KWAME NKRUMAH UNIVERSITY OF SCIENCE AND
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DEPARTMENT OF PHARMACOGNOSY

LARVICICAL AND ANTI-PLASMODIAL CONSTITUENTS OF *CARAPA PROCERA* DC. (MELIACEAE) AND *HYPTIS SUAVEOLENS* L. POIT (LAMIACEAE)

BY

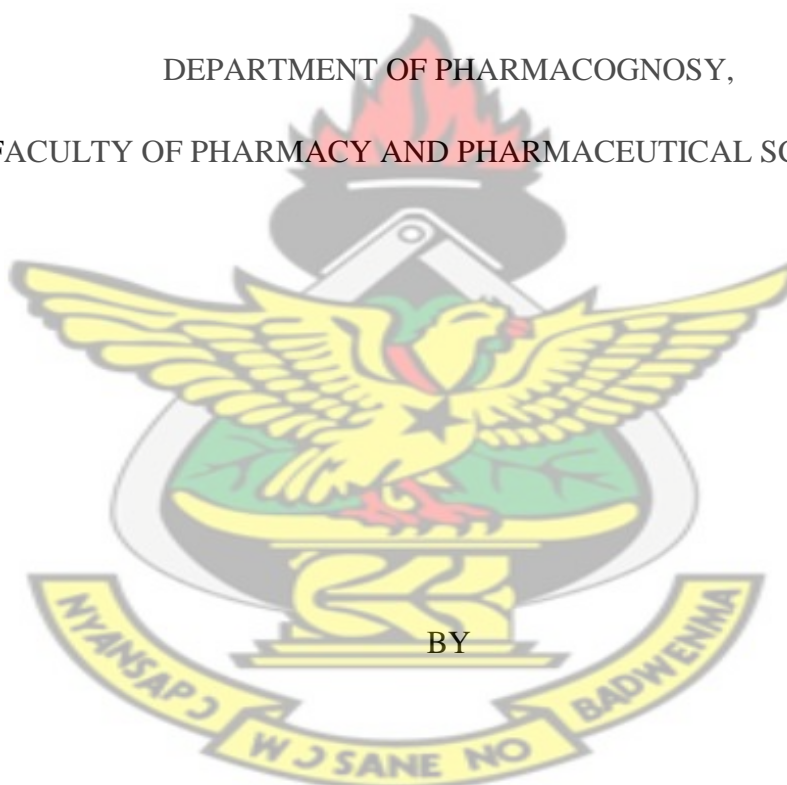BEATRICE MISSAH

SEPTEMBER, 2014

LARVICICAL AND ANTI-PLASMODIAL CONSTITUENTS OF *CARAPA PROCERA* DC. (MELIACEAE) AND *HYPTIS SUAVEOLENS* L. POIT (LAMIACEAE)

A THESIS SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS

FOR THE DEGREE OF

MPHIL PHARMACOGNOSY

IN THE

DEPARTMENT OF PHARMACOGNOSY,

FACULTY OF PHARMACY AND PHARMACEUTICAL SCIENCES

BY

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KWAME NKRUMAH UNIVERSITY OF SCIENCE AND TECHNOLOGY,

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SEPTEMBER, 2014

DECLARATION

I hereby declare that the experimental work described in this thesis is my own work towards the award of an MPhil and to the best of my knowledge, it contains no material previously published by another person or material which has been submitted for any other degree of the university, except where due acknowledgement has been made in the test.

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DEDICATION

I dedicate this work to my dear father Mr. Bernard Missah for his immense support and care for me.

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ABSTRACT

Malaria is a serious health problem worldwide due to the emergence of parasite resistance to well established antimalarial drugs. This has heightened the need for the development of new antimalarial drugs as well as other control methods. Plant based antimalarial drugs continue to be used in many tropical areas for the treatment and control of malaria and hence the need for scientific investigation into their usefulness as alternatives to conventional treatment.
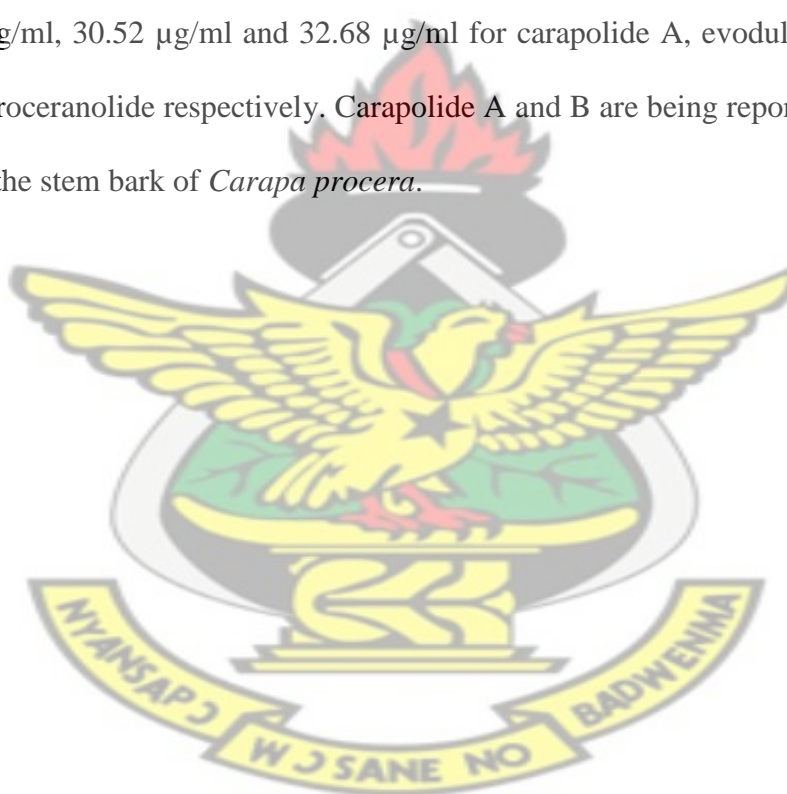
The work presented in this thesis involves the scientific investigation of the traditional uses of *Carapa procera* and *Hyptis suaveolens* as insecticidal agents. *Carapa procera* was also investigated for its antiplasmodial activity. The petroleum ether, ethyl acetate and 70% ethanol extracts of stem bark of *Carapa procera* and aerial parts of *Hyptis suaveolens* were investigated for their larvicidal and antiplasmodial activities.

The larvicidal activities of the plant extracts and isolates were investigated using the method described by WHO against third instar *Anopheles gambie* larvae. The larvae were exposed to various concentrations of the extracts and the percentage mortality was recorded after 24 hours. Temephos, a synthetic insecticide was used as the standard drug. All extracts exhibited dose dependent increase in larvicidal activity with the Petroleum ether extracts of both *Hyptis suaveolens* and *Carapa procera* having the highest activity (EC_{50} 34.96 mg/ml and 16.91 mg/ml respectively) followed by ethyl acetate and ethanol extracts.

For the antiplasmodial assay, the Lactate dehydrogenase method was used against chloroquine resistance strains of *Plasmodium falciparum*. The ethyl acetate extract of *Carapa procera* showed the highest activity (EC_{50} 18.60 µg/ml) followed by

petroleum ether (EC_{50} 24.71 µg/ml) and ethanol extracts (EC_{50} 95.46 µg/ml) as compared to artesunate the standard drug with EC_{50} of 4.90 µg/ml.

Carapolides A and B, evodulone and proceranolide were isolated from the bioactive petroleum ether extract of *Carapa procera* stem bark. They exhibited various degrees of larvicidal activities against the third instar *Anopheles gambie* larvae with EC_{50} values of 15.01mg/ml, 53.74mg/ml, 198.9mg/ml and 273.6mg/ml for Carapolide A, proceranolide, evodulone and carapolide B respectively. They also exhibited dose dependant antiplasmodial activities with EC_{50} values of 23.96 µg/ml, 26.66 µg/ml, 30.52 µg/ml and 32.68 µg/ml for carapolide A, evodulone, carapolide B and proceranolide respectively. Carapolide A and B are being reported for the first time in the stem bark of *Carapa procera*.

ACKNOWLEDGEMENT

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ABBREVIATIONS

^{13}C-NMR	Carbon-13 Nuclear Magnetic Resonance
^{1}H-NMR	Proton Nuclear Magnetic Resonance
ART	Artemisinin
DDT	Dichlorodiphenyltrichloroethane
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic Acid
DOT	Directly Observed Therapy
ELISA	Enzyme-linked Immunosorbent Assay
ELSD	Evaporative Light Scattering Detectors
GC	Gas Chromatography
GDP	Gross Domestic Product
GHS	Ghana Health Service
HIV	Human Immunodeficiency Virus
HPLC	High Performance Liquid Chromatography
IM	Intra Muscular
IPT	Intermittent Preventive Treatment
IV	Intra Venous
IRS	Inside Residual Spraying
IVM	Integrated Vector Management
LC	Liquid Chromatography
LLIN	Long Lasting Impregnated Nets
LSM	Larval Source Management

MDG'S	Millennium Development Goals
MS	Mass Spectroscopy
NCE'S	New Chemical Entities
NMCP	National Malaria Control Program
PDA	Photodiode Assay
PLDH	Parasite Lactate Dehydrogenase
RBC	Red Blood Cell
RDT'S	Rapid Diagnostic Test
RI	Reactive Index
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
SP	Sulphadoxime-pyrimmethamine
TLC	Thin Layer Chromatography
UNDP	United Nations Development Project
UNICEF	United Nations Children's Fund
UV	Ultra Violet
WHO	World Health Organization

CHAPTER ONE

INTRODUCTION

1.1. GENERAL INTRODUCTION

Insect transmitted diseases remain a major cause of illness and death worldwide. Parasitic, bacterial and viral pathogens may be transmitted by blood sucking arthropods such as ticks causing lyme disease, sand flies causing leishmaniasis, tsetse fly causing trypanasomiasis and fleas causing bubonic plague (Gubler & Wilson, 2005). Mosquitoes are the most important single group of insects in terms of public health importance. They transmit a number of diseases such as malaria, filariasis, dengue fever and Japanese encephalitis, causing millions of death every year (WHO, 2008).

Malaria is one of the most serious pathogenic diseases in endemic areas of the world. According to the World Health Organization (WHO), approximately 225 million cases of malaria were estimated to have occurred in 2009 worldwide leading to about 781,000 deaths. Ninety one percent of these deaths occur in endemic areas which include Africa, Asia and Latin America (Ravikumar *et al*., 2012; Sha'a *et al*., 2011). In Africa alone, it is estimated that more than 300 million people are infected annually by the parasite *Plasmodium falciparum* and over one million deaths have been recorded in children under five years (Ramazani *et al.,* 2010; Zofou *et al*., 2011). Malaria contributes significantly to the poor health situation in Africa. It is estimated that between 20 and 40% of outpatient visits and between 0.5 and 50% of hospital admissions in Africa are attributed to malaria (Chima *et al*., 2002) and this puts a big strain on the health system. Malaria is estimated to cost Africa more than US$12 billion every year in lost GDP even though it could be controlled with a fraction of

that sum (WHO, 2007). In 2006, malaria accounted for 38.6% of all out patient illness and 36.9% of all hospital admissions in Ghana. The disease prevalence per 1000 population was 171 and 2,835 malaria-attributed deaths representing 19% of all deaths recorded. The annual income burden of malaria is estimated to be 1 – 2 % of the Gross Domestic Product (GDP) in Ghana (UNICEF, 2007).

The effect of malaria on people of all ages is quite immense. However, this is very severe in pregnant women and children. When malaria infection is not properly treated in pregnant women, it can cause anaemia and lead to miscarriages, stillbirths, underweight babies and maternal deaths. In Ghana, as many as 13.7% of all admissions of pregnant women in 2006 was as a result of malaria whilst 9% of them died from the disease (UNICEF, 2007). Malaria in school children is a major cause of absenteeism in endemic countries. It is estimated that about 2% of children who recover from cerebral malaria suffer brain damage including epilepsy (UNICEF, 2003). Among young children, frequent episodes of severe malaria may lead to several neurological damages and developmental defects (Alonso *et al.,* 2011). The debilitating effects of malaria on adult victims are very much disturbing. This is because in addition to time and money spent on treating and preventing malaria, it causes considerable pain and weakness among its victims and this can reduce people's working abilities. Malaria therefore is not only a public health problem but also a developmental problem. At the national level, apart from the negative effect of loss of productivity on major sectors of the economy, malaria has negative effect on the growth of tourism, investment and trade especially in endemic regions. Malaria presents a major socioeconomic challenge to African countries since it is the most affected region. This challenge cannot be allowed to go unnoticed since good health is

not only a basic human need but also a fundamental human right and a prerequisite for economic growth (Alonso *et al*., 2011).

The millennium development goal number six seeks to combat HIV/AIDS, malaria and other diseases hence there has been a strong advocacy for an integrated approach for malaria control. This involves the use of medicines, prompt diagnosis, insecticide sprays and insecticide-treated bed nets, improved community based systems, proper case management, improved health information systems and proper environmental management geared towards vector control plus a continuous search for effective vaccine. There are effective antimalarial drugs which are readily available. However, there is a major problem of drug resistance to the conventional anti malaria drugs like sulfadoxine-pyrimethamine and recently artemisinin derivatives (Sha *et al*., 2011; Zofou *et al.*, 2011). Resistance to these drugs have been reported to be as high as 40-60% in some African and Asian countries hence new drugs against malaria are urgently needed.

Nature remains an ever evolving source of bioactive compounds for the treatment of malaria since natural compounds have provided the best antimalarial drugs used till date (Madureira *et al.,* 2002; Kraft *et al*., 2003). Quinine and artemisinin based compounds used in Ghana for the treatment of both complicated and uncomplicated malaria were either derived from plants or developed using plant derived structures as templates.

Between the 1940s and 1960s, malaria eradication was achieved in the USA, USSR, Southern Europe and most Caribbean Islands mainly by vector control. Much progress was also made in the Indian subcontinent and parts of South America. Now

in the 2000s emphasis needs once again to be placed on vector control (Walker & Lynch, 2007).

 Many synthetic insecticides such as organophosphates have been used in the control of mosquitoes in several countries for the last 30 years (Curtis, 1994). One major drawback with the use of these chemical compounds is their non-selectivity and hence could be harmful to other organisms in the environment. The toxicity problem together with the growing incidence of insect resistance warrants the need for the development of effective insecticides which are environmentally safe, target specific and biodegradable. Eradication of the vector can be achieved by larval and adult control of the female anopheles mosquito. The control of mosquito at the larval stage is however efficient in integrated mosquito management because during the immature stages, mosquitoes are immobile (Walker & Lynch, 2007).

The continuous search for novel and effective antimalarial compounds as well as larvicides especially from medicinal plants is of utmost importance in combating malaria.

1.2. JUSTIFICATION

In the absence of an effective vaccine, the fight against malaria relies mainly on drug therapy and vector control. The search for new antimalarial drugs remains indispensible in the face of resistant strains of *Plasmodium falciparum* (Camacho *et al*., 2000).

Vector control has been directed at adult mosquito population however, further suppression of transmission could be achieved by targeting the vector population in the environment by reducing larvae of vector (Ernest *et al*., 2006). Mosquitoes in the

larval stage are attractive for pesticide control especially in water bodies which have defined boundaries. However, larval source management (LSM) although one of the oldest tools in vector control remains forgotten and often dismissed intervention for malaria control in Africa (National Malaria Control Program, GHS). Despite lack of its application in Africa for over half a century, LSM has been the main focus of mosquito control programs for decades in the United States of America, Canada, throughout Europe, Brazil and Singapore. The use of conventional chemical pesticides has however resulted in the development of resistance, undesirable effects on non- target organs and fostered environmental and human health concerns.

Medicinal plants play a key role in malaria control in Africa, especially in the remote areas where health facilities are limited. For over hundreds of years, plants have constituted the basis of traditional medicine systems and natural products have been a good source of drug development. Some examples are quinine and artemisinin, which has been used successfully against sensitive and resistant strains of malaria parasites (Woodrow *et al*., 2005). The world's poorest countries are the worst affected by malaria and many of these people resort to the use of traditional medicines (WHO, 2000). Some Ghanaians living in rural areas depend on traditional or herbal medicines for the treatment of many diseases including malaria.

The reputed efficacies of these plants have been exploited and passed on from one generation to another. It is however essential to do rigorous evaluation of these plants to develop more reproducible active compounds. This will provide policy makers with the relevant information on these plants and can guide the introduction of new measures to improve the current strategies of malaria control and prevention.

Insecticides of botanical origin may serve as suitable alternative to conventional insecticides in the control of mosquitoes. Although several plants have been reported to have mosquitocidal activity, only a few botanicals have moved from the laboratory to use. This is because they are poorly characterized and in most cases active principles are not determined.

The present study is aimed at investigating the larvicidal activity of two Ghanaian medicinal plants, *Carapa procera* DC. (Meliaceae) and *Hyptis suaveolens* L. Poit (Lamiaceae) which are used in ethnomedicine as pesticides against mosquitoes. Again, *Carapa procera* is used in ethnomedicine to treat malaria hence the *in vitro* antiplasmodial activity will be investigated in this study.

1.3. AIMS AND OBJECTIVES

The aim of this research is to investigate the larvicidal constituents of *Carapa procera* and *Hyptis suaveolens* against *Anopheles gambiae* as well as investigate the antiplasmodial constituents of *Carapa procera* against *plasmodium falciparum.*

To achieve these aims, the following specific objectives were set;

1. Extract the plant materials using petroleum ether, ethyl acetate and ethanol successively.
2. Screen the extracts for larvicidal activity.
3. Screen the extracts of *Carapa procera* for antiplasmodial activity.
4. Fractionate and isolate active constituents from the bioactive fractions.
5. Perform antiplasmodial and larvicidal activity tests on isolated compounds.

