it was held for 10 min. The mass spectrometer had a scan range of

m/z 45–500 and a solvent delay of 5 min was used with splitless injections of 1.0 µL for 1.0 min at 250°C. The ion source was set to 230°C, and the transfer line temperature to 250°C.

4.2.4 Larval mortality assay (LMA)

Larval mortality assay as described by (Williams, Fryganas, Ramsay, Mueller-Harvey, & Thamsborg, 2014) was used. Female *A. suum* were collected from pigs in a slaughterhouse (Danish Crown, Ringsted, Denmark) and the eggs were harvested and left for 2 months at room temperature, with aeration once per week, to embryonate. They were then stored as a suspension (about 25,000 eggs/mL) in 1 M H_2SO_4 solution at 4°C until use in the bioassay.

Egg hatching was initiated using the procedure described by (Bonde et al., 2021). Briefly, 10 mL of stored egg suspension was placed into a 15 mL falcon tube and washed thoroughly in Hank's balanced salt solution (HBSS; Thermofisher Scientific, Denmark), followed by centrifugation at 500 G for 2 min. The supernatant was discarded and the process was repeated 4 times to ensure removal of all H_2SO_4. After the final centrifugation, the egg pellet was pipetted into a conical flask containing a magnetic spinning bar. Glass beads (2 mm diameter) were added to cover the highest point of the magnet, and the flask was covered with aluminium foil. The flask containing eggs, stirring bar and glass beads was placed on a magnetic stirrer inside a CO_2 incubator (10% CO_2) at 37°C. The contents were slowly agitated for 40 min to mimic the churning and grinding motion of the pig intestines and thus promote egg hatching.

The contents of the flask were checked using an inverted microscope to verify most eggs had been hatched. A Baermann funnel was setup under a 20 µm sieve and the contents of the flask was poured into the sieve. The funnel was flooded with HBSS containing penicillin, streptomycin and amphotericin B to inhibit bacterial and fungal growth. The top of the Baermann funnel was sealed with plastic wrap and placed inside the CO_2 incubator overnight to allow the larvae to migrate to the bottom of Baermann funnel.

Larvae were harvested using a 5 mL pipette and placed in a falcon tube containing Roswell Park Memorial Institute (RPMI) 1640 Medium with 2 mM L-glutamine (Sigma, Denmark) and infused with 100 U/mL penicillin and streptomycin. The solution was centrifuged at 500 G for 2 min and the supernatant discarded. The process was repeated three times to wash the larvae thoroughly. The required concentration of 100 larvae per 150 µL was achieved by counting the average number of larvae in 5 µL drops under a microscope and then diluting the solution with RPMI medium as needed.

The assay was conducted in 96-well (flat bottom) plates with each well containing 150 µL of adjusted larval solution and 1.5 µL of the plant fraction/compound to be tested. For negative controls, 1.5 µL of dimethyl sulfoxide (DMSO) was used and run in duplicate wells. The plates were sealed and incubated at 37 °C in a CO_2 (10%) incubator.

After 24 h, live and dead larvae in each well were counted. Larvae that were motile and curled were considered alive, whereas the straight and stationary larvae were considered dead. Counting was limited to 15 min periods because the larvae die if left too long outside the incubator. Thus, plates were replaced inside the CO_2 incubator for at least another 15 min before the counting resumed. If enough activity were not observed during 24 h, the plates were again similarly counted after 48 h. Percentage survival for each well was calculated as ((alive/ (alive+dead)) ×100) and the result corrected according to the negative controls to acquire the mortality rate as:

$$Mortality\ rate = \left(1 - \frac{survival\%\ of\ larvae\ exposed\ to\ extract}{survival\ \%\ of\ negative\ control}\right)$$

To confirm the activity of the fatty acids that were subsequently identified in the active fractions by GCMS, commercially available linoleic acid, oleic acid and palmitic acid (>99%, Sigma, Denmark) were purchased and used in the larval mortality bioassay.

The LD_{50} of the tested fractions, compounds and standard fatty acids were determined using 2-fold serial dilutions at concentrations from 2000 µg/mL to 15.6 µg/mL which was performed in triplicates.

4.2.5 Statistical methods

Statistical software Prism 9 was used to calculate the mean and standard deviation and non-linear regression was used to calculate the LD_{50} of isolated and test compounds.

4.3. Results

The active methanolic leaf extract was separated by RSF into 10 fractions and subjected to LMA. The results from the RSF fractions showed that Fractions 3 and 4 were the most active, killing approximately 90-100% of larvae in 24 h (Fig. 4.3).

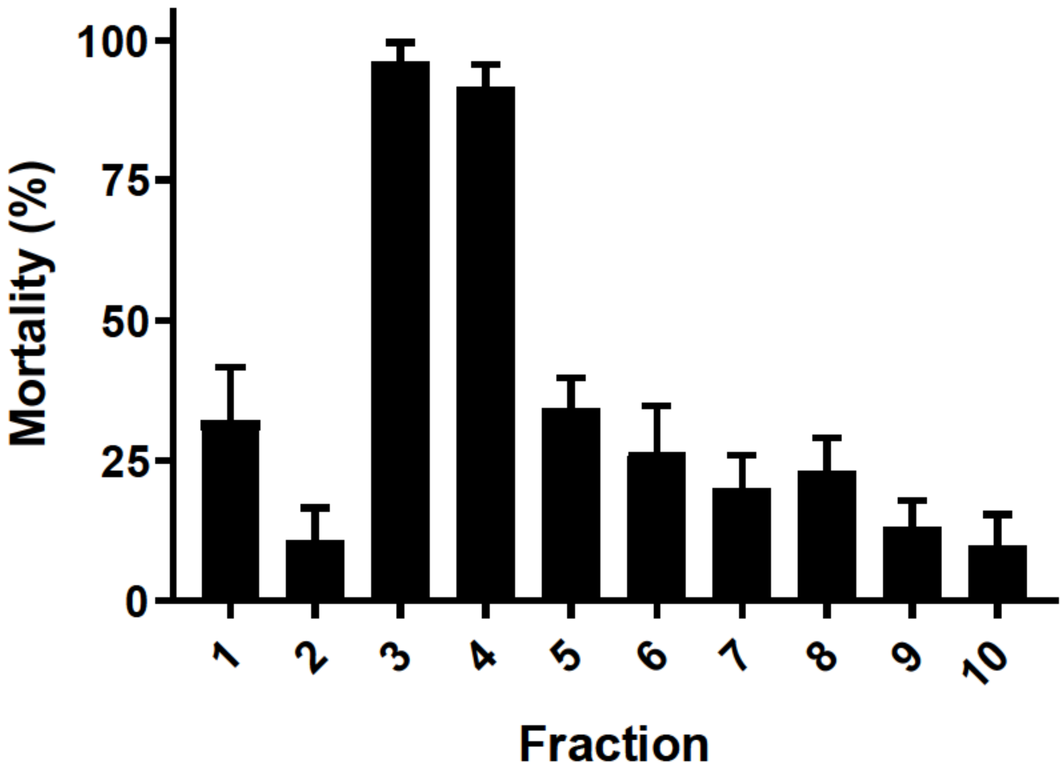

Figure 4.1. Anthelmintic activity of RSF fractions from K. senegalensis leaf in larval mortality assays.

Due to their activity, fractions 3 and 4 were pooled and separated using RSC to obtain a further 60 fractions. To make the numbers of fractions manageable for the bioassay, we initially pooled 5 consecutive sub-fractions (e.g., sub-fractions 1-5 combined, 6-10 combined and so on) but left the bulk of the fractions as individual fractions for later testing. A sample of each combined fraction (400 µL× 5 = 2 mL) was subjected to the bioassay to determine where the activity resided. Larval mortality assay showed that most of the activity were concentrated in pooled sub-fractions 16-20, 36-40, 41-45, 46-50, and 56-60. So individual fractions from these pools were then re-tested in the bioassay and the results are presented in Figure 4.4. From this follow up testing, fractions 16 and 17 were the most active, causing 100% mortality, while later eluting fractions also showed activity over 80-90% mortality levels (e.g. fractions 38, 42 and 57).

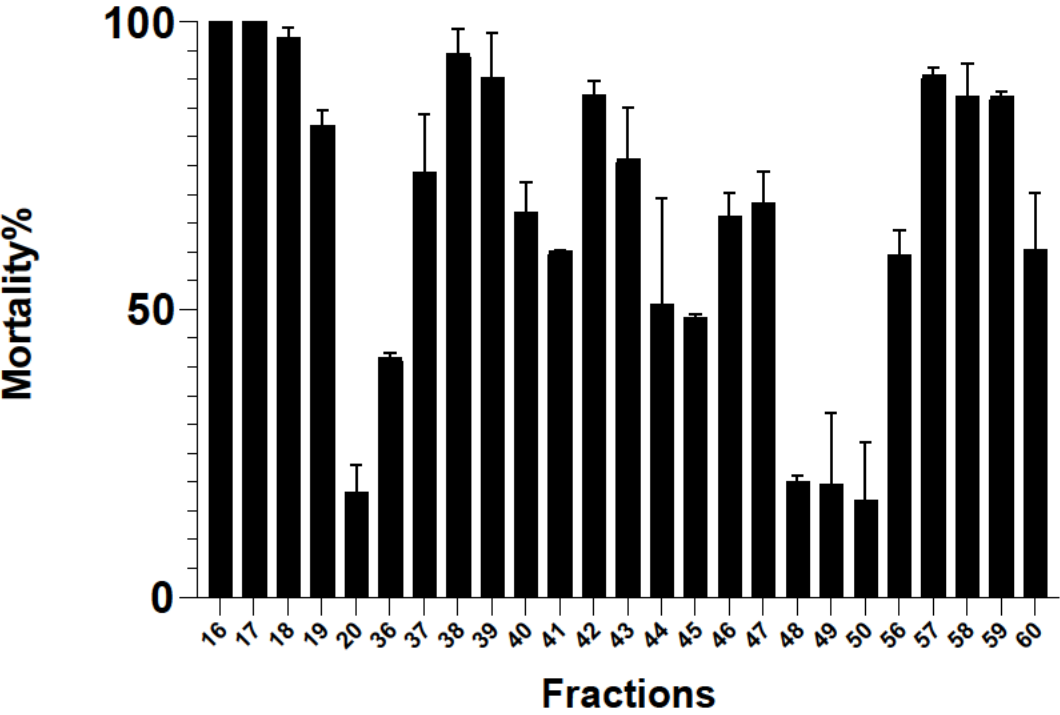

Figure 4.2. Anthelmintic activity of the most active RSC fractions from K. senegalensis leaf in larval mortality assays.

The later-eluting active fractions are the subject of Chapter 5, and here we will focus on the early-eluting, highly active Fraction 16. Fraction 16 was separated by semi-preparative HPLC using a gradient mobile phase (80-90% ACN/H_2O over 30 mins, followed by 100% for 10 mins) to generate 40 new fractions. Again for convenience in the bioassay, we pooled consecutive sub fractions (e.g., 1-2, 3-4 and so on) and the resulting 20 sub-fractions (named 16.1, 16.2 and so on) were dried under nitrogen before being tested in the larval mortality assay. The subsequent LMA revealed that sub-fractions 16.10 and 16.14 were the most active, killing 100% of the *A.suum* larvae in 48 h (Figure 4.5).

