INVESTIGATION OF THE EFFICACY AND CHEMISTRY OF SELECTED TRADITIONAL MEDICINES USED IN THE TREATMENT OF SCHISTOSOMIASIS AND THEIR ANTITRYPANOSOMAL POTENTIAL

BY

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DEDICATION

I dedicate this thesis to my parents, Mr. Alexander Afrani Twumasi and Mrs. Ama Sapoma Twumasi and to my siblings Mary, Emmanuel and Samuel for their unfailing love, prayers and support throughout.
DECLARATION

I hereby declare that this thesis is my original work which was carried out under strict supervision in the laboratories of the University of Ghana, Chemistry Department for the award of Master of Philosophy Degree in Chemistry. Apart from other works which have duly been acknowledged, I personally undertook all research work pertaining to this project and it has not been presented elsewhere for any other degree.

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........................................ ........................................
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(SUPERVISOR) (SUPERVISOR)
ACKNOWLEDGMENT

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My deepest gratitude goes to my supervisors Prof. Dorcas Osei-Safo and Dr. Kwaku Kyeremeh for their immense supervision, encouragements, and continuous support. I really appreciate all their sacrifices and it has been a wonderful experience learning from them.

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ABSTRACT

This research investigated the efficacy and chemistry of selected traditional medicines (TM) used in the treatment of schistosomiasis and also explored their antitrypanosomal potential.

Crude extracts were obtained by successive extraction of dried herbs with dichloromethane and methanol while aqueous herbal preparations were extracted with dichloromethane and butanol. Biological activity was conducted by screening the crude extracts against the schistosomula (NTS) and adult worms of *Schistosoma mansoni* and bloodstream forms of *Trypanosoma brucei brucei*. At 100 μM, two crude extracts, NTD_B4 DCM and NTD_B7 DCM completely killed the NTS and demonstrated greater than 70% effect against adult worms of *S. mansoni*. Furthermore, all crude extracts exhibited antitrypanosomal activity with IC<sub>50</sub> ranging from 6 – 19 μg/mL, with NTD_B4 DCM recording the highest IC<sub>50</sub> of 6 μg/mL as compared to the 0.54 μg/mL of the standard, diminazene aceturate. Since NTD_B4 DCM displayed the highest antischistosomal and antitrypanosomal activities, it was selected for fractionation, chromatographic separations and further biological activity testing.

Fractionation of NTD_B4 DCM yielded 6 fractions of which F5 (100% ethyl acetate) and F1 (100% petroleum ether + PE:EtOAc/9:1) displayed the highest antitrypanosomal activity with IC<sub>50</sub> values of 7.4 and 8.5 μg/mL, respectively. Antischistosomal assay on the fractions is currently ongoing. Chromatographic separations of the two fractions led to the isolation of 4 solid compounds and 4 oils from F1 and 2 solid compounds from F5. Spectroscopic (IR and NMR) and spectrometric (MS) data led to the identification of 5,7-dihydroxy-6-methyl-2-tricosyl-4H-chromen-4-one and a fatty acid from F1, and β-sitosterol-3-O-glucoside and ethyl 4-hydroxybenzoate from F5. The structure elucidation of the remaining compounds was inconclusive due to the paucity of material.

All the isolated compounds, apart from 5,7-dihydroxy-6-methyl-2-tricosyl-4H-chromen-4-one (81 μg/mL), were not active against *T. brucei brucei*. The oily compounds, however, demonstrated profound antitrypanosomal activity with IC<sub>50</sub> < 32.2 μg/mL, with one oil (F1/HML) exhibiting 1.3-fold activity (IC<sub>50</sub> <0.0977 μg/mL) as compared to the positive standard diminazene aceturate (IC<sub>50</sub> = 0.13 μg/mL). Initial cytotoxicity tests against RAW murine macrophage cells indicated that F1/HML was not cytotoxic and was selective in killing trypanosomes. The GC-MS analysis of F1/HML led to the identification of phytane (6.31%) 1,4,5,8-tetrahydro-4a,8a-
naphthalenedicarboximide (10.30%), 2-butylbenzothiazole (4.26%), 4α-Methyl-24-ethyl-5α-cholest-8(9)-en-3β-ol (4.61%), and 11-(2,5-dimethylphenyl)-10-heneicosene (2.49%) as the major constituents.

This research confirmed the efficacy of 2 of the schistosomiasis remedies, NTD_B4 and NTD_B7, and provided scientific justification for their use. In addition, NTD_B4 and NTD_2 exhibited remarkable antitrypanosomal potential confirming these traditional medicines have activity against *Trypanosoma brucei brucei*. 
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<tr>
<td>$^{13}$C NMR</td>
<td>Carbon 13 NMR</td>
</tr>
<tr>
<td>1D</td>
<td>One dimensional</td>
</tr>
<tr>
<td>$^1$H NMR</td>
<td>Proton NMR</td>
</tr>
<tr>
<td>2D</td>
<td>Two dimensional</td>
</tr>
<tr>
<td>Calcd.</td>
<td>Calculated</td>
</tr>
<tr>
<td>CDC</td>
<td>Centers for Disease Control and Prevention</td>
</tr>
<tr>
<td>CDCl$_3$</td>
<td>Deuterated chloroform</td>
</tr>
<tr>
<td>COSY</td>
<td>$^1$H- $^1$H Correlation Spectroscopy</td>
</tr>
<tr>
<td>DCM</td>
<td>Dichloromethane</td>
</tr>
<tr>
<td>DEPT</td>
<td>Distortionless enhancement by polarization transfer</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>EtOAc, EA</td>
<td>Ethyl acetate</td>
</tr>
<tr>
<td>GC-MS</td>
<td>Gas chromatography- Mass Spectrometry</td>
</tr>
<tr>
<td>Hex</td>
<td>Hexane</td>
</tr>
<tr>
<td>HMBC</td>
<td>Hetero-nuclear Multiple Bond Correlation</td>
</tr>
<tr>
<td>HPLC</td>
<td>High Performance Liquid Chromatography</td>
</tr>
<tr>
<td>HRESIMS</td>
<td>High resolution –Electrospray ionization mass spectrometry</td>
</tr>
<tr>
<td>HRMS</td>
<td>High resolution mass spectrometry</td>
</tr>
<tr>
<td>HSQC</td>
<td>Hetero-nuclear Single Quantum Coherence</td>
</tr>
<tr>
<td>IC$_{50}$</td>
<td>Half maximal inhibitory concentration</td>
</tr>
<tr>
<td>IR</td>
<td>Infrared</td>
</tr>
<tr>
<td>MDA</td>
<td>Massive drug administration</td>
</tr>
<tr>
<td>MeOH</td>
<td>Methanol</td>
</tr>
<tr>
<td>Mult.</td>
<td>Multiplicity</td>
</tr>
<tr>
<td>n-BuOH</td>
<td>n-Butanol</td>
</tr>
<tr>
<td>NECT</td>
<td>Nifurtimox eflornithine combination therapy</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>--------------------------------------------</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
</tr>
<tr>
<td>NTD</td>
<td>Neglected Tropical Diseases</td>
</tr>
<tr>
<td>NTS</td>
<td>Newly transformed schistosomula</td>
</tr>
<tr>
<td>PE</td>
<td>Petroleum ether</td>
</tr>
<tr>
<td>PZQ</td>
<td>Praziquantel</td>
</tr>
<tr>
<td>R&lt;sub&gt;F&lt;/sub&gt;</td>
<td>Retention factor</td>
</tr>
<tr>
<td>TLC</td>
<td>Thin layer chromatography</td>
</tr>
<tr>
<td>TM</td>
<td>Traditional medicine</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>v/v</td>
<td>volume per volume</td>
</tr>
<tr>
<td>WHO</td>
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CHAPTER ONE
INTRODUCTION

1.1 Background
The World Health Organization (WHO) defines traditional medicine as the entirety of the skills, knowledge and practices based on beliefs, theories and experiences of diverse cultures used in the prevention, diagnosis, improvement or treatment of physical or mental illnesses. Traditional medicine is also known as ethnomedicine or complementary and alternative medicine. It is a primeval and culturally bound method of healing that has been utilized by man to deal with countless diseases that endangered his survival and existence. It encompasses a wide range of practices with herbal medicine forming 70-80% of traditional medicine. It is significant to note that about 80% of the world’s population depend on ethnomedicine with as high as 95% of this group coming from developing countries.

1.2 Blockbuster drugs from plant sources
Many modern drugs were derived from plant sources. Morphine [I], isolated from Papaver somniferum L. in 1805, and used to alleviate pain, was the first pharmacologically active compound to be obtained from plants in its pure form. Its structure was finally deduced in 1923. Codeine [II], which doubles as an antitussive and analgesic was also obtained from P. somniferum. Atropine [III], isolated from Atropa belladonna exhibits great anticholinergic activity. Administration of atropine injection during anaesthesia and surgery keeps the heartbeat normal. Atropine sulphate blocks or reverses the adverse effects produced by some drugs and pesticides.
It is also used as a mydriatic in ophthalmology. German scientists isolated reserpine [IV], from Rauwolfia serpentina (L.) Benth ex. Kurz in 1949. Reserpine is presently used as a sedative and an antihypertensive drug. Chemical investigation of the Pacific Yew, Taxus brevifolia Nutt. led to the isolation of paclitaxel [V], which is currently used in cancer chemotherapy to treat ovarian, breast, pancreatic, cervical and lung cancers. The vinca alkaloids (vincristine [VI] and vinblastine [VII]), obtained from Catharanthus roseus, are used in the treatment of paediatric leukaemia and Hodgkin’s disease. Digitoxin [VIII] and digitalin [IX], isolated from Digitalis purpurea L. are used for the treatment of congestive heart failures. The bark extract of Cinchona tree has been used as therapy for malaria since at least 1632. Phytochemical investigation of the bark extract of
Cinchona tree by Pierre Pelletier and Joseph Caventou led to the isolation of the active compound, quinine [X] in 1820. Extracts of Artemisia annua have been used to cure fever for more than 2000 years. In 1972, Tu Youyou, a Chinese scientist, discovered artemisinin from A. annua. Artemisinin [XI] and its semi-synthetic analogues have exhibited profound antimalarial activity against Plasmodium falciparum. They have also been very effective in the treatment of other helminth infections\(^\text{10}\). Caffeine [XII] (stimulant), ephedrine [XIII] (bronchodilator) and cocaine [XIV] (anaesthetic) were isolated from Coffea arabica, Ephedra spp., and Erythroxylum coca, respectively\(^5\). The structures of the compounds afore-mentioned are indicated in Figure 1.1.
Some plants are also well known as herbal medicines. *Mentha spp.* are used to soothe soreness and to ease muscular tension. *Allium sativum* (garlic) which is believed to be native to Siberia but has spread globally is used for curing several conditions related to the cardiovascular system. It is used to treat hypotension, coronary heart disease, hypertension, high cholesterol and normalizes narrow blood vessels. It is also used as a complementary drug in the treatment of hookworm, pinworm and roundworm infections. *Ephedra spp.* are known traditionally for their therapeutic effect on asthma, common cold and hay fever.

People living in developing countries in tropical and subtropical regions harness the exceptional medicinal properties of diverse plants for therapeutic purposes against several diseases. Amongst the diseases treated traditionally are those termed as the neglected tropical diseases.

### 1.3 Neglected Tropical Diseases

Neglected tropical diseases (NTDs) consist of a varied group of infectious diseases that occur in tropical and subtropical regions worldwide. They affect about 1.4 billion people, representing 18% of the world’s populace who live below the world bank figure of $1.25 per day. They are mostly endemic in poverty-stricken areas where there are insufficient sanitary conditions; and those in close contact with domestic animals and infectious vectors. The group now consists of 20 communicable diseases. These diseases have inimical effects on the productivity of the workforce, child development and the general well-being of men and women. They cause chronic disabilities in children, impair their growth and cognitive development.

The world’s greatest concentration of poverty is in Africa, with more than 500 million people (about 40% of the people in sub-Saharan Africa) affected with trachoma, lymphatic filariasis, soil-transmitted helminth infections, schistosomiasis and onchocerciasis. These infectious diseases are transmitted by contact with the discharge of infected people, mosquitoes, through faeco-oral route, freshwater snails and blackflies, respectively. Research indicates that trachoma and onchocerciasis are the first and second chief causes of blindness associated with parasitic infections. Lymphatic filariasis is characterized by swelling of the limbs and genitals. The psychological and social stigma associated with this disease is immense. Soil-transmitted helminth infections; can result in anaemia, malnutrition, growth retardation and lowered resistance to
infections. Schistosomiasis is characterized by blood in stools or urine, fever and abdominal discomfort.

The WHO endorsed an integrated modus operandi to overcome the worldwide impact of NTDs through 5 interventions; innovative and intensified disease management, chemoprophylaxis, veterinary public health service, vector ecology and management and provision of potable water, sanitation and hygiene. In the treatment of NTDs, most of the affected populace use traditional medicine, often the only source of medication in the tropical and subtropical regions due to the fact that they are readily available, affordable and accessible and the belief that they have little side effects. Usually, there is strong folkloric evidence to back the effectiveness of traditional medicine. Some practitioners with years of experience employ certain standards and dosages in the administration of herbal medicine and hence attain the desired effects.

1.4 Schistosomiasis
Schistosomiasis is commonly known as bilharzia and is caused by parasitic Schistosoma trematode flatworms that live in selected species of freshwater snails. It affects over 207 million people, with 85% of all cases occurring in sub-Saharan Africa. Globally, mortality due to schistosomiasis is projected to be 15,000 deaths per year excluding indirect mortality associated with schistosomiasis infections such as liver disease, hypertension and cervical cancer. If all these are taken into account, it results in over 200,000 deaths per year. In terms of the impact of the disease, it ranks second to malaria as the most devastating cause of parasitic deaths worldwide.

The prevalence of the disease is about 54.8% to 60.0% in Ghana and is still a major problem in some parts of the country especially in rural areas. Urinary schistosomiasis which is the most common type in Ghana is characterized by haematuria – blood in the urine. WHO recommends praziquantel (PZQ) as the first line of treatment.

1.5 Human African Trypanosomiasis
Human African trypanosomiasis (HAT) is spread by the bite of an infected tsetse fly belonging to the genus Glossina. It is caused by two subspecies of Trypanosoma brucei: T. b. gambiense and T. b. rhodesiense. The common name, ‘sleeping sickness’ originates from meningoencephalitis
which occurs at the second stage of the disease. It eventually leads to coma and or death if not treated\textsuperscript{27}.

About 65 million people are presently at risk of infection in 36 countries in sub-Saharan Africa including Ghana. The intensity of endemicity varies with different countries but about 85\% of all entire cases occur in Angola, Sudan and the Democratic Republic of Congo\textsuperscript{28}.

In the treatment of HAT, drugs are prescribed based on the stage of the disease and the specific causative subspecies. WHO recommends pentamidine and suramin for treating the first stage and nifurtimox eflornithine combination therapy (NECT) for treating the second stage of the disease\textsuperscript{29}.

1.5.1 Some Traditional Medicines Used in the Treatment of Schistosomiasis and HAT

Investigations show that oils obtained from garlic (\textit{Allium sativum}) and onion (\textit{A. cepa}) significantly normalized liver function enzymes and enhanced the antioxidant status in \textit{Schistosoma mansoni} with a subsequent decrease in egg count and worm burden\textsuperscript{30}. The leaves of \textit{Vernonia amygdalina} are used to treat haematuria caused by \textit{Schistosoma haematobium} and stomach distresses caused by \textit{S. mansoni} infection in South Africa. A follow-up study on the petroleum ether and ethanol extract of the leaves of \textit{V. amygdalina} showed curative and prophylactic activities against liver, kidney and spleen experimental schistosomiasis in mice\textsuperscript{30,31}. In southeastern Uganda, traditional healers use various medicinal plants as therapeutics for HAT. Nine of these medicinal plants were collected for research purposes and extracts from \textit{Ehretia amoena}, \textit{Entada abyssinica}, \textit{Securinga virusa} and \textit{Albizia gummifera} showed remarkable activity against \textit{T. brucei brucei} with IC\textsubscript{50} <1 \(\mu\text{g/mL}\).\textsuperscript{32}

These traditional medicines have been effective as therapeutics in the treatment of schistosomiasis and human African trypanosomiasis in Africa and follow-up investigations of the TM by scientists confirmed the efficacy of some of these medicines.

1.6 Problem Statement and Justification

The WHO set goals in May 2012 to control the morbidity of schistosomiasis by 2020 and eliminate it as a public health concern by 2025. Other goals were set in Geneva in 2018 to eliminate HAT
as a public health concern in 2020. In this regard, chemical prophylaxis of schistosomiasis through massive drug administration and nifurtimox-eflornithine combination therapy were freely distributed in endemic areas to control the disease. As a result, cases of HAT have reduced remarkably over the years and schistosomiasis is under strict management\(^\text{33,34}\).

In Ghana, as is the case for many African countries, most people opt for traditional medicines (TM) due to the fact that they are affordable, readily accessible and available since the continent is endowed with abundant flora\(^\text{35}\). For some of these TM, especially for priority diseases and common ailments such as malaria, hypertension, diabetes and diarrhoea, there is evidence of long-term use of traditional medicine and proven safety and efficacy as well as stability under various conditions. As a result, several standardized phytopharmaceuticals for these diseases are officially registered for sale and use from accredited pharmacies and shops. This has been approved by the Foods and Drug Authorities (FDA) and also included in the essential TM list for dispensing at clinics and health centres. In contrast, this is not the case for TM of less common but debilitating diseases such as schistosomiasis, where there is lack of structures for efficacy testing, quality assurance and standardization processes. Hence, regardless of their patronage by affected people in endemic communities and beyond, their effectiveness is usually backed mainly by traditional, historical and anecdotal evidence.

If Ghana is to succeed in meeting her schistosomiasis elimination target, it is imperative to investigate these TM to substantiate their efficacy due to their wide consumption. This will provide a scientific rationale for their use and confirm that they are not mere placebos but effective medicines. Additionally, it is important to establish that they are not acutely and/or chronically toxic. Once non-toxicity has been determined, TM practitioners should be supported to develop their products into usable and well-standardized formulations while further scientific investigations continue for identification of novel active ingredients for development into suitable drug substitutes for PZQ. Reports from Egypt and Senegal, two schistosomiasis endemic populations, confirm the incidence of drug resistance of some strains of \textit{S. mansoni} to PZQ. The quest for new drug leads from TM will, therefore, prove useful especially in the event of resistance\(^\text{36}\).
1.7 Hypothesis
Traditional medicine is widely used in Ghana for the treatment of schistosomiasis. However, the proof of efficacy that is currently available is based mainly on empirical experience. Consequently, subjecting these remedies to the requisite screening platforms will provide a scientific rationale for their use. Isolation and purification of the remedies could lead to the identification of novel compounds for the development into drug candidates with novel mechanisms of action for the treatment of schistosomiasis. Furthermore, the traditional medicines used for the treatment of schistosomiasis could possess antitrypanosomal activities as well since medicinal plants often exhibit other biological activities.

1.8 Aim and Objectives

1.8.1 Aim
This research sought to evaluate and validate the efficacy of a selection of TM used in the treatment of schistosomiasis in Ghana through biological activity screening against schistosomulae and adult worms of *S. mansoni*. The same cohort of TM was also screened for their antitrypanosomal potential against bloodstream forms of *T. brucei brucei* followed by isolation of compounds present in active samples through bioassay-guided fractionation. The antitrypanosomal activity of the isolated compounds and oils was also evaluated.