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CHAPTER TWO

LITERATURE REVIEW

2.1. MALARIA

Malaria is a vector-borne infectious disease caused by five species of an eukaryotic protozoan of the genus *Plasmodium* (*Plasmodim malariae, Plasmodium knowlesi, Plasmodium. falciparum, Plasmodium vivax, Plasmodium ovale*). It is widespread in tropical and sub-tropical regions, including part of America, Asia and Africa (Snow *et al.*, 2005). Malaria is the single most important cause of ill health, death and poverty in Sub-Saharan Africa (Sachs & Malaney, 2002; Kilama, 2005; UN, 2005). The disease is believed to be a major obstruction to social and economic development in Africa. About 3.3 billion people (half of the world's population) are at risk of malaria. Every year, this leads to about 250 million malaria cases and nearly one million deaths. People living in the poorest countries are the most vulnerable (WHO, 2009).

2.1.1. Prevalence

Globally, an estimated 3.3 billion people were at risk of malaria in 2011 with populations living in sub-Saharan Africa having the highest risk of acquiring malaria. Approximately 80% of cases and 90% of deaths are estimated to occur in African regions with children under 5 years of age and pregnant women most severely affected. An African child has on the average between 1.6 and 5.4 episodes of malaria each year. Malaria is hyper endemic in Ghana. It is the number one cause of morbidity accounting for over 40% of outpatient attendance in public health facilities with annual reported cases of about 2.2 million between 1995 and 2001 (UNDP, 2000). Malaria accounts for an average of 13.2% of all mortality cases in Ghana as

well as 22% of mortalities in children under five years. It is estimated that malaria prevalence (notified cases) is 15,344 per 100 000 with a malaria death rate for all ages being 70 per 100 000. In the case of the 0–4 years, it is 448 per 100 000 reported for the year 2000 (UN, 2003).

2.1.2. Malaria Distribution

Malaria is distributed worldwide, found in tropical areas, throughout Sub-Saharan Africa and to a lesser extent in South Africa, South east Asia and the pacific islands, India, central and South America (Luxemburger *et al.,* 1997). Where malaria is prominent depends mainly on climatic factors such as temperature, humidity and rainfall. Malaria is rampant in tropical and sub-tropical regions where *Anopheles* mosquitoes can survive and multiply and also where malaria parasites can complete their growth cycle in the mosquitoes (extrinsic incubation period). Temperature is particularly critical as some species of *Plasmodium* for example; *Plasmodium falciparum* cannot complete its growth cycle in the *Anopheles* mosquito at temperatures below 20°C and thus cannot transmit malaria. Also in many malaria-endemic countries, transmission occurs at specific areas; at very high altitudes, during colder seasons and in desert places. Transmission is also high in regions close to the equator. *Plasmodium falciparum* is the most prominent species in most endemic areas with exceptions being India and South America where *Plasmodium vivax* is more common. *Plasmodium ovale* is mainly found in West Africa. The spread of malaria is becoming even more serious as the parasites that cause malaria develop resistance to the drugs used to treat the condition.

Plasmodium falciparum has developed resistance to most conventional antimalarials some of which include chloroquine, sulphodoxime-pyrimethamine and mefloquine

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monotherapies. In all endemic areas, children and pregnant women are at higher risk of malaria and are more susceptible to severe disease (McGready *et al.,* 2004).
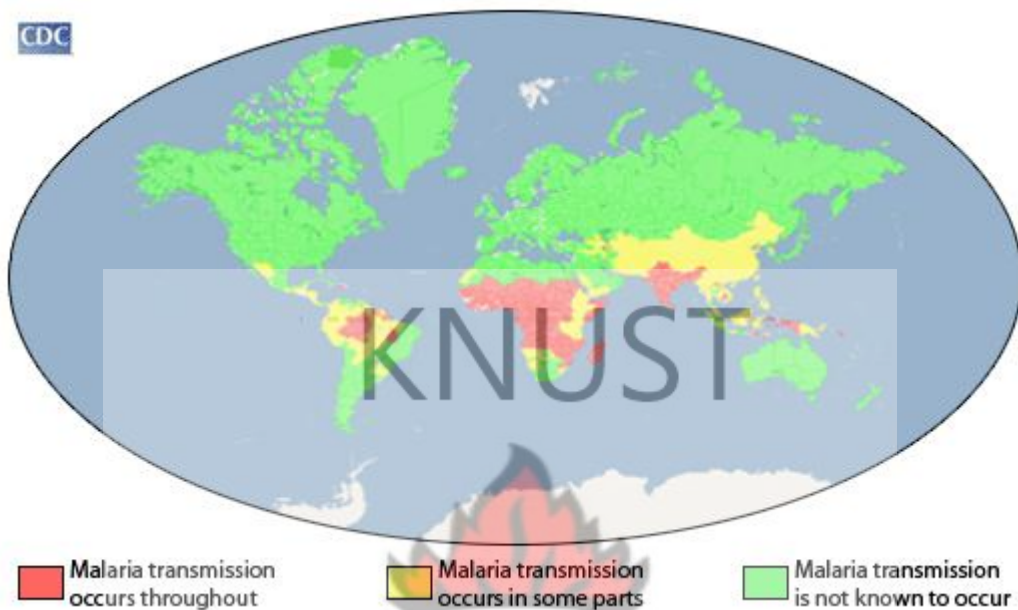
Figure 1: Malaria Distribution in the World

2.1.3. Pathophysiology

Human malaria is caused by four different species of *Plasmodium* parasite: *Plasmodium falciparum* (the most deadly), *Plasmodium vivax*, *Plasmodium malariae,* and *Plasmodium ovale*. A person gets malaria when bitten by a female mosquito that is looking for a blood meal and is infected with the malaria parasite. Malaria in humans develops via two phases; an exoerythrocytic and erythrocytic phase.

2.1.3.1. Exoerythrocytic Phase

A bite from an infected anopheline mosquito transmits malaria. Within 30 minutes of being introduced into the host, the sporozoites travel from the salivary glands of the mosquito through the bloodstream of the host to the liver, where they invade

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hepatocytes. These cells divide many 1000-fold until mature tissue schizonts are formed, each containing thousands of daughter merozoites. This exoerythrocytic stage is asymptomatic (Murray & Perkins, 1996). Upon rapture of their host, these merozoites escape into the bloodstream and infect red blood cells (Bledsoe, 2005). The parasites escape from the liver undetected by wrapping itself in the cell membrane of the infected host liver cell (Sturm *et al*., 2006). Some *Plasmodium vivax* and *Plasmodium ovale* sporozoites do not immediately develop into exoerythrocytic phase merozoites but produce hypnozoites that remain dormant for periods ranging from several months to as long as three years after which they reactivate and produce merozoites. Hypnozoites are thus responsible for the long incubation and late relapses in these two species of malaria (Cogswell, 1992).

2.1.3.2. Erythrocytic Stage

The merozoites released from the liver attach and enter the red blood cells by multiple receptor ligand interactions and this happens in few seconds. The quick disappearance from the circulation into the red blood cells minimizes the exposure of antigens on the surface of the parasite from host immune response. Within the red blood cells, the parasites numbers expand rapidly with a sustained cycling of the parasite population. The erythrocytic stage occurs every 24 hours in the case of *Plasmodium knowlesi*, 48 hours for *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium ovale* and 72 hours for *Plasmodium malariae*. During the cycle, each merozoite grows and divides within the vacoule into 8-32 fresh merozoites through the stages of ring trophozoite and schizont. At the end of the cycle, the infected red cells rapture, releasing new merozoites that in turn infect more RBC'S (Brian *et al.,* 2008). All clinical features of malaria are caused by these developments in the blood. The growing parasites

progressively consume and degrade intracellular proteins, particularly haemoglobin. This results in the formation of the 'malarial pigment' and haemolysis of the infected red blood cell. The rupture of red blood cells by merozoites releases certain factors and toxins which could directly induce the release of cytokines such as TNF and interleukin-1 from macrophages. This results in chills and high grade fever. It has been observed that in primary attack of malaria, the symptoms may appear with lesser degree of parasitemia or even with sub microscopic parasitemia. However, in subsequent attacks and relapses, a much higher degree of parasitemia is needed for onset of symptoms (Kakkilaya, 2011).

2.1.4. Signs and Symptoms

The amount of time between the mosquito bite and the appearance of symptoms varies, depending on the strain of parasite involved. The incubation period is usually between 8 and 12 days for *falciparum* malaria, but it can be as long as a month for the other types. Symptoms from some strains of *Plasmodium vivax* may not appear until 8 to 10 months after the mosquito bite occurred. The primary symptom of all types of malaria is the "malaria ague" (chills and fever). In most cases, the fever has three stages, beginning with uncontrollable shivering for an hour or two, followed by a rapid spike in temperature (as high as 38°C), which lasts three to six hours. Then, just as suddenly, the patient begins to sweat profusely, which will quickly bring down the fever. Other symptoms may include fatigue, severe headache, or nausea and vomiting. As the sweating subsides, the patient typically feels exhausted and falls asleep. In many cases, this cycle of chills, fever, and sweating occurs every other day, or every third day, and may last for between a week and a month. Those with the chronic form of malaria may have a relapse as long as 50 years after the initial infection. Malaria

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caused by *Plasmodium falciparum* is far more severe than other types of malaria because the parasite attacks all red blood cells, not just the young or old cells, as do other types. It causes the red blood cells to become very "sticky." A patient with this type of malaria can die within hours of the first symptoms (Ashley *et al*., 2006). Many red blood cells are destroyed that they block the blood vessels in vital organs (especially the kidneys), and the spleen becomes enlarged. There may be brain damage, leading to coma and convulsions. The kidneys and liver may fail. Malaria in pregnancy can lead to premature delivery, miscarriage, or stillbirth. The more common kinds of mosquitoes in the United States do not transmit the infection (Luxemburger *et al., 2001*).

2.2. MALARIA CONTROL

Malaria control involves a complex chain of measures that complement each other (Kakkilaya, 2011).

There are four basic technical elements to the malaria control strategy. The first element is to provide drugs and treatment to those infected. Second, to implement sustainable and effective preventive measures, including vector control, the third one is to prevent or detect and contain epidemics in high-risk areas and the fourth is to strengthen local capacities in research and development. The national malaria control program (NMCP) in Ghana since its establishment in the 1950's has been making progress in the control of malaria. The NMCP aims at reducing the burden of malaria till it ceases to be a disease of public health importance. Multiple strategies were employed some of which include residual insecticide application against adult mosquitoes, mass chemoprophylaxis with pyrimethamine medicated salt and improvement of drainage systems. The roll back malaria policy in 1999 (RBM) was

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aimed at reducing malaria specific morbidity and mortality by 50% by the year 2010. Though some progress was made with this policy, the burden of the disease was still huge. Also it was recognized that malaria cannot be controlled by the health sector alone nor can a single intervention help achieve results. It was therefore necessary that partnership among key stakeholders be encouraged and multiple interventions adopted for the fight against malaria. In view of this, the current malaria control program aims at reducing death and illness due to malaria disease by 75% by the year 2015 in line with the attainment of the Millennium Development Goals (MDG's). This goal is to be achieved through the overall health sector development, improved strategic investment in malaria control, increased coverage towards universal access to malaria treatment and prevention.

2.2.1. Malaria Treatment

Treatment of malaria depends on early diagnosis. In Africa, most cases of malaria are diagnosed based on clinical symptoms and the treatment is presumptive rather than laboratory confirmation. However, malaria caused by *Plasmodium falciparum* can be diagnosed almost accurately using rapid diagnostic test (RDT's) which detect plasmodial antigens or enzymes. In many areas of the world, people with uncomplicated malaria are treated as outpatients with antipyretics and antimalarial agents such as artemisinin based combination therapies.

The WHO however recommend the use of combination therapy as this helps in improving efficacy and also delay the development of resistance.

In Ghana, treatment of uncomplicated malaria involves the use of Artesunate/Amodiaquine as first line therapy. Alternative first line treatment involves the use of Arthemether/Lumefanthrine and Dihydroartemisinin/Piparaquine for

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patients who cannot tolerate Artesunate/Amodiaquine. Oral quinine is recommended as the drug of choice for the management of uncomplicated malaria in the case of treatment failure.

In the case of complicated malaria, parenteral treatment is required to provide adequate blood serum concentrations as quickly as possible and subsequently revert to oral treatment when the patient's condition permits. The drug of choice is IM quinine or IM arthemether. Other supportive treatments are given when necessary.

Malaria during pregnancy represents a life threatening infection for both mother and foetus. Therapy is therefore directed towards eradicating the infection. Malaria treatment during the first trimester of pregnancy involves the use of oral quinine or a combination of oral quinine and clindamycin. During the second and third trimesters however, oral quinine or a combination of Arthemether/Lumefantrine or Artesunate/Amodiaquine can be used. In the case of complicated malaria in pregnancy, IV or IM quinine is used in all trimesters until the patient can take oral preparations. The most preferred intervention however of malaria in pregnancy is the use of intermittent preventive treatment. Sulphadoxime-Pyrimmethamine (SP) is the drug of choice for IPT in Ghana and is given under directly observed therapy (DOT). Pregnant women who cannot tolerate SP may be put on proguanil beginning from the first trimester of pregnancy.

2.3. ANTIMALARIA AGENTS FROM PLANT SOURCES

The use of medicinal plants for the treatment of parasitic diseases is well known and documented since ancient times. A great number of plants have been identified by various cultures as having antimalarial properties since the recognition of malaria. For example, use of *Cinchona succiruba* (Rubiaceae) for the treatment of malaria

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infection is known for centuries. Current antimalarial therapy consists substantially of natural products and related derivatives (quinine, chloroquine, mefloquine, arteether, artemether and artesunate) (Kaur *et al.*, 2009).

Several classes of plant secondary metabolites are responsible for antimalarial activity and some include flavonoids, alkaloids, peptites, terpenes, xanthones, naphthopyrones, benzophenones, macrolides and halogenated compounds. Notable among these ones are alkaloids, terpenoids and flavonoids.

Alkaloids; these are physiologically active nitrogen bases derived from many biogenetic precursors. Alkaloids are one of the oldest classes of compounds with antimalarial activity. It is believed that the nitrogen atom contained in these alkaloidal drugs contribute immensely to their antimalarial activity. A number of naturally occurring alkaloids belonging to different groups have been found to have antimalarial properties, notable among them are quinine and cryptolepine.

Quinine, a 4-methanol quinoline is the first antimalarial drug isolated from the back of *Cinchona sp* (Rubiaceae) (Pelleties & Caventou, 1820). It is the most important lead compound against malaria and was used as a template for chloroquine (4-aminoquinolone) and mefloquine (8-aminoquinolone). The mechanism of action of quinine and quinolone compounds is believed to involve the inhibition of parasite heme detoxification in the food vacuole of the parasite.

Chloroquine was the prototype antimalaria drug used widely to treat all types of malaria infections. It acts by inhibiting the parasitic enzyme heme polymerase that converts the toxic heme into non-toxic hemazion resulting in the accumulation of the toxic heme in the parasite vacuole. It is also believed to interfere with the biosynthesis of nucleic acids.

Primaquine is an essential co-drug with chloroquine. It is effective against the gametocytes of all *Plasmodium sp*. Primaquine also act by generating reactive oxygen species or interfering with the electron transport in the parasite (Moretti *et al*., 1994).

Quassinoids; these are heavily oxygenated lactones with majority having C20 basic skeleton named as picrasane. However C18, C19 and C25 quassinoids are also known. Different oxygen containing groups have been found on all other carbon atoms except C5, C9 and methyl groups at C4 and C10. Some plant derived quassinoids with antimalarial properties include brusatol, bruceantin and brucein. Although the exact mechanism of action of the single agents remains unclear, some agents are found to affect protein synthesis in general or specifically HIF-1 and MYC, membrane polarization and the apoptotic machinery (Fiaschetti *et al*., 2011).

Cedronin belongs to the few quassinoids with C19 skeleton and its activity has been found to be comparable to both chloroquine resistant and chloroquine sensitive strains. This suggests that quassinoids may act upon malaria parasites by means of a mechanism different from that of chloroquine (Moretti *et al*., 1994).

Sesquiterpenes; Artemisinin (ART) also known as qinghaosu is an endoperoxide sesquiterpine isolated in1972 from the leaves of *Artemisia annua* L. (Asteraceae). This compound has been used as a parent compound or many derivatives. The use of the parent compound has been superseded by the more potent semi synthetic dihydroartemisinin and its derivative; artemether, artemotil and artesunate. The endoporoxide linkage has been found to be essential for the antimalarial activity of ART and its derivatives (Sebisubi & Tan, 2010). The discovery of ART led to the investigation of some other naturaly occurring peroxides for their activity (Klayman, 1993). These compounds open in the *Plasmodium* and release singlet oxygen and

form a free radical, a strong cytotoxin. Artemisinin is also found to cause diminution of the nucleic acid synthesis but the effect on this process is slow (Gu *et al.,* 1983; Li *et al.,* 1983).

2.3.1. *In-vitro* **Antimalarial Assays**

In-vitro screening of compounds for antimalaria activity is based on the ability to culture *Plasmodium falciparum in-vitro* in human erythrocytes. Typically, parasites are propagated in leukocyte-free erythrocytes at 2–5% haematocrit at 37°C under reduced oxygen (typically 3–5% O_2, 5% CO_2, 90–92% N_2) in tissue culture (RPMI 1640) media containing either human serum or Albumax (a lipid-rich bovine serum albumin). Multiple drug-resistant and drug-sensitive isolates from around the world have now been culture-adapted and can be obtained from the Malaria Research and Reference Reagent Resource Center (Noedl *et al.,* 2003). Trager and Jensen (1976) reported the continuous *in vitro* cultivation of the human malaria parasite, *Plasmodium falciparum*. This has served as a pioneer for the development of micro-titre plate assays for the determination of antiplasmodial activity.