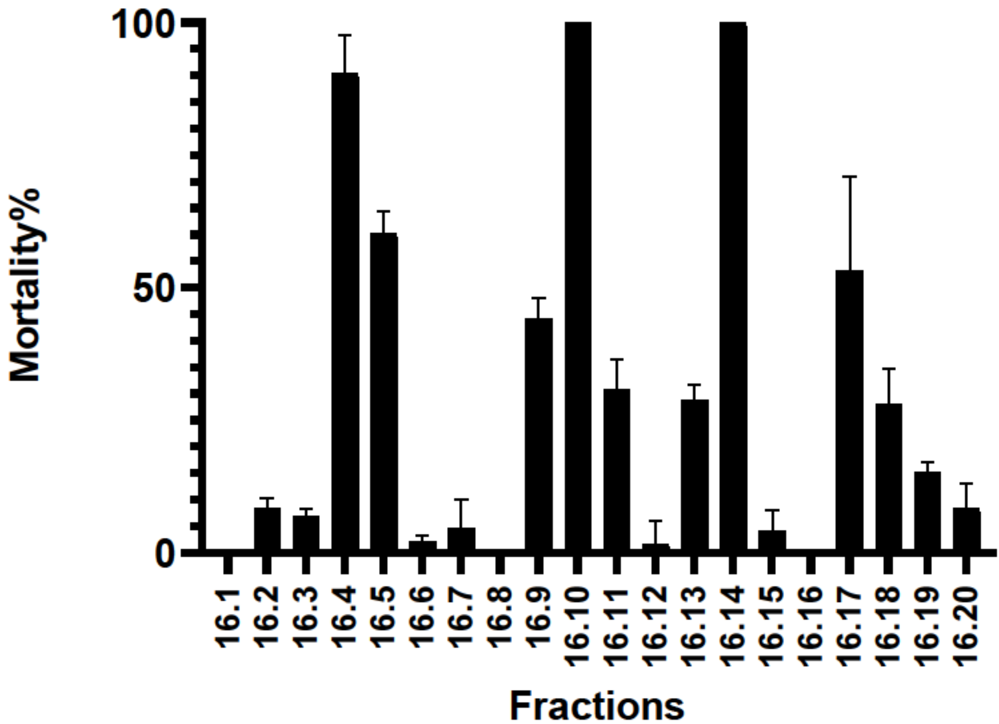

Figure 4.3. Anthelmintic activity of the semi-preparative HPLC fractions of K. senegalensis leaf in larval mortality assays.

Nuclear magnetic resonance (NMR) spectroscopy analysis of these fractions indicated the presence of unsaturated fatty acids. They were then subjected to GCMS analysis to aid in determining their structure. The GC-MS analysis showed fraction 16.10 to be composed mainly of 9,12-octadecadienoic acid (Z,Z)- (Fig. 4.1), commonly known as linoleic acid (retention time 17.8, SI 92, molecular weight 280, retention index: 2183). This identity was confirmed when standard linoleic acid was subjected to GC-MS and presented the same retention time and mass spectra as the compound in Fraction 16.10.

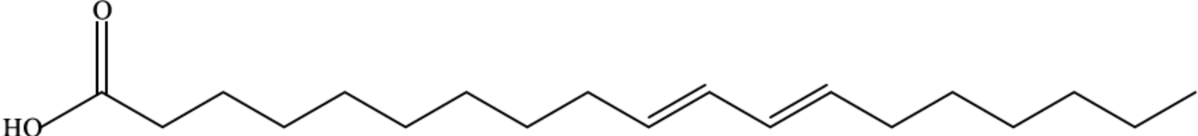

Figure 4.4. Structure of linoleic acid identified in Fraction 16.10 from K. senegalensis leaf.

Similarly, analysis indicated that the other highly active, Fraction 16.14, predominantly comprised palmitic acid (retention time 16.15, SI: 94, molecular weight: 256, retention index: 1968) and oleic acid (retention time 17.7, SI: 95, molecular weight: 282, retention index: 2175), the structures of which are shown in Figure 4.2. Once again, identity was confirmed

by matching retention time and mass spectra with a standard of both fatty acids.

O

HO

Oleic acid

O

HO

Palmitic acid

Figure 4.5. Structures of oleic and palmitic acids identified in Fraction 16.14 from K. senegalensis leaf.

When the pure compounds (linoleic acid and oleic acid) were tested in the bioassay, both showed some anthelmintic activity, whereas palmitic acid had no effect on the larvae. Linoleic acid was the most active (Figure 4.6): it killed 88% of larvae in 48 h at a concentration of 2 mg/mL and showed LD_{50} value of 496 µg/mL (95% CI: 421 to 586; R^2 = 0.95). The LD_{50} value for oleic acid was 1562 µg/mL (95% CI: 1341 to 1893; R^2 = 0.91; Figure 4.6).

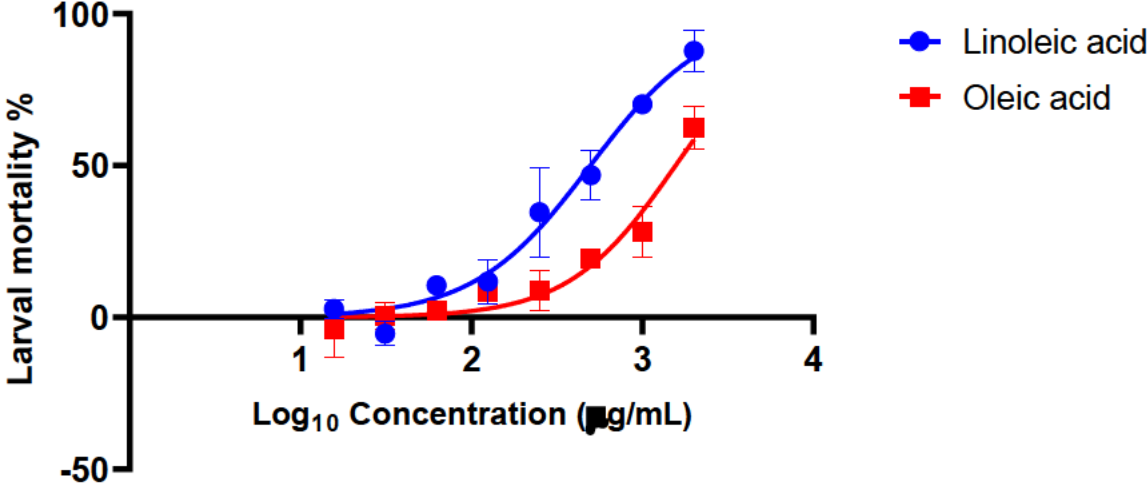

Figure 4.6. Estimation of larvicidal properties- linoleic acid and oleic acid.

4.4. Discussion

The methanolic extract of the leaf of *K. senegalensis* appears to contain many active compounds and exhibited *in vitro* larvicidal activity against *A. suum.* In this study we conducted bioassay guided isolation and showed that the activity can be separated into different fractions. Unsaturated and saturated fatty acids; linoleic acid, palmitic and oleic acids were identified from the most active fraction. In addition to fatty acids, the preliminary screening of fractions revealed many other fractions with notable levels of anthelmintic activity, so it seems likely that *K. senegalensis* produces many more compounds with anthelmintic properties that would be worth investigating further.

Previous studies have detected fatty acids in *K. senegalensis* using gas chromatography and infra-red spectroscopy (Okieimen & Eromosele, 1999). Oleic acid was found to be the predominant fatty acid (65 mg/100 g), followed by palmitic acid (21 mg/100 g), stearic acid (10 mg/100 g) and an unidentifiable acid (4 mg/100g). Similarly, when extract of stem bark of *K. senegalensis* was analysed using GC-MS, various unsaturated and saturated fatty acids, including alpha-linoleic acid, oleic acid and palmitic acid, were reported (Aguoru, Bashayi, & Ogbonna, 2017). Our results confirm the abundance of unsaturated and saturated fatty acids in *K. senegalensis* and add data suggesting that they are at least partly responsible for the anthelmintic activity of the plant (Ademola et al., 2004; China et al., 2016).

Unsaturated fatty acids have long been associated with a plethora of health benefits, but their direct anthelminthic effects have not been investigated widely. Previously, linoleic acid had been found in an acetone extract of *Clonostachys candelabrum*, and when it was tested against *H. contortus in vitro*, the estimated EC_{90} value was 930 µg/mL (Ayers et al. 2010). The present study is the first report of the toxicity of linoleic towards *A. suum*, a helminth that infects pigs and humans, it is the only study we know of that demonstrates a direct anthelmintic activity of oleic acid. The closest study we have been able to find was done with the Indian earthworm (*Pheretima posthuma*) which, when exposed to pure oleic acid at a concentration of 25 mg/mL, died within 33 min (Nilani, Pinaka, Duraisamy, Dhamodaran, & Jeyaprakash, 2012).

In recent study, linoleic acid was also isolated from the Indian plant, *Holigarna caustic*, and identified as the major compound responsible for the nematicidal activity against *Caenorhabditis elegans* (Panda, Das, Mai, De Borggraeve, & Luyten, 2020). The anti-parasitic potential of fatty acids was also revealed by (Bonde et al., 2021) who used bioassay-

guided fractionation to narrow down the wide variety of compounds in Nordic seaweeds and demonstrate important anti-parasitic activity in C18-20 poly-unsaturated fatty acids, namely stearidonic acid, eicosapentaenoic acid, alpha-linolenic acid, docosahexaenoic acid and arachidonic acid.

Our findings are thus consistent with those from a variety of other studies and also encourage us to consider the therapeutic use of unsaturated fatty acids, particularly linoleic acid, to combat livestock helminths. The active nematocidal concentration seems to be about 0.2 µg/mL (Panda et al., 2020), notably more than 10-fold lower than the widely used anthelmintic drug levamisole (IC_{50} of 3.6 µM) (Risi et al., 2019). Moreover, linoleic acid is an essential fatty acid with no known toxicity in mammals (Panda et al., 2020), an important factor when considering its therapeutic potential (Melariri, Campbell, Etusim, & Smith, 2012). On the contrary, feeding unsaturated fatty acids to ruminants can increase its deposition in muscle, perhaps offering an opportunity to manipulate human diet and improve human health (Wang et al., 2020).

The exact mechanism of anthelmintic action of fatty acids is not clear, but there are suggestions of a detergent effect that damages the larval cuticle or hypodermis, perhaps by interacting directly with lipophilic regions in the plasma membrane of the parasite (Bonde et al., 2021; Davis, Meyers, Dullum, & Feitelson, 1997; Panda et al., 2020). However, nematodes vary in their structure, composition and physiology, and further studies are needed on the mechanisms by which linoleic acid affects the larvae of *A. suum.*

In conclusion, linoleic, oleic and palmitic acids were found in the most anthelmintic fractions of *K. senegalensis* leaf exact, with linoleic and oleic acids demonstrating particularly potent action against *A. suum*. The observations strongly support the contention that *K. senegalensis* has great potential as a source of anthelmintic therapeutic treatments for livestock and, furthermore, provide a solid foundation for studies to confirm such activity *in vivo*.

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CHAPTER 5: EXPERIMENTAL CHAPTER

This chapter has been formatted for submission to *Fitoterapia*, *The journal for the study of medicinal plants* [Elsevier].

Note: the work and findings described here could be broken down into multiple papers, but have been retained as a single chapter to better integrate the story.