1.8.2 Objectives
The following objectives were employed to achieve the aim of this research.

- To enquire about TM used in the treatment of schistosomiasis in Ghana from practitioners and collect representative samples;
- To conduct biological activity (antischistosomal and antitrypanosomal) screening of the TM;
- To isolate compounds from active remedies through chromatographic techniques;
- To identify isolated compounds using IR, UV, NMR and MS data;
- To further conduct antitrypanosomal activity screening on isolated compounds.
CHAPTER TWO

LITERATURE REVIEW

2.1 Traditional Medicine

The association between plants, animals and man originated from the beginning of life. Over time, man learnt to identify plants with medicinal properties and other characteristics that met their daily necessities. Knowledge of the medicinal properties of plant acquired over time was transfused into culture and practices, for the survival and longevity of the people. This, as a result, became part of their tradition and was transferred across generations through oral tradition such as Ghanaian traditional medicine or through well-documented literature like Chinese and Egyptian traditional medicine. Despite, the Kingdom Plantae is still an unexploited reservoir of new compounds with promising therapeutic properties, since only 28,187 of the 350,000 known plant species, that is, about 8% have been investigated from phytochemical and pharmacological perspectives. Plants serve as rich sources of compounds that can be employed as scaffolds in drug synthesis. Due to this, natural products and their synthetic derivatives account for over 40% of newly listed drugs in the drug discovery process.

Traditional medicine (TM) constitutes various practices that make up the indigenous health care and traditions of the world. It has been used in the diagnosis, treatment, prevention and management of a plethora of diseases since ancient times. They are sourced from plants, animal products and mineral substances. Usually, it takes a holistic and personalized approach to the body, spirit and mind concept of health. The patient participates actively in the healing process by practicing disease prevention strategies. This is seen in the great traditions of Hippocratic-Galenic medicine of ancient Greece; Chinese medicine of Asia; Ayurveda of India; Unani Tibb of Arabia; and numerous traditions in Africa, Asia, Europe and the Americas. In Africa, 90% of Ethiopians, 85% of South Africans, 75% of Malians and 70% of Ghanaians depend on TM for their basic healthcare.

Generally, TM encompasses herbal medicine, psychotherapy, therapeutic fasting and dieting, psychiatry, therapeutic occultism, spinal manipulation and massage, radiant healing therapy, hydrotherapy, surgery and bone-setting, phlebotomy and preventive medicine. In various traditions, two aspects are considered in the healing process. In the physical aspect of TM, plants,
animals or mineral substances may be used whilst the metaphysical division employs prayers, invocations or incantations to mysterious and powerful forces\textsuperscript{43}.

The extensive use of TM in Africa may be linked to cultural and economic reasons. Regardless, WHO encourages member states to assimilate TM in their health care systems and has expressed keen interest in keeping records of medicinal plants used by indigenes from different countries. Plants used by TM practitioners contain different phytochemicals that may act individually, cumulatively or synergistically to ameliorate the health of patients\textsuperscript{44}. For instance, \textit{Khaya senegalensis} is used by some traditional healers in Africa as a purgative, abortifacient, and to treat leprosy, angina, chickenpox and syphilis. A decoction of the bark is used to treat malaria, diarrhea, dysentery, anemia and serves as an emmenagogue, anthelmintic and anodyne. The extract of the stem bark is also used by the Nupes of Niger state in Nigeria to treat jaundice, dermatoses and hookworm infections\textsuperscript{45,46}. Also, extracts from the leaves of \textit{Azadirachta indica} are used in curing jaundice, skin diseases and for expelling intestinal worms. The cake which remains after the oil has been extracted from the seed is used as fertilizer; it kills nematodes and mitigates insect attack. Extracts from \textit{A. indica} have also been used in the treatment of leprosy, stomach upset, intestinal worms, skin ulcer, cardiovascular diseases, fever, liver problems, stomach and intestinal ulcer and malaria\textsuperscript{47}.

The use of TM in Ghana is extensive and greatly varied due to cultural and plant diversity. This has an effect on the local economy and the conservation of biodiversity in the country. The rich history of herbal medicine and innovative use of plants as sources of medicine in Ghana has been passed down to generations and it is still highly patronized. Over 70\% of Ghanaians depend on TM for their primary healthcare due to its availability, accessibility and affordability\textsuperscript{48}. Apart from their belief that TMs have no side effects, people use TMs due to the high costs of some conventional medicines, their sheer lack of faith in them, possible delays at hospitals and unfavorable interactions with orthodox medicines. However, there have been reports on the side effects of TMs such as allergic reactions, asthma, headaches, nausea, vomiting and diarrhoea\textsuperscript{49}.

Populations dwelling in developing countries in the tropics are exposed to several infectious diseases including malaria, hepatitis B and C, measles and others. Amongst the infectious diseases are those classified as neglected tropical diseases which affect people living in impoverished rural and urban communities with poor sanitation in tropical regions.
2.2 Neglected Tropical Diseases

In the early years following the implementation of the Millennium Development Goals (MDGs), WHO together with a community of scientists published series of papers on the contemporary structure for reviewing a cohort of parasitic and associated infections as Neglected Tropical Diseases (NTDs)\textsuperscript{50}. NTDs comprise diverse infectious diseases which are widespread in tropical and subtropical regions. These diseases were termed ‘neglected’ due to the fact that they affected penurious populations living in conflict zones, remote rural areas and urban slums. They usually persist in conditions of paucity and are concentrated solely in impecunious populations worldwide\textsuperscript{51}. Usually, populations with insufficient sanitation and close to infectious vectors and domestic animals suffer the most. They were initially 17 diseases which included Buruli ulcer, Chagas disease, onchocerciasis, dengue fever, echinococcosis, rabies, schistosomiasis, trachoma, leishmaniasis, yaws, leprosy (Hansen’s disease), fascioliasis, soil-transmitted helminth infections, dracunculiasis (guinea worm), lymphatic filariasis, human African trypanosomiasis (HAT) and cysticercosis\textsuperscript{52}. The number was increased to 20 when the Strategic and Technical Advisory Group for NTDs, in their tenth meeting in 2017 received suggestions on the inclusion of snakebite envenoming, chromoblastomycosis and deep mycoses, scabies and other ectoparasites to the NTDs folder\textsuperscript{15}.

Research shows that about 1.4 billion of the population of the world is presently infected with NTDs with 1 billion more still at risk of being infected. Some of the causative infectious agents are bacteria (leprosy, yaws, trachoma, Buruli ulcer), viruses (dengue, rabies), protozoa (leishmaniasis, Chagas disease, human African trypanosomiasis) and helminth parasites (onchocerciasis, lymphatic filariasis, echinococcosis, schistosomiasis, intestinal worms, guinea worm). These infectious diseases are spread by contact with infected people, blackflies, sandflies, tsetse flies, mosquitoes, water snails and faeco-oral route\textsuperscript{17}. Figure 2.1 shows the distribution of NTDs in the world.
2.2.1 NTDs in Africa

Research shows that over 40% of NTD cases are found in Africa. In a statement made by Dr. Tedros Adhanom Ghebreyesus, the Director-General for WHO since 2017, he indicated that efforts must be intensified to beat NTDs if stakeholders are serious about universal health coverage. He mentioned that efforts through massive drug administration (MDA) seek to prevent/control common NTDs and they are backed by WHO and other pharmaceutical companies which donate drugs required to control and eliminate the diseases. Five of the most common NTDs that can be treated by chemoprophylaxis through MDA are lymphatic filariasis, soil-transmitted helminthiases, trachoma, schistosomiasis and onchocerciasis. Several steps have been instituted by the African Union to ensure the control and eradication of the diseases. They include committing member states to provide domestic health financing, regularly monitoring the endemicity of the diseases by setting up a task force to facilitate knowledge across the continent and celebrating countries as they achieve their elimination goals.
2.2.2 NTDs in Ghana

Investigations carried out in 2007 indicated that Ghana was endemic to ten out of the 17 neglected tropical diseases worldwide\(^\text{20}\). These were lymphatic filariasis, Buruli ulcer, yaws, trachoma, guinea worm, soil-transmitted helminth infections, leprosy, rabies, human African trypanosomiasis and cutaneous leishmaniasis. A tactical plan for the NTD program was established with a vision to free the country from the ancient diseases and afflictions that have burdened humanity for centuries. The goal was to ameliorate the potential of the Ghana Health Service and to integrate NTD programs to enhance their capability to avert, control and eradicate NTDs by 2020. During that period, it was observed that yaws and Buruli ulcer were prevalent in the humid forest zones. Populations close to rivers and lakes were susceptible to onchocerciasis (along fast-flowing tributaries) and schistosomiasis (areas with more stagnant water). The coastal and dry areas were prone to lymphatic filariasis which was transmitted by the mosquito bite\(^\text{20}\). Several disease control programs were established to target the treatment of the major NTDs; trachoma, soil-transmitted helminthiasis, schistosomiasis, lymphatic filariasis and onchocerciasis. Through this program, Ghana successfully eliminated trachoma as a public health concern in June 2018\(^\text{56}\).

2.2.3 Traditional medicines used in the Treatment of some NTD

In a research work published by Sanaa Ahmed Ali, mention was made of the fact that most developing countries relied heavily on TM for their basic healthcare and most of these were from plant sources. Several traditional medicines, specifically herbs have been used to treat NTDs in ethnomedicine\(^\text{30}\).

In a review of South African plants by Cock et al, it was highlighted that the leaves of *Acacia sieberiana* DC var. walii, locally known in Zulu as ‘umkhamba’ are used traditionally as vermifuge and taenifuge. The leaves of *Clerodendrum glabrum* E. Mey are also used to treat intestinal parasites, especially roundworm and tapeworm and to fend off parasites from wounds\(^\text{57}\).

*Allium sativum* (garlic) has been used in folk medicine to treat parasitic worms in diverse cultures in India, East Asia, Saudi Arabia, North America and Peru. Furthermore, the seeds from *Cucurbita pepo* are effective against tapeworms\(^\text{30}\).
An *in vitro* assay of the ethanolic extract of *Neurolaena lobata* shows significant inhibitory activity on the motility of adult worms of *Brugia phangi*. Phytochemical investigations of cinnamon led to the isolation of cinnamic acid [XV] which exhibited inhibitory activity against the adult worms of the parasitic roundworm (*Brugia phangi*) *in vitro*. This confirmed the traditional use of these plant extracts as anthelmintics.[58]

In Gabon, an infusion of the leaves of *Alchornea cordifolia* is taken orally, twice a day to effectively expel filarial worms, the causative organism for lymphatic filariasis. A decoction of the leaves of *Senna alata* L. Roxb. from the family Leguminosae is taken orally and also via the dermal route by washing one’s self with the herbal preparation to cure helminthiases.[59]

A follow-up on the phytochemical investigation of some plants used traditionally led to the isolation of berberine [XVI], an isoquinoline alkaloid from the stem bark of *Berbers aquifolium* and *Hydrastis candensis* amongst others. It stimulates the protective mechanisms in patients with clinically active trachoma lesions. Patients clinically treated with this compound experienced no relapse even after a year of treatment. Biochanin A [XVII] was isolated from red clover extract and it exhibits growth inhibitory activity with an IC$_{50}$ of 12 μM against *Chlamydia trachomatis*, the causative agent of trachoma. Chemical investigation of the root bark of *Cussonia zimmermannii* led to the isolation of polyacetylene which displayed antitrypanosomal activity with IC$_{50}$ of 0.4 μM against the amastigotes of *Trypanosoma cruzi* with a high selectivity index of 150.[60]

Dihydrochalcone [XVIII], which was isolated from *Piper elongatum* exhibited notable activity against *Leishmania braziliensis* with an IC$_{50}$ of 2.98 μg/mL. Piperine [XIX] was isolated from the same genus and it exhibited antitrypanosomal activity *in vitro* with an IC$_{50}$ value of 7.36 μM and 4.91 μM against the epimastigotes and amastigotes of *T. cruzi*, respectively.[61]

Asparagalin [XX], a triterpene saponin isolated from the roots of *Asparagus stipularis* exhibited antischistosomal activity against *Schistosoma mansoni*. It retarded worm growth and suppressed egg-laying at an IC$_{50}$ of 200 μg/mL.[60] **Figure 2.2** shows the structures of compounds isolated from some of these traditional medicines.
Figure 2.2: Structures of Active Compounds from some traditional medicines

2.3 Schistosomiasis (Bilharzia)

Schistosomiasis is a parasitic disease caused by blood flukes (trematode) belonging to the genus *Schistosoma*. Reports indicate that over 261 million people worldwide are affected and 91.4% of these cases are found in Africa. About 700 million people are in danger of infection in 76 countries where schistosomiasis is declared endemic. Deprived and pastoral communities, especially those who engage in agriculture and the fishing business are mostly affected. In the performance of domestic chores such as doing laundry in infected water bodies, women and girls get exposed to female genital schistosomiasis which is associated with genital lesions and may be a risk factor for HIV. Two main forms of schistosomiasis occur. These are intestinal and urogenital schistosomiasis and are caused by certain specific species of *Schistosoma*.

Intestinal schistosomiasis is caused by *Schistosoma mansoni, Schistosoma japonicum, Schistosoma guineensis, Schistosoma intercalatum* and *Schistosoma mengkoni*. They can be found in the Middle East, China, Africa, Indonesia, the Caribbean, Brazil, Philippines, Cambodia, Lao
Peoples Democratic and the Central African Republic. It is characterized by abdominal pain, diarrhoea and blood in faeces. In chronically advanced stages, the liver enlarges due to periportal fibrosis resulting from the inflammatory effects of the parasite on hepatic venous outflow. This leads to portal hypertension and results in the collection of fluid in the peritoneal cavity. Spleen enlargement can also occur. Urogenital schistosomiasis occurs in Africa, Corsica (France), the Middle East, and is caused by *S. haematobium*. The classical symptom is haematuria and can lead to damage to the bladder, ureter and kidney in advanced cases. Bladder cancer complications can occur in the later stages of the disease. Urogenital schistosomiasis may be associated with genital lesions, vaginal bleeding and pain during sexual intercourse in women. It can initiate diseases of the prostate, seminal vesicles and other organs in men. Long term irreversible effects include infertility.

### 2.3.1 Transmission and Life Cycle of Schistosomiasis

This cycle starts with the introduction of eggs into water bodies through faeces and urine of infected people. Miricidia are released as the eggs hatch and infiltrate specific water snail intermediate hosts (*Biomphalaria spp.*.) under optimal conditions. Cercariae are produced after two generations of sporocysts occur in the water snail. The infectious cercariae swim and infiltrate the skin of human hosts and break off their forked tail to become schistosomulae. During this period, they cause cercarial dermatitis, characterized by itching at the site of entry and urticarial wheals. The schistosomulae stay in the skin for a number of days and eventually enter the venous circulation. They travel through the circulatory system to the hepatoporal circulation where they develop into adult worms and mate. The female adult worms lay thousands of eggs that cause major damage to the intestine, bladder, kidneys and lungs. The adult female worms of *S. haematobium* are usually found in the venous plexus of the bladder but can also occur in the rectal venules. Eggs of *S. japonicum* and *S. mansoni* are progressively moved toward the lumen of the intestine whilst those of *S. haematobium* move towards the bladders and ureters and eventually get eliminated through faeces and urine respectively.

*S. japonicum* and *S. mansoni* infections are characterized by hepatic perisinusoidal egg granulomas, Katayama fever, portal hypertension and embolic egg granulomas in the brain and the spinal cord. Symptoms of *S. haematobium* infection include visible blood in urine also called
haematuria, calcification, bladder cancer, scarring and occasional ectopic egg granulomas in brain or spinal cord\textsuperscript{65}. The life cycle of schistosomiasis is summarized in Figure 2.3.

![The Life cycle of Schistosomiasis- CDC\textsuperscript{64}](image)

**Figure 2.3:** The Life cycle of Schistosomiasis- CDC\textsuperscript{64}

### 2.3.2 Diagnosis

Schistosomiasis is diagnosed by the identification of eggs in stools or worm antigens in the blood serum through sensitive rapid point-of-care tests for people living in endemic areas\textsuperscript{68}. People suffering from intestinal schistosomiasis are diagnosed by employing the Kato-Katz technique where thick fecal smears are made on methylene blue-stained cellophane saturated in glycerin or glass slides. Urine concentration tests are conducted in the diagnosis of urogenital schistosomiasis\textsuperscript{69}.

### 2.3.3 Treatment and Control of Schistosomiasis

A lot of measures have been instituted to control schistosomiasis. In view of this, communities living in proximity with infected waters have been educated on improving sanitation conditions which aim at reducing or eliminating the transmission of the disease. Bathing, swimming and wading in freshwater in areas where schistosomiasis is endemic should be avoided.
Vector control
Freshwater snails have been targeted in the control of schistosomiasis. The molluscicidal property of copper sulfate was discovered in 1939 by Chandler and this was used by Khalil as a first control attempt of the freshwater snails in Egypt. The waterbody is usually treated with about 0.125 ppm to 0.25 ppm of copper sulphate to kill the snails. This was then replaced by WHO-recommended drug of choice, niclosamide [XXI] in 1959 which has been used extensively in Egypt, Kenya, Morocco and Peoples’ Republic of China\textsuperscript{70}.

![Niclosamide](image)

As a check on the increasing costs of traditional molluscicide and their harmfulness to other non-target organisms, natural substances with molluscicide properties can be researched on and developed for snail control. *Ambrosia maritima*, for example, is an Egyptian herb, it shows great efficacy in killing intermediate hosts thus *Biomphalaria alexandrina* and *Bulinus truncatus* without harming non-target organisms\textsuperscript{71}.

Chemotherapy
Many drugs have been employed in the treatment of schistosomiasis since the 20\textsuperscript{th} century. Egypt implemented its foremost mass drug administration (MDA) in 1920 in covering all age groups using tartar emetic which was introduced by Christopherson. Safe drugs such as niridazole [XXII], metrifonate [XXIII], oxamniquine [XXIV] and praziquantel [XXV] as shown in Figure 2.4 refocused the control strategy on chemotherapy\textsuperscript{72}.

![Drugs](image)

\textbf{Figure 2. 4:} Drugs used in the treatment of schistosomiasis
Metrifonate is an organophosphorus compound that was initially intended as an insecticide. It has been very effective in the curing of urinary schistosomiasis caused by *S. haematobium*. It is well
tolerated and therefore used in mass chemotherapy programs involving school children. The main precaution taken in its administration is to avoid MDA in communities newly exposed to insecticides or other agricultural chemicals with anti-cholinesterase action. It has been found to be embryotoxic in pregnant women and so an alternative drug is preferred during treatment\textsuperscript{73}. In 2000, WHO stopped recommending metrifonate for the treatment of urinary schistosomiasis because it was not as effective as the drug of choice, praziquantel (PZQ)\textsuperscript{74}.

Oxamniquine is a tetrahydroquinoline derivative that has selective schistosomicidal activity against \textit{S. mansoni} but not the other \textit{Schistosoma} spp. About 60-90\% of cure rates have been observed in Egypt and Southern Africa. Resistant strains which were reported from South America were effectively treated with PZQ\textsuperscript{75}.