Several *in-vitro* methods are now commonly used for the assessment of *Plasmodium falciparum* growth inhibition. These include:

2.3.1.1. **Schizont Counting On Thick Films**

This test is based on the evaluation of the morphology of the parasite growth (ring form to schizonts) (Plummer & Pinto, 2008). The parasites are counted using a light microscope. The WHO mark 111 microtest assay is a low cost alternative standardized by WHO. This experiment is based on the maturation of *Plasmodium falciparum* in a 24 to 36 hour microculture. The number of parasites that develop into

schizonts are counted microscopically in a Giemsa attained thick films (Ferreira *et al*., 2001).

This method is economical and simple to perform in the field, however it is labour intensive and prone to individual variability (Le Bras *et al*., 1984).

2.3.1.2. DNA Dye Intercalating Assays

The malaria parasites have both DNA and RNA however, the host erythrocytes lack DNA and RNA. This phenomenon is employed in the dye-intercalating assay. The parasites are stained with dyes that fluoresce in the presence of nucleic acids. Measurement of the different DNA based dyes have been used such as DAPI (4, 6-diamidino-2-phenylindole), Pico green, YOYO-1 and SYBR green 1 but YOYO-1 and DAPI have been successful in the screening of a number of compounds. This method is relatively inexpensive and simpler compared to radioactive methods (Weisman *et al*., 2006; Baniecki *et al*., 2007).

2.3.1.3. Isotopic assays

This method has been used as a gold standard in *Plasmodium falciparum* drug sensitivity (Desjardins *et al*., 1979). In most applications, parasites are cultured in the presence of different concentrations of test compound in media containing reduced concentrations of hypoxanthine, after which 3H-hypoxanthine is added for an additional incubation period before cell harvesting and measurement of radioactive counts. IC_{50} values can be determined by linear regression analyses on the linear segments of the dose–response curves (Fidock *et al*., 2004). Some other precursors such as palmitate, serine, choline, inositol, isoleucine and ethanolamine have been used in this assay. This method is expensive due to the high purchase cost of the

needed equipment such as liquid scintillation counters and harvesting machines. Again regulations regarding handling of these radioactive materials have made this method applicable only at domains where there are no alternatives (Nogueira *et al.,* 2010).

2.3.1.4. Quantification of parasite proteins

Two Enzyme-linked immunosorbent assays (ELISAs) which measure the *Plasmodium falciparum* – specific antigen histidine in rich protein 2 (HRP2) or lactate dehydrogenase protein (pLDH) are widely used. The levels of pLDH, the last enzyme in the glycolytic pathway and HRP2, a highly stable protein secreted through the membrane of infected red blood cells are proportional to the extent of parasite growth. The pLDH enzyme activity assay is based on the observation that the LDH enzyme of *Plasmodium falciparum* has the ability to rapidly use 3-acetyl pyridine NAP (APAD) as a co-enzyme in the reaction, which leads to the formation of pyruvate to lactate. On the other hand, human red blood cell carries out this reaction at a slow rate in the presence of APAD. The development of APADH is measured and there is a correlation between levels of parasitemia and the activity of parasite LDH (Basco *et al.,* 1995; Makler *et al*., 1993). The HRP2 assay is based on the measurement in the increase of HRP2 concentration in *Plasmodium falciparum* culture samples. If the antimalarial drug inhibits the parasite growth, the inhibition is reflected in the increment in HRP2 levels and can therefore be easily quantified by anti-body mediated detection (Noedle *et al*., 2005).

2.3.2. Vector Control

Vector control is an important part of the global malaria control strategy. At the same time as the global malaria community is considering how to eliminate malaria, the

World Health Organization (WHO) is actively promoting Integrated Vector Management (IVM), where multiple interventions are combined to control vector-borne diseases (Ernest *et al*., 2006).

Eradication of the vector can be achieved by larval and adult control of the female *Anopheles* mosquito. Control of adult mosquitoes is not an easy task, considering their varied habits, their ability to fly all over and to hide in nooks and corners.

Certain species of mosquito prefer to feed at twilight or night-time while others bite mostly during the day. Some mosquito species are zoophilic (preferring to feed on animals) while others are anthropophilic (preferring human blood). In some mosquito species, seasonal switching of host provides a mechanism for transmitting disease from animal to man (Miike, 1987).

Control of adult mosquitoes involves the following measures: preventing entry of adult mosquitoes into human dwellings, using mosquito nets, personal protection and using adult insecticides (Kakkilaya, 2009).

The present global malaria control strategy aims at protecting individuals and communities using long-lasting impregnated nets (LLINs) and indoor-residual spraying. In order to maintain this momentum and aim for further reductions in malaria transmission, supplementary tools for vector control need to be added to the current arsenal (Ernest *et al*., 2006). Since LLINs and IRS are directed against the adult vector population that enters houses, further suppression of transmission could be achieved by targeting the aquatic stages by reducing vector larval habitats, thus attacking both outdoor and indoor biting vectors. This may be particularly important in areas targeted for elimination where malaria foci or 'hot spots' persist (Smith *et al*., 2005).

2.3.3. Larval Source Management

Source reduction involves preventing development of mosquito larvae. The female mosquitoes need a blood meal from a vertebrate host to nourish their eggs. To develop, mosquitoes need an environment of standing water. As a group, they have adapted to complete their life cycle in various aquatic habitats including fresh water, salt-water marshes, brackish water, water found in containers, old tires and tree holes. The life cycle of the mosquito has four stages namely, the egg, larva, pupa and adult (Clements, 1963). The Greek philosopher Aristotle first described this process. About 50-200 eggs are laid per oviposition on the surface of stagnant water and these eggs develop into adult mosquitoes in a span of about 5-14 days. High humidity and ambient temperature between 20-30ºC provide ideal conditions for breeding of anopheline mosquitoes.

The most effective method to control mosquito is by stopping the development of the eggs into adult mosquitoes, by reducing the sources of breeding as well as killing the larvae. Besides being simple and cost effective, this method is also environment friendly (Kakkilaya, 2009).

Over the past decade interest in LSM by the international scientific community has grown and its potential has been demonstrated for contemporary Africa. As a consequence, LSM has been included in the latest Global Malaria Action Plan of the Roll Back Malaria Partnership. The document outlines that in areas where malaria transmission is low to moderate, seasonal or focal the integration of LSM can be appropriate. It is viewed as a targeted approach in addition to LLINs and/or IRS. The benefit of LSM is especially anticipated during the phase of' sustained control (as opposed to 'scale-up-for-impact') (Cohen *et al*., 2010). Recent public actions also

convincingly demonstrate that as malaria declines in many African countries, driven down (partly) by the use of LLINs and IRS, outdoor biting is becoming a more important feature of malaria transmission with the more exophilic *Anopheles arabiensis* increasing in importance as vectors (Bayoh *et al*., 2010). Griffin and colleagues (Griffin *et al.,* 2010) recently presented strong evidence that outdoor biting defines the limit of what is achievable with LLINs and IRS. LSM is one of the few strategies effective against outdoor biting vectors.

Different groups of compounds are used in the control of larvae and they include: insect growth regulators, an example being methoprene, microbial larvicides example being *Bacillus thuringiensis,* organophospahtes an example being temephos and surface film oils (Connelly, 2009).

2.3.4. Larvicides of Natural Origin

Phytochemicals are botanicals which are naturally occurring insecticides obtained from floral resources. Phytochemicals in mosquito control have been in use since the 1920s (Shahi *et al.,* 2010) but the discovery of synthetic insecticides such as DDT (Dichlorodiphenyltrichloroethane) in 1939 abrogated the use of phytochemicals in these control programmes. After facing several problems due to injudicious and over application of synthetic insecticides in nature, re-focus on phytochemicals that are easily biodegradable and have no ill effects on non-target organisms is appreciated. Since then, the search for new bioactive compounds from the plant kingdom and an effort to determine its structure and commercial production has been initiated. At present, phytochemicals make up to 1% of world's pesticide market (Isman *et al*., 1997). Generally, the active toxic ingredients of plant extracts are secondary metabolites that are evolved to protect them from herbivores. The insects feed on

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these secondary metabolites potentially encountering toxic substances with relatively non-specific effects on a wide range of molecular targets. These targets range from proteins (enzymes, receptors, signaling molecules, ion-channels and structural proteins), nucleic acids, biomembranes, and other cellular components. This in turn, affects insect physiology in many different ways and at various receptor sites, the principal of which is abnormality in the nervous system (such as, in neurotransmitter synthesis, storage, release, binding, and re-uptake, receptor activation and function, enzymes involved in signal transduction pathway) (Rattan, 2010). Rattan reviewed the mechanism of action of plant secondary metabolites on insect body and documented several physiological disruptions, such as inhibition of acetylecholinestrase (by essential oils), GABA-gated chloride channel (by thymol), sodium and potassium ion exchange disruption (by pyrethrin) and inhibition of cellular respiration (by rotenone). Such disruption also includes the blockage of calcium channels (by ryanodine), of nerve cell membrane action (by sabadilla), of octopamine receptors (thymol), hormonal balance disruption, mitotic poisioning (by azadirachtin), disruption of the molecular events of morphogenesis and alteration in the behaviour and memory of cholinergic system (by essential oil), *etc*. (Senthil-Nathan *et al*., 2008).

Several studies have documented the efficacy of plant extracts as the reservoir pool of biotoxic agents against mosquito larvae. Only a few have been commercially produced and extensively used in vector control programmes. The main reasons behind the failure in laboratory to land movements of bioactive toxic phytochemicals are poor characterization and inefficiency in determining the structure of active toxic ingredients responsible for larvicidal activity. For the production of a green biopesticide, the wide range of phytochemicals used ethnobotanically as pesticides needs to be investigated into and characterised.

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2.4. THE FAMILY MELIACEAE

The Meliaceae, or the Mahogany family, is a flowering plant family of mostly trees and shrubs (and a few herbaceous plants). Alternate, usually pinnate leaves without stipules and bisexual flowers borne in panicles, cymes, spikes, or clusters characterize them.

Most species are evergreen, but some are deciduous, either in the dry season or in winter. The family includes about 50 genera and 550 species, with a pan tropical distribution; one genus (Toona) extends north into temperate China and south into southeast Australia, and another (Melia) nearly as far north. Various species are used for vegetable oil, soap-making, insecticides and highly prized wood (mahogany). Some economically important species belong to this family. Examples includes sapele (*Entandrophragma cylindricum*) found in tropical Africa.

Members of the Meliaceae family have been used for generations in Africa, India and tropical America to treat malaria. In tropical America *Cedrela odorata, Carapa guianensis* and *Swietenia mahagoni* have been used while in Africa and India the 'Neem' tree or *Azadirachta indica* is used (MacKinnon *et al*., 1997).

2.4.1. The Genus *Carapa*

The genus *Carapa* comprise of small to medium-sized trees. Plants of this genus grow naturally in rain forest of western tropical Africa, South America, and the West Indies. Common species found in Africa are *Carapa augustifolia*, *Carapa batesii*, *Carapa macrantha, Carapa parviflora* and *Carapa procera* (Forget *et al*., 2009). Plants of this genus provide many ecological and economic benefits to both humans and animals. Frugivors and granivores as well as rodents and elephants depend on the

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fruits and seeds as a stable source of food. The trees are used for timber, which is of great benefit to humans. The oil extracted from the seeds are used as mosquito repellent, in ethno medicine or cosmetics (Degen *et al*., 2001).

2.4.2. Phytochemistry and biological activities of the genus *Carapa*

Plants in this genus are poorly investigated for their biological activities. However *Carapa guianensis* is widely investigated. It is also known as andiroba tree and taste bitter due to the presence of a group of terpenes called meliacin. Gedunin is a type of meliacin and has been found to have antiparasitic as well as antimalarial effect equal to that of quinine (Forget & Kenfack, 2006). Oil from the seeds (andiroba oil) exhibited anti-inflammatory and pain-relieving properties using zymosan-induced arthritis in mice model (Penido, 2006). It has also been found to have antiparasitic and/or insecticidal actions (Mackinnon, 1997; Mesquita, 2005).

Anti-bacterial and anti-tumor properties have also been attributed to the stem bark (Hammer & Johns, 1993). The major components found in andiroba oil include andirobin, arachidic acid,acetoxy-gedunins, epoxyazadiradiones, deacetoxygedunins, hydroxylgedunins, gedunins,hexadecenoic acid, linoleic acid, linolenic acid, oleic acid, palmitic acid, palmitoleic acid, and stearic acid (Mulholland *et al*., 2000).

2.4.3. *Carapa procera* DC.

Carapa procera is a common riverside tree belonging to the family Meliaceae. Synonyms include; *Carapa touloucouna* Guill & Perr., and *Carapa. gummiflua* CDC. Common names: It is known as tallicoonah oil tree or kunda oil tree. In Nigeria, it is called irrere,obi, agogo and as monkey kola in Ghana (Burkill, 1985).

2.4.5. Ecological and geographical distribution

Habitat range of the plant includes lake shores and mid altitude forest especially where drainage is impeded (Forget *et al,* 2004). It has also been recorded to grow on sandy soils and generally at sea level. The species are distributed from Senegal to Angola in East Africa. It is also found in Sierra Leone, Liberia, Mali, Ivory coast, Nigeria and Ghana (Kenfack, 2011).

2.4.6. Botanical description

Carapa tree is usually a small sprawling tree in swamp forest but sometimes reaches up to25m high in lowland rain forest (Burkill & Abbiw, 1990). It has smooth blackish brown to red bark. It has characteristic fruits, which are brown in colour. It is globose to elliptic in shape, shortly beaked, green, becoming brownish later at maturity. It is about 10 cm long with ridges at the corners. Its seed are big (3.5-4.5cm), dark brown and shiny. The kernel contains 55% oil; the leaves are long clustered at the end of twigs and are glaborous. Leaf shape is obovate, rounded apex or shortly uspidate. Leaves bear greenish-yellow or rarely pinkish flowers which have 5, sometimes 6 petals and are glaboruous (Kenfack, 2011).

2.4.7. Uses

Medicinal: different parts of the plant are used for various medical therapies. The bark has been used in eye treatments, as genital stimulants/depressant, in paralysis, epilepsy, convulsion and spasms. The leaves together with the bark are also used in malnutrition, stomach are, arthritis and rheumatism. The bark has also been used externally for cutaneous and subcutaneous parasitic infections. Also used as emetics, febrifuge, in leprosy, pulmonary troubles and in venereal diseases. The seed oil is

used as a laxative and vermifuge. It's used as an antidote in venomous stings and bites, as pain killers and in yaws (Kenfack, 1995).

Non medicinal: the plant is an ornamental shade tree (Burkil, 1990). The good quality reddish brown timber known as Uganda crabwood resembles true mahogany and is used for furniture (Kenfack, 1995). The fruit is edible and oil can be extracted from seeds for various purposes such as for fuel and lighting. Twigs are used as chewing sticks.

2.4.8. Phytochemistry of *Carapa procera*

Carapa procera wood has been found to contain several limonoids of evodulone, obacunol, mexicanolide and phragmalin groups as well as the carapolides (Sondengam *et al.,* 1981; Mikolajckak, 1988; Titanji *et al.,* 1990; Kimbu *et al.*, 1984). Limonoids are tetranortriterpenoids of considerable interest due to their structural varieties and biological activities, such as insecticidal, antibacterial, anti-fungal, antimalarial, anticancer and antiviral. *Carapa procera* has also been reported to contain tannins, glycosides, triterpnoids (limonoids), fatty acids and a bitter principle tulukinin (Busia, 2007).