Structural elucidation and anthelmintic evaluation of limonoids isolated from the leaf extract of *Khaya senegalensis*

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Abstract

A methanolic extract of leaves of Khaya senegalensis *was subjected to bioassay-guided fractionation using high resolution mass spectrometry, nuclear magnetic resonance spectroscopy and a bioassay for anthelmintic activity based on* Ascaris suum*. One new limonoid, 16-oxodelevoyin B, and two known limonoids, methyl angolensate and mahonin, were identified. Mahonin showed the most anthelmintic activity, killing a significant number of third stage larvae at concentrations as low as 60 µg/mL with an LD_{50} value of 38 µg/mL. This study shows that limonoids from* Khaya senegalensis *have potential for combatting gastrointestinal parasites in livestock.*

5.1. Introduction

In Australia, the gross value of livestock and livestock products was about Au$30 billion for 2019-20 (Australian Bureau of Statistics). In these industries, helminths are a major production constraint, reducing growth rates and meat production (Fitzpatrick, 2013), and imposing significant financial and production losses. Indeed, the cost of dealing with gastrointestinal parasites in sheep and cattle is around $1 billion annually (Sackett, Holmes, Abbott, Jephcott, & Barber, 2006). For sheep farmers in Western Australia (WA), helminths cause an estimated loss of around $430 million annually (Lane, Jubb, Shephard, Webb-Ware, & Fordyce, 2015), with the most common species, *Trichostrongylus* and *Teladorsagia spp.*, often presenting as mixed infections and considered to be the main cause of diarrhoea (‘scouring’; (Cotter, 2017). These two species are most problematic in south-western WA because it is a winter rainfall zone (Besier & Love, 2003).

Gastrointestinal helminths are also a major problem in non-ruminants, including humans. Pigs, for example, are frequently troubled by *Ascaris suum*, *Oesophagostomim spp*., *Trichostrongylus axei*, *Trichuris suis* (Roepstorff et al., 1998) and cestode-like *Taenia solium* (Dorny et al., 2004; Sah et al., 2017). *A. suum* is fairly common in pigs world-wide (Nansen & Roepstorff, 1999) and causes economic loss mainly due to liver condemnation, altered carcass composition and reduced feed conversion rate (Thamsborg, Nejsum, & Mejer, 2013). *A. suum* closely resembles the highly prevalent human helminth, *Ascaris lumbricoides*, that infects more than 800 million people worldwide (Pullan, Smith, Jasrasaria, & Brooker, 2014).

The control of helminths currently relies heavily on synthetic anthelmintic drugs that generally fall into three classes: i) benzimidazoles; ii) nicotinic acetylcholine agonists like levamisole; and iii) macrocylic lactones, like ivermectin. Initially, these drugs were very effective but, due to extensive and indiscriminate use, there has been a steady increase in the prevalence of organisms resistant to them (Kaplan, 2004). For *A. suum*, there is an additional complexity in that the eggs can remain viable in the environment for years, greatly increasing the probability of reinfection and the costs of medication. Simultaneously, as anthelmintic resistance has been increasing, consumers have been becoming more concerned about drug residues in animal-sourced food. This situation made it imperative to introduce strategies for parasite control that are clean, green and ethical (CGE; (Martin et al., 2004).

In the search for an alternative approach to helminth control, options are appearing among the vast array of organic compounds synthesised by plants. These compounds are generally classified as primary and secondary metabolites. Primary metabolites are directly involved in essential functions such as photosynthesis, respiration, growth and development. Secondary metabolites, on the other hand, are involved in non-essential functions that assist the survival of the plant – for example, by providing chemical defence against herbivores and infections. These defence metabolites can be toxic to other organisms so are potential sources of natural antimicrobials, herbicides and insecticides (cited by (Crozier, 2006). Several secondary metabolites are known to have anthelmintic properties, including tannins (Athanasiadou, Kyriazakis, Jackson, & Coop, 2001; A. Molan, Attwood, Min, & McNabb, 2001; Payne et al., 2018), flavonoids (Barrau, Fabre, Fouraste, & Hoste, 2005), saponins (Ali et al., 2011; Doligalska et al., 2011), terpenes (A. L. Molan, Duncan, Barry, & McNabb, 2003; Foster, Cassida, & Turner, 2011), lectins (Ríos-de Álvarez et al., 2012) and alkaloids (Satou et al., 2002; Wang et al., 2010).

In the present study, we focus on *Khaya senegalensis* (commonly known as African mahogany), a member of the Meliaceae family that is native to tropical and subtropical Africa. It is valued for its timber, fuelwood and medicinal properties (CABI, 2013). The therapeutic properties of *K. senegalensis* have been exploited by many communities. It is extensively used to treat fever and malaria and, in cattle, it is used for the treatment of diarrhoea, ulcers, and gastrointestinal worms (Iwu, 2014). Wakirwa et al. (2013) demonstrated the presence of saponins, flavonoids, tannins and alkaloid glycosides in the plant.

Extracts from *K. senegalensis* have previously been found to have anthelminthic in activity. Ademola, Fagbemi, & Idowu (2004) reported that an ethanolic extract from *K. senegalensis* given to sheep (500 mg/Kg orally) reduced the faecal egg count (FEC) by 90%. Additionally, methanolic extracts *of K. senegalensis* stem were more potent than several other plants when tested against *Haemonchus contortus* as an inhibitor of egg-hatching, larval migration and adult worm motility (China, Attindehou, Gbngboche, & Salifou, 2016).

The present study used bioassay-guided fractionation to test whether i) an extract of *K. senegalensis* leaf has anthelmintic activity, and whether ii) the compound(s) responsible for that activity can be purified, isolated and identified.

5.2. Materials and Methods

5.2.1 Experimental design

Bioassay-guided fractionation was used to process a crude extract of *K. senegalensis* leaf. Rapid silica filtration (RSF), Reveleris flash chromatography (RSC) and semi-preparative High Performance Liquid Chromatography (HPLC) were used to separate compounds and Nuclear Magnetic Resonance (NMR) and high resolution mass spectrometry (HR-MS) were used for structural elucidation of individual compounds. The bioassay used in the process was based on *in vitro* mortality of *A. suum,* a helminth that is highly suited to *in-vitro* management. *A. suum* offers the major advantage that large numbers of eggs can be collected from adult helminths at abattoirs, stored for long periods at 4°C, and then hatched when needed to provide fresh L3 larvae or the assay. This system overcomes the need to maintain a herd of experimentally infected animals as a source of larvae. All of the chemistry was performed at the University of Western Australia whereas the parasite bioassay was performed at the University of Copenhagen.

5.2.2 Plant material

Leaves of *K. senegalensis* were collected from a plantation in tropical north-Western Australia (Bioactive Solutions Pty Ltd, Floreat, WA). They were harvested from an individual, 19-year-old tree on 13 July 2017, packed in airtight plastic bags and transported to The University of Western Australia. They were freeze-dried and ground to pass 1 mm screen using a cyclone grinder (CYCLOTECH 1093 Sample Mill; Tecator, Hoganas, Sweden). The powder was immediately stored in airtight plastic containers at –20°C until processing.

5.2.3 Fractionation and isolation of compounds

5.2.3.1 Solvent extracts

Leaf powder (20 g) was added to 400 mL of methanol and stirred overnight at room temperature. The mixture was then filtered through a Whatman No 1 filter and the extracts dried under reduced pressure in a water bath not exceeding 40°C.

5.2.3.2 Rapid Silica Filtration (RSF)

The dried extracts were reconstituted using a minimal amount of methanol (ca. 2 mL) and run through a silica column (Davisil chromatographic silica media LC60A 40-63 µm, Grace Discovery Sciences, internal diameter 4 cm; height 8 cm). The column was loaded with 200 mL of 100% hexanes initially and then eluted with increasing concentrations of ethyl acetate (in hexane, v/v) at 20%, 40%, 60%, 80% and 100% with 200 mL volumes. This was followed by increasing the concentration of methanol in ethyl acetate from 10% to 100%.

5.2.3.3 Reveleris Silica Chromatography (RSC)

The most bioactive fractions from RSF were pooled and adsorbed onto celite and seperated through a flash silica chromatography column (Silica 40µm, 80 g, p/n 145146133, Reveleris, BUCHI) using a Reveleris flash chromatography instrument. The fractions were separated using a gradient solvent system, from 20% ethyl acetate in hexanes to 60% ethyl acetate in hexanes, run over 30 min at a flow rate of 60 mL/min.

5.2.3.4 High Performance Liquid Chromatography (HPLC)

Semi-preparative and analytical HPLC were performed using either an Agilent 1200 HPLC system with a diode array detector (DAD) and fraction collector, or a Hewlett-Packard 1050 equipped with a DAD and Pharmacia Biotech RediFrac fraction collector. Analytical work was conducted using an Apollo C18 reversed-phase column (250 mm × 4.6 mm, 5 µm, Grace Discovery Sciences, Bannockburn, Illinois) with a flow rate of 1.0 mL/min, and semi-

preparative HPLC was undertaken with an Apollo C18 reversed-phase column (250 mm × 10 mm, 5 µm, Grace Discovery Sciences) with a flow rate of 4.0 mL/min (80–90% acetonitrile over 30 mins and 100% acetonitrile for 10 mins).

4.2.4 Larval mortality assay (LMA)

The process as described by (Williams et al., 2016) was used. Female *A. suum* were collected from pigs in a slaughterhouse (Danish Crown, Ringsted, Denmark) and the eggs were harvested and left for 2 months at room temperature, with aeration once per week, to embryonate. They were then stored as a suspension (about 25,000 eggs/mL) in 1 M H_2SO_4 solution at 4°C until use in the bioassay.

Egg hatching was initiated using the procedure described by (Bonde et al., 2021). Briefly, 10 mL of stored egg suspension was placed into a 15 mL falcon tube and washed thoroughly in Hank's balanced salt solution (HBSS; Thermofisher Scientific, Denmark), followed by centrifugation at 500 G for 2 min. The supernatant was discarded and the process was repeated 4 times to ensure removal of all H_2SO_4. After the final centrifugation, the egg pellet was pipetted into a conical flask containing a magnetic spinning bar. Glass beads (2 mm diameter) were added to cover the highest point of the magnet, and the flask was covered with aluminium foil. The flask containing eggs, stirring bar and glass beads was placed on a magnetic stirrer inside a CO_2 incubator (10% CO_2) at 37°C. The contents were slowly agitated for 40 min to mimic the churning and grinding motion of the pig intestines and thus promote egg hatching.

The contents of the flask were checked using an inverted microscope to verify most eggs had been hatched. A Baermann funnel was setup under a 20 µm sieve and the contents of the flask was poured into the sieve. The funnel was flooded with HBSS containing penicillin, streptomycin and amphotericin B to inhibit bacterial and fungal growth. The top of the Baermann funnel was sealed with plastic wrap and placed inside the CO_2 incubator overnight to allow the larvae to migrate to the bottom of Baermann funnel.

Larvae were harvested using a 5 mL pipette and placed in a falcon tube containing Roswell Park Memorial Institute (RPMI) 1640 Medium with 2 mL-glutamine (Sigma, Denmark) and infused with 100 U/mL penicillin and streptomycin. The solution was centrifuged at 500 G for 2 min and the supernatant discarded. The process was repeated three times to wash the larvae thoroughly. The required concentration of 100 larvae per 150 µL was achieved by

counting the average number of larvae in 5 µL drops under a microscope and then diluting the solution with RPMI medium as needed.

The assay was conducted in 96-well (flat bottom) plates with each well containing 150 µL of adjusted larval solution and 1.5 µL of the plant fraction/compound to be tested. For negative controls, 1.5 µL of dimethyl sulfoxide (DMSO) was used and run in duplicate wells. The plates were sealed and incubated at 37 °C in a CO_2 (10%) incubator.

After 24 h, live and dead larvae in each well were counted. Larvae that were motile and curled were considered alive, whereas the straight and stationary larvae were considered dead. Counting was limited to 15 min periods because the larvae die if left too long outside the incubator. Thus, plates were replaced inside the CO_2 incubator for at least another 15 min before the counting resumed. If enough activity were not observed during 24 h, the plates were again similarly counted after 48 h. Percentage survival for each well was calculated as ((alive/ (alive+dead)) ×100) and the result corrected according to the negative controls to acquire the mortality rate as:

$$Mortality\ rate = \left(1 - \frac{survival\%\ of\ larvae\ exposed\ to\ extract}{survival\ \%\ of\ negative\ control}\right)$$

The LD_{50} of the isolated compounds were determined using 2-fold serial dilutions at concentrations from 500 µg/mL to 15.6 µg/mL which was performed in triplicates.