Artemether is effective against 3 major \textit{Schistosoma} spp.; \textit{S. mansoni}, \textit{S. haemotobium} and \textit{S. japonicum}. Combination therapy involving PZQ and artemether or artesunate has been used in the treatment of schistosomiasis since the late 1990s. It is particularly suitable for treating patients with repeated exposure to schistosome infested water\textsuperscript{76}. This combination therapy is very effective since PZQ has a short half-life (1-1.5 hours) and cannot eliminate the schistosomula stage of the parasite. So this is complemented by artemisinin and its derivatives since they show activity against the schistosomula stage of the parasite. The combination can also be used in places where malaria coexists with schistosomiasis; solving two dire ailments with one cure\textsuperscript{77}. However, there is a high concern with artemisinin and its derivatives therapy due to the emergence of resistant strains of \textit{Plasmodium} spp in malaria-endemic areas\textsuperscript{78}.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{artemether_artesunate.png}
\caption{Structures of Artemether and Artesunate.}
\end{figure}

Praziquantel is a pyrazino-isoquinoline anthelmintic used in the treatment of schistosomiasis and other cestode infections\textsuperscript{79}. It was developed in the 1970s and subsequently replaced other
antischistosomal drugs thereby becoming the drug of choice. It has high efficacy, excellent tolerability and mild side effects. Though the effectiveness of PZQ is well documented, its mode of action is not clearly defined\textsuperscript{26,80}. It is relatively safe and efficacious against all \textit{Schistosoma} spp. in humans but it does not avert reinfection and the emergence of resistance to this drug is a major challenge\textsuperscript{68}.

In spite of the widespread accessibility of praziquantel, schistosomiasis remains the cause of substantial morbidity and death in mainly developing countries. A heavily infected individual loses a considerable amount of blood through stools and urine which leads to anaemia\textsuperscript{81}.

\subsection*{2.3.4 Traditional Remedies Used in the Treatment and Control of Schistosomiasis}

An ethnopharmacological survey of plants in Mali showed that a decoction of the root of \textit{Calotropis procera} and the leafy parts of \textit{Stylosanthes erecta} is used to cure urinary schistosomiasis. Others use the pulverized fruits of \textit{Aframomum latifolium} and \textit{Trichilia emetica} twice a day for two weeks in the treatment of urinary schistosomiasis. Also, people of Nionoko in Mali mix the berries of \textit{A. latifolium} with the pulverized root of \textit{Entoda africana} to treat intestinal schistosomiasis. A decoction of the chopped fruits of \textit{Citrus aurantiifolia} and \textit{Tamarindus indica} is also used for treating urinary schistosomiasis in Mali\textsuperscript{24}.

In Egypt, \textit{Asparagus stipularis} is used to treat schistosomiasis amongst other diseases. It significantly reduces the ability of the female worms to lay eggs in a dose-dependent manner\textsuperscript{82}. Myrrh, a gum resin from \textit{Commiphora molmol} exhibited antischistosomal activity at a dosage of 10 mg/10 kg. In three days, a cure rate of 91.7\% was observed after the first administration\textsuperscript{29}. The ethanolic extract of the stem of \textit{Vernonia amygdalina} had cercaricidal properties with LT\textsubscript{50} value of 6.72 minutes\textsuperscript{83}.

In a large clinical trial in the Democratic Republic of Congo, tea infusions from two species of wormwood, \textit{Artemisia annua} and \textit{Artemisia afra} cured schistosomiasis faster than praziquantel with no side effects. The treatment with the tea took 14 days whilst that of the standard took 21 days. Patients were considered cured when no more parasite eggs were observed in their stool samples\textsuperscript{84}. An amide piplartine, isolated from the methanolic extract of \textit{Piper tuberculatum} Jacq., showed antischistosomal activity against the eggs of \textit{S. mansoni}. At a concentration of 7.5 \mu M, it
caused the death of all schistosomula within 120 hours\textsuperscript{85}. In Zimbabwe, 286 traditional healers were interviewed to assess their knowledge about the signs and symptoms associated with urinary schistosomiasis. They pointed out haematuria as the apparent sign as well as increased urine frequency, pain on micturition and general body weakness. Amongst the 8 commonly used plant materials whose extracts were administered orally to hamsters infected with the cercariae of \textit{S. haematobium}; extracts of \textit{Abrus prectorius}, \textit{Ptecarpus angolensis}, \textit{Ozoroa insignis} were found to be lethal to adult schistosomes\textsuperscript{86}.

The ethanolic leaf extract of \textit{Terminalia catappa} displayed molluscicidal activity against some species of freshwater snails, \textit{Biomphalaria pfeifferi} and \textit{Bulinus globusus} (intermediate hosts of \textit{Schistosoma} spp)\textsuperscript{87}. An extract of powdered \textit{Capparis spinosa} and \textit{Acacia arabica} also exhibited molluscicidal activity against \textit{Biomphalaria alexandrina}. Aqueous extract of \textit{Harissonia abyssinica} showed significant molluscicidal activity with LD\textsubscript{50} of 2.43 mg/L. \textit{Rauwolfia vomitoria} has been used widely in Ghana as an emetic and is used in the treatment of parasitic skin infection. Previous investigations of the ethanolic extracts of the stem back and roots of \textit{R. vomitoria} showed activity against the cercariae and adult worms of \textit{S. mansoni} with IC\textsubscript{50} of 62.5-1000 μg/mL and 250-1000 μg/mL, respectively. Cytotoxicity tests against Chang liver and HepG2 cells using the MTT assay affirmed the extracts were safe as therapeutics\textsuperscript{86}. Also, eugenol, the major essential oil component from \textit{Syzigium aromaticum} exhibits moderate antischistosomal activity \textit{in vivo}. It reduces egg density of \textit{S. mansoni} in intestinal walls of mouse models by 11.7% as compared to 96% reduction observed in praziquantel positive control. It also reduces worm output by 19.2% \textit{in vivo} as compared to a 100% kill using PZQ\textsuperscript{87}.

\subsection*{2.4 Human African Trypanosomiasis}

HAT is a vector-borne parasitic disease caused by two subspecies of the extracellular protozoa, \textit{Trypanosoma brucei}. The acute form of the disease occurs in East Africa and is caused by \textit{T. b. rhodesiense} whilst the chronic form occurs in West and Central Africa and the causative subspecies is \textit{T. b. gambiense}\textsuperscript{88}. They belong to the family Trypanosomatidae which comprises solely parasitic organisms occurring in insects and vertebrates. They are unicellular species that are not pathogenic but have mainly co-evolved with their hosts and therefore live in a commensal association. Three main morphologically indistinguishable subspecies of \textit{Trypanosoma brucei}
occur, *T. b. brucei*, *T. b. rhodesiense* and *T. b gambiense*. Their cells comprise one central nucleus, flagellum and a single mitochondrion with its own DNA which consists of kinetoplast located at the posterior end of the cell. *T. b. brucei* affects domestic animals and causes animal African trypanosomiasis (Nagana disease), which is not pathogenic to man. They are not infective to humans due to the presence of two trypanolytic factors; apolipoprotein L-1 and hepatoglobulin-related protein which are responsible for initiating the death of trypanosomes. *T. b rhodesiense* and *T. b. gambiense* developed resistance to apolipoprotein A and can infect humans. The disease is spread by some species of *Glossina* tsetse fly and can be transferred from mother to offspring or through sexual intercourse.

Sleeping sickness (HAT) occurs in two main phases. The first (haemolymphatic) phase of the disease is characterized by the appearance of a trypanosomal chancre at the site of inoculation. It results in painful erythematous swelling with a central vesicle. This is characterized by fever, chills, pains in the head and limbs, exanthema, pruritus and lymph node swelling of the neck. In the second stage (meningoencephalitic), the parasites cross the blood-brain barrier and infect the central nervous system (CNS). It is associated with personality changes, progressive confusions and somnolence with disturbed sleep at night resulting in its popular name, sleeping sickness. Partial paralysis, hormonal imbalance in certain cases, loss of consciousness and coma occur as the disease progresses. HAT is acute when the subspecies responsible is *T. b. rhodesiense* but progresses rather slowly when the causative subspecies is *T. b. gambiense* and often kills in three years if left untreated.

### 2.4.1 Transmission and life cycle of HAT

HAT is spread when an infected tsetse fly belonging to the genus *Glossina* injects metacyclic trypomastigotes into the skin tissues of a mammalian host during a blood meal. This results in a chancre. The parasites invade the lymphatic system and travel to the bloodstream where they get transformed into trypomastigotes. They are then transported to other body fluids such as the lymph and spinal fluids and eventually invade the brain where they reproduce by binary fission. The rest of the cycle which takes approximately three weeks occurs in the tsetse flies when they become infected during blood meals taken from infected human hosts. The parasites are transformed into procyclic trypomastigotes in the insects’ mid-gut and increase by binary fission. They transform
into epimastigotes when they move from the midgut to the salivary gland where they continually multiply and transform into metacyclic trypomastigotes. The tsetse fly injects trypomastigotes into human hosts during a blood meal resulting in the continuity in the cycle. Humans and wild game animals serve as the main reservoirs for *T. b. gambiense* and *T. b. rhodesiense*, respectively. The life cycle of HAT in both the human host and the tsetse fly is summarized in Figure 2.5.

**Figure 2.5:** The Life cycle of human African trypanosomiasis

2.4.2 Treatment and Chemotherapy

The diagnosis of HAT involves the microscopic detection of trypanosomes in wet and thick films. Drugs prescribed for the treatment of HAT as illustrated in Figure 2.6 is contingent on the causative subspecies of *Trypanosoma brucei* and the phase of the disease. The stage can be better clarified by puncturing the lumbar diagnostically to ascertain the invasion of the CNS by the parasites.
The first phase of *T. b. gambiense* infection can be cured by the intravenous or the intramuscular administration of pentamidine [XXVI] over a period of two hours. Suramin [XXVII] is used to treat the first phase of *T. b. rhodesiense* infection and is also effective in the treatment of infections initiated by *T. b. gambiense* but it poses complications in patients harboring *Onchocerca volvulus* worm in addition.\(^9^4\)

Melarsoprol [XXVIII] was recommended as the first drug of choice in the treatment of the second stage of infections caused by *T. b. gambiense* due to its easy administration. It is administered intravenously and it enters the CNS in large quantities to eliminate the trypanosomes which have invaded the CNS. Research, however, indicates that it has unacceptable levels of toxicity and results in 5% drug-induced death through reactive encephalopathy.\(^9^5\) Eflornithine [XXIX] is a safer alternative and effective against the second stage of *T. b. gambiense*. The administration of this drug, however, involved the delivery of huge doses in 56 infusions over a period of 14 days in 14 L of sterile saline. It is trypanostatic with a short half-life and the tendency for non-compliance may result in the development of resistance. A combination therapy involving eflornithine and nifurtimox [XXX] (NECT) which is dosed less frequently with efficacy as high as the monotherapy is now recommended by WHO and this has replaced melarsoprol.\(^5^9\)

![Chemical structures](http://ugspace.ug.edu.gh)

**Figure 2.6:** Drugs used in the treatment of human African trypanosomiasis

### 2.4.3 Traditional Medicines used in the Treatment of HAT

Several plants have been used traditionally as therapeutic agents for HAT in Africa. In Angola, an infusion of the roots and leaves of *Vilex madensis* (Fabaceae) is widely used by TM practitioners for treating HAT and has been found to greatly improve the conditions of the patients.\(^9^7\) An
infusion of the stem and root bark of Ancistrocladus congolensis has also been reported to have activity against trypanosomiasis. Extracts from the aerial parts of Cassytha filiformis (Lauraceae) exhibit moderate activity against T. b. rhodesiense at an IC₅₀ of 17.4 μM. In Niger, dichloromethane extracts of the roots of Waltheria indica exhibited significant antitrypanosomal activity against T. b. rhodensiense with an IC₅₀ of 17.4 μg/mL. W. indica contains some quinolones such as waltheriones which are responsible for the antitrypanosomal activity⁸².

Morinda lucida has been used in ethnomedicine as a remedy for a plethora of diseases in Africa due to its outstanding medicinal properties. Following reports on its antiprotozoal properties, its antitrypanosomal activity was investigated. The results indicated that molucidin isolated from M. lucida had significant activity with an IC₅₀ of 1.27 μM against T. b. brucei⁹⁸.

The Nupes of Niger living by the Niger River use Acacia nilotica, Terminalia avicennoides, Bombax buonopozense and Zanthoxylum xanthoxyloides traditionally to treat sleeping sickness. The methanol extracts of all plant parts were investigated by Mann and his group to validate the use of these plants and to test their potency against T. b. brucei in vivo. The methanolic extracts of the stem bark of A. nilotica and B. buonopozense eliminated the T. b. brucei completely at 200 mg/Kg and 300 mg/Kg. Extracts of the fruits of T. avicennoides also showed antitrypanosomal activity⁹⁹.
CHAPTER THREE
MATERIALS AND METHODS

3.1 General Experimental Methods

Traditional medicines (TM) used for the treatment of schistosomiasis in some endemic communities were collected from the Ghana Federation of Traditional Medicines Practitioners Associations (GHAFTRAM) based on information obtained from the administration of questionnaires. Aqueous herbal preparations were extracted by successive solvent partitioning with dichloromethane (DCM) and n-butanol (n-BuOH) in 250 mL and 1 L capacity separatory funnels. Dried powdered herbs were extracted with dichloromethane and subsequently with methanol (MeOH) by cold maceration. All solvents used for the extraction processes were of HPLC grade. The extracts were concentrated to dryness in vacuo by the use of a Heidolph rotary evaporator.

The antischistosomal potential of the crude extracts was evaluated in vitro by screening them against newly transformed schistosomulae and adult worms of S. mansoni at the Swiss Tropical and Public Health Institute, Switzerland. Crude extracts, fractions and isolated compounds were also screened in vitro against bloodstream forms of Trypanosoma brucei brucei at the West African Centre for Cell Biology and Infectious Pathogens (WACCBIP) at the University of Ghana.

Due to timely feedback on the antitrypanosomal screening, separation and isolation were carried out on active samples by subjecting their crude extracts to flash and column chromatography, carried out in glass columns packed with high purity grade silica gel (SIGMA-ALDRICH) with a particle size of 130-270 mesh. By gradient elution, the chromatographic separation was achieved with mixtures of solvents of increasing polarity from petroleum ether (40-60 °C) to ethyl acetate and methanol. Further purification of the compounds was achieved by trituration and recrystallization. Solvents commonly used for the purifications were petroleum ether (40-60 °C), ethyl acetate, chloroform, hexane, acetone and methanol. All solvents used for chromatographic separations and purifications were of analytical grade.

Analytical thin layer chromatography (TLC) was carried out on aluminium foil slides (20 x 20 cm) pre-coated with 0.2 mm thickness of silica gel 60 F254 (Merck). Spots on TLC plates were
visualized under handheld UVGL-58 lamp (λ = 254 and 364 nm), iodine vapour and anisaldehyde spray.

The identification of the isolated compounds was done by determining their melting points on a Stuart Scientific melting point apparatus. They were further probed using spectroscopic and spectrometric techniques. Neat infrared spectral data were recorded on a Perkin Elmer FTIR spectrometer (attenuated total reflectance). $^1$H (500 MHz) and $^{13}$C (125 MHz) NMR spectra were acquired at the Department of Chemistry, University of Ghana, on a 500 MHz Bruker NMR spectrometer in CDCl$_3$ with reference to tetramethylsilane (TMS). High resolution mass spectrometric data were acquired on a Waters Synapt G2 QTOF Spectrometer by electrospray ionization at a cone voltage of 15 V at the Central Analytical Facilities of Stellenbosch University, South Africa.

HPLC fingerprint of oil samples was obtained on a Varian LC 920 instrument equipped with a UV-Vis detector and a Galaxie™ software apparatus HPLC. A prepacked C18 reversed-phase column (Machery-Nagel Nucleosil 120-5, 250 x 4 mm) was used for analytical HPLC with a binary isocratic elution (H$_2$O/CH$_3$CN 40:60) and a flow rate of 1 mL/min.

Oils were further analyzed using Gas Chromatograph (6890N, Agilent Technologies Network) coupled to inert XL Electron Impact/Chemical Ionization Mass Selective Detector (MSD), (Agilent Technologies Inc.) also at Stellenbosch University. The constituents of the oils were separated on a STABILWAX capillary column (60 m, 0.25 mm ID, 0.25 μm). Helium was employed as the carrier gas at a flow rate of 2 mL/min. The temperature of the injector was kept at 240 °C. About 1 μL of the sample that was prepared prior to the analysis was injected in a splitless mode. In the programming of the temperature of the oven, it was initially kept at 45 °C for 3 minutes and increased rapidly at a rate of 6 °C/min to 250 °C where it was kept for five minutes. The MSD was run in full scan mode and the temperature of the source and quad were kept at 230 °C and 150 °C, respectively. The temperature of the transfer line was maintained at 250 °C. The mass spectrometer was operated in the electron impact mode at ionization energy of 70 eV, scanning from 25 to 450 m/z.
3.2 Preparation of Reagents

1. Reagents for Screening for alkaloids
   a. Wagner’s reagent

   About 2 g of potassium iodide and 1.27 g of iodine crystals were dissolved in a small volume of distilled water in a volumetric flask and the solution topped up to 100 mL with distilled water. The presence of alkaloids was inferred from the formation of large brown amorphous and flocculent precipitate after treating the hydrochloric acid solution of the extract with some drops of the reagent.

   b. Mayer's reagent

   About 1.36 g of mercuric iodide was dissolved in 60 mL of distilled water and the resultant solution was added to 10 mL solution of 5 g of potassium iodide in 10 mL of distilled water. The mixture was topped up to 100 mL with distilled water.

   c. Dragendorff’s reagent

   About 4 g of hydrated bismuth nitrate was dissolved in 10 mL of concentrated nitric acid and the resulting solution was slowly added to a solution of 13.47 g of potassium iodide in 25 mL of distilled water. Potassium nitrate precipitate was filtered off and the ensuing filtrate topped up to 50 mL with distilled water.

2. Reagents for Screening for phenolics

   Iron (III) chloride solution

   The reagent was prepared by dissolving 10 g of iron (III) chloride in 100 mL of distilled water.

3. Reagent for screening for terpenoids

   Liebermann-Burchard Reagent

   About 5 mL of acetic anhydride was cautiously added to 5 mL of concentrated H₂SO₄ acid. The resulting mixture was slowly added while cooling in ice to 50 mL of absolute ethanol.
4. Anisaldehyde Spray reagent

Glacial acetic acid (1.5 mL) was added to 135 mL of absolute ethanol. About 5 mL of concentrated H$_2$SO$_4$ and 3.7 mL of anisaldehyde were then added to the resulting solution. The reagent was sprayed unto TLC plates, dried and heated to 110 ºC for five minutes before visualizing in visible light.

3.3 Collection of Selected Traditional Medicines used in the treatment of Schistosomiasis

Prior to the collection of the TM, there were three separate interactions with members of GHAFTRAM to probe their awareness of NTDs in general and schistosomiasis in particular. Ethical clearance required for this study was obtained from the Ethics Committee for Basic and Applied Sciences at the University of Ghana (Appendix I). The meetings were followed up with the administration of questionnaires for documentation of the practitioners’ demographic information, reasons for joining the profession, length of time in the profession, dates of manufacture and expiry, route of administration, dose regimen, constituent plants and curative rate in terms of people cured by their TM. A sample questionnaire is presented in Appendix I.

Following this exercise, 19 TM samples, 7 of which were indicated for schistosomiasis were collected, transported to the laboratory and stored at 24 ºC. The samples were either aqueous herbal preparations or dried pulverized herbs as indicated in Table 3.1. The antischistosomal remedies were coded as NTD_B1 - NTD_B7, where NTD stands for neglected tropical disease and B stands for bilharzia.