Structures
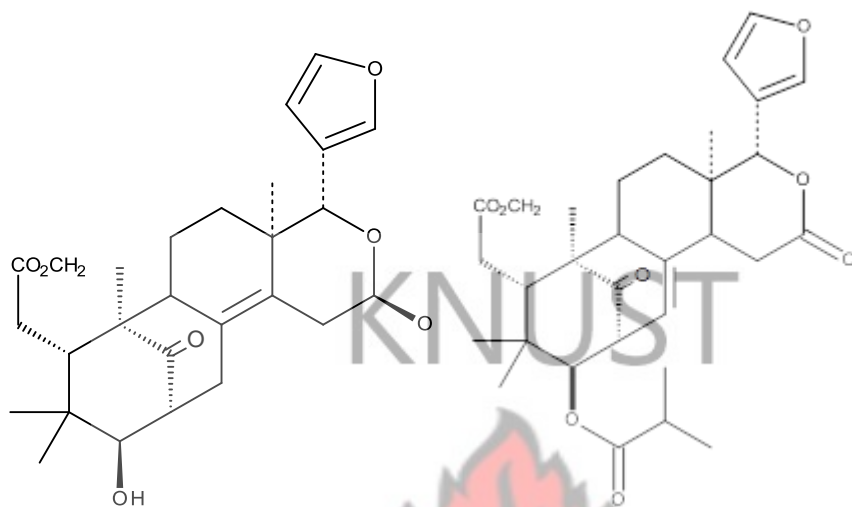
6-Deoxyswietenolide

3β-Isobutyryloxy-1-oxomeliac-8-(30)-enate
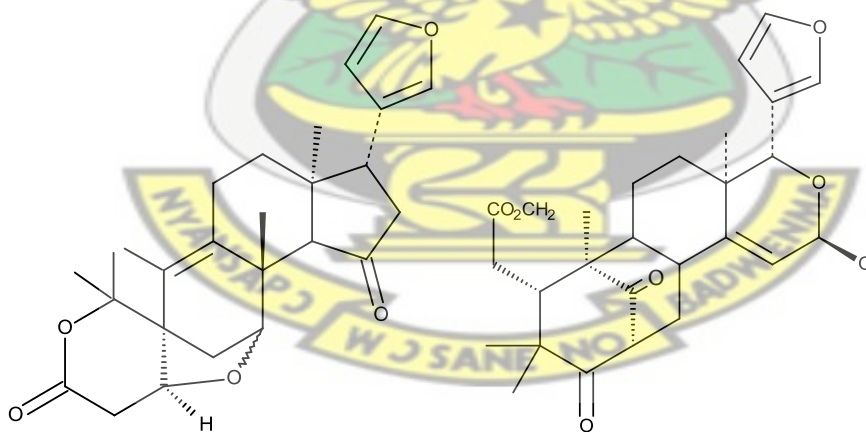
Carapa Spirolactone

Carapin

Carapolide A

KNUST
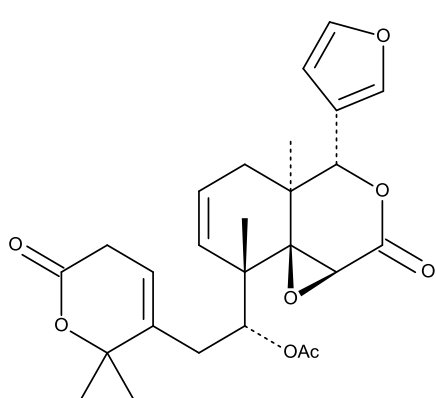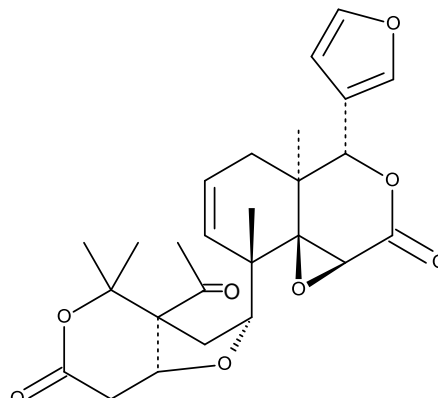
Carapolide C
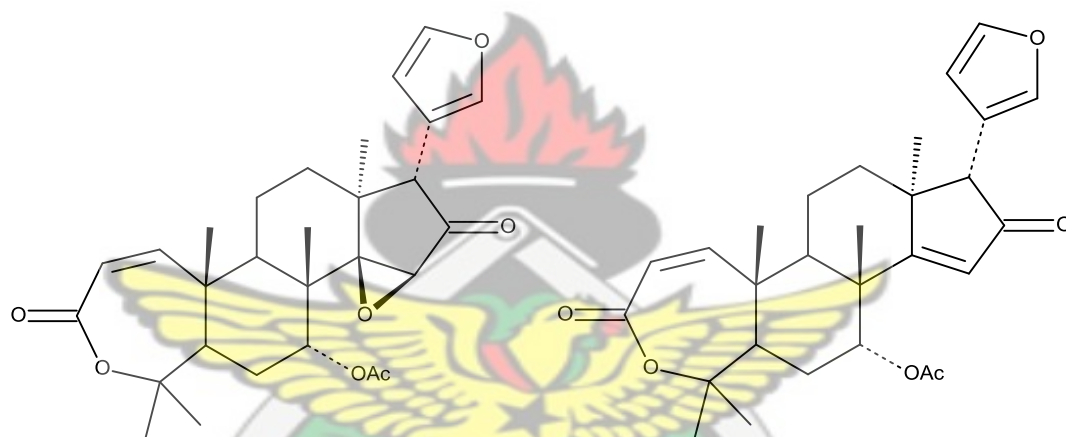
Evoduline

Proceranone

2.4.9. Biological activity of *Carapa procera*

Oil from *Carapa procera* seeds has been widely used for anti-inflammatory and insect repellent purposes. (Seignot *et al.*, 1991; Konan *et al*., 2003).

Six of the eleven *Carapa procera* limonoids showed notably strong anti-tripanosomal activity. Limonoids are characteristic phytochemicals of the Meliaceae family

including *Carapa procera* (Mulholland *et al*., 2000) and numerous limonoids have exhibited antiprotozoal activities (Khalid *et al*., 1998).

2.5. THE FAMILY LAMIACEAE

The Lamiaceae family is commonly known as the mint family and is the seventh largest flowering plant family. This family comprises of about 7000 species worldwide, including woody herbs and climbers, shrubs and trees. Species occur throughout the world with the exception of the coldest polar regions and are particularly wide spread in the tropical and temperate regions (Raymond *et al*., 2004). This family contains about 236 genera and 6900 to 7200 species (Vernon *et al.,* 2006). The largest genera are *Salvia* (900), *Scutellaria* (360), *Stachys* (300), *Plectranthus* (300), *Hyptis* (280), *Teurium* (250), *Vitex* (250), *Thymus* (220) and *Nepeta* (200) (Raymond *et al*., 2004).

The original family name is labiateae, so given because the flowers typically have petals fused into an upper lip and a lower lip. However, most botanists now use the name lamiaceae in referring to this family. Plants from this family are well known ornamental plants eg. Sages (*Salvia*) and bugle (*Ajuga*). Many of these and related species are important bee plants, providing nectar and pollen. The essential oils from these plants are commercially extracted and used medicinally (Vernon *et al.,* 2006).

2.5.1 The Genus *Hyptis*

The genus *Hyptis* is commonly known as the bushmints. They are widespread in the tropics and the warmer temperate regions of America. There are about 400 species of this genus which may be annual or perennial and small herb to large shrub (Hickey & King, 1988). Several species of this genus are aromatic and used in folk medicine to

treat digestive disorders, pain, gastrointestinal problems and skin infections (Moria *et al.,* 2005). Many pharmacological properties of this genus have been documented and some include; antimicrobial (Souza *et al.,* 2003), antiulcer and gastro protective (Barbosa & Ramos, 1992), larvicidal (Costa *et al.,* 2005), antidepressant, anti-inflammatory and anti – nociceptive (Bueno *et al.,* 2006).

2.5.2 Phytochemistry of the genus *Hyptis*

Several species belonging to the *Hyptis* genus represents an important source of bioactive compounds which are reputed for their wide range of antimicrobial, anti-cancer and insecticidal activities. The genus has been studied extensively and found to contain terpenoids including monoterpenes and sesquiterpenes, flavonoids, lactones, lignans and alkaloids (Falcao & Menezes, 2003).

2.5.3. *Hyptis suaveolens* (L. Poit)

Hyptis suaveolens (L. Poit) is commonly known as mint weed and is native to tropical America, the tropics and sub-tropics.

2.5.4. Botanical description

Hyptis suaveolens is an erect annual herb up to 3m high with a woody base. Stems are 4-angled, hairy and hollow. The plant is covered with glandular and non-glandular hairs. The leaves are hairy, ovate and 2-10cm long. Those on flowering stems are smaller with margins shallowly toothed and leaf stalk 0.5-4cm long. The fruits are lobed capsule. *Hyptis* leaves are opposite with strong mint aroma when crushed. The fruits are spread by water, in the mud and attached to animals (Mahesh, 2001).

2.5.5. Ethno medicinal uses

Various herbal communities to cure various diseases use the medicinal important plant *Hyptis suaveolens*. Almost all parts of this plant are used traditionally to treat various diseases. The leaves of *Hyptis suaveolens* have been utilized as a stimulant, carminative and as a cure for parasitic cutaneous diseases (Kirtikar & Basu, 1991). Crude leaf extract is also used as a relief for colic and stomachache. Leaves and twigs are considered to be antispasmodic and used as antirheumatic and antisuporific baths (Mahesh, 2001), an anti-inflammatory, fertility agents and also applied as an antiseptic in wounds, burns and various skin diseases. The decoction of the roots is highly valued as an appetizer and is reported to contain urosolic acid, a natural HIV-integrase inhibitor (Chatterjee & Pakrashi, 1997). Fumes of the dried leaves are also used to repel mosquitoes and control insect pest of stored grains.

2.5.6. Biological activity and phytochemistry

Steam distilled extract of *Hyptis suaveolens* leaves was found to have antibacterial and antifungal activity against *Aspergilus niger* and *Micrococcus leteus* (Mandal *et al.,* 2007). Ethanol extract of whole plant of *Hyptis* also had antimicrobial activity against *Candida albicans, Collectrotrichum capsici, Fugarium oxysporum, Klebsiella pneumoneae, Staphylococcus aereus* and *Escherichia coli.* Preliminary phytochemical screening showed the presence of volatile oils, starch, proteins, tannins, saponins, fats, alkaloids and glycosides (Pachkore *et al*., 2011). The volatile oils of *Hyptis suaveolens* have been found to contain β-caryophyllene, 1,8-cineole and sabinene, β-pinene, sesquiterpenes and monoterpenes, terpenoids and sterols (Busia, 2007).

The aqueous extract of *Hyptis suaveolens* was compared with commercial insecticide furadan (carbofuradan) for the control of *S. calamistis* on maize. The extract

compared favourably with Furadan while maize surrounded by live plant had lower *S. calamistis* densities (Adda *et al*., 2011).

Sabinene   β-Pinene   β-Caryophyllene
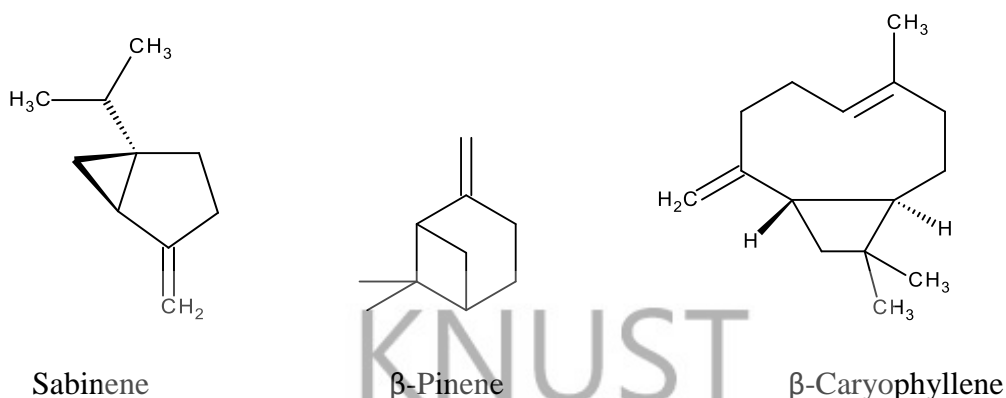
2.6. NATURAL PRODUCTS AND DRUG DISCOVERY

Plants are indispensible to man as far as life is concerned. They have been used as food, as source of shelter, medicines and even as poisons since the beginning of mankind (Cordell, 1981). Plants have been used for medicines for over thousands of years (Samuelsson, 2004), which originally took the forms of crude drugs such as tinctures, teas, poultices, powders and other herbal preparations (Bulick & Cox, 1997; Samuelsson, 2004).The plant parts used include; root, stem bark, leaves. Flowers, twigs, exudates and modified plant organs (Unijal *et al.,* 2006). Before the realization that pharmacologically active compounds present in these medicinal plants are responsible for their efficacy, the doctrine of signatures was often used to identify specific plants for treating some diseases. For instance the golden-rod with a yellow hue was used to treat jaundice, red coloured herbs were used to treat blood disorders, liverworts for liver diseases, pileworts for haemorrhoids and toothworts were used for toothache.

The plant chemicals responsible for the medicinal and other activities of the plants were found to be the secondary metabolites. These secondary metabolites are derived biosynthetically from the plants primary metabolites (e.g. Carbohydrates, amino acids and lipids) and are not directly involved in the growth, development or reproduction of the plants. The secondary metabolites can be classified into several groups according to their chemical classes; alkaloids, terpenoids, glycosides etc (Unijal *et al.,* 2006).

In 1805, morphine became the first pharmacologically active compound to be isolated in pure from a plant although its structure was not elucidated until 1923. In the 19th century, numerous alkaloids were isolated from plants and some of these includes; atropine from *Atropa belladonna*, caffeine from *Caffea arabica*, ephedrine from *Ephedra sp*, morphine and codeine from *Papaver somneferum* and other examples.

Following these discoveries, bioactive secondary metabolites from plants were utilized more widely as medicines both in their original and modified forms. It is estimated that 60% of anti-tumour and anti-infectious drugs already on the market or under clinical trial are of natural origin (Yue-ZhongShu, 1998). The vast majority of these cannot yet be synthesised economically and are still obtained from wild or cultivated plants. Natural compounds can be lead compounds, allowing the design and rational planning of new drugs, synthesis and the discovery of new therapeutic properties not yet attributed to known compounds (Hamburger & Hostettmann, 1991). In addition, compounds such as muscarine, physostigmine, cannabinoids, yohimbine, forskolin, colchicines and phorbol esters, all obtained from plants, are important tools used in pharmacological, physiological and biochemical studies (Williamson *et al.*, 1996). In recent years, there has been growing interest in alternative therapies and the therapeutic use of natural products, especially those derived from plants.

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It is estimated that, in 1997, the world market for over the- counter phytomedicinal products was US$ 10 billion, with an annual growth of 6.5% (Soldati, 1997; Vulto & Smet, 1988). The WHO considers phytotherapy in its health programs and suggests basic procedures for the validation of drugs from plant origin in developing countries (Vulto & Smet, 1988). Eastern countries, such as China and India, have a well-established herbal medicines industry. These countries have been investing in research programs in medicinal plants and the standardisation and regulation of phytomedicinal products, following the example of European countries, such as France and Germany. More recently, drug discovery techniques have been applied to the standardization of herbal medicines, to elucidate analytical marker compounds. The methods employed to acquire compounds for drug discovery includes isolation from plants and other natural sources, synthetic chemistry, combinatorial chemistry, and molecular modeling (Geysen *et al.*, 2003; Ley & Baxendale, 2002; Lombardino, 2004).

An example of drug development based on traditional medicine is Artemesinin, a sesquiterpene lactone with potent antimalarial activity against multiple-resistant *plasmodium falciparum*. It is the first line drug for the treatment of malaria in most countries. It was rescued from a millenary traditional use of the plant *Artemisia annua* L. (Gen & Lin, 1986; Philpson, 1986). This has resulted in investigation of most folk medicines used for the treatment of malaria.

CHAPTER THREE

MATERIALS AND METHOD

3.1 ANALYTICAL METHODS IN NATURAL PRODUCT SEPARATION

3.1.1 Extraction methods

Extraction is the first step of any medicinal plant study as it plays a significant role on the final result. Extraction methods employed in natural medicinal plants processing takes into account knowledge of several physicochemical properties of the compounds of interest. These include partition coefficient in water or other solvents (organic), relative polarity of the molecule, stability of the molecule in light or dark as well as the temperature employed during the extraction process (Cseke *et al.,* 2006). The efficacy of any extraction depends on the choice of solvent. The polarity of the compound of interest is the most important factor for the choice of solvent. Molecular affinity between solvent and solute, mass transfer, use of co-solvent, environmental safety, human toxicity and financial feasibility are also factors to be considered in the selection of solvents (Cowan, 1999). The commonly used traditional methods of extraction include; maceration, infusion, digestion, decoction and soxhlet extraction. In recent times, methods that are more efficient have been employed in extraction and some of these include;

1. Ultrasound extraction (sonication). This method involves the use of ultrasound with frequencies ranging from 20kHz to 2000kHz. These frequencies increase the permeability in cell wall of plant materials hence produce cavitation. This method is highly efficient but limited on large scale because of cost. Also the effect of ultrasound energy on some active constituents of medicinal plants

leads to the formation of free radicals and consequently undesirable changes in the drug molecule (Handa *et al.,* 2008).

2. Counter-current extraction; in this method, wet raw material is pulverized using toothed disc disintegrators to produce a fine slurry. This method is highly efficient, requiring less time and has no risk of high temperature effect (Handa *et al.,* 2008).

3.1.2 Chromatographic separation methods

Chromatography is an analytical method that is widely used for the separation, identification and determination of the individual components of a complex mixture. This technique is a complex one involving distribution of the components of a sample between two phases, one of which is stationary and other mobile. The following chromatographic methods are commonly used in phytochemical analysis: thin layer chromatography (TLC), liquid column chromatography (LC), gas chromatography (GC) and high-performance liquid chromatography (HPLC).

3.2. PHYTOCHEMICAL INVESTIGATIONS

3.2.1. Collection and authentication of plant materials

The stem bark of *Carapa procera* DC. (Meliaceae) was harvested around the Kwame Nkrumah University of Science and Technology Campus-Kumasi, whereas the whole plants of *Hyptis suaveolens* were harvested from the Physique garden of the Kwame Nkrumah University of Science and Technology Kumasi. Mr. George Henry Sam of the Department of Herbal Medicine, Faculty of Pharmacy and Pharmaceutical Sciences, Kwame Nkrumah University of Science And Technology identified the

plant samples. Voucher specimen with numbers KNUST/HM1/2012/S001 and KNUST/HM1/2012/WP001 for *Carapa procera* and *Hyptis suaveolens* respectively have been deposited at the herbarium.

3.2.2 Processing of plant materials

The plant materials were screened for foreign matter like insects, stones, organic and inorganic matter as well as other plant parts of the same plant. The aerial part of *Hyptis suaveolens* was washed with water and cut into smaller pieces. The stem bark of *Carapa procera* was also cut into smaller pieces and all the plant materials were air dried for a week. The materials when dried were milled coarsely with a mechanical mill and packed into brown envelops until needed for further analysis.

3.2.3 Extraction of plant materials

The dried plant materials were soxhlet- extracted successively using petroleum ether, ethyl acetate and 70% ethanol for 12 hours. The resulting extracts were evaporated on a rotary evaporator and the concentrated extracts were kept in desiccators until needed for further analysis.

3.2.4. Chromatography

The results of the bioassays of the stem bark extracts of *Carapa procera* (petroleum ether, ethyl acetate and ethanol) indicated that the petroleum ether and ethyl acetate fractions had larvicidal and antiplasmodial activities. Based on these, the petroleum ether extract was fractionated using various chromatographic methods to isolate the chemical constituents present and were identified by their spectroscopic features.