5.2.5 Synergistic effects

Synergy between the two isolated compounds was also tested in the LMA. The least potent compound was tested at a series of 2-fold serial dilutions ranging from 500 µg/mL to 3.9 µg/mL with the second compound (the most potent in our study) being added at a concentration equivalent to its LD_{20}. The LD_{20} was calculated based on the LD_{50} curve using the equation:

$$LD_{20} = \left(\frac{20}{100 - 20}\right)^{1/_{Hill\ Slope}} \times LD_{50}$$

A predicted additive effect curve was calculated using an equation as described below. The two compounds were assumed to have a synergistic effect if the mortality of the larvae in the LMA was above the predictive additive curve.

$$Predicted\ additive\ effect = \left(1 - \frac{mortality\ of\ test\ larvae\ exposed\ to\ compound\ x}{100}\right) \times \left(1 - \frac{LD_{20}\ of\ compound\ Y}{100}\right) \times 100$$

5.2.6 Statistical methods

Statistical software Prism 9 was used to calculate the mean and standard deviation and non-linear regression was used to calculate the LD_{50} of isolated and test compounds.

5.3. Results

5.3.1 Bioassay guided fractionation

As described in Chapter 4, the bioassay-guided fractionation led to the identification of fatty acids from early-eluting active Fraction 16. Here, we focussed on the later-eluting Fractions 38 to 42 that showed anthelmintic activity and, on chemical analysis, revealed two known limonoids, mahonin (Compound **1**) from Fraction 38-40 and methyl angolensate (**2**) from Fraction 41-42, as the two major compounds. Further analysis of additional Fractions 47-48 with minor anthelmintic activity allowed us to also identify a novel limonoid that was assigned the trivial name 16-oxodelevoyin B (**3**).

5.3.2 Structural elucidation

High resolution electrospray ionisation mass spectrometry (HR-ESI-MS) analysis of Compound **1** in positive mode revealed a protonated molecular ion $[M+H]^+$ at *m/z* 509.2540 (calc 509.2539), consistent with a molecular formula of $C_{30}H_{36}O_7$. ^1H NMR and ^{13}C NMR of purified Compound **1** confirmed the structure of Compound **1** as mahonin, previously isolated from *Swietania mahogany* (Kadota, Marpaung, Kikuchi, & Ekimoto, 1990). HR-ESI-MS analysis of Compound **2** in positive mode revealed a protonated molecular ion $[M+H]^+$ at *m/z* 471.2384 (calc. 471.2383), compatible with a molecular formula of $C_{25}H_{34}O_7$, consistent with methyl angolensate, reported previously as a metabolite of *Khaya senegalensis*. The assignment was corroborated by ^1H and ^{13}C NMR analysis of purified Compound **2**, consistent with that previously reported for methyl angolensate (Nakatani, Abdelgaleil, Okamura, Iwagawa, & Doe, 2000).The chemical structure of the three isolated secondary metabolites along with atom numbering is presented in Figure 5.1.

Figure 5.1. Chemical structures of secondary metabolites isolated in this study from K. senegalensis. mahonin (1), methyl angolensate (2), 16-oxodelevoyin B (3) with atom numbering depicted.

HR-ESI-MS analysis of Compound **3** in positive mode revealed a protonated molecular ion $[M+H]^+$ at *m/z* 585.2702 (calc. 585.2700), consistent with a molecular formula of $C_{32}H_{40}O_{10}$ allowing us to infer a tri-acetylated tetra-nor-triterpene. ^{1}H NMR analysis (400 MHz, $CDCl_3$, assigned in Table 5.1) of the purified metabolite indicated a number of salient features including the presence of a furan system (δ_H 7.46: H-23, 7.41, H-21, 6.23: H-22), a vinylic proton (δ_H 5.93: H-15), three methine protons geminal to an esterified alcohol (δH 5.53: H-7, 5.42; H-6, 4.95: H-1), three acetyl groups (δH 2.07 1-$OCOCH_3$, 2.03: 6-$OCOCH_3$, 2.01; 7-$OCOCH_3$), and five singlet methyls integrating for three protons each (δH 1.68: H-29, 1.44: H-30, 1.43; H-28, 1.32; H-19, 0.97; H-18). ^{13}C NMR analysis (125 MHz, CDCl3, assigned in Table 5.1), aided by multiplicity-edited heteronuclear single-quantum coherence (multi-ed-HSQC) and heteronuclear multiple bond correlation (HMBC) indicated the presence of 32 distinct carbon environments, consistent with the calculated molecular formula derived from high resolution mass measurements. Notable spectral features included the presence of an alpha-beta unsaturated ketone system (δ_C 204.7; C-16, 124.7; C-15, 189.8; C-14), a furan system (δ_C 142.9; C-23, 141.7; C-21, 118.3; C-20, 111.1; C-22), four ester carbonyls (δ_C 169.9; 1-OCO, 169.6; C-3, 169.4; 6-OCO, 169.1; 7-OCO), three esterified methines (δ_C 73.5; C-1, 73.1; C-7, 70.8; C-6) and an esterified tertiary carbon (δC 85.1; C-4), as well as an allylic methine resonance at δ_C 60.9 ppm consistent with the resonance at position C-17 of Compound **1** (Kadota et al., 1990). Additionally, multi-ed-HSQC analysis permitted us to discern the presence of three diastereotopic methylene pairs (δ_H 3.03, 2.69; δC 37.9; CH_2-2, δ_H 1.80, 1.52; δ_C 16.7; CH_2-11, δ_H 1.97, 1.83; δ_C 30.9; CH_2-12), two tertiary methines

(δ_H 3.05; δ_C 32.7; CH-9, δ_H 2.74; δ_C 45.4; CH-5), five methyl groups (δ_C 32.6; CH_3-29, 27.7; CH_3-28, 27.3; CH_3-18, 24.7; CH_3, 16.8; CH_3-19), and an additional three acetylene methyls (δ_C 21.13; 1-$OCOCH_3$, 21.12; 6-$OCOCH_3$, 20.9; 7-$OCOCH_3$). Combined correlation spectroscopy (COSY) analysis and total correlation spectroscopy (TOCSY) analysis allowed us to piece together four distinct ^1H-^1H spin systems H-1 (δ_H 4.95) to H-2α/β (δ_H 3.03,2.63), H-5 (δ_H 2.74) to H-7 (δ_H 5.53), H-9 (δ_H 5.05) to H-12α/β (δ_H 1.97, 1.83) and a weak correlation from H-17 (δ_H 3.41) to the extended furan system: H-21 (δ_H 7.41) to H-23 (δ_H 7.46). Finally, HMBC analysis, with key correlations H-19 (δ_H 1.32) to C-1 (δ_C 73.5), H-30 (δ_H 1.44) to C-7 (δ_C 73.1) and C-9 (δ_C 32.7), and H-18 (δ_H 0.97) to C-11 (δ_C 16.7) permitted assembly of the planar structure of Compound **3** as depicted in Figure 5.1.

The relative stereochemistry of metabolite at positions C-5 through to C-7 was elucidated following analysis of the magnetic spin-spin coupling constants of protons H-5 to H-7 and application of the Karplus equation. The relative configuration at centres C-1, C-8 to C-10 and C-13, C-17 was determined from nuclear-Overhauser effect spectroscopy (NOESY) experiments and is in keeping with that predicted from biosynthetic theory assuming the 7-deacetylazadirone (**4**) precursor common to all meliaceous limonoids (Hodgson et al., 2019). The absolute configuration of metabolite **3** is predicted to be as depicted in Figure 5.2 on biosynthetic grounds, again, assuming a 7-deacetylazadirone (**4**) precursor.

The combined spectroscopic evidence allowed us to unambiguously assign the structure of Compound **3** as depicted in Figure 5.2 and, given the structural similarity to the known metabolite delevoyin B, we propose the trivial name 16-oxodelevoyin B. Biosynthetically, the metabolite may be envisioned to arise from a 7-deacetylazadirone (**4**) precursor via the intermediacy of mahonin (**1**) following oxidation and acetylation of **4** (fig 5.2). A subsequent Michael addition, acetylation and Bayer-Villiger type ring expansion sequence then afforded Compound **3**.

Figure 5.2. Proposed biosynthesis of 3 from 4 via the intermediacy of compound 1.

Table 5.1. NMR data for isolated compound 3 in CDCl3 (400 MHz, 125 MHz).

No.	δH , m (J in Hz)	δCa, type
1	4.95, dd (J = 6.0, 8.4 Hz)	73.5, CH
2α	3.03, dd (J = 6.0, 13.4 Hz)	37.9, CH_2
2β	2.63, dd (J = 8.4, 13.4 Hz)	
3		169.6, C
4		85.1, C
5	2.74, d (J = 11.9 Hz)	45.4, CH
6	5.42, dd (J = 11.9, 2.9 Hz)	70.8, CH
7	5.53, d (J = 2.9 Hz)	73.1, CH
8		44.7, C
9	3.05, m	32.7, CH
10		43.6, C
11α	1.80, m	16.7, CH_2
11β	1.52, m	
12α	1.97, m	30.9, CH_2
12β	1.83, m	
13		47.5, C
14		189.8, C
15	5.94, s	124.7, CH

16		204.7, C
17	3.41, s	60.9, CH
18	0.97, s	27.3, CH_3
19	1.32, s	16.8, CH_3
20		118.3, C
21	7.41, m	141.7, CH
22	6.23, m	111.1, CH
23	7.46, m	142.9, CH
28	1.43, s	27.7, CH_3
29	1.68, s	32.6, CH_3
30	1.44, s	24.7, CH_3
1-O*C*OCH_3		169.9, C
1-OCO*CH_3*	2.07, s	21.1, CH_3
6-O*C*OCH_3		169.4, C
6-OCO*CH_3*	2.03, s	21.1, CH_3
7-O*C*OCH_3		169.1, C
7-OCO*CH_3*	2.01, s	20.9, CH_3

5.3.3. Bioactivity of the isolated limonoids

Mahonin (**1**) was the most active compound in the larval mortality assay, killing 100% of larvae at concentrations as low as 250 µg/mL. With an LD_{50} value of 38 (95% CI: 35.7-41.6) µg/mL ($R^2 = 0.99$), the compound maintained some activity at concentration as low as 7.8 µg/mL. Methyl angolensate (**2**) had an LD_{50} value of 152 (95% CI: 115.2- 200.8) µg/mL ($R^2 = 0.97$), and the novel compound 16-Oxodelevoyin B (**3**) demonstrated reasonable activity with an LD_{50} value of 72.16 (95% CI: 56.7-91.8) µg/mL ($R^2 = 0.98$) (Figure 5.3).