Table 3.1: Collected Traditional Medicines used in the Treatment of Schistosomiasis

<table>
<thead>
<tr>
<th>Sample Code</th>
<th>Nature of TM</th>
<th>Dosage</th>
<th>Curative Rate (People cured)</th>
<th>Manufacturing-Expiry Date</th>
</tr>
</thead>
<tbody>
<tr>
<td>NTD_B1</td>
<td>Aqueous</td>
<td>60 mL x 3</td>
<td>&gt; 20</td>
<td>25/06/2018-N/A</td>
</tr>
<tr>
<td>NTD_B2</td>
<td>Aqueous</td>
<td>3 tbsp x 3</td>
<td>6-10</td>
<td>6/07/2018-6/07/2020</td>
</tr>
<tr>
<td>NTD_B3</td>
<td>Dried Herbs</td>
<td>1 tbsp in 250 mL of water</td>
<td>&gt; 20</td>
<td>3/07/2018-2/07/2019</td>
</tr>
<tr>
<td>NTD_B4</td>
<td>Dried Herbs</td>
<td>1 tsp in 100 mL of water</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>NTD_B5</td>
<td>Aqueous</td>
<td>4 tbsp x 4</td>
<td>&gt; 20</td>
<td>14/06/2018-N/A</td>
</tr>
<tr>
<td>NTD_B6</td>
<td>Aqueous</td>
<td>N/A</td>
<td>N/A</td>
<td>06/2018-06/2020</td>
</tr>
<tr>
<td>NTD_B7</td>
<td>Aqueous</td>
<td>5 tbsp x 3</td>
<td>&gt; 20</td>
<td>06/2018-06/2020</td>
</tr>
</tbody>
</table>

*The constituent plants have not been disclosed due to intellectual property issues  *tbsp. – tablespoon  *tsp- teaspoon  * N/A - not available
3.4 Extraction of traditional medicines

About 300 mL of aqueous herbal preparations were transferred into separatory funnels and extracted three times by solvent partitioning with 300 mL of dichloromethane (3 x 300 mL) and subsequently with n-butanol (3 x 300 mL). The extracts obtained were concentrated to dryness at reduced pressure in vacuo. Dried herbs were weighed and extracted three times with dichloromethane (3 x 300 mL) and successively with methanol (3 x 300 mL) by cold maceration in a period of 72 hours. The extracts were filtered and concentrated to dryness in vacuo. The masses of the dried extracts were then determined, and TLC was done to ascertain the complexities of the fractions. The crude extracts obtained from the extraction of the traditional remedies are illustrated in Figure 3.1.

![Sample Collection Diagram](image)

**Figure 3.1:** Crude extracts obtained from the TM samples

The dried extracts of NTD_B6 DCM and NTD_B7 DCM precipitated substantial sheet-like crystals which on filtration, followed by TLC, were found to be impure. About 150 mg of NTD_B6 DCM was therefore chromatographed on a glass column prepacated with a slurry of silica and petroleum ether. A TLC profile was obtained for fractions which precipitated solids upon elution with 95% petroleum ether: ethyl acetate. Fractions with similar R_F were combined, concentrated to dryness and purified by triturating with cold hexane to obtain colourless crystals(DP2). A TLC profile of the compound was obtained using petroleum ether: ethyl acetate/8:2. The compound was identified by acquiring 1D and 2D NMR spectral data and determining its melting point. A comparative TLC was obtained for DP2 and the crystals in NTD_B7 DCM extract.
The 14 crude extracts were evaluated for their antischistosomal and antitrypanosomal activities by screening them against the schistosomulae and adult worms of *S. mansoni* and *T. brucei brucei*, respectively. Summary of work done from sample collection to the biological activity studies: antischistosomal and antitrypanosomal activity have been illustrated in **Figure 3.2**.

**Figure 3.2:** Summary of work done on the NTD_B crude extracts

### 3.5 Chemical Investigation of NTD_ B4 DCM

At a concentration of 100 μM, NTD_B4 DCM exhibited one of the highest antischistosomal activity against NTS and adult worms of *S. mansoni* and displayed significant antitrypanosomal activity against *T. brucei brucei* (Detailed information in Chapter 4). It was therefore selected for further separations and tests.
3.5.1 Extraction of NTD_B4 DCM

About 231.4 g of NTD_B4 sample was extracted exhaustively with about 1 L of HPLC grade dichloromethane in a period of 72 hours by cold maceration. The extract was filtered and concentrated to dryness in vacuo to obtain a thick dark green crude extract of mass, 16.712 g. A small amount of NTD_B4 DCM was dissolved in chloroform and different TLC profiles were obtained using 100% PE, PE: EtOAc/10:1, PE: EtOAc/ 5:1, PE: EtOAc/ 2:1, PE: EtOAc/ 1:1 as solvent system.

3.5.2 Phytochemical Screening

Phytochemical screening was done on the crude extract of NTD_B4 DCM to determine the class of secondary metabolites present. An 80% ethanolic solution of the crude extract was screened for the presence of alkaloids, steroids, tannins, terpenoids, saponins, phenolic compounds, polyphenols, cardiac glycosides, anthraquinones and flavonoids using this protocol.

1. Test for alkaloids

About 4 mL of the 80% ethanolic solution was added to 5 mL of 2M HCl solution. This was stirred, heated and filtered. The filtrate from the extract was then divided per three test tubes. Dragendorff’s reagent was added to one portion of the test solution. To another portion Mayer's reagent and to the remaining test solution Wagner’s reagent. These were then observed for turbidity or colour change.

2. Screening for saponins

About 2 mL of the ethanolic solution was added to 4 mL of deionized water. This was vigorously shaken and left to stand for about 10 minutes. The presence of saponins was inferred from the formation of thick persistent foam.

3. Screening for cardiac glycosides (Salkowski Test)

About 2 mL of the 80% ethanolic solution was transferred into a test tube with 2mL of glacial acetic acid containing a drop of FeCl₃ solution. Concentrated H₂SO₄ was cautiously added along the wall of the test tube to form a lower layer. The aglycone moiety of the cardiac glycoside was inferred from the appearance of a reddish-brown colouration at the interface.
4. Test for terpenoids

A test tube was filled with 2 mL of the 80% ethanolic solution and 2 mL of CHCl₃ was added. About 1 mL of concentrated H₂SO₄ was cautiously added along the wall of the test tube. A reddish-brown colour at the confluence implied a positive test thus, confirming the presence of terpenoids.

5. Test for tannins

About 0.2 g of the extract was dissolved in 5 mL of aqueous methanol. The resultant mixture was split between 2 test tubes where one portion served as a blank and the other, freshly prepared FeCl₃ was added. A greenish or dark blue colouration confirmed the presence of tannins.

6. Test for steroids

About 2 mL of acetic anhydride was added to 2 mL of the 80% ethanolic solution in a test tube and boiled. Upon cooling, H₂SO₄ (2 mL) was added to the resultant mixture. The presence of steroids was confirmed by the development of a brown ring at the interface; with a green upper layer.

7. Test for polyphenols

About 1 mL of the 80% ethanolic solution was measured into a test tube. Three drops of 10% aqueous FeCl₃ and 3 drops of potassium ferrocyanide were added to the solution. The presence of polyphenols was inferred by the formation of a blue or green coloration.

8. Test for Phenolic compounds

Freshly prepared FeCl₃ solution (10%) was added to 1 mL of the 80% ethanolic in a test tube. An ensuing dark colouration implied the presence of phenolic compounds.

9. Test for Flavonoids and Leucoanthocyanins

Three test tubes were each filled with 2 mL of the ethanolic solution. The first test tube served as a blank solution. About five pieces of magnesium turnings were put in the second test tube followed by the addition of 0.5 mL of hydrochloric acid. It was observed for change in colour. About 0.5 mL of concentrated hydrochloric acid was added to the third test tube and heated on a
water bath for about 5 minutes. The presence of flavonoids and leucoanthocyanins were confirmed by the change in colour in the second and third test tubes with reference to the first test tube.

10. Test for anthraquinones and anthracene derivatives

About 5 mL of the ethanolic extract was transferred into a test tube. It was warmed in a water bath for about ten minutes and transferred into a separatory funnel. It was then extracted with 4 mL of benzene by liquid partitioning and to allowed to stand for equilibration. The benzene layer was collected and treated with about 1.5 mL concentrated ammonia. The emergence of a reddish colour in the ammonia layer established the presence of anthraquinones and anthracene derivatives.

3.5.3 Fractionation of NTD_B4 DCM

About 16.7 g of NTD_B4 DCM extract was dissolved in chloroform and mixed with about 70 g of silica gel. This was air-dried and packed into flash cartridges. It was eluted sequentially with 375 mL each of 100% petroleum ether (PE), PE: EtOAc/ 9:1, PE: EtOAc/ 8:2, PE: EtOAc/ 1:1, PE: EtOAc/ 2:8, 100% EtOAc and EtOAc: MeOH/1:1. Solids that precipitated at the tips of the column were washed with ethyl acetate into vials and their purity ascertained.

The various fractions obtained from this process were concentrated to dryness and their masses were determined. TLC profiles of all the fractions with the crude extract were obtained using mobile phases of solvent compositions as explained for the crude extracts. The 100% petroleum ether (PE) and PE: EtOAc/ 9:1 fractions were recombined due to similarities in the Rf of the TLC profiles and also for the purpose of maximizing the yield of the compounds since some compounds were common to these fractions. Fractionation of the NTD_B4 DCM is summarized in Figure 3.3.
Figure 3.3: Fractionation of B4 DCM Crude Extract

3.5.4 Column Chromatography of NTD_B4 DCM

A glass column was packed with a slurry of 100% petroleum ether and about 80 g of silica gel. This was run for about an hour and left to stand overnight to ensure the column was properly packed and there were no air bubbles. The samples were dissolved in a small amount of chloroform, mixed with silica, and air-dried. This was loaded unto the column and eluted with 100% PE. Gradient elution was employed in the separation of the components by spiking the petroleum ether with a small amount of ethyl acetate which steadily increased. It was further eluted with 100% EtOAc, EtOAc: MeOH 1:1 and finally with 100% methanol. The eluates were chromatographed on a TLC plate and combined based on Rf from their TLC profiles. The combined eluates were concentrated to dryness in vacuo and transferred into sample vials. These were kept below room temperature and observed for precipitates. Fractions with an appreciable amount of precipitates were filtered and washed with cold hexane, ethyl acetate, acetone or methanol. The choice of solvent was dependent on the insolubility of the compounds in those solvents.

3.5.4.1 Investigation of F1

The 100% PE and PE: EtOAc/ 9:1 fractions were combined and labelled as F1 due to their similar constituents as indicated by their TLC profile. Fraction F1 was dissolved in dichloromethane and
filtered to separate precipitates which were formed upon refrigeration. The residue was triturated using cold hexane and ethyl acetate. TLC analysis was done on the off-white precipitate to ascertain its purity. The filtrate was concentrated to dryness and about 3.770 g of the sample was dissolved in a small amount of chloroform, mixed with silica and air-dried. This was loaded on a column and separated by gradient elution. TLC profiles of the eluates were obtained and fractions were combined based on similar $R_F$. The combined filtrates were concentrated to dryness and purified by trituration. Compounds isolated from the column are demonstrated in Figure 3.4. Oils isolated from the column were labelled F1/HML, F1/JML and F1/KML.

![Figure 3.4: Isolation of Compounds from F1](image)

**3.5.4.2 Investigation of F3**

A small glass column was packed with a slurry of 60 g silica gel and a mobile phase with the solvent composition of PE: EtOAc/ 9:1. About 4.013 g of PE: EtOAc/ 1:1(F3) fraction was dissolved in chloroform, mixed with 3 g of silica and air-dried. This was loaded unto the column and the constituents separated by gradient elution. The eluates were combined based on $R_F$ from their TLC profiles. The samples were refrigerated and observed for precipitates. The precipitates were purified and the compounds which were obtained are indicated in (Figure 3.5).  

![Figure 3.5: Isolation of Compounds from F3](image)
3.4.4.3 Investigation of F4 and F5 Combinations

About 1.662 g of the sample resulting from the combination of PE: EtOAc/ 2:8 (F4) and 100% EA (F5) fractions was dissolved in chloroform. A small amount of silica was added and air-dried. The polarity was rapidly increased since 100% PE was too nonpolar to elute the components of these fractions. It was gradually eluted with increasing polarities of ethyl acetate and methanol. Fractions collected were purified by subjecting them to the same processes as indicated in the isolations from previously mentioned chromatographic separations (Figure 3.6).

![Figure 3.6: Isolation of Compounds from F4+F5](image)

3.5.4.4 Investigation of F6

About 2.177 g of the EtOAc: MeOH/1:1 fraction (F6) was dissolved in chloroform and small amounts of ethanol. This was air-dried and loaded onto a previously packed column. Since the fraction was very polar, the polarity mobile phase was rapidly increased by increasing portions of ethyl acetate. It was then eluted with 100% EtOAc, EtOAc: Acetone/ 1:1, 100% Acetone, Acetone: EtOH 1:1, 100% EtOH and finally with 100% MeOH. Fractions were obtained by the combination of eluates from test tubes with similar R_F were concentrated and refrigerated and further observed for precipitates.

![Figure 3.7: Isolation of Compounds from F6](image)
3.6 Acquisition of HRMS Data of F1/K
HRESIMS data was obtained for F1/K to determine the mass of the proposed structure.

3.7 Chemical Investigation of F1/HML
A TLC profile was obtained for the oily sample, F1/HML to determine the profile of the constituents. It was chromatographed in petroleum ether: ethyl acetate/ 9.5:0.5. An HPLC profile of F1/HML was also obtained using acetonitrile: water/ 60:40.

3.7.1 GC-MS Analysis of HML
About 1 mL of dichloromethane was added to F1/HML and sonicated overnight. GC-MS data of the oil was obtained to identify the constituents based on their masses and fragmentation patterns.

3.7.2 NMR Analysis of F1/HML
$^1$H and $^{13}$C NMR data were obtained for F1/HML to give an overview of the proton and carbon environments present in the oil.

3.8 Biological Assays
The 14 crude extracts obtained from the traditional medicines were tested on the schistosomulae and adult worms of *S. mansoni* to evaluate their antischistosomal activity. Their antitrypanosomal potential was also determined by screening them against the bloodstream forms of *T. brucei brucei*.

3.8.1 Antischistosomal Assay
As indicated in Chapter 2, *Schistosoma* spp. is characterized by 3 major life stages in humans: eggs, schistosomulae and adult worms. In this study, an antischistosomal screening was done on the NTS using 10 μM and 100 μM of all the 14 crude extracts. The protocol employed in the assay was a screening cascade starting with a preliminary screening of extracts on NTS of *S. mansoni*. Active extracts were then tested *in vitro* on adult-stage worms, and the most active extracts from this screen were tested *in vivo* in mice with chronic infection. Compounds that showed a decrease in worm burden greater than 80% were further characterized on mice bearing immature worms.
Newly Transformed Schistosomula Culture Medium

The newly transformed schistosomula (NTS) culture medium was prepared by supplementing M199 medium with 5% (v/v) fetal calf serum, 1% (v/v) antibacterial/antifungal mix with 45% (v/v) kanamycin, 1% (v/v) streptomycin/penicillin, 27% (v/v) penicillin G, 23% (v/v), 5-fluorocytosine and 5% (v/v) chloramphenicol. This was maintained at a temperature of 4 ºC.

Adult Worm Culture Medium

This was prepared by supplementing RPMI 1640 medium with 5% (v/v) fetal calf serum and 1% (v/v) streptomycin/penicillin and stored at 4 ºC.

Test Compounds

All crude extracts were dissolved in DMSO and the final concentration used was 1%.

Positive Control

The positive control used in the antischistosomal assay was praziquantel. It displays an IC$_{50}$ value of 2.2 and 0.1 μM against the schistosomulae and adult worms of S. mansoni.

3.8.1.1 Screening Against NTS of S. mansoni

The drug stock solution was prepared by making a solution of the crude extracts in DMSO in a supplemented M199 medium. About 175 μL of M199 medium which has been supplemented was added to each well of the 96-well plate. This was followed by the addition of 25 μL of the prepared solution to each well, and afterward, 50 μL of the 2000 NTS/mL which resulted in about 100 NTS per well. DMSO controls were prepared by the addition of 20 μL of 10% v/v DMSO in 180 μL of M199 medium which has been supplemented. They were done in triplicates and incubated at 37 ºC under 5% carbon (IV) oxide. They were then evaluated at 24, 48 and 72 hours after drug incubation.

The parasites were assessed soon after removing them from the incubator. Each well was evaluated by means of a bright-field microscope with x 4 or x 10 magnification. Scores were assigned to each well to reflect the phenotype of the majority of the well. The motility of the parasites and the integrity and morphology of the tegument were the most important parameters that were
considered. Data acquired were reported in a spreadsheet and the effect score was calculated by normalization of the scores recorded by the drugs to the scores of the control using this formula.

\[
\% \text{ Effect} = \frac{100 - \frac{\text{Average (test) \times 100}}{\text{Average control}}}{100}
\]

In the assessment of the antischistosomal activity of the extracts from the TM, a 70% effect on the motility of the parasites as well as the morphology and integrity of the tegument were considered as hits\textsuperscript{101}.

**3.8.1.2 Screening Against Adult worms of S. mansoni**

The drug stock solution was prepared by making a solution of crude extracts in 100% DMSO. The starting concentrations employed in this assay were 10 μM and 100 μM. About 15 μL of the stock solution of the crude extracts was pipetted into 24-well plate and 1485 μL of the supplemented RPMI 1640 was added. All the crude extracts were tested in triplicates. In the control experiment, 10% v/v of DMSO in RPMI 1640 medium was prepared and 15 μL of this was pipetted into the control wells to achieve 0.1% concentration. The positive control used was praziquantel. Three pairs of worms (both male and female) were carefully placed in each well using a tweezer. This was incubated at 37 °C under 5% CO\textsubscript{2} and observed. The sexes of both worms were noted and phenotypic scores assigned every 24 hours for 72 hours.

**3.8.1.3 Screening of Fractions Against NTS and Adult worms of S. mansoni**

Fractions from the flash chromatography (F1-F6) were evaluated for their antischistosomal activity using the same protocols in 3.8.1.1. Unfortunately, feedback on the antischistosomal activity of the NTD_B crude extracts took about 10 months, which implied further delays in the antischistosomal activity studies. Fractions were however submitted for their antischistosomal activity studies and the results are pending.
3.8.2 Antitrypanosomal Assay

The crude extracts (NTD B_DCM, MeOH and BuOH) were also evaluated for their antitrypanosomal potential by targeting the bloodstream forms of *T. brucei brucei*. This subspecies was used in the biological assay due to the highly identical features of the *T. brucei brucei* to the infective human *T. b rhodesiense* and *T. b. gambiense*\(^9\). The fractions and compounds were obtained by bioassay-guided fractionation\(^{101,102}\).

**Trypanosome cultures**

Wild-type bloodstream forms of *Trypanosoma brucei brucei* (GuTat 3.1 strain) were cultured in Hirumi’s Modified Iscove’s Medium-9, HMI-9 (Hirumi & Hirumi, 1994). The media was supplemented with 10% heat-inactivated fetal bovine serum (Gibco) and 1% penicillin-streptomycin. The trypanosome cultures were maintained at 37°C and 5% carbon (IV) oxide.

**Test compounds**

All crude extracts were prepared in dimethyl sulfoxide (DMSO). The final concentration of DMSO used was 1%.

**Drug sensitivity test using the Alamar Blue assay**

The extracts were evaluated on *Trypanosoma brucei brucei* (GuTat 3.1) strain using the Alamar blue assay. Briefly, 2-fold serial dilutions of the different crude extracts were performed from 250 μg/mL to 0.49 μg/mL in 100 μL HMI-9 media in a 96 well plate. Trypanosomes in their logarithmic growth phase were added into each well, excluding the media control well to which no cell was added, at a cell density of 1 × 10^5 cells/well. For each 96-well plate, there were the media and untreated controls. After 24 hours’ incubation, 20 μL of 500 μM Alamar blue dye was added and incubated for an additional 24 hours. After the final incubation, the absorbance was measured at 570 nm using the Varioskan Lux Elisa plate reader (ThermoFisher Scientific, USA).