3.2.4.1. Materials

Silica gel 60 (70-230 mesh ATSM, Merck Germany) was used as the stationary phase. Aluminium percoated silica gel plates 60F 254 (0.25 thick) was used for the analytical thin layer chromatography (TLC). For the preparative thin layer chromatography, silica gel PF254 (Merck, 0.75mm thick) prepared and activated at 105 for 30 minutes was used. All the reagents were purchased from Macherey-Nagel, Germany.

3.2.4.2. Solvents and reagents

Analytical grade methanol, ethanol, ethyl acetate and petroleum ether were used for extraction, column chromatography and TLC analysis. Anisaldehyde was used as detecting reagent. All the solvents were purchased from BDH laboratories supplies (England).

The following solvent systems were used for the column chromatography in order of increasing polarity, petroleum ether, petroleum ether and ethyl acetate, ethyl acetate, ethyl acetate and ethanol. For the analytical and preparative thin layer chromatography, the following solvent systems were used and the calculation of the Rf values were based on the respective solvent systems. These were; petroleum ether and ethyl acetate (90:10) and (95:5)

3.2.4.3. Detection of analytical TLC

The zones on TLC plates corresponding to separated compounds were detected under U.V light 254 nm and 365 nm and also by spraying with anisaldehyde 0.5% in HOAC/H$_2$SO$_4$/MEOH in the ratio 10:5:85 followed by heating for 5-10 min.

3.2.4.4. Column Chromatography

90g of silica gel 60 (70-230 mesh ASTM) was packed dry into a glass column (90cm x 5cm). 12.5g of the petroleum ether fraction of *Carapa procera* was dissolved in about 50ml of chloroform and mixed with 30g of silica gel 60 (70-230 mesh ASTM), allowed to dry to attain same consistency as the silica gel in the column and then spread gently on top of the packed column. A wad of cotton wool was placed on top of the packed column in order not to disturb the surface of the packing. The column was eluted with a gradient of petroleum ether, ethyl acetate and ethanol. Aliquots were collected based on the bands of the eluent. By means of thin layer chromatography and anisaldihyde as detecting reagent, similar fractions were bulked together based on their TLC profiles and six fractions were obtained.

The fractions were then weighed and subjected to thin layer chromatography to ascertain their chromatographic fingerprint.

Table 1: Weights of Bulked Fractions

BULKED FRACTIONS	ALIQUOT NUMBER	WEIGHT/g
CP1	1-18	0.65
CP2	19-36	1.05
CP3	37-53	2.30
CP4	54-59	1.87
CP5	60-71	1.55
CP6	72-80	2.98
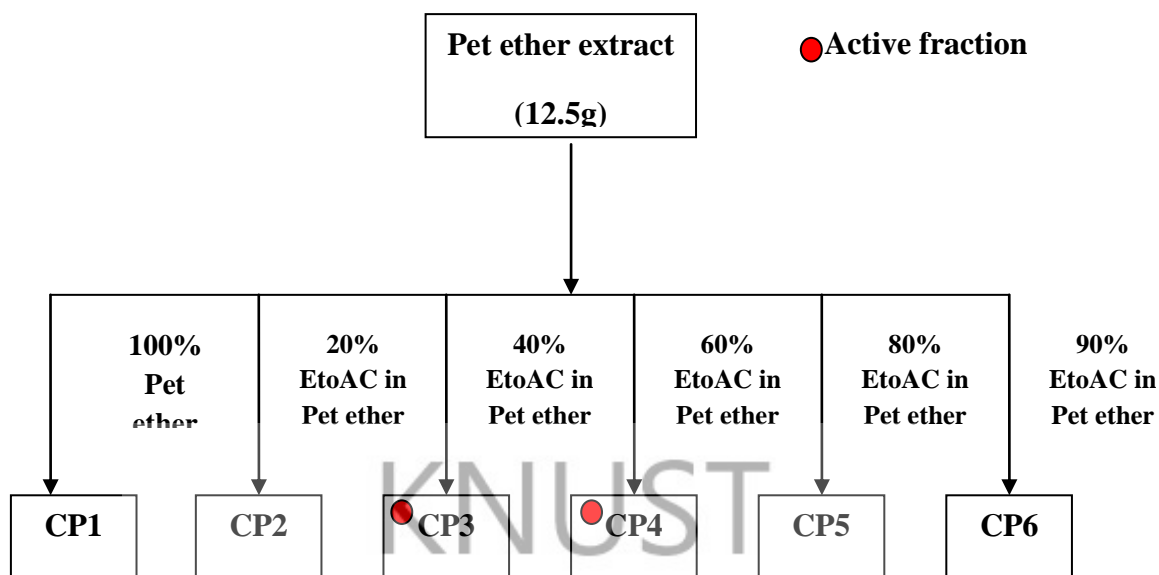
Figure 2: Schematic presentation of the fractionation of petroleum **ether extract of** *Carapa procera.*

3.2.4.5. Column chromatography of active fractions (CP3)

3g of silica gel was packed into a column using the wet packing method. 2.30g of fraction CP3 was dissolved in 3ml ethyl acetate and the solution applied gently on top of the packed column. The column was eluted in gradiently starting with petroleum ether and ending with 100% ethyl acetate. 20x 150ml aliquots were collected and concentrated under reduced pressure.

3.2.4.6. Isolation of compounds C1 and C2

Based on the TLC profiles of the 20 different aliquots, CP3-1 to CP3-6 were bulked (0.52g) and designated 3A. Aliquots CP3-7 to CP3-9 was bulked (0.42g) and designated 3B. Aliquots CP3-10 to CP3-14 was bulked (0.33g) and designated 3C. Aliquots CP3-14 to CP3-17 was bulked (0.24g) and designated 3D.

Chromatographic studies to ascertain the chromatographic fingerprint of each fraction was performed and the figures below illustrate the column chromatographic procedure of the active fraction CP3. Compounds C1 and C2 were isolated from sub fraction 3B and 3D respectively by preparative TLC.
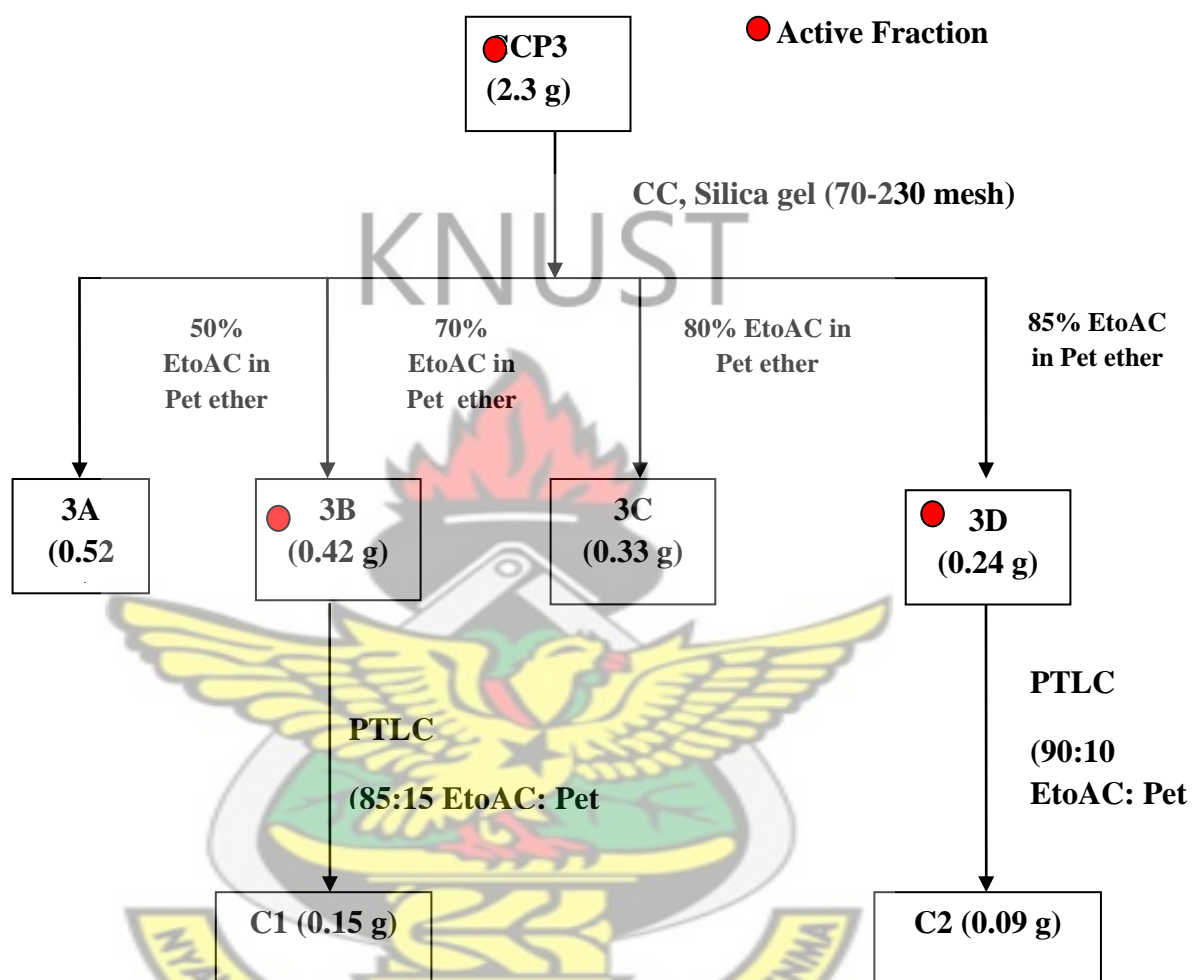

Figure 3: Schematic representation of isolation of compounds **C1** and **C2**

3.2.4.7. Isolation of compounds C3 and C4

Compounds C3 and C4 were isolated from the sub fraction CP4 of the pet ether fraction using preparative TLC.
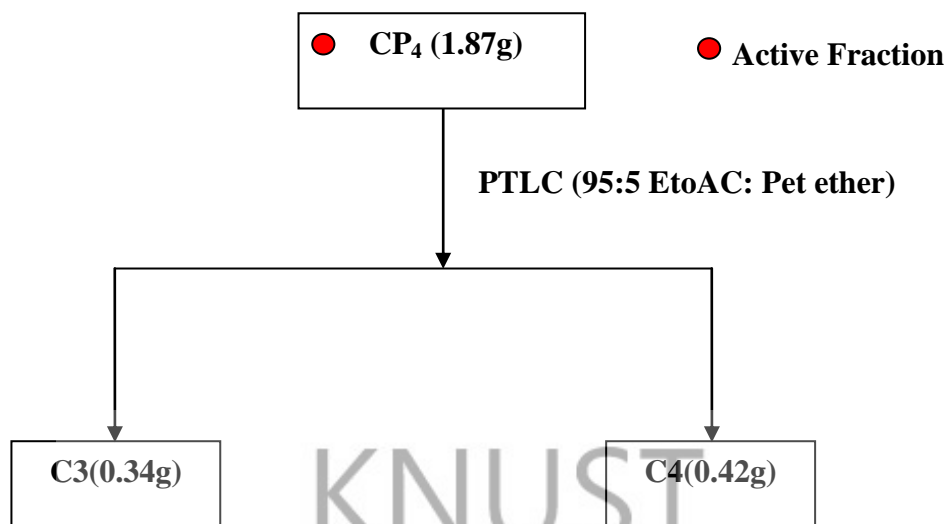
```
                    ┌─────────────────────┐
                    │  ● CP₄ (1.87g)      │        ● Active Fraction
                    └─────────────────────┘
                              │
                    PTLC (95:5 EtoAC: Pet ether)
                              │
              ┌───────────────┴───────────────┐
              ▼                                 ▼
      ┌───────────────┐               ┌───────────────┐
      │  C3(0.34g)    │  KNUST        │  C4(0.42g)    │
      └───────────────┘               └───────────────┘
```

Figure 4: **Schematic representation of isolation of compounds C3 and C43.**

3.3. LARVICIDAL ACTIVITY OF EXTRACTS

3.3.1. Test mosquitoes

Larvae of *Anopheles gambiea* were obtained from a laboratory colony maintained in the insectory of the Department of Animal Science, Ghana Atomic Energy Commission. The colony was kept free from exposure to pathogens, insecticides or repellents and maintained at a temperature of $25\text{-}30^{o}C$ and a photoperiod of 12 hour light and 12 hour darkness. The larvae were fed with dog biscuit. Adults were simultaneously fed with 10% sucrose and 10% multivitamin syrup and were periodically blood fed on retained rats.

3.3.2. Larvicidal Bioassay

The larvicidal bioassay followed the World Health Organization (WHO) protocols with slight modifications. Batches of 25 third instar larvae were transferred by means of small droppers into small disposable test cups each containing 100ml of distilled water. 1ml of the appropriate dilution of each extract was added to each test cup to

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obtain the required concentration. Equal number of controls where 1ml of DMSO was added to 100ml of distilled water was set up. Temephos was used as the positive control and five different concentrations of it were prepared. Each test was run three different times on three different days with different batches of mosquitoes. The test containers were held at between 25-28°C temperature and a photoperiod of 12 hour light and 12 hour darkness. Larval mortality was recorded after 24 hours and results analysed using graph pad prism 5.

3.4. ANTIPLASMODIAL ACTIVITY

3.4.1 Plasmodium culture and maintenance

Plasmodium falciparum were grown and maintained in culture using the method of Trager and Jensen with some modifications. Cultures consisted of a 4% hematocrit suspension of O+ human erythrocytes in RPMI 1040 medium supplemented with gentamicin solution at 0.01mg/ml, 25Mm HEPES buffer, 25mM NaHCO and 1% Albumax11. Cultures were fed with a gas mixture containing 5% CO_2 and incubated at 37°C. The estimation of the parasitaemia as well as parasite visualization before incubation was done using both fluorescence (Acridine orange) and normal light (Giemsa stain) microscopes.

3.4.2 Antiplasmodial Bioassay

The antiplasmodial activity of each extract was evaluated against chloroquine resistance strains of *Plasmodium falciparum* using the parasite lactate dehydrogenase assay as described by (Makler *et al.,* 1993).

A stock solution of 5µg/ml was prepared in 10% methanol (in deionized water). Artesunate was used as the standard drug (positive control). The extracts were serially

diluted two fold in complete medium up 0.0024 µg/ml to using a flat bottomed, 96-well microtitre plate (Geiner Bio-one).

Unparasitised erythrocyte (RBC) was added to column 1 (blank) which had no drug, while parasitized red blood cells were added to column 2-12. The plates were gassed 4 min (93% N, 45CO_2 and 3% O_2) and incubated for 48 hours. A final hematocrit and parasitemia of 2%was used for all experiments. The absorbance of each well was read using a microplate reader at 590 nm. The percentage parasite survival and the concentration that inhibits the growth of parasites by 50% were determined using Graph pad prism 5.

CHAPTER FOUR

RESULTS

4.1. EXTRACTION OF PLANT MATERIALS

The stem bark of *Carapa procera* as well as the whole plant of *Hyptis suaveolens* were extracted with petroleum ether, ethyl acetate and ethanol successively. The percentage yield of the extracts is presented in table 2 below.

Table 2: Percentage Yield

SOLVENTS	%YIELD OF EXTRACTS/g	
	CARAPA PROCERA	*HYPTIS SUAVEOLENS*
Petroleum ether	4.3	4.8
Ethyl acetate	10.2	14.2
Ethanol	17.4	16.4

4.2. PHYTOCHEMICAL EVALUATION

The presence of secondary metabolites (saponins, reducing sugars, flavonoids, glycosides, steroids, terpenoids and alkaloids) in the powdered samples was investigated following qualitative methods as described by Evans (2009). Table 3 shows the results of the phytochemical screening.

Table 3: Results of Phytochemical Screening

	CARAPA PROCERA	*HYPTIS SUAVEOLENS*
GLYCOSIDDES	Present	Absent
ALKALOIDS	Present	Present
TANNINS	Present	Present
FLAVONOIDS	Present	Absent
ANTHRAQUINONE	Present	Present
STEROIDS	Present	Absent

4.3. IDENTIFICATION OF COMPOUNDS

4.3.1. Identification of compound C1 as Carapolide B

Compound C1 was compared to literature data and was identified as a tetranortriterpenoid. C1 was obtained in the form of yellow powder with melting point between 194-197 {literature 194-196 (Kimbu *et al*., 1984)}.