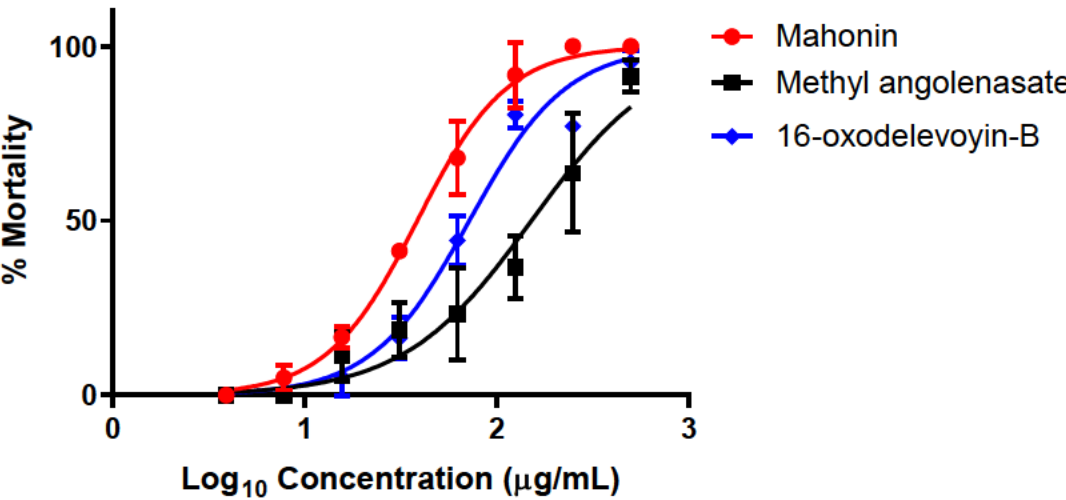

Figure 5.3. Anthelmintic activity of mahonin (1), methyl angolensate (2) and 16-oxydelevoyin B (1) in larval mortality assay.

The activity of methyl angolensate (**2**) and mahonin (**1**) combined in the LMA suggests they do not have a synergistic effect as the activity of the combination was not always higher than the predicted value (fig. 3). However, the combination clearly seems to be better than the single compound and we can conclude the metabolites have an additive effect.

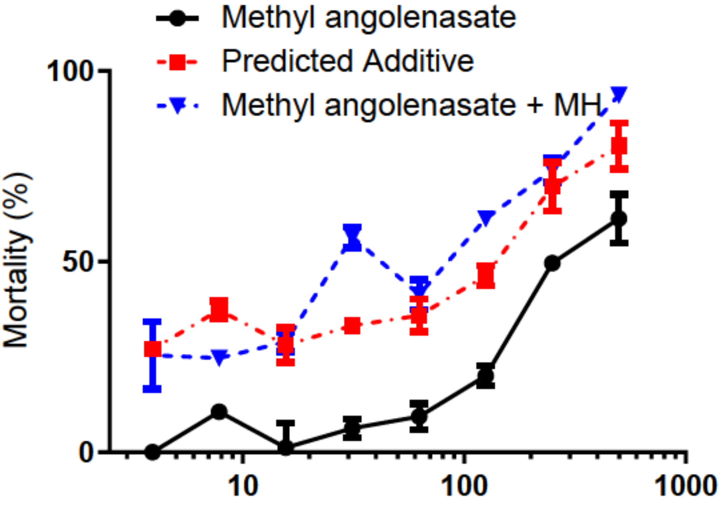

Figure 5.4. Synergestic effect of methyl angolensate (2) with mahonin (1). Percentage mortality of Ascaris suum L3 subjected to pure compound methyl angolensate (2) with and without LD_{20} of pure compound mahonin (1). Results are the mean (± SEM) of triplicates, MH= methyl angolensate.

5.4. Discussion

In addition to the fatty acids (Chapter 4), leaf extract of *K. senegalensis* produces three limonoids that offer anthelmintic activity: mahonin (**1**), methyl angolensate (**2**) and the novel compound 16-oxodelevoyin B (**3**). These findings add weight to the early report from (Ademola, Fagbemi, & Idowu, 2004) who reported that an ethanolic extract from the bark of *K. senegalensis* killed first stage larvae of sheep helminths *in vitro* with a LC_{50} of 0.52 µg/mL. The authors also performed an *in vivo* study which revealed that an oral dose (500 mg/Kg) of an ethanolic extract of *K. senegalensis* reduced FEC by 90% in sheep. That study was limited to a crude extract from the bark, and we have able to progress to isolation and identification of the active compounds.

All three anthelmintic compounds identified in the present study are limonoids. Limonoids are tetranortriterpenoids, highly oxygenated triterpenes formed by the loss of terminal four carbon atoms in the side chain and the remainder of the side chain cyclized into a furan ring. Until now, there has been little information about any effect of limonoids on animal helminths.

Limonoids are widely reported to have a diverse spectrum of biological activities in humans, including anti-inflammatory, anti-cancerous, anti-viral, antimicrobial and many other

pharmacological effects (Zhang, Wang, Chen, Androulakis, & Wargovich, 2007; Maneerat, Laphookhieo, Koysomboon, & Chantrapromma, 2008; Ribeiro et al., 2008; Petrera & Coto, 2014; Akihisa et al., 2017). In an agricultural context, limonoids are known to have insect antifeedant, insecticidal and growth regulatory activities (Nakatani et al., 2001; Koul, 2008). In fact, azadirachtin, a limonoid from the neem tree (*Azadirachta indica*), is such a potent insecticide and antifeedant that it is used as a standard for comparison of antifeedants (Drijfhout & David Morgan, 2010). Recently 108 triterpenoids were screened for potential antiviral activity against SARS-CoV-2, MERS and SARS coronaviruses, and it was found that citrus limonoids (citrusin, obacuone, glaucin B, limonin, ruaevin) were potential inhibitors of Mpro of SARS-CoV-2 based on their non-covalent bonding to at least two of the active site amino acid residues (Sundar Jeyaraj & Aarthy, 2020). The mode of action of the three limonoids isolated in the present study are not known, but other studies show that limonoids exert inhibitory effects on growth, plasma membrane pumps, and parasite enzymes, and can even interfere with metabolic pathways in parasites (Squires, Foster, Lindsay, Caudell, & Zajac, 2010).

Mahonin (**1**) had the strongest anthelmintic activity of the three limonoids isolated in this study, and has previously been found to have other strong bioactive effects following its initial isolation from the cotyledons of *Swietenia mahogany* (Kadota et al., 1990). After extraction from the seeds of *Chisocheton siamensis,* mahonin also showed cytotoxic activity against two cancer cell lines, NCI-H187 (human small cell lung cancer; IC_{50} 15.6 µg/mL) and MCF-7 (breast cancer; IC_{50} 18.4 µg/mL). In addition, mahonin has shown strong activity against the malaria parasite, *Plasmodium falciparum*, with an impressive IC_{50} value of 2.9 µg/mL, and moderate activity against *Mycobacterium tuberculosis* (Maneerat et al., 2008). Finally, there is also some evidence that it can help control Type 2 diabetes (Vigneshwaran & Lalitha, 2016).

Methyl angolensate (**2**), the least active of the three limonoids, was previously isolated and identified in *K senegalensis* (Adesogan & Taylor, 1968; Thioune, Pousset, & Lo, 1999; Nakatani et al., 2000). It has shown some antifeedant activity against third-instar larvae of *Spodoptera littoralis* and it has been found to exert a dose-dependent gastro-protective action against indomethacin-induced ulceration in mice (Njar, Adesanwo, & Raji, 1995).

The identification of the novel limonoid, 16-oxodelevoyin B (**3**), confirms other studies showing that *K. senegalensis* is a rich source of limonoids. Khalid et al (1998) identified two

limonoids, 2,6-dihydroxyfissinolide and methyl 3ß-acetoxy-6-hydroxy-1-oxomeliac-14-enoate, in *K. senegalensis* bark and both compound exhibited moderate antiprotozoal activity against *Plasmodium falciparum* and the promastigote of *Leishmania major*. Similarly, (Yuan, Zhang, Yang, & Yue, 2010) identified seven new limonoids (khayalenoids C to I), three new triterpenoids (senegalenes A to C) and eight other known limonoids in an extract of *K. senegalensis* stem, but did not assess their biological activity.

We also tested whether a combination of mahonin (**1**) and methyl angolensate (**2**), at fixed concentrations, would act synergistically to improve on the anthelmintic activity of the individual compounds. No clear synergy between two compounds was observed but there was certainly an additive effect. The same concept has been tested with similar results from fatty acids isolated from seaweed, each of which only showed moderate activity against L3 of *A. suum*. When they were tested in combination, synergism was clearly evident (Bonde et al., 2021). Similarly, when synergistic interactions were tested among essential oils for anthelmintic effects against *Haemonchus contortus in vitro*, the best result arose with the combinations rather than individual oils (Katiki et al., 2017).

Given the findings in Chapter 4 and 5, we can address the practicality of using *K. senegalensis* for anthelmintic therapy in sheep. The ethanolic extract, given orally at 500 mg/Kg, reduces FEC by 90% (Ademola et al., 2004) so, for an adult weighing 50 kg, only 25 g of crude extract would be required. This is certainly feasible. Importantly, the toxicity of *K senegalensis* for mammals is very low, as shown by (Nwosu, Hassan, Abubakar, & Ebbo, 2012) who found no evidence of renal or liver damage, or histopathological changes, in rats were fed an aqueous extract of *K. senegalensis* (600-3000 mg/Kg). In addition to potency and risk, we need to assess the ease with which *K. senegalensis* can be sourced. Fortunately, as African mahogany is harvested for its timber, the leaves are generally by-products and can be cheaply acquired in large quantity.

In summary, three limonoids, including the novel compound 16-oxodelevoyin B (**3**), were isolated from the leaf of *K. senegalensis* and all three showed reasonable anthelmintic activity. They significantly inhibited the infective larval stage of *A. suum in vitro,* suggesting that there is potential for incorporating *K senegalensis* into parasite control methods in livestock. However, a better understanding can only be achieved after verifying these results under *in vivo* conditions.

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CHAPTER 6: GENERAL DISCUSSION

The general hypothesis tested in this thesis is that *Khaya senegalensis* produces bioactive secondary metabolites that exhibit anthelmintic activity that could be applied in the livestock industries. This hypothesis was supported because even crude extracts from the plant showed strong anthelmintic activity in our *in-vitro* bioassay based on helminth larval mortality. Using the *in-vitro* bioassay for guidance, we were also able to progress beyond that initial, critical observation by processing the extracts through chromatography and mass-spectroscopy so individual compounds with anthelmintic activity could be isolated and identified. These studies showed that *K. senegalensis* produced several metabolites, including limonoids and unsaturated and saturated fatty acids that exerted powerful anthelmintic activity. To add to that, these compounds have either no known or a very low toxicity in mammals (Nwosu, Hassan, Abubakar, & Ebbo, 2012; Panda et al., 2020). None of these compounds had been seriously considered important in the field of animal parasitology. Importantly, one of the limonoids identified was a completely novel compound. Equally as exciting is the clear evidence, from the outputs of the chromatography and mass-spectroscopy, that *K. senegalensis* produces many more potentially useful anthelmintic compounds. This thesis has thus added significant weight to the pioneering work indicating the potential of *K. senegalensis* as an anthelminthic treatment for livestock (Ademola, Fagbemi, & Idowu, 2004; China, Sabbas, Gbangboche, & Salifou, 2016).

We discovered three limonoids in the late-eluting fractions of the methanolic extract of leaves of *K. senegalensis*: mahonin (**1**), methyl angolensate (**2**) and, a major highlight of this project, the novel compound 16-oxodelevoyin B (3). All three limonoids showed anthelmintic bioactivity, with Mahonin (**1**) being most active. Limonoids are widely reported to present a diverse spectrum of potential therapeutic uses in humans, including anti-inflammatory, anti-cancerous, anti-viral and antimicrobial activities, as well as many other pharmacological effects (Zhang, Wang, Chen, Androulakis, & Wargovich, 2007; Maneerat, Laphookhieo, Koysomboon, & Chantrapromma, 2008; Ribeiro et al., 2008; Petrera & Coto, 2014; Akihisa et al., 2017). However, as far as we can tell, this is the first report that they show anthelmintic activity relevant to livestock health. Moreover, we were also able to demonstrate additive effects of the limonoids: when mahonin (**1**) was added to methyl angolensate (**2**) at a fixed concentration, the activity of methyl angolensate was increased. When the combined effect of two or more agents are more than the expected additive effect, the phenomenon is called

synergy (Greco, Faessel, & Levasseur, 1996). This phenomenon is fairly common with natural compounds. Synergism has been reported among fatty acids isolated from seaweed, all of which showed only moderate anthelmintic activity (Bonde et al., 2021). The discovery of a novel limonoid, and demonstration of strong anthelmintic activity in limonoids has opened a new avenue for future research on plants rich in limonoids and might be a stepping stone towards new drug discovery for the livestock industries.