**Statistical Analysis**

Plate readouts for the Alamar blue assay were analysed using non-linear regression analysis (log₁₀ inhibitor versus response – variable slope) for growth inhibition on GraphPad Prism version 8.0.1. The IC₅₀ value of the compounds/fractions was determined for three biological replicates, each with triplicate determinations.
3.8.2.1 In vitro Screening of crude extracts for Anti-Trypanosome Activity

About 5 mg of each of the crude extracts were dissolved in DMSO and evaluated for their antitrypanosomal activity using the protocol in 3.8.2.

3.8.2.2 Antitrypanosomal Activity Evaluation of Fractions from Flash Chromatography

About 5 mg of each of the fractions (F1-F6) obtained from the flash chromatography were dissolved in DMSO and evaluated for their antitrypanosomal activity. Diminazene aceturate and DMSO were used as positive and negative controls, respectively. The samples were subjected to the same antitrypanosomal protocol used for the evaluation of the antitrypanosomal activity of the crude extracts.

3.8.2.3 Antitrypanosomal Activity Screening of Compounds and Oils of NTD_B4 DCM

Antitrypanosomal screening of the compounds and the oils was carried out using a modified Alamar blue assay. Compounds that were insoluble in DMSO were warmed and vortexed to enhance solubility. The addition of water to the DMSO solution greatly improved the solubility of compounds. Serial dilutions of the compounds/fractions were prepared on a 96-well plate with a starting concentration of 100 µg/mL to 0.1953 µg/mL for all the compounds/fractions. The final concentration of DMSO in the well with the highest percentage of DMSO was 0.5%.

For diminazene aceturate and F1/HML, the assay was conducted again with concentrations ranging from 25 µg/mL to 0.0488 µg/mL.
Scheme of Work – NTD_B

Sample Collection

Aqueous herbal preparations (5 Samples)
- Liquid-liquid partitioning
  - Dichloromethane extracts
  - n-Butanol extracts

Dried herbs (2 Samples)
- Cold maceration
  - Dichloromethane extracts
  - Methanol extracts

Biological Activity Screening

Antischistosome activity

Antitrypanosome activity
Scheme of Work - NTD_B4 DCM

NTD_B4 DCM

Extraction (Dichloromethane)

Cold maceration, 25°C, 24 hours, x3

Crude Extract

Phytochemical Screening

Flash Chromatography

Antischistosome Assay

Antitrypanosome Assay

100% PE

PE: EtOAc/ 9:1 Combination

PE: EtOAc/ 8:2

F1/S

Column Chromatography

Trituration

F1/Z

F1/B

F1/H

F1/J

F1/K

F1/HML

F1/JML

F1/KML

PE: EtOAc/ 1:1

F3/A

F3/C

F3/E

F3/G

F3/L

PE: EtOAc/ 2:8

Combination

100% EA

EA: MeOH/1:1

Column Chromatography

EA P1

EA P2

EA P3

EM 1

EM 2

EM 3
CHAPTER FOUR
RESULTS AND DISCUSSION

4.1 Summary

Traditional medicines (TM) with different phytochemical constituents used for the treatment of schistosomiasis were obtained from GHAFTRAM. Liquid-liquid partitioning of the aqueous herbal preparations successively with dichloromethane and n-butanol and cold maceration of the dried herbs with dichloromethane followed by methanol gave 14 crude extracts.

Antischistosomal activity studies were carried out on the crude extracts in vitro to determine their efficacy. At 100 μM, the methanol extract of NTD_B3 and dichloromethane extracts of NTD_B4 and NTD_B7 were found to be very effective against the newly transformed schistosomulae of S. mansoni and subsequent tests of these extracts on the adult worms induced greater than 60% reduction in the worm viability and motility. In the antitrypanosomal activity studies, all the crude extracts exhibited good to moderate activity against the bloodstream forms of T. brucei brucei with IC₅₀ values ranging from 6-19 μg/mL. The dichloromethane extract of NTD_B4 showed the highest antitrypanosomal activity with an IC₅₀ value of 6 μg/mL as compared to the 0.13 μg/mL of diminazene aceturate.

Further work which focused on the isolation of compounds from the active crude extract, NTD_B4 by chromatographic processes led to the isolation of 8 compounds of which 4 were identified as 5,7-dihydroxy-6-methyl-2-tricosyl-4H-chromen-4-one, β-sitosterol glucoside, a fatty acid and ethyl 4-hydroxybenzoate. Compound F1/K (5,7-dihydroxy-6-methyl-2-tricosyl-4H-chromen-4-one) had a molecular formula of C_{33}H_{54}O_{4}, and on the basis of HRESIMS, 7 degrees of unsaturation were deduced from its molecular ion [M⁺] (m/z 514.2398, calcd. m/z 514.4022). This was accompanied by homologues C_{24}H_{37}O_{4}⁺ and C_{26}H_{41}O_{4}⁺ with m/z 389.34 and 417.24, respectively.

Further antitrypanosomal activity studies on the isolates showed that, with the exception of F1/K (IC₅₀ = 81 μg/mL), all pure compounds from F1 (100% PE + PE: EA/9:1) exhibited no antitrypanosomal activity. The oils obtained, on the other hand, demonstrated notable activity. With the exception of oil F1/JML (IC₅₀ = 32.2 μg/mL), all the IC₅₀ values of the oils were less than 13 μg/mL. F1/HML exhibited quite significant activity with IC₅₀ < 0.0977 μg/mL as compared to the 0.13 μg/mL of diminazene aceturate. The GC-MS analysis of F1/HML with confirmation
from its NMR fingerprint revealed phytan, 4α-Methyl-24-ethyl-5α-cholest-8(9)-en-3β-ol, 1,4,5,8-tetrahydro-4a,8a-naphthalenedicarboximide and 5α, 8α, 14-cholestan as the major components.

4.2 Extraction
Liquid-liquid partitioning of 300 mL of 5 aqueous herbal preparations with 300 mL of dichloromethane and successively with 300 mL of n-butanol, and cold maceration of 2 dried herbs with dichloromethane and followed by methanol gave 14 crude extracts. A comparative TLC profile of the isolated crystals (DP2) from NTD_B6 DCM and the crystals from NTD_B7 DCM gave an R_F of 0.5 which showed the crystals were similar.

4.3 Investigation of NTD B4_DCM
4.3.1 Extraction of NTD_B4 DCM
In both antischistosomal and antitrypanosomal assays, NTD_B4 DCM exhibited profound antischistosomal activity at 100 μM with 100% and 78.40% effect against the schistosomulae and adult worms of _S. mansoni_, respectively. It also displayed an antitrypanosomal activity against bloodstream forms of _T. brucei brucei_ with an IC_50_ of 6 μg/mL as compared to 0.54 μg/mL of the standard, diminazene aceturate.

Further extraction of 231.4 g of NTD_B4 upon cold maceration with dichloromethane in a total period of 72 hours gave a crude extract which weighed 16.712 g. This implied a 7.22% yield.

4.3.2 Phytochemical Screening of NTD_B4 DCM
The crude extract of NTD_B4 DCM was screened to ascertain the various classes of secondary metabolites present. It was tested for the presence of alkaloids, saponins, cardiac glycosides, steroids, flavonoids and leucoanthocyanins, polyphenols, tannins, anthraquinones and anthracenes, and phenolic compounds. Table 4.1 summarizes the outcome of the analyses.
Table 4.1: Results from Phytochemical Screening of NTD B4_DCM

<table>
<thead>
<tr>
<th>SECONDARY METABOLITE</th>
<th>TEST</th>
<th>INFEERENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaloids</td>
<td>Dragendorff</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Mayer</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Wagner</td>
<td>+</td>
</tr>
<tr>
<td>Saponins</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>Cardiac glycosides</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>Steroids</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>Terpenoids</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>Flavonoids and leucoanthocyanins</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>Phenolic</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>Polyphenols</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>Anthraquinones and Anthracenes</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>Tannins</td>
<td></td>
<td>+</td>
</tr>
</tbody>
</table>

Legend: (+) = Positive test; (-) = Negative test

Results from the phytochemical analysis indicated that NTD_B4 DCM constituted terpenoids which included cardiac glycosides, steroids and phenolic compounds such as tannins, flavonoids and leucoanthocyanins. Also, the Dragendorff, Mayer and Wagner tests confirmed the presence of alkaloids. Amongst the phytochemicals screened, saponins and anthraquinones were not present in the NTD_B4 DCM.

4.3.3 Fractionation of NTD_B4 DCM

Fractions obtained by flash chromatography with their respective yields are indicated in Table 4.2.

Table 4.2: Fractionation of NTD_B4 DCM Crude Extract

<table>
<thead>
<tr>
<th>Label</th>
<th>Fractions</th>
<th>Mass</th>
<th>Percentage Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>100% PE</td>
<td>4.112 g</td>
<td>24.6%</td>
</tr>
<tr>
<td></td>
<td>PE: EA/ 9:1</td>
<td>0.951 g</td>
<td>5.7%</td>
</tr>
<tr>
<td>F2</td>
<td>PE: EA/ 8:2</td>
<td>2.122 g</td>
<td>12.7%</td>
</tr>
<tr>
<td>F3</td>
<td>PE: EA/ 1:1</td>
<td>4.065 g</td>
<td>24.3%</td>
</tr>
<tr>
<td>F4</td>
<td>PE: EA/ 2:8</td>
<td>1.323 g</td>
<td>7.9%</td>
</tr>
<tr>
<td>F5</td>
<td>100% EA</td>
<td>0.529 g</td>
<td>3.2%</td>
</tr>
<tr>
<td>F6</td>
<td>EA: MeOH/1:1</td>
<td>3.374 g</td>
<td>20.2%</td>
</tr>
</tbody>
</table>
Elution of NTD_B4 DCM with 100% PE and PE: EA/ 9:1 gave the highest yield, which was 5.063 g, 30.3% (F1). This was followed closely by PE: EA/ 1:1 and EA: MeOH/1:1 which gave yields of 4.065 g (24.3%) and 3.374 g (20.2%), respectively. The least yield obtained by fractionation was 0.529 g (F5). Compounds isolated from these fractions with their retention factors and their IR data (APPENDIX III C, D, E, G) are summarized in Table 4.3.

Table 4.3: Compounds isolated from F1, F3, F4+F5 and F6

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Solvent Systems</th>
<th>Rf</th>
<th>IR (cm⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>OH</td>
<td>-C-Hstr</td>
</tr>
<tr>
<td>F1/S</td>
<td>Hex: EtOAc/ 9.5:0.5</td>
<td>Rf 0.20</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Hex: CHCl3/7:3</td>
<td></td>
<td>Rf 0.88</td>
</tr>
<tr>
<td>F1/Z</td>
<td>Hex: EtOAc/ 9.5:0.5</td>
<td>Rf 0.20</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Hex: CHCl3/7:3</td>
<td></td>
<td>Rf 0.88</td>
</tr>
<tr>
<td>F1/B</td>
<td>Hex: EtOAc/9.5:0.5</td>
<td>Rf 0.83</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Hex: CHCl3/7:3</td>
<td></td>
<td>Rf 0.88</td>
</tr>
<tr>
<td>F1/I</td>
<td>Hex: CHCl3: Ace./ 5:5:1</td>
<td>Rf 0.65</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Rf 0.75</td>
<td></td>
</tr>
<tr>
<td>F3/A</td>
<td>Hex: EtOAc/ 9.5:0.5</td>
<td>Rf 0.18</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Hex: Acetone/ 8:2</td>
<td>Rf 0.63</td>
<td></td>
</tr>
<tr>
<td>F3/C</td>
<td>PE:EtOAc/ 7:3</td>
<td>Rf 0.20</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Hex: CHCl3: Acet./ 5:5:1</td>
<td>Rf 0.95</td>
<td></td>
</tr>
<tr>
<td>F3/E</td>
<td>Hex: Acetone/ 8:2</td>
<td>Rf 0.63</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Hex: CHCl3/7:3</td>
<td>Rf 0.65</td>
<td></td>
</tr>
<tr>
<td>F3/G</td>
<td>PE:EtOAc/ 1:1</td>
<td>Rf 0.28</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Rf 0.65</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Rf 0.78</td>
<td></td>
</tr>
<tr>
<td>F3/L</td>
<td>Hex: Acetone/ 8:2</td>
<td>Rf 0.15</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Hex: CHCl3:Acet</td>
<td>Rf 0.23</td>
<td></td>
</tr>
<tr>
<td>EA P1</td>
<td>Hex: Acetone/ 1:1</td>
<td>Rf 0.65</td>
<td></td>
</tr>
<tr>
<td>EA P2</td>
<td>Hex: EtOAc/ 2:8</td>
<td>Rf 0.8</td>
<td></td>
</tr>
<tr>
<td>EA P3</td>
<td>Hex: Acetone/1:1</td>
<td>Rf 0.45</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Hex:EtOAc/ 2:8</td>
<td>Rf 0.13</td>
<td></td>
</tr>
<tr>
<td>EM 1</td>
<td>PE:EtOAc/ 2:8</td>
<td>Rf 0.85</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Rf 0.93</td>
<td></td>
</tr>
<tr>
<td>EM 2</td>
<td>PE:EtOAc/ 2:8</td>
<td>Rf 0.80</td>
<td></td>
</tr>
<tr>
<td>EM 3</td>
<td>Hex: Acetone/ 1:1</td>
<td>Rf 0.43</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Hex: EtOAc/2:8</td>
<td>Rf 0.1</td>
<td></td>
</tr>
</tbody>
</table>

From the IR data of the compounds isolated from F1, the predominant functional groups deduced from F1/S, F1/Z, F1/B and F1/I were C-H stretch of alkanes with their accompanying bending vibrations occurring around 2900 and 1400 cm⁻¹, respectively. In addition, F1/S, F1/B and F1/I had absorption bands occurring around 1700 and 1100 cm⁻¹ from which C=O and C-O were inferred. An ester functionality was inferred from these deductions. F1/I also had a weak absorption band occurring at 3477.89cm⁻¹ which could be attributed to a secondary amine.
functionality. Based on deductions from the IR absorption bands and $^1$H and $^{13}$C NMR, F1/S, F1/B, F1/Z were mainly aliphatics with carbonyl functionalities, specifically carboxylic acids and esters.

The retention factors of the rest of the compounds indicated that different compounds had been isolated and some of the isolates such F1/I and EM2 consisted of two or three compounds. The various RF indicated that most of the compounds isolated from F1 were quite non-polar as compared to the highly polar compounds isolated from F6. Structure elucidation of other compounds isolated from the chromatographic separation of F1 (F1/J and F1/K) and a combination of F4 and F5 (EA P2 and EA P3) have been discussed in detail.

4.4 Structure Elucidation of Compounds from NTD_B4 DCM
Structure Elucidation of F1/K

Compound F1/K was isolated as a cream coloured powdery substance. Its TLC profile using different solvent systems with corresponding RF values are indicated in Table 4.4. A yellow colour was observed when the spot was observed under UV light and with anisaldehyde spray reagent. The melting point of F1/K was found to be 105-107 ºC.

Table 4.4: Retardation Factor of F1/K Using Different Solvent Systems

<table>
<thead>
<tr>
<th>Solvent System</th>
<th>RF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hexane: Acetone/ 8: 2</td>
<td>0.63</td>
</tr>
<tr>
<td>Hexane: Chloroform/ 1:1</td>
<td>0.23</td>
</tr>
<tr>
<td>Hexane: Chloroform/Acetone/ 5:5:1</td>
<td>0.70</td>
</tr>
</tbody>
</table>

In the IR spectrum (Appendix III A), absorption bands observed at 3394.14, 2914.38, 1646.25 and 1629.69 cm$^{-1}$ were attributed to OH stretch, an intense C-H stretch, α, β-unsaturated C=O and C=C functionalities, respectively. Weak absorption bands observed at 1348.71 and 1185.89 cm$^{-1}$ were ascribed to a phenol (C-O) stretch and a C-O-C stretch, respectively.

From the $^{13}$C NMR spectral data (Figure 4.1), all signals were observed from 7.1 - 182.6 ppm. A total of 23 signals were seen in the spectrum of which 9 were $sp^2$ carbons and the remaining, $sp^3$ carbons.
From the DEPT 135° spectrum (Figure 4.2), 7 quaternary carbons were deduced at δC 182.6, 170.3, 159.9, 159.7, 155.9, 106.8 and 103.4. A cluster of methylene carbons occurred around δC 29.7 (C12-C19) which was suggestive of a considerable aliphatic character. The rest of the methylene carbons labelled C10, C11, C20 and C21 were observed at δC 34.2, 31.9, 26.8 and 22.7, respectively. Also, 2 methine carbon signals occurring at δC 107.8 and 93.2 were labelled as C6 and C9, respectively. The methyl carbons, C22 and C23 occurred at 14.2 and 7.2 ppm, respectively.
From the $^1$H NMR spectrum (Figure 4.3) a signal at $\delta_H 0.87$ occurred as a triplet and integrated for 3 protons which implied it was methyl protons adjacent to methylene protons. Also, a sharp singlet that integrated for 3H occurred at $\delta_H 2.13$. These supported the two methyl carbons identified by the DEPT experiment. Two singlets occurring at $\delta_H 6.02$ and 6.34, integrating for one proton each, were attributed to protons attached to alkene/aromatic carbons and confirmed the 2 methine carbons. A broad signal observed at $\delta_H 1.25$ integrated for 40 protons, corroborating the aliphatic moiety which was observed in the $^{13}$C NMR spectrum. At $\delta_H 1.68$, a signal which occurred as quartet integrated for two protons. Another signal at $\delta_H 2.56$ occurred as a triplet and integrated for 2H suggesting that it was in a deshielded environment. A sharp signal observed at $\delta_H 13.00$ accompanied by a very weak broad signal at $\delta_H 5.71$ integrated for one proton each.
Figure 4.3: Full $^1$H NMR Spectrum of F1/K

From the HSQC spectrum, the $sp^2$ methine carbons, $\delta_C$ 93.2 (C6) and 107.8 (C9) correlated with $\delta_H$ 6.34 (H2) and 6.03 (H3), respectively. The methyl carbons, $\delta_C$ 7.1 (C23) and 14.1 (C22) were directly attached to $\delta_H$ 2.13 (H6) and 0.87 (H9), respectively. The methylene carbons, $\delta_C$ 34.1, 32.0, 26.7 and 22.7 were directly attached to $\delta_H$ 2.55 (H5), 1.24 (H7), 1.69 (H6) and 1.28 (H7), respectively. Also, there were direct correlations between methylene carbons occurring around $\delta_C$ 29.3 - 29.7 and protons around $\delta_H$ 1.25 (H5) and these constituted the long chain moiety. It was observed that there were no correlations between $\delta_C$ 182.6, 170.3, 159.9, 159.7, 155.9, 106.8 and 103.4 which confirmed the quaternary deductions from the DEPT spectrum (Figure 4.4).
In the COSY spectrum, (Figure 4.5), two main sets of spin systems were observed. A cross peak between $\delta_H$ 2.55 (H5) and 1.68 (H7) indicated that these protons were attached to adjacent carbons. Also, cross peaks were observed between the terminal methyl protons of $\delta_H$ 0.87 (H9) and the methylene, $\delta_H$ 1.25.