The ^{1}HNMR spectrum and the ^{13}C-NMR spectrum were identical to that of Carapolide B as described by Kimbu *et al*., 1984. The ^{1}HNMR spectrum showed resonance for a secondary acetate, (δH2.01, 5.07), a methylene group (δH 2.60 and 2.02), a methyl ketone (δH 2.26) and an unsaturated lactone ring (δH 7.15, 6.19).
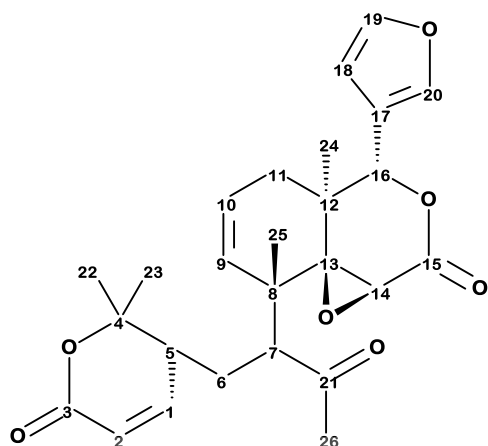
Carapolide B [C1]

4.3.2. Identification of compound C2 as Proceranolide

C2 was obtained as cream powder with melting point between 192-195°C [literature:192-196°C] (Sondengam *et al.*, 1980)]. The [1] HNMR spectra was characteristic of a bicyclononanolide with four methyls (δH 0.72, 0.80, 1.02 and 1.12) , one methyl – esther group (δH 3.68) and a double bond without vinylic protons (δH 7.44). According to Sondengam *et al.*, (1979) ^{13}C-NMR spectrum showed 27 carbon atom signals which was similar to that of fissinolide except that there was no acetate group. It was therefore concluded that proceranolide is 3β-deacetylfissinolide (Sondengam *et al.*, 1979).
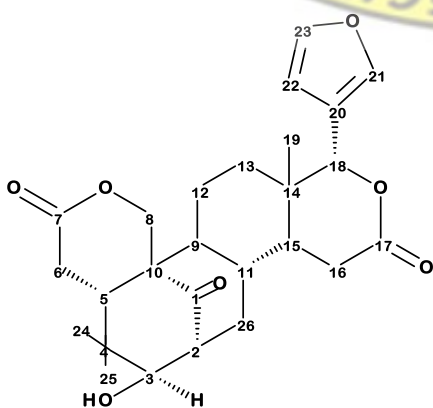

Proceranolide [C2]

4.3.3. Identification of compound C3 as Carapolide A

The spectra for C3 appeared to be identical (^{1}HNMR and ^{13}C-NMR) with Carapolide
B of Kimbu *et al*., 1984. C_3 was obtained as white powder with melting point between
195-196[o]C [literature: 194-196[o]C (Kimbu *et al*., 1984)].

The ^{1}HNMR and ^{13}C-NMR spectrum (Apendix 3) exhibited four tertiary methyl
groups (δH1.47, 1.45. 1.16 and 0.92) and secondary acetate (δH2.02, 5.53). The
spectra also showed one conjugated double bond which is trisustituted (δH 5.51, δC
118.4 and δC 136.6). The presence of an acetate group indicates that carapolide A has
a 24 carbon skeleton (Kimbu *et al.,* 1984).
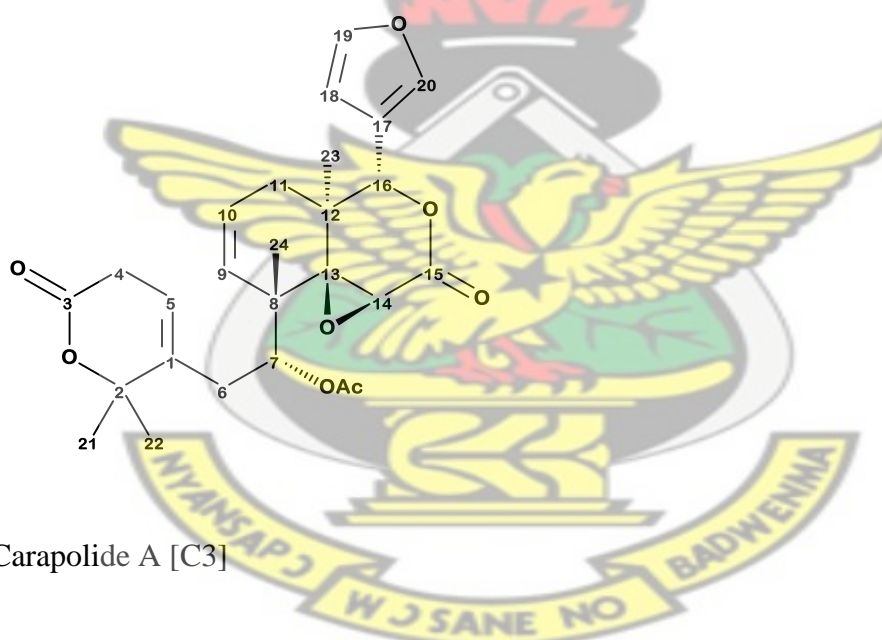
Carapolide A [C3]

4.3.4. Identification of compound C4 as Evodulone

Compound C4 was identified as evodulone, a tetranortritepenoid by comparison with
literature data. The compound was obtained as white powder and had a melting point
of 198-200 as compared to 199-200 in literature. The compound also displayed
characteristic signals in the ^{1}H-NMR spectrum for teranortriterpeniods, a β-substituted
furan (δH6.25, 7.41, 7.60), five tertiary methyl groups (δH1.04, 1.20, 1.35, 1.38 and

1.44) and a secondary acetate (δH3.45, 3.92, 5.91). The ^{13} C-NMR spectrum showed signals at 157.3, 121.4, 167.5 and 84.8.
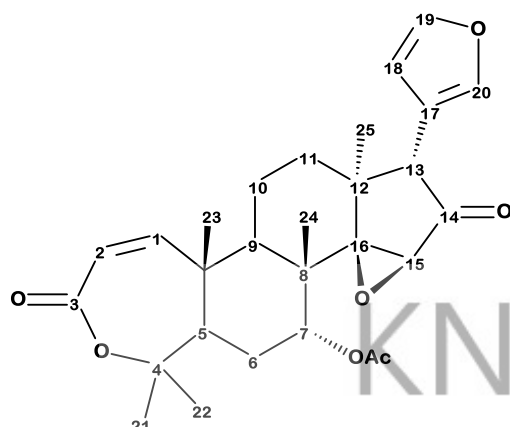
Evodulone [C4]

4.4. LARVICIDAL ACTIVITY OF THE EXTRACTS

The effect of the extracts on the growth and development of the *Anopheles gambiae* larvae were determined by adding various concentrations of the extracts to about 25 larvae in a container and counting the number of dead larvae after 24 hours. The effective concentration at which 50% of the larvae died was used as a standard measure of larvicidal efficacy of the extracts against *Anopheles gambiea*. The percentage mortality was calculated and used to calculate the EC_{50} using graph pad prism. Percentage mortality was analyzed according to the formula;

mortality % = number of dead larvae/total number of larvae x 100%.

In cases where the negative control mortality was between 5% to 20%, the test mortality was corrected using the Abbort formula shown below.

Mortality % = X –Y/X

Where X = percentage survival in untreated (control)

Y = percentage survival in treated sample.

4.4.1. Larvicidal activity of *carapa procera* extracts

Petroleum ether, ethyl acetate and ethanol extracts of *Carapa procera* stem bark were tested for their larvicidal activity as stated in section 3.3.2. at concentrations of 0001, 001, 0.01, 0.1 and 1%w/v. The effect of the extracts at various concentrations on the growth of the *Anopheles* larvae was observed after 24 hours. Figure 5 shows the dose dependent larvicidal effect of the stem bark extracts at the various concentrations employed in this study. The different extracts showed various degrees of growth inhibition against the *Anopheles gambiea* larvae. The petroleum ether extract showed a significant larvicidal activity (EC_{50} 16.91mg/ml) against the larvae which was comparable to that of temephos (5.76 mg/ml). This was followed by the ethyl acetate (EC_{50} 77.14mg/ml) and ethanol extracts (EC_{50} 186.4mg/ml) respectively as shown in figures 6 and 7. Table 4 Shows the EC_{50} values of the extracts.

Table 4: In-vitro larvicidal activity of extracts of *Carapa procera*

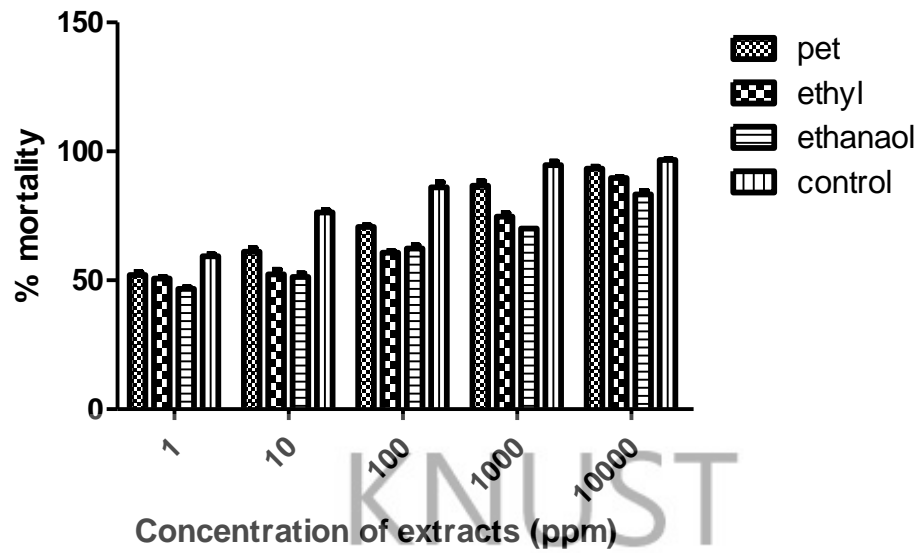| EXTRACTS	EC_{50} VALUES(mg/ml)
Petroleum ether	16.91
Ethyl acetate	77.14
Ethanol	186.4

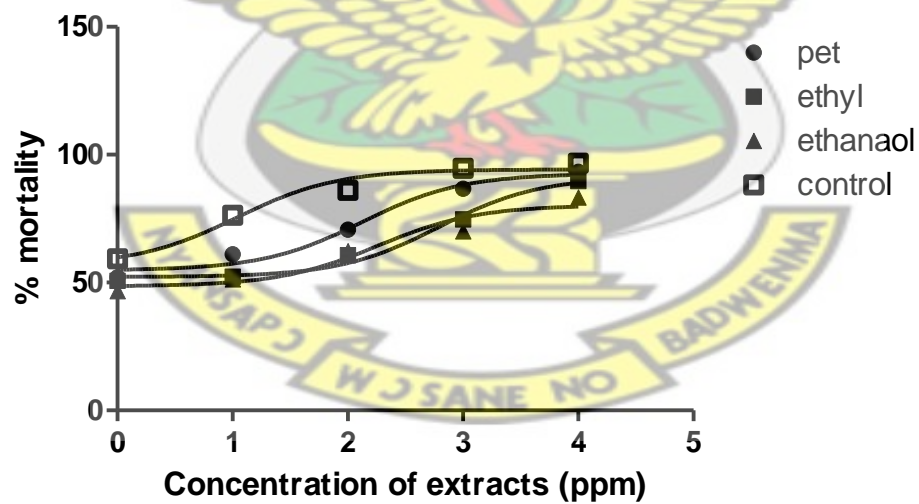**Figure 5: Effects of Concentration on Percentage Mortality of *Carapa procera* extracts.**

Figure 6: Effect of *Carapa procera* extracts on percentage mortality after 24 hours of exposure.
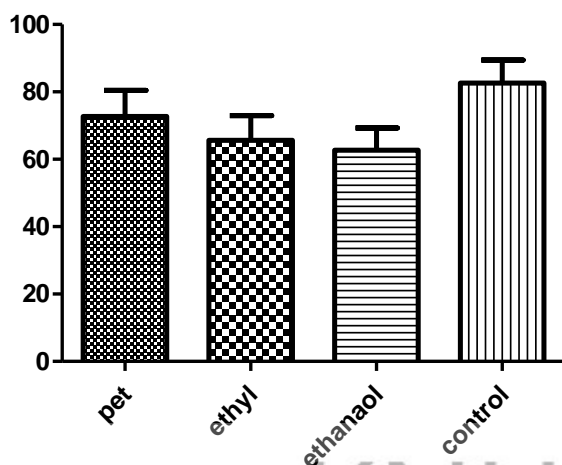
Figure 7: Effect of *Carapa procera* extracts on total mortality after 24 hours compared to control group (Values are mean± S.E.M).

4.4.2. Larvicidal activity of *Hyptis suaveolens* extracts

Petroleum ether, ethyl acetate and ethanol extracts of *Hyptis suaveolens* whole plant were tested for their larvicidal activity as stated in section 3.3.2 at concentrations of 0001, 001, 0.01, 0.1 and 1%w/v. The effect of the extracts at various concentrations on the growth of the *Anopheles* larvae was observed. Figure 8 shows the dose dependent larvicidal effect of the various extracts of *Hyptis suaveolens* at the various concentrations employed in this study. The extracts showed various degrees of mortality against the third instar *Anopheles gambie* larvae with the pet ether extract showing the highest activity with EC_{50} value of 34.96mg/ml as compared to temephos the control. This activity was followed by the ethyl acetate extract (EC_{50} 159.8mg/ml) then the ethanol extract (249.7mg/ml) as shown in figures 9 and 10.
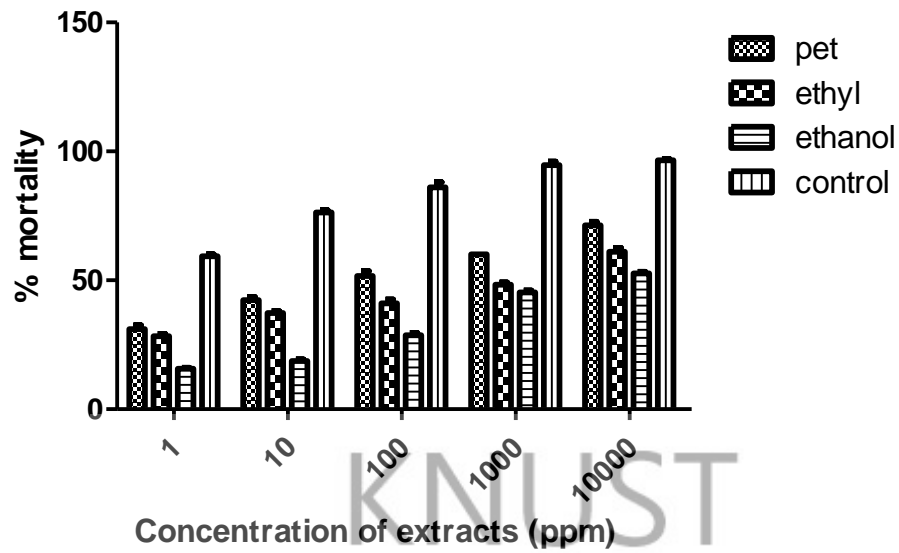
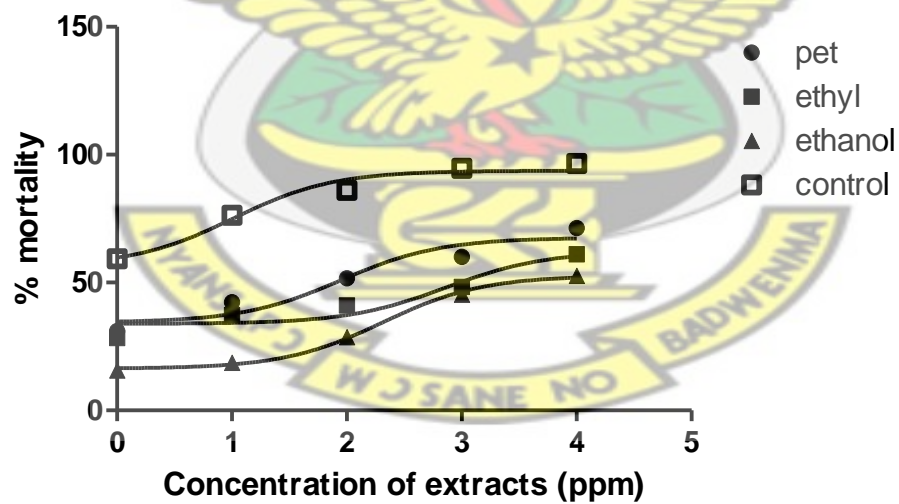**Figure 8: Effect of concentration on the percentage mortality of *Hyptis suaveolens* extracts**

Figure 9: Effect of *Hyptis suaveolens* extract on percentage mortality after 24 hours of exposure.
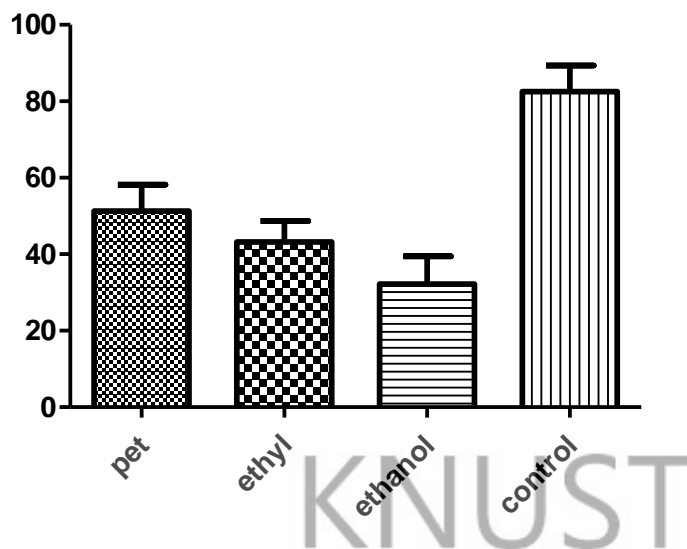
Figure 10: Effect of *Hyptis suaveolens* extracts on total mortality after 24 hours compared to control group (Values are mean ± S.E.M)

4.4.3. Larvicidal activity of compounds

Compounds C1, C2, C3 and C4 of *Carapa procera* stem bark were tested for their larvicidal activity as stated in section 3.3.2 at concentrations of 0001, 001, 0.01, 0.1 and 1%w/v. The effect of the extracts at various concentrations on the growth of the *Anopheles* larvae was observed. The different compounds showed various degrees of growth inhibition against the *Anopheles gambiea* larvae. The inhibition however did not increase with increasing concentration as was observed in the extracts. Compound C3 showed a significant larvicidal activity (EC_{50} 15.01mg/ml) against the larvae which was comparable to that of temephos (EC_{50} 5.76mg/ml). This was followed by compounds C2, C4 and C1 with EC_{50} 53.74, 198.9 and 273.6 mg/ml respectively as shown in figures 11 and 12.

Figure 11: Effects of coumpounds on the percentage mortality after 24hours of exposure.