When we concentrated on an early-eluting fraction of the methanolic extract of *K. senegalensis* that had shown strong anthelmintic activity, we identified two sub-fractions that could kill 100% larvae *in vitro* within 48 h. The GC-MS analysis showed that one sub-fraction comprised mainly linoleic acid and the other comprised predominantly palmitic acid and oleic acid. Interestingly, using the same bioassay to study anti-parasitic compounds in Nordic seaweeds, 18-20 poly-unsaturated fatty acids were identified (Bonde et al., 2021). Moreover, linoleic acid isolated from Indian plant, *Holigarna caustic*, was identified as the major compound responsible for nematicidal activity against *Caenorhabditis elegans* (Panda, Das, Mai, De Borggraeve, & Luyten, 2020). Some fatty acids have previously been identified in extracts of the seed and stem of *K. senegalensis* (Okieimen & Eromosele, 1999; Aguoru, Bashayi, & Ogbonna, 2017), but the observations in this thesis are probably the first demonstration of their anthelmintic activity. Of course, these compounds are essential fatty acids and therefore important in the nutrition of livestock and humans. Importantly, linoleic acid has no known toxic effects in mammals (Melariri, Campbell, Etusim, & Smith, 2012; Panda et al., 2020). Thus, they are valuable in a human food context as well as for their potential anthelmintic properties.

In addition to the limonoids and fatty acids, the preliminary screening of fractions revealed many other fractions with notable levels of anthelmintic activity, none of which could be pursued due to time constraints, so it seems likely that *K. senegalensis* produces many more molecules with anthelmintic properties that might be investigated further. The fact that the leaf contains a wide variety of classes of compounds with varying degrees of anthelmintic activity is a very favourable scenario because, when a therapy is based on a mix of several complex molecules, it is more difficult for helminths to evolve resistance to the treatment. Importantly, the earlier fractions that comprised mixtures of compounds were generally more potent in the bioassay than the single pure molecule isolated from that the same fraction, indicating synergistic or additive effects that might become a valuable aspect of therapeutic

formulations. This concept is supported by the interaction between mahonin (**1**) and methyl angolensate (**2**). Arguably, a crude extract, or even the incorporation of fresh leaf in livestock feed, could present a better approach to health management than investing in the isolation of pure compounds. Clearly, we need *in vivo* studies that include measures of the potential nutritional and anti-nutritional aspects of the plant or its molecules.

One critical aspect of the bioassay-guided methodology is the decision to use a bioassay based on *A. suum*, rather than a helminth species that is relevant to the type of livestock of interest. There are three arguments against this view:

a) *A. suum* is relevant because it presents major issues for the pig industry, as well as human health, for which it is widely used as a model parasite (Shao et al., 2014; Midha et al., 2018).
b) *A. suum* is ideally suited to *in-vitro* management – one major advantage is that large numbers of eggs can be collected from adult helminths at abattoirs, stored for long periods at 4°C, and then hatched when needed to provide fresh L3 larvae or the assay; this system overcomes the need to maintain a herd of experimentally infected animals as a source of larvae; moreover, the *A. suum* bioassay is extremely sensitive, requiring as little as 1.5 µL of test compound, a useful situation when only small amounts of compounds can be isolated (a common situation when studying natural products);
c) Anthelminthic molecules usually have a broad mode of action so will affect a very wide variety of helminth species so it is likely that the molecules we discovered will be effective against sheep helminths as well. For example condensed tannins have been reported to be effective against variety of helminths species of small ruminants as well as *A. suum* (Athanasiadou, Kyriazakis, Jackson, & Coop, 2001; Williams, Fryganas, Ramsay, Mueller-Harvey, & Thamsborg, 2014). Similarly linoleic acid which was effective against *A. suum* in our study has been shown to have some anthelmintic activity against *Haemonchus contortus* (Pineda-Alegría et al., 2020).

A second aspect of the methodology is the choice of solvent for extraction of the plant material because the nature of the solvent greatly influences the types and yield of compounds extracted. The obvious is polarity, with solvents like methanol, ethanol or water producing different extract mixes to non-polar solvents like hexane and dichloromethane (Pandey & Tripathi, 2014; Altemimi, Lakhssassi, Baharlouei, Watson, & Lightfoot, 2017). For the studies in this thesis, a polar solvent (methanol) was used for

the initial crude extraction, although during subsequent fractionation, a variety of polar and non-polar solvents were used.

The value of a polar solvent is that it is more relevant to the aqueous environment of the gut of grazing livestock. Bioactive non-polar compounds might not get utilized directly or would seem less likely to be utilized directly by the animal, although we should probably consider exploring them in future.

Having identified interesting compounds with good anthelmintic potency *in vitro*, it is now imperative that we confirm their potential with *in vivo* trials. The complexity of the gut environment is difficult to mimic in the laboratory, so we cannot be sure that *in vitro* results will translate to the whole animal in its normal environment. Having said that, there are multiple examples in which *in vitro* findings translate faithfully to animals. For example, an ethanolic extract of *K. senegalensis* with *in vitro* bioactivity against a variety of ovine helminths was also effective when administered orally, reducing the faecal egg count by 90% (Ademola et al., 2004). Similarly, (Athanasiadou et al., 2001) corroborated the *in vitro* findings of (Paolini, Fouraste, & Hoste, 2004) demonstrating that treating sheep with condensed tannins reduced intestinal helminth load. There will always be an argument about the legitimacy of industry recommendations based only on *in vitro* parasitological tests, but we cannot disagree on a fact that *in-vitro* studies provide strong first-level scientific evidence and confidence to pursue expensive and complex animal trials.

In conclusion, the main aim of the project has been achieved. Helminths have always been a major problem in livestock production, a situation that has been exacerbated by the absence of effective vaccines against helminths, the difficulty of providing high standards of sanitation on farm, and the emergence of resistance to anthelminthic medications. Industry has been completely reliant on a limited number of chemical classes of anthelmintic medications to break the life cycle of helminths, so there is a desperate need for alternatives. Despite that need, since 2000, only three new drug classes have been approved for animal use and none have been approved for humans (Nixon et al., 2020). The research in this thesis has identified new potential options based on *K. senegalensis*, either through incorporation of the plant or its extracts in animal feed, or through the use of bioactive molecules, the limonoids and essential fatty acids, as therapeutic agents. Importantly, African mahogany is harvested for its timber, so leaf material is a by-product that is both readily available and inexpensive. The option must be explored further.

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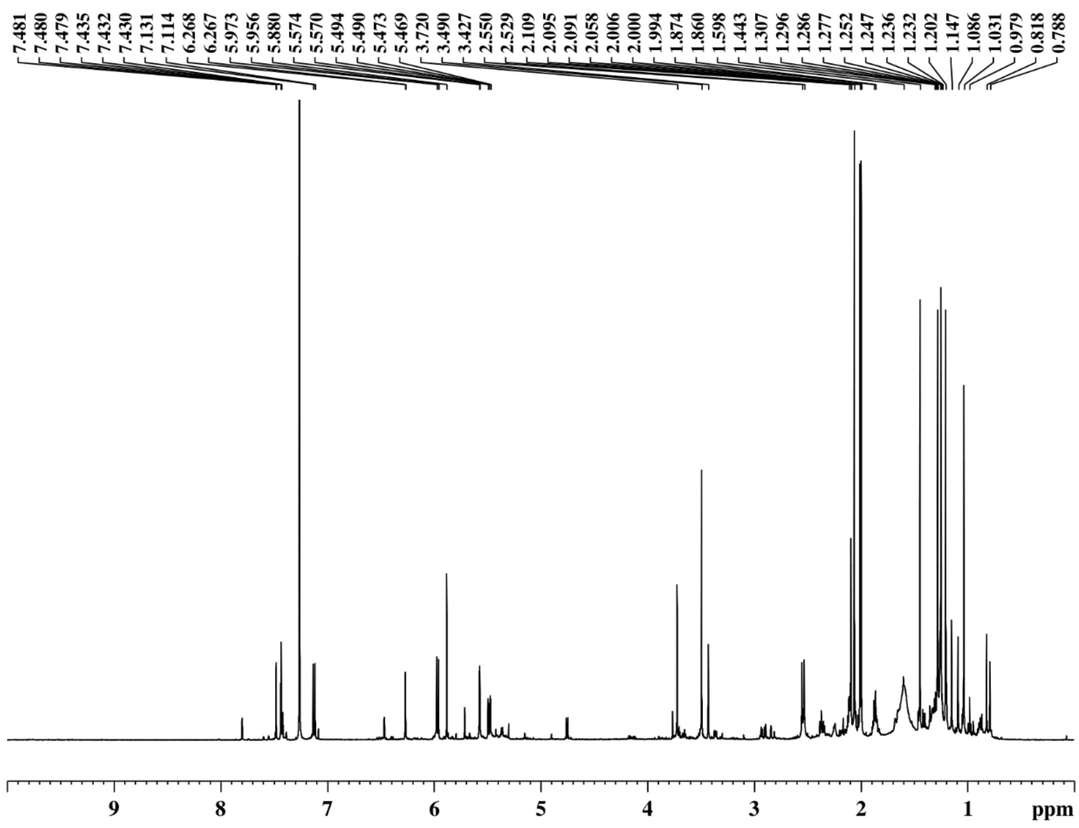
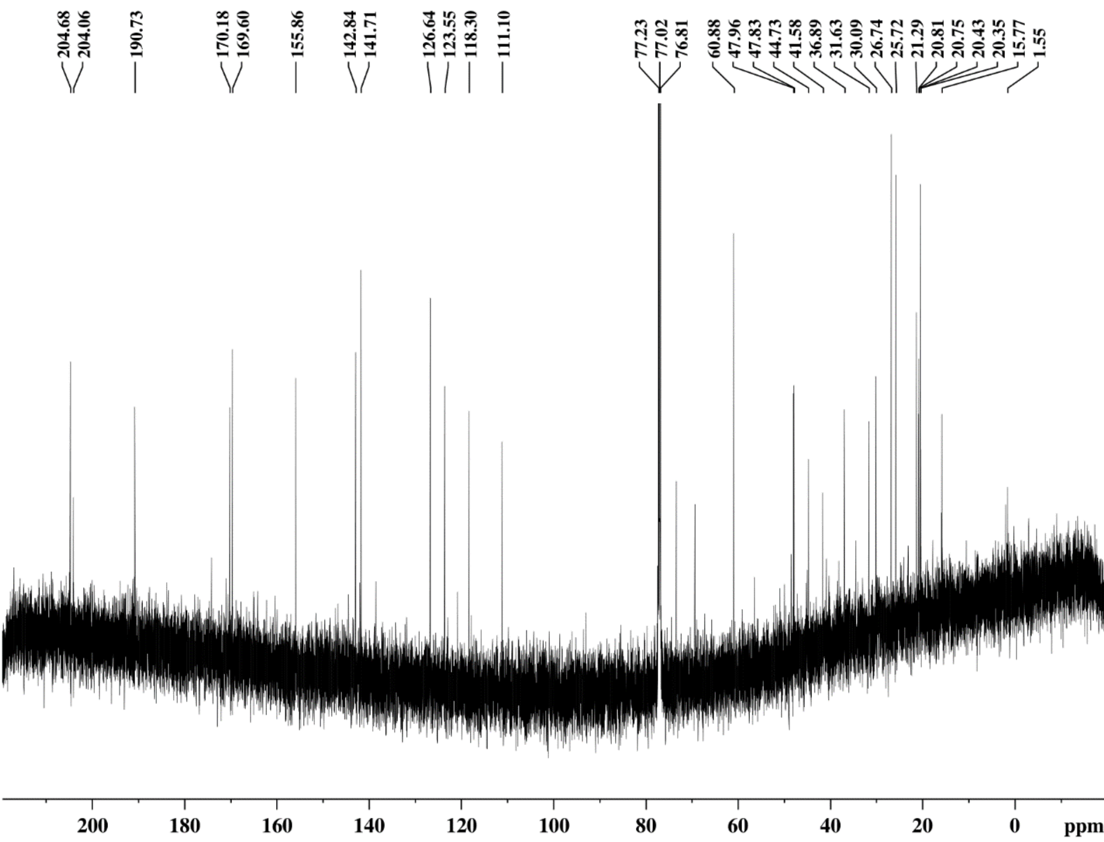
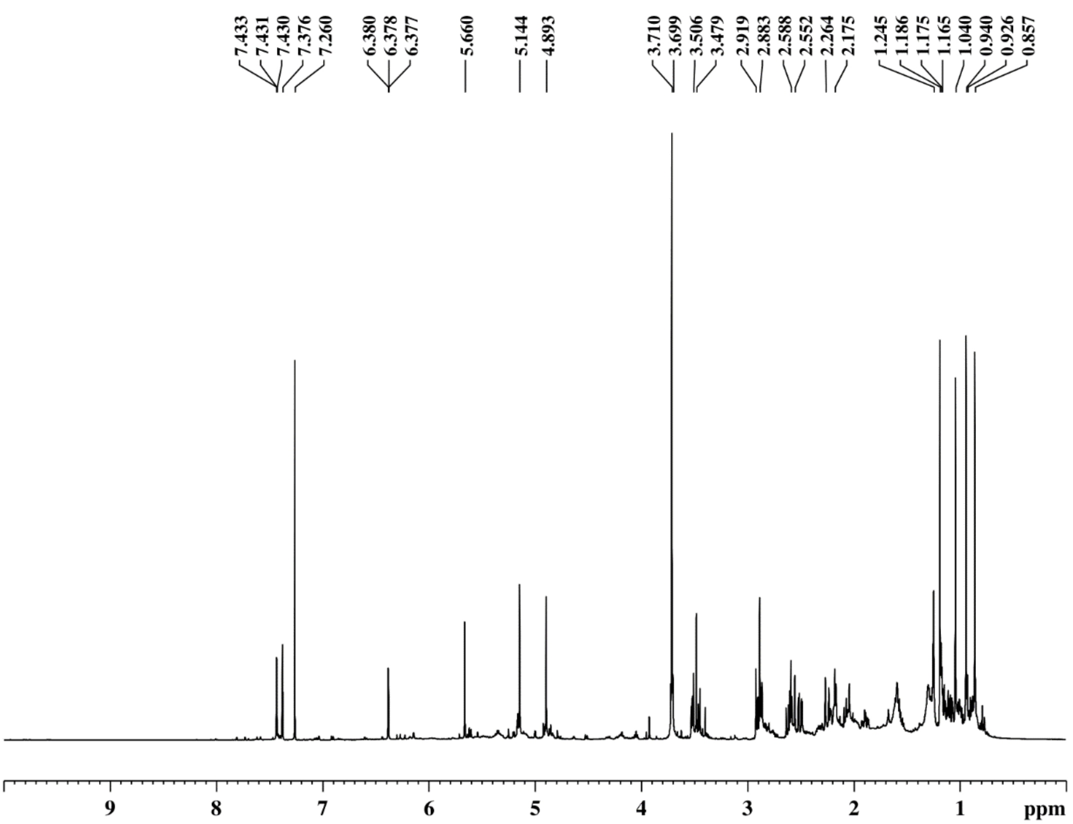
Appendix