In the HMBC (Figure 4.7), the methine proton at $\delta_H$ 6.34 (H2) showed correlations with $\delta_C$ 155.9 (C5) and 159.6 (C4) (FIGURE 4.7 A). The strongly bonded hydroxyl proton $\delta_H$ 13.00 (H1)
coupled by $^{3}J_{\text{CH}}$ to $\delta_{C}$ 159.9 (C3), 159.6 (C4) and 106.8 (C7) (Figure 4.7 D). The singlet proton at $\delta_{H}$ 2.13 (H6) showed strong correlations with the quaternary $\delta_{C}$ 106.8 (C7). $\delta_{H}$ 2.13 (H6) also coupled strongly to adjacent oxygenated aromatic carbons at $\delta_{C}$ 159.6 (C4) and 159.9 (C3) (Figure 4.7 C). This established the connectivity of the methyl to the benzene ring. The quaternary carbon $\delta_{C}$ 103.2 (C8) coupled weakly to the hydroxyl, $\delta_{H}$ 13.00 (H1), the aromatic proton $\delta_{H}$ 6.34 (H2) and the methyl protons at $\delta_{H}$ 2.13 (H6 (Figure 4.7 B).

A vinylogous ester carbonyl moiety in conjugation with an aromatic ring was deduced from $\delta_{C}$ 180.2 (carbonyl-C1), vinyl ester at $\delta_{C}$ 107.8 (C6) and 170.3 (C2), and quaternary carbons connecting the ester to the aromatic ring at $\delta_{C}$ 103.4 (C8) and 155.9 (C5). These were characteristic of a chromen-4-one skeleton. The $\alpha, \beta$-unsaturation as well as the ether of the chromen-4-one (Figure 4.6) was confirmed in the IR spectrum. A strong coupling between the protons of the methine singlet $\delta_{H}$ 6.01 (H3) and triplet at $\delta_{H}$ 2.56 (H5) to $\delta_{C}$ 170.3 (C2) established the connectivity of the long chain (Figure 4.7 E) with the chromenone.

![Figure 4.6: $\alpha, \beta$-unsaturation of a 4H-chromen-4-one nucleus](image)

The carbonyl ketone which is mostly observed after 200 ppm was more upfield due to the alpha-beta unsaturation and the extended conjugation in the benzene ring. The methine protons appeared as singlets since they were no coupling neighbouring protons. The $^1$H, $^{13}$C, COSY and HMBC NMR data of F1/K are summarized in Table 4.5 and Figure 4.8.
Figure 4.7: Expanded HMBC spectrum for F1/K
Table 4.5: Summary of $^1$H, $^{13}$C, COSY and HMBC NMR data of F1/K

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Figure 4.8: $^1$H-$^1$H COSY and HMBC of F1/K

In the F1/K mass spectrum (HRESIMS) (Figure 4.9), the highest mass was found at $m/z$ 515.1523 which corresponded to a molecular formula of C$_{33}$H$_{55}$O$_4$ $^+$ ($\Delta = +0.12$ ppm and 7 degrees of unsaturation) for the pseudo molecular ion [M+H] $^+$. An M$^+$ ion which was observed at $m/z$
514.2398, calcd. $m/z$ 514.4022 corresponded to the molecular formula, $C_{33}H_{54}O_4^{+}$ ($\Delta = +0.16$ and 7 degrees of unsaturation) (Figure 4.10).

The base peak, $M^+$ occurred at $m/z$ 389.40 corresponding to a molecular formula of $C_{24}H_{36}O_4^{+}$, respectively. This was accompanied by a minor peak at $m/z$ 417.24. In addition to $m/z$ 515.15 (F1/K), 417.24 and 388.40 form common daughter ions at $m/z$ 291.92 ($C_{17}H_{22}O_4^{+}$) by losing $C_{16}H_{33}$, $C_{9}H_{19}$ and $C_{7}H_{15}$ fragments, respectively (Figure 4.11).

From the NMR data with confirmation from the mass data, F1/K was identified as 5,7-dihydroxy-6-methyl-2-tricosyl-4H-chromen-4-one.

Figure 4.9: HRESIMS of F1/K
Figure 4.10: Structure of Compound F1/K

Figure 4.11: Fragmentations of F1/K from HRESIMS
Cooke R. J & Down J. G, (1970) isolated a similar chromen-4-one attached to a long chain moiety from *Dianella revoluta* and *Stypandra grandis*. The compound $m/z$ 598 ($C_{39}H_{66}O_{4}$) was accompanied by homologues occurring at $m/z$ 570 ($C_{37}H_{62}O_{4}$) and 626 ($C_{41}H_{70}O_{4}$) (Figure 4.12). In the biosynthesis of this chromen-4-one, a polyketide attached to the required number of acetate units to an appropriate long chain fatty acid was proposed.

In the biosynthesis of this chromen-4-one, a polyketide attached to the required number of acetate units to an appropriate long chain fatty acid was proposed.

Figure 4.12: Chromen-4-one compounds isolated from *Dianella revoluta* and *Stypandra grandis*

Chromenones belong to the family of flavonoids which constitute flavones, isoflavones and coumarins. The basic skeleton has benzopyran with a substituted keto group on the pyron. This class of compounds is known to exhibit antibiotic, antibacterial, antiviral, anticoagulant and anti-HIV activities. The synthetic derivative, 3-hydroxy-6-(hydroxymethyl)-2-(2-phenyl-4H-chromen-4-yl)-4H-pyran-4-ones exhibits potent antimicrobial activities on some bacteria strains. It is cytotoxic against human neuroblastoma and human gastric cancer cells.
Structure Elucidation of Compound EA P3

Compound EA P3 was isolated as a white flaky compound.

In the $^{13}$C NMR spectrum (Figure 4.13), 35 signals were observed from $\delta_C$ 11-141. Six of the signals occurring from $\delta_C$ 62-101 were characteristic of a sugar moiety. The aglycone portion had 29 signals of which 3 were quaternary, 9 methines, 11 methylene and 6 methyl carbons.

![β-sitosterol glucoside](image)

**Figure 4.13:** Full and expanded $^{13}$C NMR Spectrum of EA P3

Signals which occurred at $\delta_C$ 140.3 (C5), 122.0 (C6), 79.0 (C3), 56.7 (C14), 55.9 (C20), 50.1 (C9), 46.8 (C24), 42.2 (C13), 39.7 (C12), 38.6 (C4), 37.2 (C1), 36.6 (C9), 36.0 (C20), 33.8 (C22), 31.8 (C8), 31.8 (C7), 29.6 (C2), 29.1 (C25), 28.1 (C16), 26.1 (C23), 24.1 (C15), 23.0 (C28), 21.0 (C11), 19.6 (C27), 19.2 (C19), 18.9 (C26), 18.6 (C21), 11.8 (C18) and 11.7 (C29) were characteristic of β-sitosterol.

The angular methyls, C18, C19 and C21 of the aglycone occurred at $\delta_C$ 11.6, 19.2 and 18.6. Signals occurring at $\delta_C$ 19.6, 18.9 and 11.7 were assigned to the other three methyl carbons, C27, C26 and C29, respectively. The quaternary carbons labelled C5, C10 and C13 occurred at $\delta_C$141.3, 36.6
and 42.2. The methylene carbons C1, C2, C4, C7, C11, C12, C15, C16, C22 and C28 occurred at $\delta_C$ 37.2, 29.6, 38.6, 31.8, 21.0, 39.7, 24.1, 28.1, 33.8 and 23.0, respectively.

The sugar moiety constituting 6 carbon signals occurred at $\delta_C$ 100.1 (C1’), 73.5 (C2’), 76.5 (C3’), 70.0 (C4’), 75.8 (C5’) and 63.8 (C6’). These $\delta_C$ values were consistent with those of a glucose moiety. The anomeric carbon of the sugar occurred at $\delta_C$ 100.1 due to its highly deshielded environment.

The $^{13}$C NMR data of compound EA P3 and $\beta$-sitosterol-3-O-glucoside (Figure 4.14) isolated by other research groups are summarized in Table 4.6. From the cited literature, the $^{13}$C NMR data of the glucoside was acquired by Peshin and Kar on a Bruker R-32 (400 MHz) using CDCl$_3$ and CD$_3$OH as deuterated solvents whilst Delporte and Cassels acquired their $^{13}$C NMR data on a Bruker 500 MHz spectrometer using CDCl$_3$ and CD$_5$D$_5$N as deuterated solvents. The different conditions accounted for the slight differences in the chemical shift values.

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![β-sitosterol-3-O-glucoside](image.png)

**Figure 4.14:** β-sitosterol-3-O-glucoside

Earlier investigations by other research groups indicated that at 200 μg/mL, β-sitosterol-3-O-glucoside exhibits antibacterial activity against some gram-positive and gram-negative strains including *Staphylococcus aureus*, *Bacillus subtilis*, *Salmonella typhi*, *Klebsiella pneumonia*, *Proteus mirabilis* and *Escherichia coli* which are known to cause skin infections, pneumonia and infections of the urinary tract and diabetic foot. It also displayed antifungal activity against *Candida albicans* and *C. tropicalis*.107

β-sitosterol and its glucoside were also found to exhibit analgesic activity in murine models by increasing the tolerance to pain by 300% as compared to 157% of the drug standard, mefenamic acid. An anthelminthic evaluation of β-sitosterol against *Ascaris suum* revealed that its activity approximated that of the standard mebendazole. Other research studies confirmed the antioxidant, antidiabetic, anti-inflammatory, angiogenic effects of β-sitosterol and its glucoside.108,109
Structure Elucidation of Compound F1/J

Compound F1/J was isolated as a white amorphous compound with a melting point of 72-75 °C. In the IR spectrum (Appendix III B), absorption bands observed at 2916.05 and 2848.89 cm\(^{-1}\) were due to C-H stretch of alkanes. Weak absorption bands occurring at 1463.09 were attributed to bending vibrations of CH\(_3\). A carbonyl functionality (C=O) was observed at 1704.81 cm\(^{-1}\). Absorption bands occurring at 719 cm\(^{-1}\) were due to OH in the swinging mode which was characteristic of a fatty acid.

From the \(^{13}\)C NMR spectrum (Figure 4.15), all carbon signals occurred from δ\(_C\) 14.1-177.7. A total of 14 signals were observed of which one was \(sp^2\) and the remaining, \(sp^3\) carbons.

![Figure 4.15: \(^{13}\)C NMR Spectrum of F1/J](http://ugspace.ug.edu.gh)
From the DEPT 135 spectrum (Figure 4.16), one quaternary and one methyl carbon were deduced at $\delta_C$ 177.1 and 14.1 and were labelled as C1 and C14, respectively. The remaining signals were methylene carbons, and a broad peak occurring $\delta_C$ 29.8 (C5) implied several CH$_2$ carbons occurring in the same chemical environment.

**Figure 4.16:** DEPT 135 Spectrum of F1/J

From the $^1$H NMR spectrum (Figure 4.17), the signal occurring at $\delta_H$ 2.32 (H1) as a triplet integrated for two protons which implied it was methylene attached to another methylene. A signal at $\delta_H$ 1.62 (H2) integrating for 2 protons occurred as a pentet. The broad signal at $\delta_H$ 1.24 (H3) was due to the protons attached to the long continuous chain of carbons occurring in that chemical environment. A signal at $\delta_H$ 0.86 (H4) integrated for 3H and occurred as a triplet which was suggestive of methyl protons adjacent to methylene protons.
In the HSQC spectrum (Appendix IV A), a methyl occurring at δC 14.1 (C14) was directly attached to δH 0.86 (H4). The long chain methylene carbons (C4-C11) were attached to protons occurring at δH 1.24 (H3). The methylene carbon occurring at δC 33.8 (C2) and 31.9 (C3) were directly attached to δH 2.32 (H1) and 1.24 (H3), respectively. Methylene carbons occurring at δC 24.7 (C12) and 22.6 (C13) were directly attached to protons occurring at δH 1.61 (H2) and δH 1.24 (H3), respectively.

In the COSY spectrum (Appendix IV B), two main spin systems were observed. Strong correlations were seen between δH 2.32 (H1) and 1.64 (H2), which were both methylene protons with the former being in a more deshielded environment. Also, cross peaks were observed between the terminal methyl protons δH 0.86 (H4) and the methylene protons at 1.24 (H3).

In the HMBC spectrum (Appendix IV C), the carbonyl δC 177.7 (C1) showed strong correlations with δH 2.32 (H2). This implied C1 was directly attached to a CH2 (δC 33.8, δH 2.32) which was also attached to CH2 (δC 24.7-C11, δH 1.67 -H2) as established by the 1H-1H COSY and HMBC.

Figure 4.17: 1H NMR Spectrum of F1/J
Cross peaks were observed between carbon signals occurring at $\delta_C$ 31.9 (C3) and 22.6 (C13) and terminal methyl protons $\delta_H$ 0.86 (H4). From the $^{13}$C and DEPT spectrum, a long chain of methylene carbons with a terminal methyl attached to a carbonyl was identified.

The 1D and 2D NMR data of F1/J were suggestive of a fatty acid. A singlet proton observed from 9-12 ppm usually confirms the acidic proton of the carboxylic acid functionality. However, it does not usually appear due to the acidic nature of the deuterated chloroform and the subsequent proton exchange with the solvent and hence the absence of the singlet peak. The 1D and 2D data are summarized in Table 4.7.

To confirm the length of the aliphatic chain, the sample has been sent for HRMS analysis and currently awaiting the results.

Table 4.7: Summary of $^1$H, $^{13}$C, COSY and HMBC NMR data of F1/J

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<td>H1-H2</td>
<td>H3</td>
</tr>
<tr>
<td>3</td>
<td>31.9</td>
<td>CH$_2$</td>
<td>(H3) 1.24</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>29.7</td>
<td>CH$_2$</td>
<td>(H3) 1.24</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>29.6</td>
<td>CH$_2$</td>
<td>(H3) 1.24</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>29.6</td>
<td>CH$_2$</td>
<td>(H3) 1.24</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>29.6</td>
<td>CH$_2$</td>
<td>(H3) 1.24</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>29.4</td>
<td>CH$_2$</td>
<td>(H3) 1.24</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>29.3</td>
<td>CH$_2$</td>
<td>(H3) 1.24</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>29.2</td>
<td>CH$_2$</td>
<td>(H3) 1.24</td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>29.1</td>
<td>CH$_2$</td>
<td>(H3) 1.24</td>
<td>H3</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>24.7</td>
<td>CH$_2$</td>
<td>(H2) 1.61 (2H, p, J= 7.4)</td>
<td>H2-H1</td>
<td>H2, H1</td>
</tr>
<tr>
<td>13</td>
<td>22.6</td>
<td>CH$_2$</td>
<td>(H3) 1.24</td>
<td>H3-H4</td>
<td>H4</td>
</tr>
<tr>
<td>14</td>
<td>14.1</td>
<td>CH$_3$</td>
<td>(H4) 0.86 (3H, t, J=6.0)</td>
<td>H4-H1</td>
<td></td>
</tr>
</tbody>
</table>

Figure 4.18: Proposed Structure of F1/J
Structure Elucidation of EA P2

Compound EA P2 was isolated as a white amorphous solid.

From the $^{13}$C NMR spectrum (Figure 4.19), all carbon signals were observed from $\delta_c$ 167.8 to 14.3. Five of the signals occurred in the $sp^2$ region with the remaining two occurring in the $sp^3$ region.

Figure 4.19: Full $^{13}$C NMR Spectrum of EA P2
From the DEPT experiment (Figure 4.20), signals occurring at $\delta_C 167.8$ (C1), 156.7 (C2) and 132.0 (C3) were deduced as quaternary carbons. Two methine carbon signals, $\delta_C 131.1$ (C4) and 129.0 (C5) with high relative intensities representing 2 carbons each, one highly deshielded methylene carbon and one methyl carbon were also observed at $\delta_C 61.8$ and 14.3, respectively.

Figure 4.20: Full DEPT 135 Spectrum of EA P2

In the $^1$H NMR (Appendix IV D), 4 different proton environments were observed. Protons occurring as dd at $\delta_H 7.72$ (H1) and $\delta_H 7.53$ (H2) integrated for two protons each and coupled with each other. Proton signals observed at $\delta_H 4.37$ were characteristic of alkoxy protons. It integrated for two protons which was indicative of an ethoxy and coupled to the methyl protons which occurring at $\delta_H 1.32$ (H4).

In the HSQC spectrum (Appendix IV E), carbons occurring at $\delta_C 130.9$ (C4) and 128.7 (C5) were coupled to protons occurring at $\delta_H 7.72$ (H1) and 7.53 (H2), respectively. The methylene carbon, $\delta_C 61.8$ was directly connected to protons occurring at $\delta_H 4.37$ (H3). The methyl carbon occurring at $\delta_C 14.1$ (C7) was directly attached to $\delta_H 1.32$ (H4).
Two sets of spin systems were observed in the COSY spectrum (Appendix IV F). Protons occurring at δ\textsubscript{H} 7.72 (H1) and δ\textsubscript{H} 7.53 (H2) coupled with each other. Cross peaks were also observed between 4.37 (H3) and 1.3 (H4). This confirmed the information gained from the \textsuperscript{1}H NMR.

In the HMBC (Appendix IV G), \textsuperscript{3}J\textsubscript{CH} coupling was observed between δ\textsubscript{C} 167.8 C and ethoxy protons occurring at δ\textsubscript{H} 4.37. Strong correlations were also observed between carbons occurring at δ\textsubscript{C} 130.1 and 129.0 and protons at δ\textsubscript{H} 7.72 and 7.53. Weak correlations were also observed between these same carbons and their direct protons.

The NMR data of F3/F and their subsequent interpretations as summarized in Table 4.8 were consistent with those of a para-substituted ethyl 4-hydroxybenzoate ester.

<table>
<thead>
<tr>
<th>Atom</th>
<th>δ\textsubscript{C}/ppm</th>
<th>δ\textsubscript{C}mult</th>
<th>δ\textsubscript{H}/ppm J(Hz)</th>
<th>COSY</th>
<th>HMBC</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>167.8</td>
<td>C</td>
<td>-</td>
<td>-</td>
<td>C1-H5</td>
</tr>
<tr>
<td>2</td>
<td>156.7</td>
<td>C</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>132.0</td>
<td>C</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>130.9</td>
<td>2CH</td>
<td>(H1) 7.55 dd, J = 5.7, 3.3 Hz, 2H</td>
<td>H3-H4</td>
<td>C4-H5</td>
</tr>
<tr>
<td>5</td>
<td>128.7</td>
<td>2CH</td>
<td>(H2) 7.72 J = 5.7, 3.3 Hz, 2H</td>
<td>H4-H3</td>
<td>C5-H4</td>
</tr>
<tr>
<td>6</td>
<td>61.4</td>
<td>CH\textsubscript{2}</td>
<td>(H3) 4.37 q, J = 7.1 Hz, 2H</td>
<td>H5-H6</td>
<td>C6-H7</td>
</tr>
<tr>
<td>7</td>
<td>13.8</td>
<td>CH\textsubscript{3}</td>
<td>(H4) 1.32 t, J = 7.2 Hz, 3H</td>
<td>H6-H5</td>
<td>C7-H6</td>
</tr>
</tbody>
</table>

![Ethyl 4-hydroxybenzoate](image)

**Figure 4.21:** Proposed Structure of EA P2

Ethyl 4-hydroxybenzoate results from the condensation of 4-hydroxybenzoic acid with ethanol. It occurs as in vegetables such as onions, grapes, cocoa beans amongst others. Ethylparaben, as is
commonly known, is highly effective as an antimicrobial and it inhibits bacterial and fungal growth. It mainly used in the pharmaceutical, food and cosmetic industries\textsuperscript{110}.

**Other compounds from NTD_B6 DCM**

Fine crystals that were observed in the DCM extracts of NTD_B6 necessitated a chromatographic separation which led to the isolation of compound DP2. It was isolated as colourless crystals with a melting point of 121-122 °C.