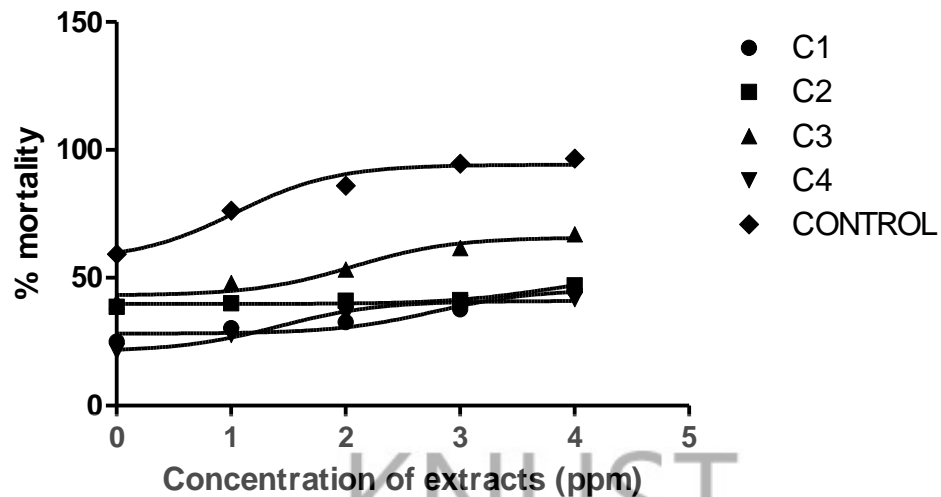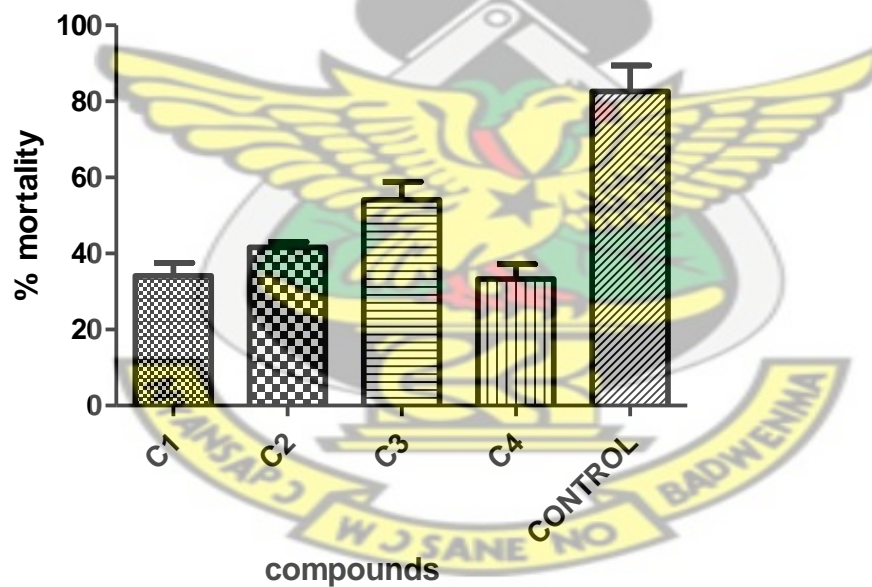**Figure 12: Effects of compounds C1, C2, C3 and C4 as compared to temephos on total mortality after 24 hours of exposure (Values are mean ± S.E.M).**

4.5. ANTIPLASMODIAL ACTIVITY OF *CARAPA PROCERA* EXTRACTS

Petroleum ether, ethyl acetate and ethanol extracts of stem bark of *Carapa procera* were tested for their antiplasmodial activity as described in section 3.4.2 at concentrations of 5 µg/ml to 0.0024 µg/ml. The effect of the extracts at the various concentrations on the growth of the malaria parasite was observed. Figure 13 shows the dose dependent chemosupressive effect of the stem back extracts at various concentrations employed in the study. The extracts showed various degrees of inhibition against the *Plasmodium falciparum* parasites as shown by the area under the curve (AUC) in figure 14. The ethyl acetate extract showed a significant level of activity as compared to artesunate the positive control. This was followed by the petroleum ether then the ethanol extract respectively as shown in figure 14. The EC_{50} values were 18.60 µg/ml, 24.71 µg/ml and 95.46ug/ml for ethyl acetate, petroleum ether and ethanol extracts respectively.
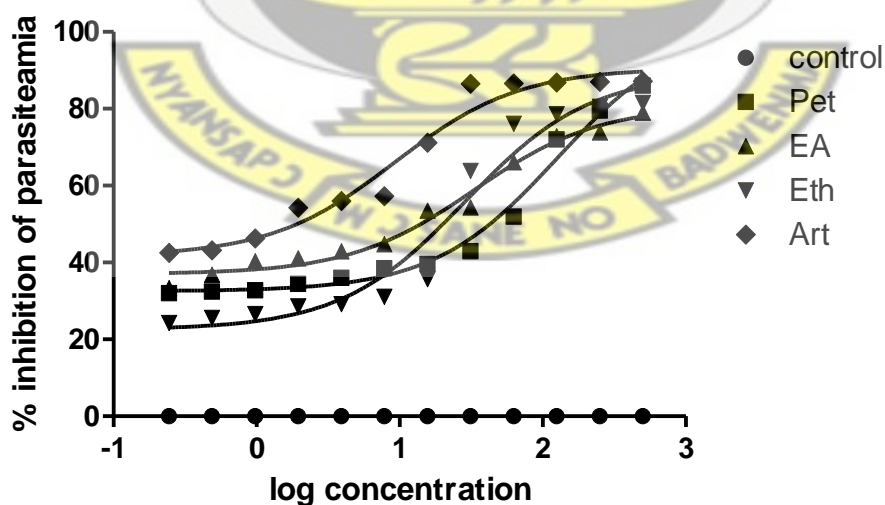
Figure 13: Effect of concentration on total parasiteamia after 48 hours of exposure to *Carapa procera* extracts.

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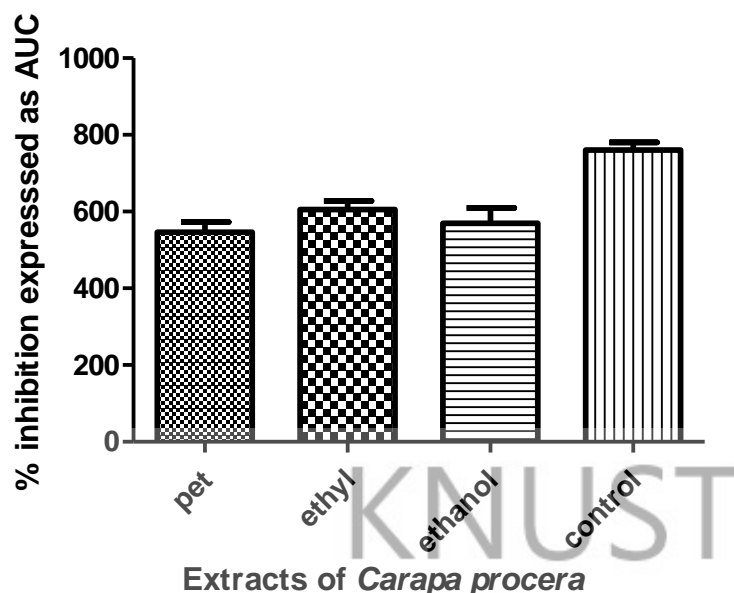
Figure 14: Effect of *Carapa procera* extracts as compared to artesunate on total parasiteamia after 48 hours (Values are mean ± S.E.M.)

4.5.1. Antiplasmodial activity of compounds

Compounds C1, C2, C3 and C4 from petroleum ether extract of *Carapa procera* were tested for their antiplasmodial activity as described in section 3.3.2 at concentrations of 5 µg/ml to 0.0024 µg/ml. The effect of the extracts at the various concentrations on the growth of the malaria parasite was observed. Figure 15 Shows the dose dependent chemosupressive effect of the stem back extracts at various concentrations employed in the study. The extracts showed various degrees of inhibition against the *Plasmodium falciparum* parasites as shown by the area under the curve (AUC) in figure 16. Compound C3 showed a significant level of activity as compared to artesunate (EC$_{50}$ 4.90µg/ml) the positive control. This was followed by C4, C1 and C2 respectively as shown in figure 16. The EC$_{50}$ values were 23.96 µg/ml, 26.66 µg/ml, 30.54, µg/ml 32.68 µg/ml for C3, C4, C1and C2 respectively.

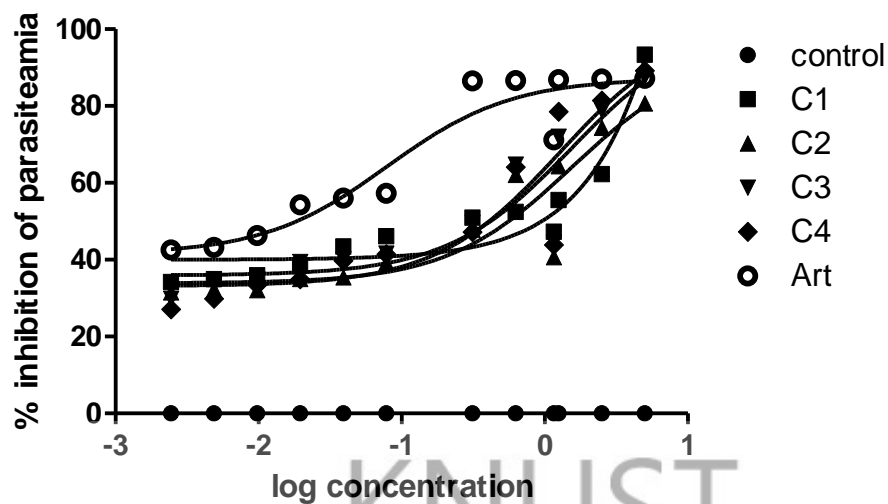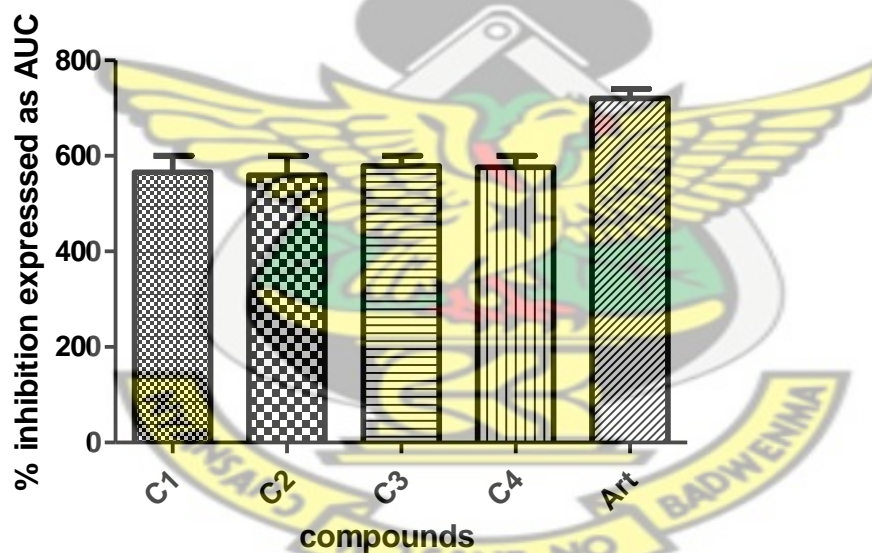**Figure 15: Effect of concentration on parasiteamia after 48 hours of exposure to compounds C1, C2, C3 and C4.**

Figure 16: Effect of Compounds C1, C2, C3, and C4 on total parasiteamia as compared to artesunate (Values are mean ± S.E.M.)

CHAPTER FIVE

DISCUSSION

5.1. LARVICIDAL ACTIVITY OF PLANT EXTRACTS

In the present study, the larvicidal potential of the stem bark of *Carapa procera* (Meliaceae) and aerial part of *Hyptis suaveolens* (Lamiaceae) were investigated against third instar larvae of *Anopheles gambie*.

Carapa procera is used indigenously as a mosquito repellent and insecticide activity. From the results, the petroleum ether extract had the highest activity followed by the ethyl acetate then ethanol extracts with EC_{50} values of 16.91mg/ml, 77.14mg/ml and 186.4mg/ml respectively.

Hyptis suaveolens is also used traditionally as an insecticide for stored grains and the fumes also used to repel mosquitoes. From the results the various extracts exhibited different degrees of activities against the *Anopheles gambie* larvae. The petroleum ether extract had the highest activity followed by the ethyl acetate then ethanol extracts with EC_{50} values of 34.96mg/ml, 159.8mg/ml and 249.7mg/ml respectively.

It was evident from both results that a rise in concentration of the different extracts as well as the different solvents used in extraction had an effect on the larvicidal activity against the mosquitoes. Increase in concentration increased the activity and the petroleum ether extracts had the highest activities. In a previous work, the larvicidal activity of petroleum ether, ethyl acetate and methanol extracts of *Citrullus vulgaris* leaves against *Anopheles stephansi* was investigated. The EC_{50} values were 48.51mg/ml, 49.57mg/ml and 50.32mg/l for petroleum ether, ethyl acetate and ethanol respectively and the activity was dose dependent (Bilal *et al*., 2012).

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Plants are found to contain a complex mixture of bioactive metabolites which affect insect behaviours. Notable among these compounds are limonoids and other plant volatile constituents (Bilal & Hanson, 2012). These compounds are mostly found in the non-polar extacts of plant materials hence the isolation of the possible mosquito larvicides from the petroleum ether extract of *Carapa procera*.

5.2. ANTIPLASMODIAL ACTIVITY OF PLANT EXTRACTS

The emergence and spread of resistance of plasmodium parasites to conventional antimalaria drugs has increased the search for new drugs. Although continued attempts have been made to develop a machine is ongoing, drugs remains the only option for malaria treatment (Nwaka & Hudson, 2006). Plants have always been considered a rich source of new drugs as most of the commonly used antimalarial drugs such as quinine and artemisinin were either obtained directly from plants or developed by modification of plant-derived compounds (Basco *et al.,* 1994). The majority of populations living in many tropical countries depend on plant-based remedies for many ailments including malaria (Zirihi *et al*., 2005). However, there is lack of scientific evidence for most of these plants used in the treatment of malaria.

This study was therefore designed to investigate the antimalarial potential of the stem bark of *Carapa procera* which is used in Ghana for the treatment of malaria.

The lactate dehydrogenase method was employed in these assays using chloroquine resistant strains of *Plasmodium falciparum*. Three different extracts; petroleum ether, ethyl acetate and ethanol of both plants were used.

Previous studies on the *in vitro* antiplasmodial activity of furoquinolie and acridone alkaloids reported EC_{50} values in µg/ml using the following criteria; <10ug/ml (good activity) 10-50 (moderate), 50-100µg/ml (low activity) and >100µg/ml (inactive) (Basco *et al.,* 1994). This criterion was used in this work.

From the results, the ethyl acetate extract of *Carapa procera* stem bark had the a good activity with EC_{50} value of 18.60µg/ml followed by the petroleum ether also having a moderate activity with EC_{50} 24.71µg/ml then ethanol extracts having a low activity with EC_{50} value of 95.46µg/ml Respectively.

This result is comparable to that of a previous study in which the antiplasmodial activity of *Carapa procera* bark was investigated against *Plasmodium falciparum*. From the results, the petroleum ether extract had a moderate activity with EC_{50} value of 19.52µg/ml whereas the ethanol extract also had a moderate activity with EC_{50} of 33.35µg/ml.

Many plants in the genus *Carapa* have been investigated for their antiplasmodial activities. The common and widely investigated of them is *Carapa guinensis* and *Carapa odorata*. These extracts had good activities with EC_{50} values between 1.0 and 10.0µg/ml (Khalid *et al*., 1986; Phillipson & Wright, 1991; Bickii *et al*., 2000). *Carapa guinensis* has been found to contain a number of limonoids which are believed to be responsible for the anti-malarial activity (Miranda *et al.,* 2012). The antimalarial effect of the petroleum ether extract in this work may be attributed to the presence of one or more of these limonoids, thus the isolation of active compounds from the petroleum ether extract.

5.3. BIOLOGICAL ACTIVITY OF ISOLATED COMPOUNDS

Four compounds namely C1 (carapolide B), C2 (proceranolide), C3 (carapolide A) and C4 (evodulone) were isolated from the petroleum ether fraction of *Carapa procera* stem bark.

All four compounds have been previously isolated from the seeds of *Carapa procera*. Proceranolide and evodulone have also been isolated from the stem bark of *Carapa procera* but to the best of our knowledge, Carapolide A and B are being reported for the first time in the stem bark of *Carapa procera*. The four compounds were evaluated for their larvicidal and antiplasmodial activities. All four compounds exhibited various degrees of larvicidal activity but the activities were not dose dependent with EC_{50} values of 15.01mg/ml, 53.74mg/ml, 198.9mg/ml and 273.7mg/ml for C3, C2, C4 and C1 respectively.

For the antiplasmodial activity, all the four compounds had antimalarial activities with the effects increasing as concentration increased. In this case also, compound C3 had the highest activity with EC_{50} of 23.96 µg/ml as compared to artesunate which had an EC_{50} value of (4.90 µg/ml). This activity was followed by that of compounds C4, C1 and C2 with EC_{50} values of 26.66µg/ml, 30.52µg/ml and 32.68µg/ml respectively. The antimalarial activity of the limonoids in this study is comparable to those of other limonoids and purified compounds obtained from other plant families with the highest activity observed with 7α-obacunylacetate (Noster & Kraus, 1990; Koumaglo *et al.*, 1990; Nkunya, 1991; Bickii *et al.*, 2000). The four compound share the same basic structure but slight differences in their side chains may account for the variation in the level of activity between them.