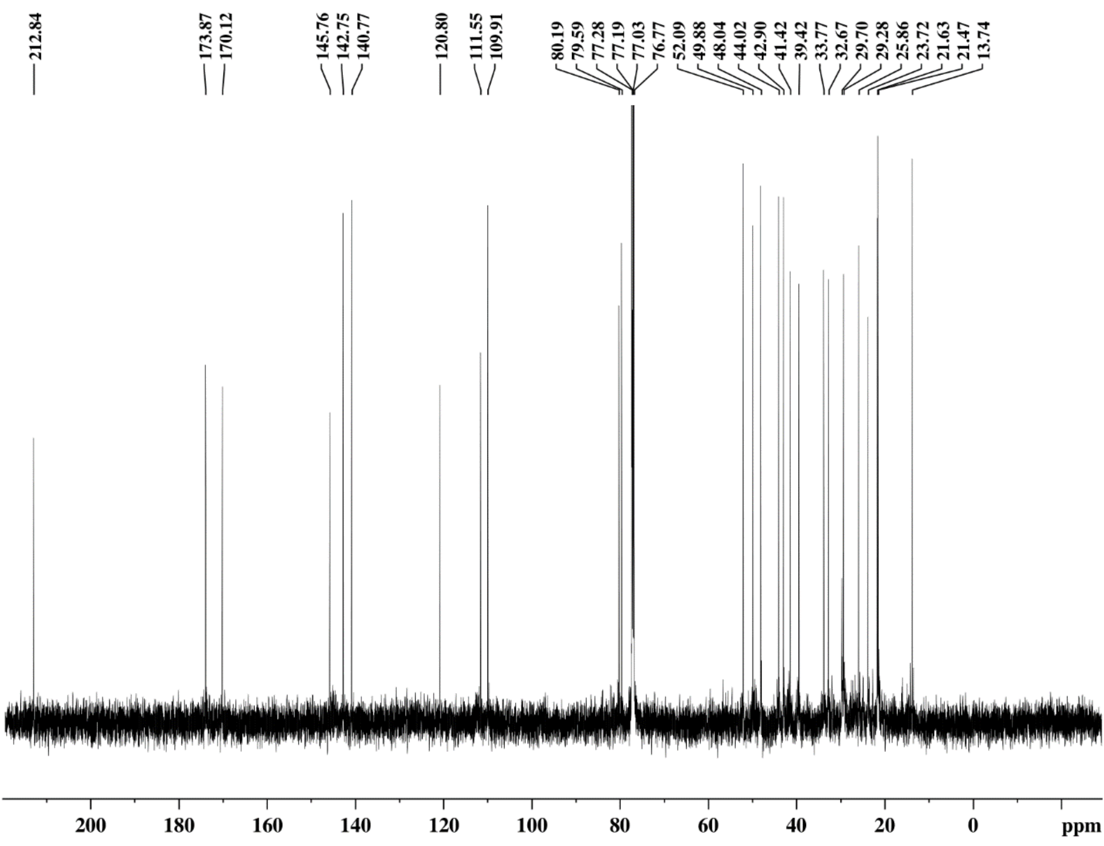
Appendix 1: ^{1}H NMR of Mahonin (1) (600MH$_{Z}$, CDCL3)



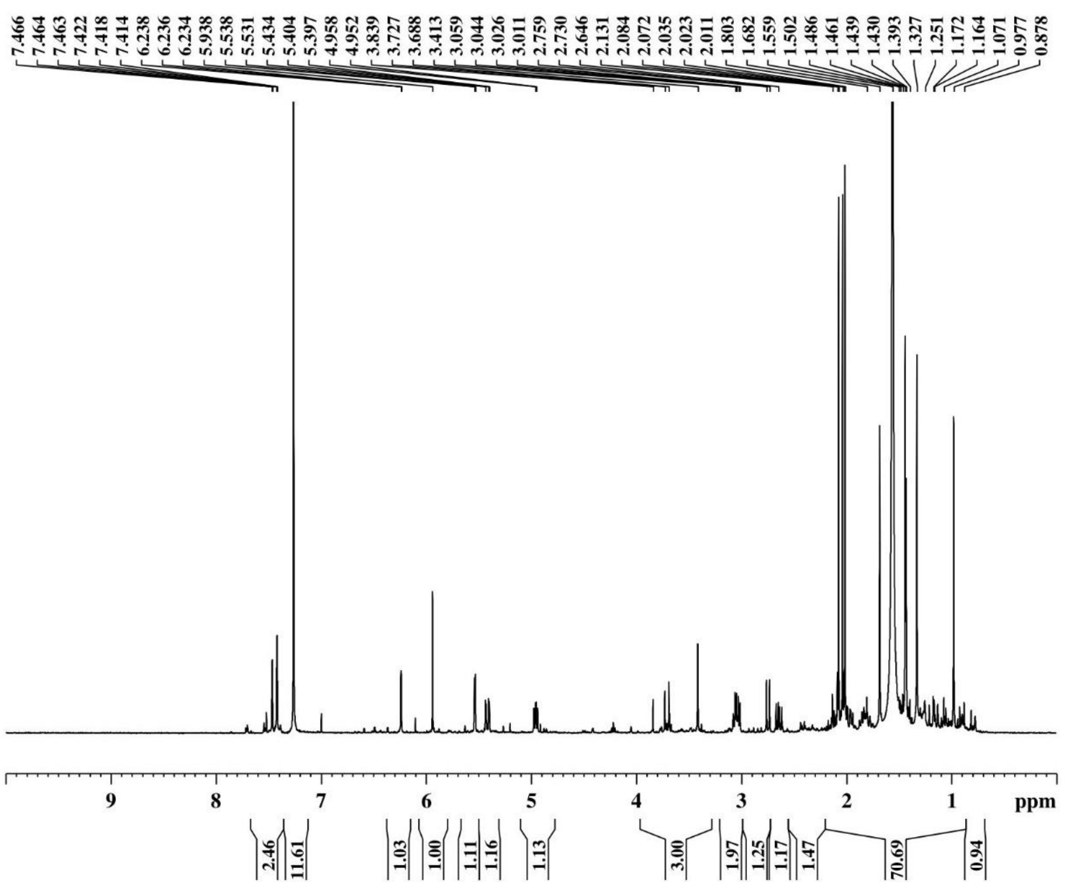
Appendix 2: ^{13}H NMR of Mahonin (1) (150 MH$_Z$, CDCL3)