From the $^{13}$C NMR spectrum (Appendix IV H), all signals were observed from δ$_C$128.5 -174.4. The peak heights at δ$_C$130.2 and 128.5 were about twice the heights of the peaks in its surroundings which implied 2 carbons with the same chemical environment. The peak which appeared downfield at δ$_C$172.4 was attributed to a carbonyl. The absence of peaks in the $sp^3$ region from δ$_C$50-80 indicated that the carbonyl was neither an amide nor an ester.

From the DEPT experiment with comparison to the $^{13}$C spectrum, 3 peaks appeared in the methine/methyl phase which showed there were 3CH environments that represented five CH. Also, 2 quaternary carbons were deduced from the spectrum.

From the 1D $^1$H NMR spectrum (Appendix IV I), all signals were observed from δ$_H$ 7.47-8.15. Signals observed at δ$_H$ 7.49 (t, $J = 7.4$ Hz, 1H), δ$_H$ 7.62 ppm (t, $J = 7.4$ Hz, 1H) and δ$_H$ 8.13 (dd, $J = 8.3$, 1.5 Hz, 2H) were attributed to aromatic Hs. The signals integrated for a total of 5Hs. This was characteristic of a monosubstituted benzene ring.

<table>
<thead>
<tr>
<th>Atom</th>
<th>δ$_C$/ppm</th>
<th>$^{13}$C mult.</th>
<th>$^1$H/ppm J</th>
<th>Drugbank\textsuperscript{111}</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>172.4</td>
<td>C</td>
<td>-</td>
<td>172.8</td>
</tr>
<tr>
<td>2</td>
<td>134.0</td>
<td>CH</td>
<td>7.62 (1 H, t, $J = 7.4$ Hz)</td>
<td>133.8</td>
</tr>
<tr>
<td>3</td>
<td>130.4</td>
<td>2CH</td>
<td>7.49 (1 H, t, $J = 7.8$ Hz)</td>
<td>130.2</td>
</tr>
<tr>
<td>4</td>
<td>129.5</td>
<td>C</td>
<td>-</td>
<td>129.4</td>
</tr>
<tr>
<td>5</td>
<td>128.6</td>
<td>2CH</td>
<td>δ 8.14 (dd, $J = 8.3$, 1.5 Hz)</td>
<td>128.5</td>
</tr>
</tbody>
</table>

No signal appeared in the $^1$H NMR spectrum to confirm the carbonyl was a carboxylic acid but this was also expected due to the proton exchange of the benzoic acid with the deuterated solvent, CDCl$_3$. The melting of 121-122 °C was consistent with 122 °C of benzoic acid in literature. The
yield of the benzoate was 38 mg which was 25.3% of the extract loaded on the column. A comparative TLC profile of the benzoate and the crystals from B7 DCM indicated that benzoate was also present.

Benzoic acid is known to occur in cranberries, cloves and raspberries\textsuperscript{112}. However, the quantity isolated from NTD_B6 DCM was unusually high. Further inquiries from the traditional medicine practitioners confirmed their use of benzoic acid and sodium benzoate in their aqueous herbal preparations as preservatives. It is one of the oldest and most widely used preservatives due to its antimicrobial and antifungal properties. The World Health Organization, however, limits the amounts of benzoic acid that can be incorporated in consumables to 0.03-0.2%\textsuperscript{113}.

\section*{4.5 Chemical Investigation of F1/HML}

A TLC profile of the oil, F1/HML upon visualization with a UV lamp and anisaldehyde spray reagent indicated that it had both UV and non-UV active constituents. An HPLC fingerprint (Figure 4.22) of F1/HML was suggestive of the presence of more than 7 major compounds with retention times (t\textsubscript{R}) occurring from 5.8 to 20.2 minutes.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{hplc_profile.png}
\caption{HPLC Profile of F1/HML}
\end{figure}
4.5.1 GC-MS Analysis of F1/HML

The basic principle which was employed in the analysis of F1/HML was the fact every compound isolated by the gas chromatograph has unique retention times (t<sub>R</sub>) with specific masses and fragmentation patterns. The spectrum (Figure 4.23) indicates the gas chromatogram of F1/HML.

![Figure 4.23: GC profile of F/HML](image)

From the chromatogram, it was observed that most of the compounds were eluted from 21-43 minutes with the most abundant compounds occurring at 23.9 and around 35-37 minutes. The eluates gave specific masses with specific MS data which led to the identification of constituent compounds as indicated in Table 4.10.
Table 4.10: Compounds Identified in F1/HML by GC-MS

<table>
<thead>
<tr>
<th>Retention time (min)</th>
<th>Compound</th>
<th>Area</th>
</tr>
</thead>
<tbody>
<tr>
<td>12.41</td>
<td>Myrcene</td>
<td>0.0280</td>
</tr>
<tr>
<td>12.92</td>
<td>Allylamine 3-chloro-n-isopropyl-2-methyl</td>
<td>0.0196</td>
</tr>
<tr>
<td>13.55</td>
<td>5α-pregnane-12,20-dione</td>
<td>0.0349</td>
</tr>
<tr>
<td>15.34</td>
<td>Hydrocinnamic acid, 3,4-dimethoxy-, methyl ester</td>
<td>0.0184</td>
</tr>
<tr>
<td>17.19</td>
<td>1-amino-3-hydroxyamino-isoquinoline</td>
<td>0.0280</td>
</tr>
<tr>
<td>21.15</td>
<td>Cyclohexadecane</td>
<td>0.0846</td>
</tr>
<tr>
<td>23.17</td>
<td>Chola-5,22 Z-dien-3β-ol</td>
<td>0.0303</td>
</tr>
<tr>
<td>23.90</td>
<td>2-(2'-nitro-3'-thienyl)pyrimidine</td>
<td>3.0048</td>
</tr>
<tr>
<td>24.95</td>
<td>Octahydro-α-camphorene</td>
<td>0.0974</td>
</tr>
<tr>
<td>31.72</td>
<td>11-(2,5-dimethylphenyl)-10-heneicosene</td>
<td>0.2746</td>
</tr>
<tr>
<td>32.07</td>
<td></td>
<td>1.1466</td>
</tr>
<tr>
<td>38.73</td>
<td></td>
<td>1.0896</td>
</tr>
<tr>
<td>30.52</td>
<td>5α, 13α- D-homoandrostan</td>
<td>0.8742</td>
</tr>
<tr>
<td>32.34</td>
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<td>1.3742</td>
</tr>
<tr>
<td>34.34</td>
<td></td>
<td>1.3796</td>
</tr>
<tr>
<td>32.22</td>
<td>Cholest-5-en-3β-ol</td>
<td>0.2243</td>
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<tr>
<td>38.84</td>
<td></td>
<td>1.0261</td>
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<td>41.05</td>
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<td>0.9410</td>
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<td>32.91</td>
<td>Stigmaster-5-en-3β-ol</td>
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<tr>
<td>32.78</td>
<td>22, 22-dimethyl-ergost-5-en-3β-ol</td>
<td>0.3205</td>
</tr>
<tr>
<td>33.81</td>
<td>Cycloproa-7,8-cholestan-3-one</td>
<td>3.8201</td>
</tr>
<tr>
<td>34.07</td>
<td>4α-Methyl-24-ethyl-5α-cholest-8(9)-en-3β-ol</td>
<td>2.2243</td>
</tr>
<tr>
<td>37.45</td>
<td></td>
<td>2.3921</td>
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<tr>
<td>34.71</td>
<td>Cholestan-3,11-dione</td>
<td>1.7800</td>
</tr>
<tr>
<td>35.80</td>
<td>2-butyl-benzothiazole</td>
<td>4.2563</td>
</tr>
<tr>
<td>36.12</td>
<td>Phytan</td>
<td>6.3188</td>
</tr>
<tr>
<td>36.53</td>
<td>5α, 8α, 14β-cholestan</td>
<td>6.1091</td>
</tr>
<tr>
<td>37.15</td>
<td>1,4,5,8-tetrahydro-4α,8α-napthalenedicarboximide</td>
<td>10.3027</td>
</tr>
<tr>
<td>37.72</td>
<td>2-chloro-2-styryl-4-methylstilbene</td>
<td>3.2902</td>
</tr>
<tr>
<td>39.34</td>
<td>n-tetradecane</td>
<td>2.9057</td>
</tr>
<tr>
<td>39.71</td>
<td>Urs-12-en-28-al</td>
<td>0.5879</td>
</tr>
<tr>
<td>40.07</td>
<td>5α, 14β-cholestan</td>
<td>2.0782</td>
</tr>
<tr>
<td>40.60</td>
<td>5α-stigmaslane</td>
<td>1.4028</td>
</tr>
</tbody>
</table>

4.5.2 NMR ANALYSIS OF F1/HML

$^1$H and $^{13}$C NMR spectral data were obtained to confirm the GC-MS data in terms of the distribution of carbons in the $sp^2$ and $sp^3$ regions.
The $^{13}$C NMR spectrum indicated that F1/HML was dominated by mainly aliphatic $sp^3$ carbons occurring from $\delta_C 10$ to 50. A few $sp^2$ carbons occurred around $\delta_C 122 – 155$. The signals occurring further downfield can be attributed to further deshielding by heteroatoms and hence carbonyls which may be esters, carboxylic acids and amides were speculated to be present in the oil. The full $^{13}$C NMR data of F1/HML is displayed in Figure 4.24.

![Figure 4.24: Full $^{13}$C NMR Spectrum of F1/HML](image)

From the GC-MS analysis, most of the compounds identified were terpenoids and long chain aliphatic compounds. The methyls and methylenes of these groups of compounds usually occur from 14-38 ppm. Carbons in the same chemical environment in the long chain moiety resulted in an intense signal at $\delta_C 29.5$ and this was typical of aliphatic chains of either fatty acids or saturated long chain compounds. Some of the aliphatics identified in the GC-MS analysis were phytan, n-tetradecane and cyclohexadecane which occurred at $t_R$ of 36.12, 39.34 and 21.15 minutes with phytan being the most abundant constituent. The methyls and methylenes of the triterpenoids usually occur from 11-38 ppm. Some of the peaks occurring around $\delta_C 40-57$ could be attributed to $sp^3$ methine and quaternary carbons.
Signals occurring at δC 120-190 could be ascribed to the aromatic and carbonyl carbons of the most abundant constituent 1,4,5,8-tetrahydro-4a,8a-napthalenedicarboximide (tR=37.12 10.30%). The sp² carbons of 2-chloro-2-styryl-4-methylstilbene (tR=37.72) were also known to occur within this region. Some of these carbons could also be attributed to sp² carbons of the triterpenoids.

In the ¹H NMR spectrum (Figure 4.25), most of the signals appeared in the δH 0.8-2.4 ppm range. This implied most of the protons were in the aliphatic region and confirmed protons of the long chain carbon moieties as well as the methyls and methylenes of the terpenoids as indicated in the GC-MS results. Tiny peaks occurring at δH 4.14-5.34 were consistent with protons attached to alkene carbons or heteroatoms such as oxygen or nitrogen present in that environment.

Figure 4.25: Full ¹H NMR Spectrum for F1/HML
Figure 4.26: Compounds Identified from F1/HML by GC-MS
Figure 4.27: Mass Spectrum of 4α-Methyl-24-ethyl-5α-cholest-8(9)-en-3β-ol

Figure 4.28: Mass Spectrum of 11-(2,5-dimethylphenyl)-10-heneicosene
Figure 4.29: Mass spectrum of $5\alpha, 8\alpha, 14\beta$-cholestane

Figure 4.30: Mass spectrum of urs-12-en-28-al
Some of the major compounds identified by the GC-MS analysis have been isolated from plants and their synthetic derivatives have a broad spectrum of biological activities.

2-butyl-benzothiazole consists of a bicyclic ring. It is usually found in nuts and is the flavor component of roasted cashew nut and coconut. Synthetic derivatives of benzothiazole exhibited antileishmanial, anti-inflammatory and antimicrobial activity against *S. aureus*, *E. coli* and *C. albicans*. They also exhibited significant anticonvulsant activity against various types of induced seizures in mouse models and possess anthelmintic and antitrypanosomal activity. The phenyl substituted benzothiazoles have antitumor activities\textsuperscript{114,115}.

2-chloro-2-styryl-4-methylstilbene belongs to the family of stilbenes which constitute a small group of phenylpropanoids (mostly saturated bibenzyls) and are characterized by 1, 2-diphenyl ethylene backbone. They have been isolated from sorghum, peanuts, grapes and pine. Analysis of the common stilbenes, resveratrol isolated from grapes shows that it prevents or delays the occurrence and progression of cancer and other cardiovascular diseases. Derivatives of stilbenes exhibit antifungal, antibacterial, anticancer, antiallergic properties. One of the derivatives diethylstilbestrol was medically used to prevent threatened abortion and to treat prostate and breast cancer\textsuperscript{116,117}.

According to the GC-MS data, the most abundant component (10.3\%) was 1,4,5,8-tetrahydro-4a,8a-naphthalenedicarboximide and it occurred at $t_R = 37.12$. The base peak, $m/z$ 203.1 corresponded to the molecular formula of C$_{12}$H$_{13}$NO$_2$, which implied 7 degrees of unsaturation. Fragments peaks occurring at $m/z$ 188.1, 161.1, 131.1 and 77.0 were due to the loss of amine and carbonyl fragments of the dicarboximide and C$_4$H$_6^{2+}$ of the ring by McLafferty rearrangements and retro Diels Alder fragmentations as indicated in Figure 4.31. A signal occurring at $m/z$ 218 which by difference from the base peak gave $m/z$ 15 may be attributed to a methyl moiety which was lost to obtain the base peak, $m/z$ 203.1. The N-methyl derivative of 1,4,5,8-tetrahydro-4a,8a-naphthalenedicarboximide which has a calculated mass of $m/z$ 218 has also been reported.
Figure 4.31: Mass spectrum of 1,4,5,8-tetrahydro-4a,8a-naphthalenedicarboximide

1,4,5,8-tetrahydro-4a,8a-naphthalenedicarboximide is a tetrasubstituted succinimide which is also known as 1,4,5,8-tetrahydro-4a,8a-(methanoiminomethano)naphthalene-9,11-dione. Wagner and Rudzik investigated the anticonvulsant activity of the synthetic 1,4,5,8-tetrahydro-4a,8a-naphthalenedicarboximide against pentylenetetrazole induced convulsions. It exhibited an activity ED$_{50}$ of 22 mg/Kg and low toxicity at LD$_{50}$ of 620 mg/Kg\textsuperscript{118}. Further investigations by Wagner and Davisson demonstrated the analgesic activity of ED$_{50}$ of 50 mg/Kg in HCl writhing test but was found to be quite toxic at LD$_{50}$ of 100 mg/Kg\textsuperscript{119}.

GC-MS data is currently being acquired in other laboratories to confirm the structures of the constituent compounds predicted by the GC-MS analysis. And also, since F1/HML was found to be very active, larger quantities are being obtained. This will further be separated by chromatographic processes and subjected to antitrypanosomal and antischistosomal activity screening to identify the specific compound(s) responsible for the observed efficacy.
4.6 Results from Biological Activity Screening

4.6.1 Antischistosomal Activity

About 5 mg of the 14 crude extracts from the traditional medicines were tested against newly transformed schistosomula and adult worms of *S. mansoni* and the results obtained are indicated in Table 4.11.

Table 4.11: The Antischistosomal Activity of Crude Extracts Against NTS and Adult worms of *S. mansoni*

<table>
<thead>
<tr>
<th>Crude Extracts</th>
<th>NTS Effect in %</th>
<th>NTS Effect in %</th>
<th><em>S. mansoni</em> adult Effect in %</th>
<th><em>S. mansoni</em> adult Effect in %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Test conc. 100uM</td>
<td>Test conc. 10uM</td>
<td>Test conc. 100uM</td>
<td>Test conc. 10uM</td>
</tr>
<tr>
<td>NTD_B1 DCM</td>
<td>62.50</td>
<td>10.41</td>
<td>57.14</td>
<td>8.33</td>
</tr>
<tr>
<td>NTD_B1 BuOH</td>
<td>53.57</td>
<td>19.83</td>
<td>30.36</td>
<td>16.67</td>
</tr>
<tr>
<td>NTD_B2 DCM</td>
<td>32.14</td>
<td>12.50</td>
<td>32.14</td>
<td>12.50</td>
</tr>
<tr>
<td>NTD_B2 BuOH</td>
<td>30.36</td>
<td>16.67</td>
<td>30.36</td>
<td>16.67</td>
</tr>
<tr>
<td>NTD_B3 DCM</td>
<td>57.14</td>
<td>8.33</td>
<td>60.07</td>
<td>30.60</td>
</tr>
<tr>
<td>NTD_B3 MeOH</td>
<td>89.59</td>
<td>12.50</td>
<td>60.07</td>
<td>30.60</td>
</tr>
<tr>
<td>NTD_B4 DCM</td>
<td>100.00</td>
<td>14.58</td>
<td>78.40</td>
<td>22.90</td>
</tr>
<tr>
<td>NTD_B4 MeOH</td>
<td>35.72</td>
<td>14.58</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NTD_B5 DCM</td>
<td>32.15</td>
<td>12.50</td>
<td>25.00</td>
<td>18.75</td>
</tr>
<tr>
<td>NTD_B5 BuOH</td>
<td>55.36</td>
<td>12.50</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NTD_B6 DCM</td>
<td>25.00</td>
<td>18.75</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NTD_B6 BuOH</td>
<td>20.84</td>
<td>12.50</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NTD_B7 DCM</td>
<td><strong>100.00</strong></td>
<td><strong>18.75</strong></td>
<td><strong>84.30</strong></td>
<td><strong>16.70</strong></td>
</tr>
<tr>
<td>NTD_B7 BuOH</td>
<td>25.00</td>
<td>18.75</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Praziquantel (standard)  
NTS IC$_{50}$ = 2.2 μM  
Adult worms IC$_{50}$ = 0.1 μM

The starting concentration for the assay was 100 μM. At this concentration, NTD_B4 DCM and NTD_B7 DCM showed very promising results by killing the NTS completely within 24 to 72 hours. The methanol crude extract of NTD_B3 also showed good activity by 89.9% effect. The remaining crude extracts exhibited low to moderate activity with the least activities observed in the NTD_B6 extracts. At 10 μM, the activity of the extracts mostly reduced by five to ten-fold. They all exhibited a dose-dependent inhibition of the schistosomulae. The butanol extract of NTD_B1 recorded the highest activity of 19.8% at this test concentration with a ten-fold reduction in the activities exhibited by NTD_B7 DCM and NTD_B6 DCM.
After the NTS screening, crude extracts which showed greater than 70% effect at 100 μM test concentration were selected and further tested on the adult worms of *S. mansoni in vitro*. NTD_B7 DCM reduced the worm viability significantly by exhibiting an 84.30% effect. NTD_B4 DCM also showed great potential by displaying a 78.40% reduction in worm viability, respectively. At 10 μM, the activity of NTD_B3 MeOH decreased by 2-fold. The dose-dependency was also observed in the DCM extracts of NTD_B4 and NTD_B7.

Praziquantel exhibited an IC₅₀ value of 2.2 and 0.1 μM against the schistosomulae and adult worms of *S. mansoni*, respectively which implied a 50% effect at the test organisms. Considering NTD_B4 DCM and NTD_B7 DCM in terms of IC₅₀, about 50 μM of the extract would have been required to induce a 50% effect on the test organisms which implied a moderate activity as compared to praziquantel.