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Compound C3 (Carapolide A) was found to be more active in both bioassays than the extract from which it was isolated. The other three had activities far lower than that of the extract from which they were isolated. This may imply that Carapolide A may be responsible for both the larvicidal and antiplasmodial activities of *Carapa procera*. The other compounds may complement its effect.

Although the four compounds have been previously isolated from the seeds of *Carapa procera*, no biological activity has been assigned to them yet. Proceranolide has also been isolated from the seeds of *Quivisia papinae* which also belongs to the Meliaceae family (Coombes *et al*., 2005). There is no recorded biological activity assigned to the compound. The Meliaceae family however has been found to be rich in limonoids which are tetranortriterpenoid compounds. These compounds have been reported to have a wide range of activities including insecticidal, antifeedant, growth regulators on insects and antimalarial (Roy & Saraf, 2006). Gudinin isolated from *Azadiracta indica, Carapa odorata, Khyaha grandiflora* have been reported to have antiplasmodial activity against *Plasmodium falciparum* (Bickii *et al.,* 2000; Saxena *et al*., 2003). Previous reports have showed that andirobin and 7-oxogudunin which are limoniods isolated from *Carapa guinensis* have antiplasmodial activities against *Plasmodium falciparum* with EC_{50} values of 15µg/ml and 47.3 µg/ml respectively (Pereira *et al*., 2014). In 1979, Sondengam *et al* reported the presence of andirobin and 7-oxogudunin in the hexane extract of seeds of *Carapa procera*. (Sondengam *et al.,* 1979). These compounds may also be found in the stem bark thereby contributing to its antimalaria effect.

The antiplasmodial mechanism of action of limonoids is not known but Tchouya *et al* established that the alpha beta unsaturated ketone ring is an important feature for the antimalarial activity of limonoids (Tchouya *et al.,* 2013).

For the larvicidal activity, *Azadiracta indica* from the family Meliaceae has been widely investigated against various species of mosquitoes. The active compounds found are azadirachtin and a number of limonoids (Warthen, 1979; Schmutterer, 1981).

The mode of action of most larvicides is not known but earlier studies suggest that phytochemicals either react with DNA of the larvae or generate ROS (reactive oxygen species) thereby causing protein and lipid damage in the larvae (Gupta *et al*., 2010). It was also found in another study that phytochemicals affect the midgut epithelium and secondarily affect the gastric caeca and the malpaghian tubules in mosquito larvae (Rey *et al*., 1999; David *et al.,* 2000).

5.4. CONCLUSION

This work has demonstrated that extracts of the stem bark of *Carapa procera* and aerial part of *Hyptis suaveolens* possess larvicidal activity against third instar larvae of *Anopheles gambie*. The petroleum ether extracts showed the highest activities in both plants with the extracts of *Carapa procera* showing higher activities compared to the same extracts from *Hyptis suaveolens*.

The extracts of *Carapa procera* also showed antiplasmodial activities against *Plasmodium falciparum* with the ethyl acetate extract showing the highest activity.

Four tetranortriterpenoids namely **carapolide A** and **B, proceranolide** and **evodulone** were isolated from the stem bark of *Carapa procera*. All four compounds showed larvicidal and antiplasmodial activities with Carapolide A having the highest activity in both bioassays.

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This study has therefore provided scientific evidence for the folklore uses of *Carapa procera* and *Hyptis suaveolens* as antimalarial and larvicidal agents.

Carapolide A and B are being reported for the first time in the stem bark of *Carapa procera*.

5.5. RECOMMENDATIONS

Based on the results from this study the following may be considered for future work on the two plant specimen.

1. Structural modification of carapolide A should be considered to improve its larvicidal and antiplasmodial activities.

2. *Carapa procera* stem bark and *Hyptis suaveolens* aerial part can be formulated into mosquito larvicides for the control of mosquito larvae in their breeding sites.
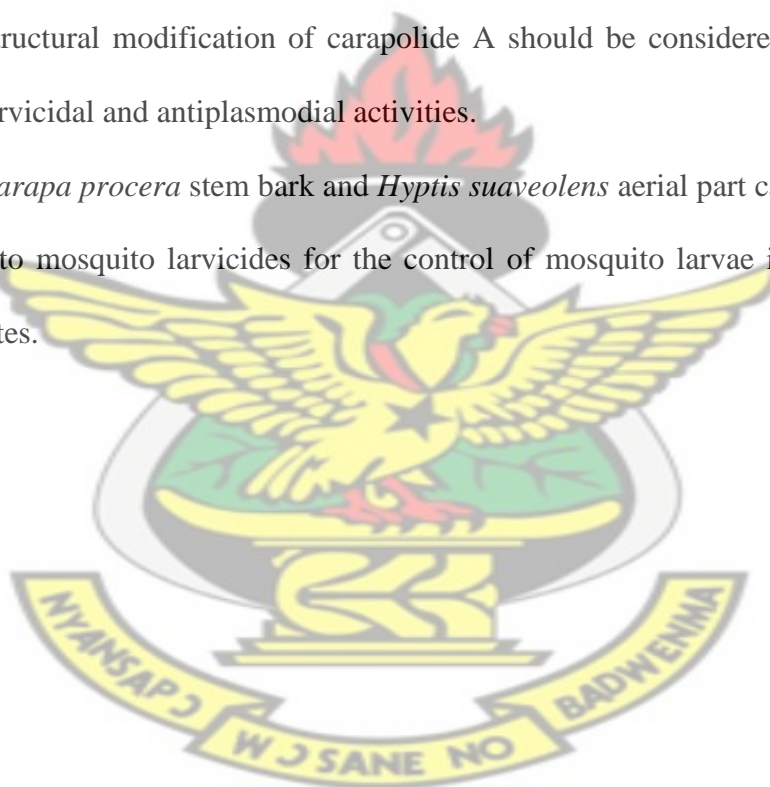
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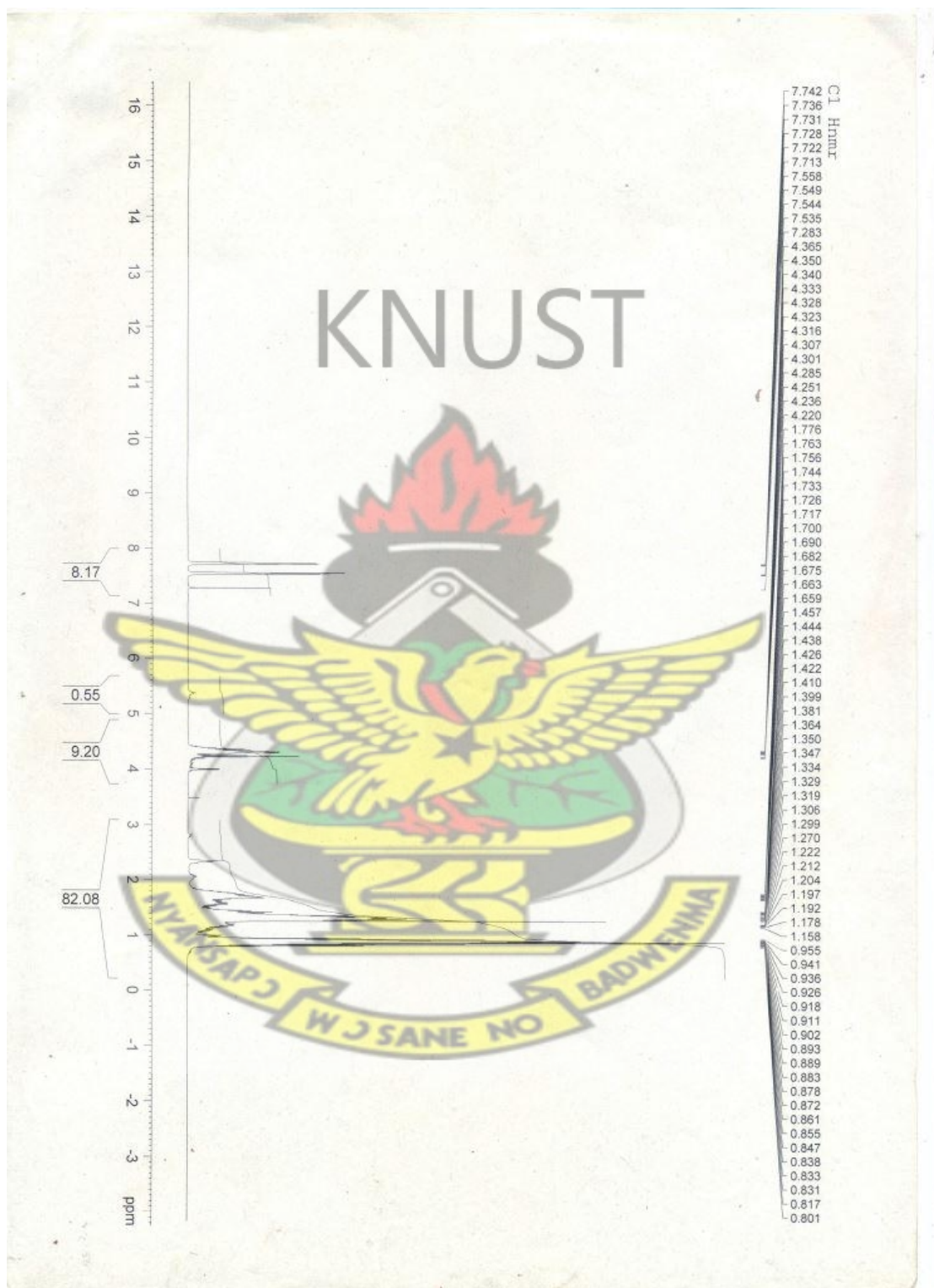
APPENDIX 1

Figure A- 1 ¹H-NMR SPECTRA OF COMPOUND C1

APPENDIX 2

Figure B- 1 ^{13}C-NMR SPECTRA OF COMPOUND C2

Figure B- 2 ^{1}H-NMR SPECTRA OF COMPOUND C2

APPENDIX 3

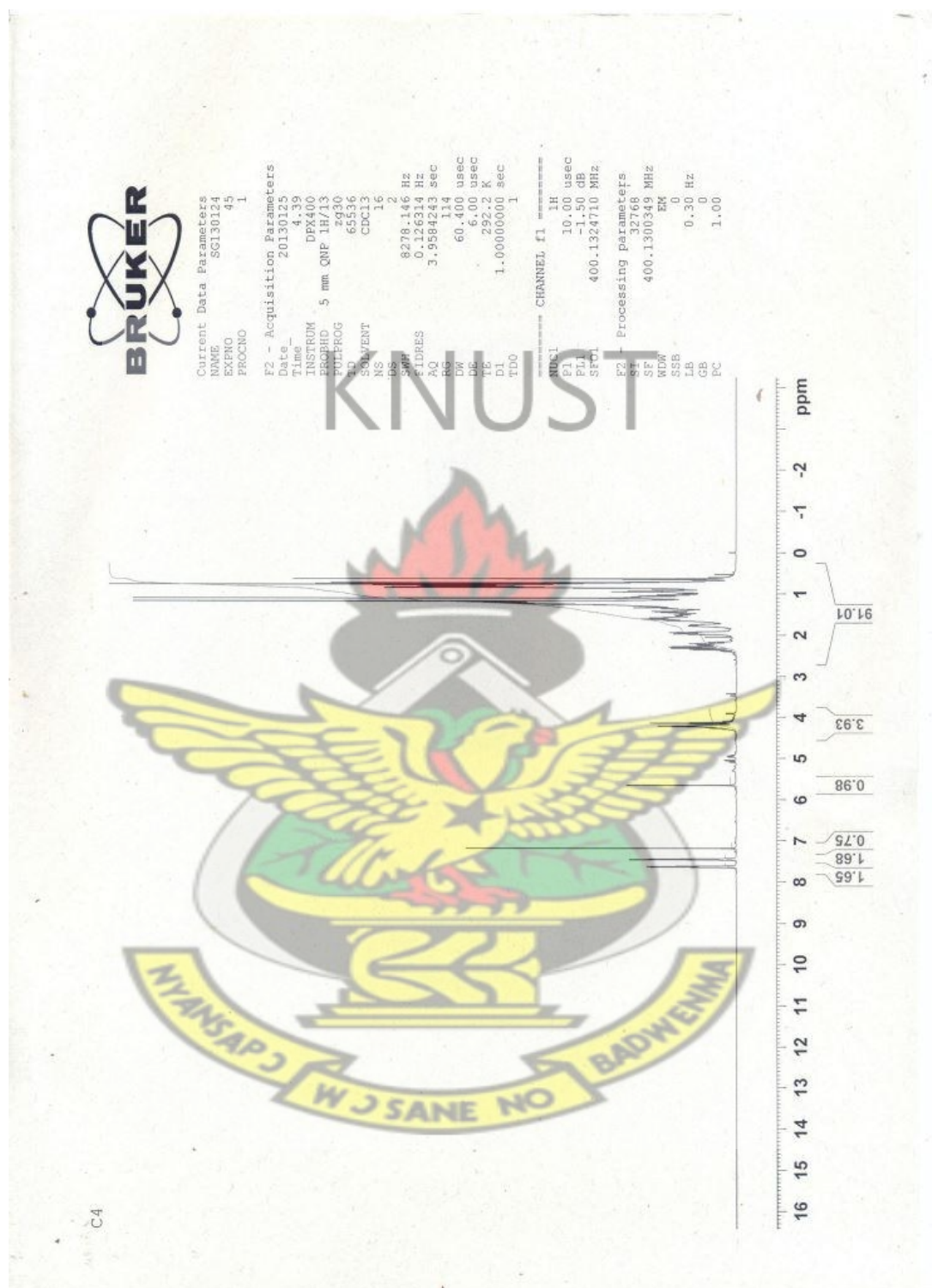**Figure C- 1 ^{13}C-NMR SPECTRA OF COMPOUND C3**
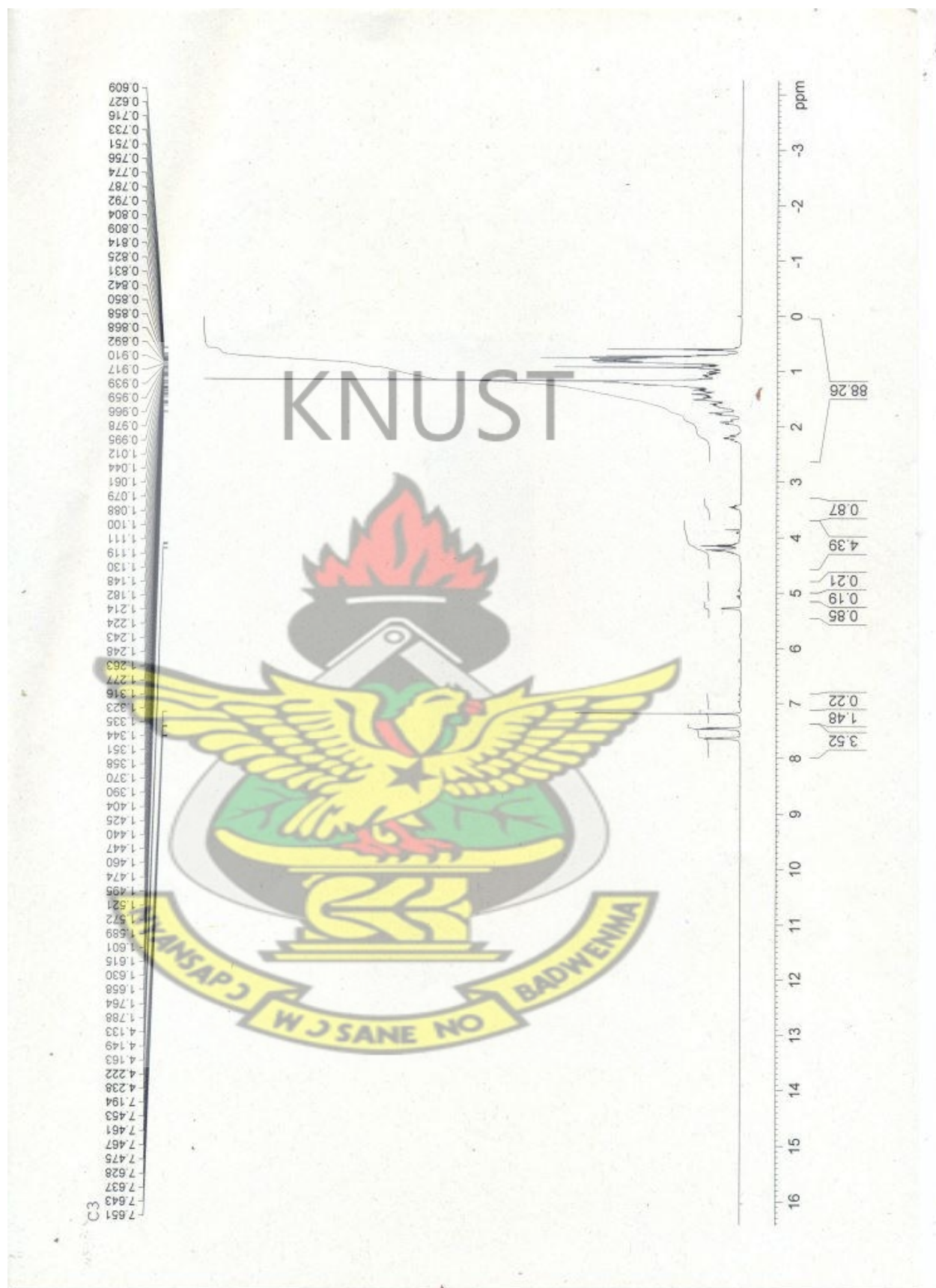
Figure C- 2 ^{1}H-NMR SPECTRA OF COMPOUND C3

APPENDIX 4

Figure D- 1 ^{1}H-NMR SPECTRA OF COMPOUND C4

Figure D- 2 ^{13}C-NMR SPECTRA OF COMPOUND C4