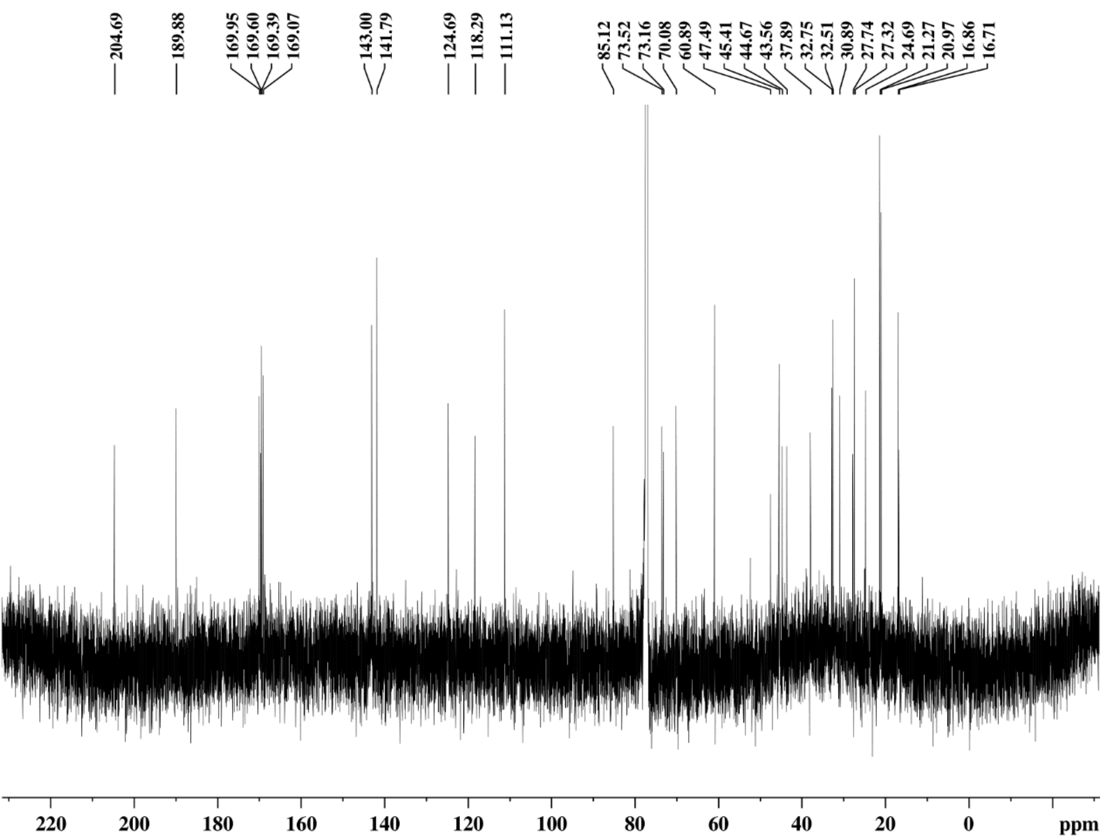
*Appendix 3: ^{1}H NMR of Methyl angolensate (**2**) ($600MH_Z$, $CDCL_3$)*



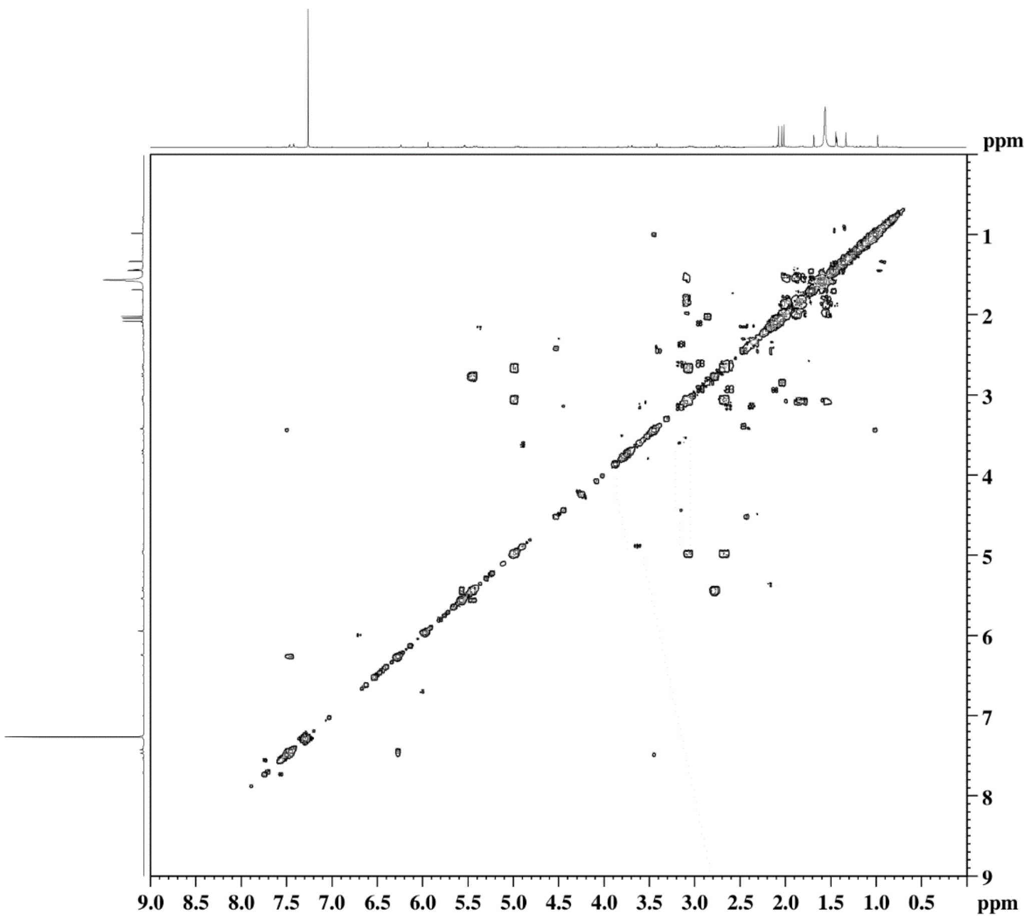
*Appendix 4: ^{13}H NMR of Methyl angolensate (**2**) (150 MH_Z, $CDCL_3$)*



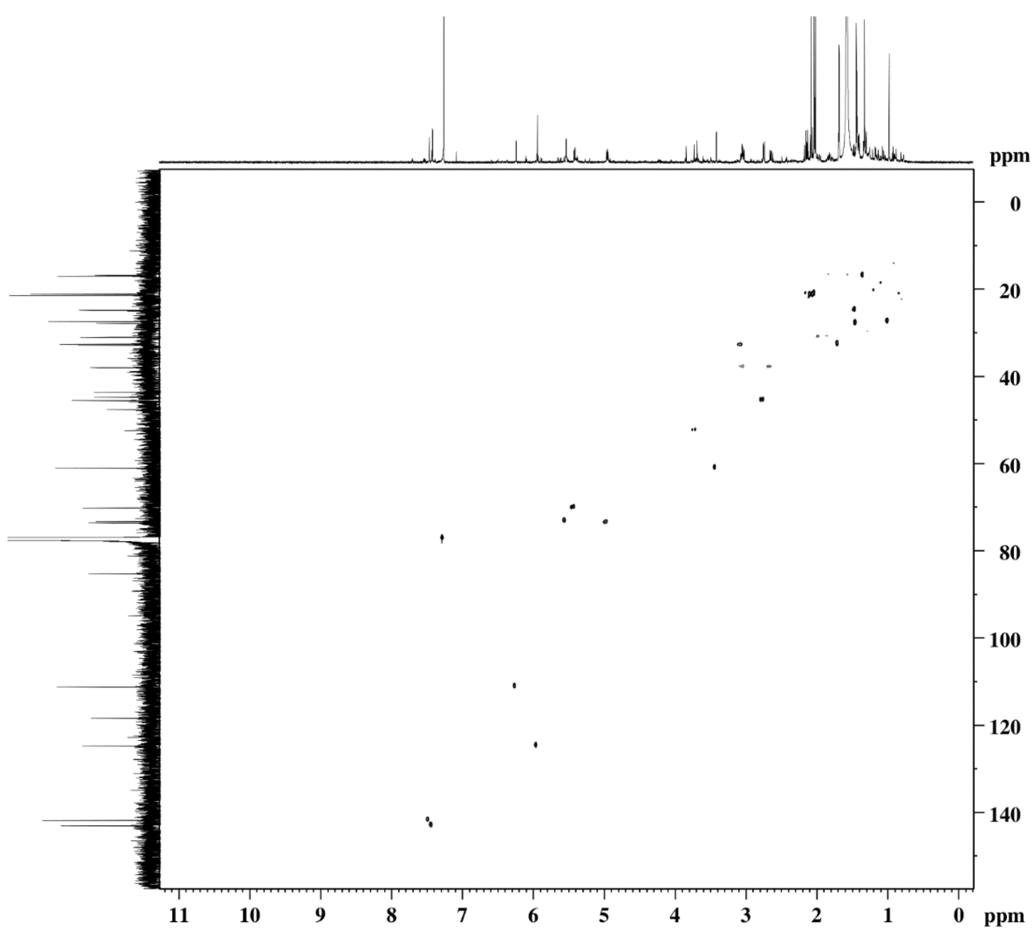
*Appendix 5: ^{1}H NMR of 16-oxodelevoyin B (**3**) (600MH$_Z$, CDCL$_3$)*



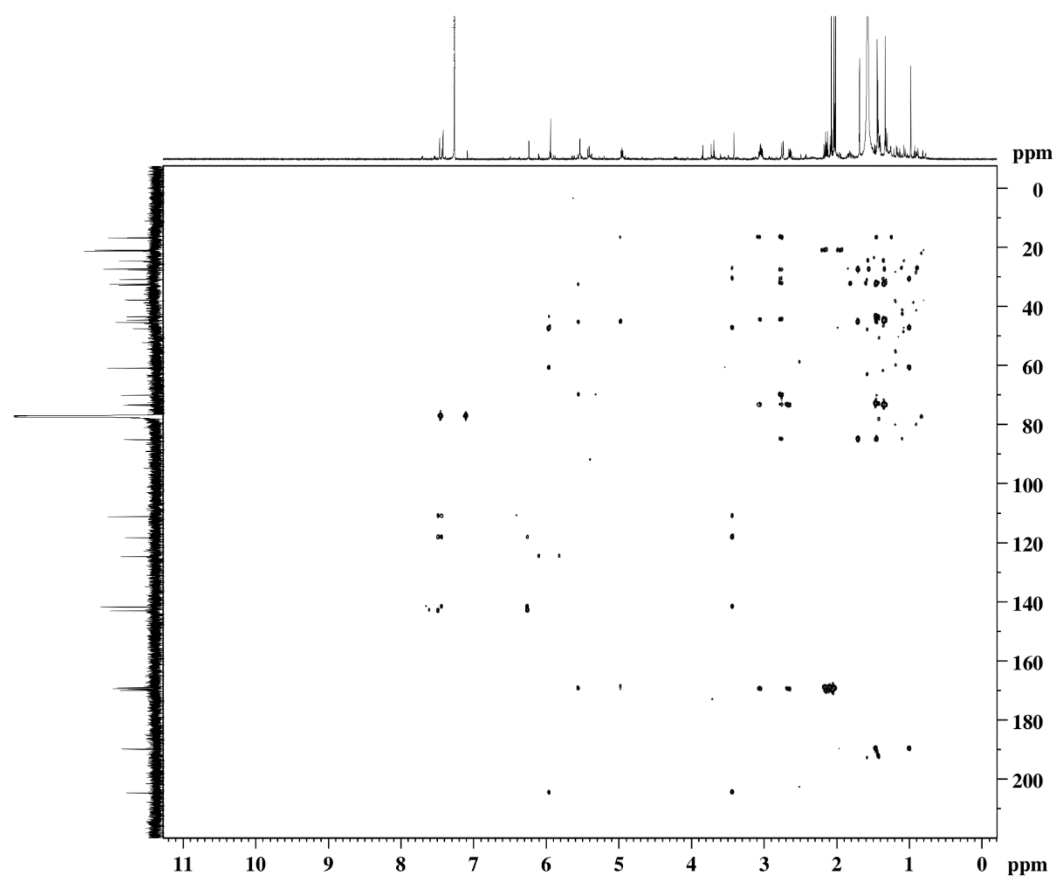
*Appendix 6: ^{13}H NMR of 16-oxodelevoyin B (**3**) (150 MH_Z, $CDCL_3$)*



*Appendix 7. COSY spectrum of 16-oxodelevoyin B (**3**) (600 MH_Z, $CDCL_3$)*



*Appendix 8. HSQC spectrum of 16-oxodelevoyin B (**3**) (600 MH$_Z$, 150 MH$_Z$, CDCL$_3$)*



*Appendix 9. HSQC spectrum of 16-oxodelevoyin B (**3**) (600 MHz, 150 MHz, CDCL3)*