### 4.6.2 *In vitro* Screening of Crude Extracts for Antitrypanosomal Activity

The 14 crude extracts were also screened against the bloodstream forms of *T. brucei brucei* at WACCBIP. *T. brucei brucei* was employed in this research due to its highly identical features to *T. b. gambiense* and *T. b. rhodesiense* and the fact that it has been studied thoroughly. The rationale was also to screen the crude extracts on readily available screening platforms at the University of Ghana, on the basis that the causative organisms in both cases – schistosomiasis and trypanosomiasis – are parasites. It is possible that a medicinal plant with a certain biological activity may exhibit other biological activities. For instance, *Artemisia annua* which is an antimalarial also exhibits profound activity against the three main species of *Schistosoma*⁷⁶. The results obtained from the assay are indicated in **Table 4.12**.

<table>
<thead>
<tr>
<th>Crude Extract</th>
<th>EXPT 1 IC₅₀ (μg/mL)</th>
<th>EXPT 2 IC₅₀ (μg/mL)</th>
<th>EXPT 3 IC₅₀ (μg/mL)</th>
<th>MEAN IC₅₀ (μg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NTD_B1 DCM</td>
<td>15.23</td>
<td>12.30</td>
<td>14.61</td>
<td>14±1</td>
</tr>
<tr>
<td>NTD_B1 BuOH</td>
<td>12.91</td>
<td>9.89</td>
<td>11.40</td>
<td>11±2</td>
</tr>
<tr>
<td>NTD_B2 DCM</td>
<td><strong>8.34</strong></td>
<td><strong>7.66</strong></td>
<td><strong>5.35</strong></td>
<td><strong>7±1</strong></td>
</tr>
<tr>
<td>NTD_B2 BuOH</td>
<td>10.33</td>
<td>13.10</td>
<td>13.90</td>
<td>12±1</td>
</tr>
</tbody>
</table>

**Table 4.12**: Antitrypanosomal Activity of NTD Crude Extracts against *T. brucei brucei*
<p>| | | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>NTD_B3 DCM</td>
<td>12.22</td>
<td>10.72</td>
<td>12.90</td>
<td>12±1</td>
</tr>
<tr>
<td>NTD_B3 MeOH</td>
<td>17.60</td>
<td>20.23</td>
<td>18.55</td>
<td>19±1</td>
</tr>
<tr>
<td><strong>NTD_B4 DCM</strong></td>
<td><strong>6.53</strong></td>
<td><strong>4.76</strong></td>
<td><strong>5.60</strong></td>
<td><strong>6 ±1</strong></td>
</tr>
<tr>
<td>NTD_B4 MeOH</td>
<td>16.35</td>
<td>10.35</td>
<td>23.69</td>
<td>17±5</td>
</tr>
<tr>
<td>NTD_B5 DCM</td>
<td>10.56</td>
<td>8.88</td>
<td>13.24</td>
<td>11±2</td>
</tr>
<tr>
<td>NTD_B5 BuOH</td>
<td>12.85</td>
<td>7.70</td>
<td>22.03</td>
<td>14±5</td>
</tr>
<tr>
<td>NTD_B6 DCM</td>
<td>13.21</td>
<td>12.89</td>
<td>14.90</td>
<td>14±1</td>
</tr>
<tr>
<td>NTD_B6 BuOH</td>
<td>14.29</td>
<td>15.33</td>
<td>13.40</td>
<td>14±1</td>
</tr>
<tr>
<td>NTD_B7 DCM</td>
<td>12.51</td>
<td>10.26</td>
<td>9.88</td>
<td>11±1</td>
</tr>
<tr>
<td>NTD_B7 BuOH</td>
<td>15.94</td>
<td>20.05</td>
<td>12.30</td>
<td>16±3</td>
</tr>
</tbody>
</table>

*DA

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><em>DA</em> Diminazene aceturate- Positive control</td>
<td>0.54</td>
</tr>
</tbody>
</table>

All the extracts showed a broad spectrum of activity against the bloodstream forms of *T. brucei brucei* with IC$_{50}$ values ranging from 6 to 19 μg/mL. NTD_B4 DCM showed the highest activity with IC$_{50}$ of 6 μg/mL. This was quite significant when it was compared to the IC$_{50}$ value of 0.54 μg/mL of the standard, diminazene aceturate. NTD_B2 DCM also showed promising antitrypanosomal activity with IC$_{50}$ of 7 μg/mL. The rest of the crude extracts displayed moderate antitrypanosomal activity, with NTD_B3 MeOH displaying the least activity.

### 4.6.3 Antitrypanosomal Activity of Fractions Obtained from Flash Chromatography

The fractions obtained from the flash chromatography were tested against the bloodstream forms of *T. brucei brucei* using diminazene aceturate as standard. The resulting IC$_{50}$ values are summarized in **Table 4.13**.
Table 4.13: Antitrypanosomal Activity of Fractions from Flash Chromatography

<table>
<thead>
<tr>
<th>Sample Code</th>
<th>Mean ± Standard Deviation (µg/mL)</th>
<th>95% Confidence Interval (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1 (100% PE + PE: EA 9:1)</td>
<td>9 ± 1</td>
<td>7.311 – 9.686</td>
</tr>
<tr>
<td>F2 (PE:EA 8:2)</td>
<td>64 ± 7</td>
<td>46.59 – 82.36</td>
</tr>
<tr>
<td>F3 (PE:EA /1:1)</td>
<td>20.0 ± 0.1</td>
<td>18.87 – 21.04</td>
</tr>
<tr>
<td>F4 (PE:EA 2:8)</td>
<td>12 ± 3</td>
<td>4.50 – 18.71</td>
</tr>
<tr>
<td>F5 (100% EA)</td>
<td>7 ± 1</td>
<td>5.32 – 9.42</td>
</tr>
<tr>
<td>F6 EA: MeOH /1:1</td>
<td>31 ± 1</td>
<td>28.26 – 32.98</td>
</tr>
<tr>
<td>DA</td>
<td>0.13 ± 0.02</td>
<td>0.08 – 0.19</td>
</tr>
</tbody>
</table>

*DA- Diminazene aceturate

All fractions obtained from the flash chromatography displayed good to moderate activity against *T. brucei brucei*. Amongst the six fractions tested, only two fractions exhibited significant antitrypanosomal activity: fractions F1 (IC$_{50}$ value of 8.5 µg/mL) and F5 (IC$_{50}$ value of 7.4 µg/mL). Fraction F5 showed the greatest antitrypanosomal activity. This was followed closely by F1 which displayed an IC$_{50}$ of 8.5 µg/mL. Fractions F2 and F6 showed the lowest activities with IC$_{50}$ values of 64 and 31 µg/mL, respectively. Comparison of the IC$_{50}$ values of the test fractions against the standard showed that they were not as active. Further chromatographic separations were nonetheless continued in order to identify the compound(s) responsible for the observed activity and ascertain synergistic or additive effects resulting in the observed efficacy.

4.6.4 Antitrypanosomal Activity Studies of Compounds and Oils

For the antitrypanosomal activity studies of the flash chromatographic process, F5 showed the highest activity with an IC$_{50}$ value of 7.4 µg/mL. Due to paucity of F5 and time constraint, F1 which showed the next highest activity with an IC$_{50}$ of 8.5 µg/mL was selected for further isolations and tests. Antitrypanosomal activity evaluation of the compounds and oils obtained from the chromatographic separations of F1 are indicated in Table 4.14.
Quite unexpectedly, the pure compounds did not exhibit significant antitrypanosomal activity (IC$_{50}$ values >50 µg/mL). Compound F1/K, however, demonstrated the moderate activity with an IC$_{50}$ of 81 µg/mL. Comparing this to the standard in terms of their structure-activity relationship (Figure 4.32), diminazene aceturate has aromatic groups which probably interacts with the binding site by Van der Waal’s forces. Also, it has 7 nitrogens which can undergo dipole-dipole interactions and function as hydrogen bond acceptors and donors. F1/K also has an aromatic ring with two hydroxyl groups which can undergo hydrogen bonding. The carbonyl functionality could also be used for dipole-dipole interaction at the binding site of the *T. brucei brucei*. The long chain substituent, however, makes it too lipophilic and hence lowers the activity of F1/K. This observation is further confirmed by the inactivity of the other pure compounds which are mainly fatty acids and their esters from their IR and NMR data.

**Figure 4.32:** Comparison of Diminazene aceturate with F1/K
The oils (ML) showed incredible antitrypanosomal activity with IC$_{50}$ values ranging from 0.0977 µg/mL to 32.2 µg/mL. F1/HML showed the highest activity with IC$_{50}$ less than 0.0977 µg/mL which was very impressive since it was more active than the standard, diminazene aceturate which had an IC$_{50}$ of 0.13 µg/mL. Preliminary cytotoxicity tests revealed that F1/HML is non-toxic to murine macrophage cells (RAW 264.7) and this indicates that the compound would highly selectively kill trypanosomes sparing host cells.

Further analysis (GC-MS and NMR) of F1/HML revealed phytan, 4α-Methyl-24-ethyl-5α-cholesta-8(9)-en-3β-ol, 1,4,5,8-tetrahydro-4a,8a-napthalenedicarboximide and 5α, 8α, 14-cholestane as the major constituents of the oils. Terpenoids formed about 25% of F1/HML. More studies would be required to separate the individual components of the F1/HML and identify which constituent is responsible for the observed activity.

Most of the pure compounds isolated from F1 were not active against the trypanosomes. All the oils on the contrary exhibited profound antitrypanosomal activity. TLC profiles showed the oils (ML) indicated they had quite a number of constituents, both UV and non-UV active. Their notable activity could be attributed to the synergistic effect of the collection of compounds present in the oils, which served as the basis of herbal medicines; to harness the effects of all the components to effect greater activity. Further investigation of the oil will be required to confirm the synergism assertion.
CHAPTER FIVE
CONCLUSION AND RECOMMENDATIONS

5.1 CONCLUSION

In the present study, the efficacy of 7 traditional medicines marketed as schistosomiasis remedies was investigated and their antitrypanosomal potential evaluated.

Two out of the 7 remedies, NTD_B4 DCM and NTD_B7 DCM, at 100 μM, completely killed the newly transformed schistosomulae of *S. mansoni* while NTD_B3 MeOH displayed 89.9% effect on NTS. NTD_B4 DCM and NTD_B7 DCM further exhibited significant antischistosomal activity against the adult worms of *S. mansoni* by 78% and 84% effects, respectively. Also, all the remedies exhibited a broad spectrum of antitrypanosomal activity against *T. brucei brucei* with IC\textsubscript{50} values ranging from 6 to 19 μg/mL. The highest IC\textsubscript{50} value of 6 μg/mL was displayed by NTD_B4 DCM. Since NTD_B4 DCM displayed the highest antischistosomal and antitrypanosomal activities, it was selected for fractionation, chromatographic separations and further biological activity testing.

From the 7 fractions obtained from NTD_B4 DCM, fraction F5 (100% ethyl acetate) and fraction F1 (100% petroleum ether + PE:EtOAc/9:1) displayed the highest antitrypanosomal activity with IC\textsubscript{50} values of 7.4 and 8.5 μg/mL, respectively. Isolation of compounds from the fractions and subsequent characterization by spectroscopic and spectrometric techniques led to the identification of 5,7-dihydroxy-6-methyl-2-tricosyl-4H-chromen-4-one (F1/K) and a fatty acid (F1/J) from F1, and β-sitosterol-3-O-glucoside and ethyl 4-hydroxybenzoate from F5.

Due to the paucity of F5, only isolates from F1 were tested for their antitrypanosomal activity and among the compounds screened, only F1/K exhibited antitrypanosomal activity with IC\textsubscript{50} of 81 μg/mL. However, the oils displayed promising antitrypanosomal activity with IC\textsubscript{50} < 31 μg/mL. The oily compound F1/HML displayed significant activity with IC\textsubscript{50} <0.0977 μg/mL, which was 1.3 fold superior to the standard. This was significantly superior to that of the standard, diminazene aceturate which had an IC\textsubscript{50} value of 0.13 μg/mL. Preliminary cytotoxicity studies against RAW murine macrophage cells indicated that F1/HML was not cytotoxic and it selectively killed trypanosomes. The notable activity of the oils as compared to the pure compounds could be attributed to the synergistic effects of the constituents of the oils on the *T. brucei brucei*. 
GC-MS analysis of F1/HML revealed phytan (6.31%), 5α, 8α, 14β-cholestane (6.32%), 2-butylbenzothiazole (4.26%), 4α-methyl-24-ethyl-5α-cholest-8(9)-en-3β-ol (4.61%), cyclopropa-7,8-cholestan-3-one (3.80%), 2-chloro-2-styryl-4-methylstilbene (3.29%), 1,4,5,8-tetrahydro-4a,8a-napthalenedicarboximide (10.30%), 5α, 13 α-D-homoandrostan (3.63%) as the major components identified based on their m/z and their fragmentation patterns.

This study has provided scientific backing to the efficacy of 2 out of the 7 selected traditional medicines used in the treatment of schistosomiasis. Further antitrypanosomal activity studies demonstrated that all the traditional medicines, especially NTD_B4 DCM can be recommended as remedies for human African trypanosomiasis. Also, it was deduced that the antitrypanosomal activity of the drugs was dependent on synergism of the constituents. These findings affirm the need for herbal drug development as well as compound isolation for drug discovery and development.

5.2 RECOMMENDATIONS

- Cytotoxicity studies against cancer cell lines are recommended for compound F1/K (5,7-dihydroxy-6-methyl-2-tricosyl-4H-chromen-4-one) since compounds with the chromen-4-one nucleus mostly possess significant anticancer activity.
- A confirmatory GC-MS analysis on different GC-MS equipment is required to confirm the constituents of the oil of F1/HML.
- F1/HML should be further separated by LC-MS with UV and light scattering detectors to isolate both UV and non-UV constituents and to ascertain their masses and fragmentation patterns.
- The antischistosomal screening of pure compounds should be pursued to identify active compounds.
- Identification of the active constituent(s) will provide new drug leads for both human and animal African trypanosomiasis.
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APPENDIX I

Sample Questionnaire

Ethical clearance
QUESTIONNAIRE

A. DEMOGRAPHIC INFORMATION

1. Name: ________________________________________________________________

2. Sex: [ ] Male [ ] Female

3. Age (years): [ ] 20-29 [ ] 30-39 [ ] 40-49 [ ] 50-59 [ ] above 60

4. Educational Background: [ ] Basic [ ] Secondary [ ] Tertiary [ ] Informal

5. Marital Status: [ ] Married [ ] Single [ ] Widowed

B. INFORMATION ABOUT TRADITIONAL MEDICINE PRACTICE

6. How did you get into this practice of medicine?
   [ ] Inheritance [ ] Formal Training [ ] Divine Means

7. For how long have you been practicing (years)?
   [ ] 1-5 [ ] 6-10 [ ] 10-20 [ ] above 20

C. PRODUCT SUBMISSION

8. Name of product: ________________________________________________________

9. Indication: ____________________________________________________________

10. Route of administration: ______________________________________________

11. Dosage: ______________________________________________________________

12. Active ingredients: ____________________________________________________

============================================================================
13. Date of manufacture: -----------------------------------------------

14. Address: -----------------------------------------------------------------

---------------------------------------------------------------------------

D. CASE-SPECIFIC QUESTIONS

15. Below is a list of some neglected tropical diseases in Ghana. Tick against the ones that you know about? Indicate the local name.

   **Local name**

(a) Bilharzia (Schistosomiasis) [ ] ------------------------------------------
(b) River blindness (Onchocerciasis) [ ] -------------------------------------
(c) Elephantiasis (Lymphatic filariasis) [ ] --------------------------------
(d) Intestinal worms (Helminthiasis) [ ] -----------------------------------
(e) Eye infection (Trachoma) [ ] ------------------------------------------

16. Which of these diseases do you specialize in treating/managing? ----------------

17. How many patients have you treated for these diseases?

   1-5 [ ] 6-10 [ ] 10-15 [ ] 15-20 [ ] above 20 [ ]

18. How do you treat your patients?

   Herbs only [ ] Herbs and spiritual means [ ]
Ethical Approval

Ethical approval for the study was provided by the Ethics Committee for Basic and Applied Sciences, University of Ghana, with reference number ECBAS 045/17-18.
APPENDIX II

Antischistosomal activity of crude extracts against NTS of *Schistosoma mansoni*

Antischistosomal activity of crude extracts against adult worms of *Schistosoma mansoni*

Antitrypanosomal activity of crude extracts against *Trypanosoma brucei brucei*

Antitrypanosomal activity of fractions against *T. brucei brucei*
Antischistosomal Activity of Crude Extracts Against Newly Transformed Schistosomula of *Schistosoma mansoni*

![Antischistosomal Activity of Crude Extracts Against NTS](image-url)
Antischistosomal Activity of Crude Extracts Against Adult Worms of Schistosoma mansoni

Antischistosomal Activity of Crude Extracts Against Adult Worms against S. mansoni

- NTD B3- MeOH
- NTD B4- DCM
- NTD B7- DCM

S. mansoni adult Effect in % Test conc. 100 μM
S. mansoni adult Effect in % Test conc. 10 μM
Antitrypanosomal Activity of Crude Extracts Against *Trypanosoma brucei brucei*

Antitrypanosomal Activity of Crude Extracts IC\(_{50}\) (μg/mL)
Antitrypanosomal Activity of Crude Extracts Against Bloodstream Forms of *T. brucei brucei*

**Antitrypanosomal Activity of B4 DCM Fractions (μg/mL)**

- F1: 8.5
- F2: 64
- F3: 20
- F4: 12
- F5: 7.4
- F6: 31
- DA: 0.13
APPENDIX III

IR Spectrum of F1/K - A
IR Spectrum of F1/J - B
IR Spectrum of F1/S - C
IR Spectrum of F1/Z - D
IR Spectrum of F1/H - E
IR Spectrum of F1/I - G
IR Spectrum of F1/K

- 3394 cm⁻¹, 92.24% T
- 1667.50 cm⁻¹, 92.24% T
- 1407.66 cm⁻¹, 90.33% T
- 1348.71 cm⁻¹, 87.65% T
- 1028.68 cm⁻¹, 85.91% T
- 1646.25 cm⁻¹, 83.31% T
- 1472.35 cm⁻¹, 85.62% T
- 1429.50 cm⁻¹, 87.65% T
- 1307.70 cm⁻¹, 82.49% T
- 1196.59 cm⁻¹, 91.08% T
- 1095.59 cm⁻¹, 91.08% T
- 837.65 cm⁻¹, 94.76% T
- 719.64 cm⁻¹, 92.61% T
IR SPECTRUM OF F3/C

2816 cm⁻¹, 51.02%T
2343 cm⁻¹, 90.17%T
1733 cm⁻¹, 99.60%T
1576 cm⁻¹, 95.02%T
1402 cm⁻¹, 50.32%T
1177 cm⁻¹, 93.17%T
719 cm⁻¹, 95.02%T

%T

4000 3500 3000 2500 2000 1500 1000 500

cm⁻¹
APPENDIX IV

HSQC Spectrum of F1/J – A

$^1$H- $^1$H COSY Spectrum of F1/J - B

HMBC Spectrum of F1/J - C

$^1$H NMR Spectrum of EA P2 - D

$^1$H- $^1$H COSY Spectrum of EA P2 - E

HSQC Spectrum of EA P2 – F

HMBC Spectrum of EA P2 - G

$^{13}$C NMR Spectrum of DP2 - H

$^1$H NMR Spectrum of DP2 - I
Appendix IV A

HSQC OF F1/J (Fatty acid)
Appendix IV B
Appendix IV E
Appendix IV F

$^1$H-$^1$H COSY Spectrum of EA P2
Appendix IV G

HMBC Spectrum of EA P2
Appendix IV H
Appendix IV I