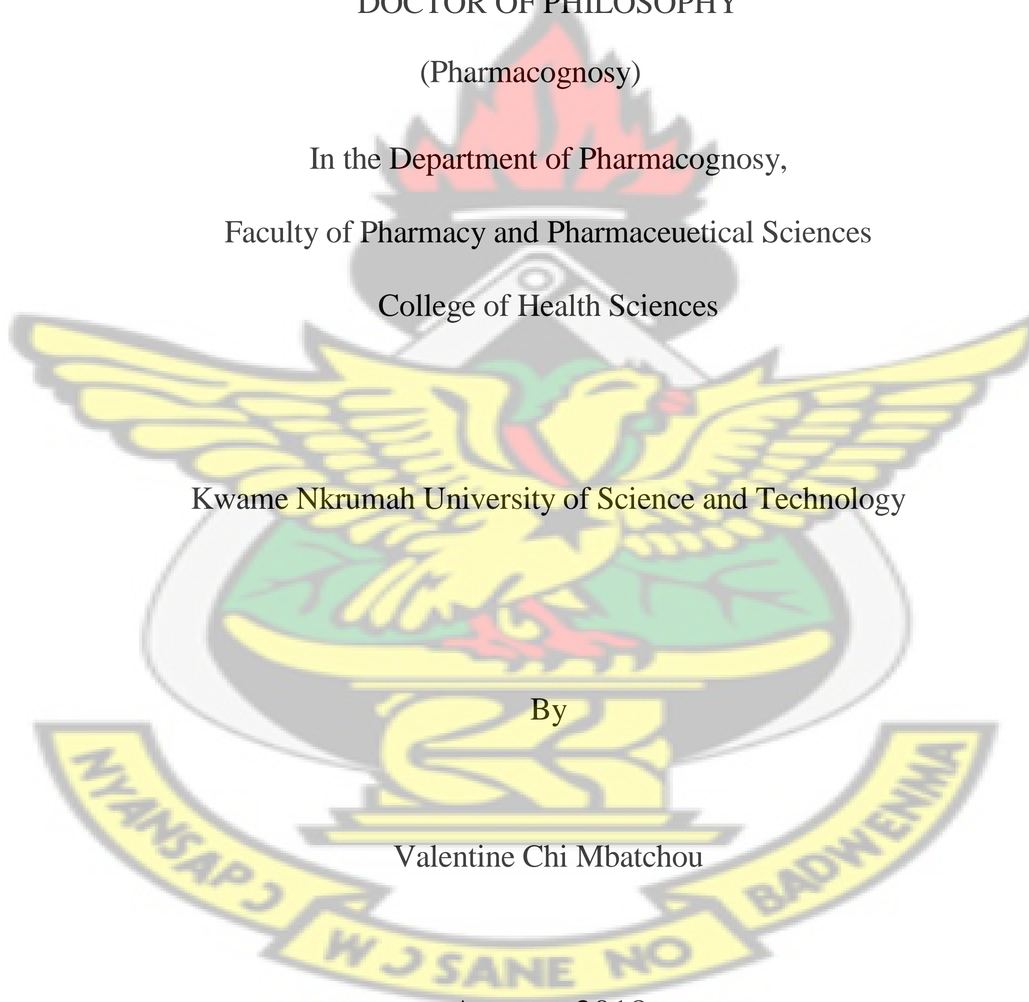
PESTICIDAL AND GERMINATING ENHANCING RELEVANCE OF ANTHRAQUINONES FROM *CASSIA TORA* (LINN) SEEDS

KNUST

A Thesis submitted in partial fulfillment of the requirements for the degree

of



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By

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DECLARATION

I hereby declare that the research work described in this thesis is my work submitted for the award of PhD and to the best of my knowledge, it does not contain materials published by another person or materials which have been accepted for the award of any other degree of the University, except where due acknowledgement has been made in the text.

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DEDICATION

I dedicate this thesis to my late and beloved mother Mrs. Rebecca Queenkem Mbatchou

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ABSTRACT

Vigna unguiculata Lam (Cowpea) seeds is the major essential leguminous crop cultivated in sub-Saharan Africa and occupies a greater area of land than any other legume. The leaves, green pods, green peas and dry grains of cowpea are eaten all over the continent in different dishes for being nutritious to both humans and animals. *Callosobruchus maculatus* (Cowpea weevils) are known to destroy up to about 70 % of the seeds in storage. Pre-treatment of the seeds before storage includes the use of neem oil and cinnamaldehyde but both have been observed on the field to be toxic to the seeds and also human health. Seeds treated in this way have shown very low germinability. Thus, alternative safe and ecofriendly pesticides are needed to replace these standard agents. *Cassia tora* is grown on farmlands alongside food crops in the South-West Region of Cameroon as a protectant to seeds/grains against insect pests. Therefore the present study sought to investigate its edible seeds for ecologically friendly pesticides against *Callosobruchus maculatus* (cowpea weevils) and its potential as enhancers of cowpea seed-germination and seedling-vigor as folklore suggest. The study also explores the most active *C. tora* seed extract and its constituents for larvicidal activity against *Anopheles gambaei* third-fourth instar larvae. The petroleum ether (pet-ether), ethyl acetate and methanol extracts of *C. tora* were tested for insecticidal, oviposition deterrent, feeding inhibition, germination and seedling vigor enhancement effect on cowpea seeds using neem oil and cinnamaldehyde reference compounds. Pet ether extract of *C. tora* seeds showed the highest insecticidal activity (LC_{50}= 8.33±0.6 μg/mL) against adult weevils, followed by the ethyl acetate and methanol extracts respectively. The ethyl acetate showed

the highest germination and seedling vigor enhancement effect. The ethyl acetate extract was selected and assessed for mosquito larvicidal activity against the larvae of the malaria vector *Anopheles gambiae*. It exhibited larvicidal activity that was comparable to the positive control azadirachtin. It was then subjected to column chromatographic purification to study its bioactive compounds. The anthraquinones aurantio-obtusin, obtusin and a novel compound cassiatorin were isolated and assessed for various pesticidal activities. Aurantio-obtusin and cassiatorin showed comparable antifeedant activity as the reference compounds. Aurantio-obtusin and cassiatorin were the most effective enhancers of germination and vigor of the cowpea seeds and seedlings respectively when compared with the negative control. *Cassia tora* seed extracts and its anthraquinones aurantio – obtusin and obtusin possess larvicidal activity against *Anopheles gambiae* third-fourth instar larvae, and could serve as eco-friendly larvicides for control or management of mosquito population. Thus the present study gives credence to the folkloric use of *C. tora* seeds for crop/grain-protection from insect pest infestations as well as crop yield-enhancement. Unlike the reference drugs, *C. tora* seed extracts and isolated compounds were not toxic to the seeds and showed over 70% germination and seedling vigor enhancement activities. These activities are being reported in the plant (*C. tora*) for the first time. Even though the anthraquinones aurantio-obtusin and obtusin have been isolated from *C. tora* and other cassia species, this is the first report of their insecticidal, oviposition deterrent, antifeedant, germination and seedling vigor enhancement activities. A novel compound, 1, 3, 8-trihydroxy-2, 7-dimethoxy-6-methyl-9, 10-anthraquinone is being reported for the first time and is given the trivial name

‘cassiatorin’ in conformity with the naming of anthraquinones from *Cassia species* using their botanical origin.

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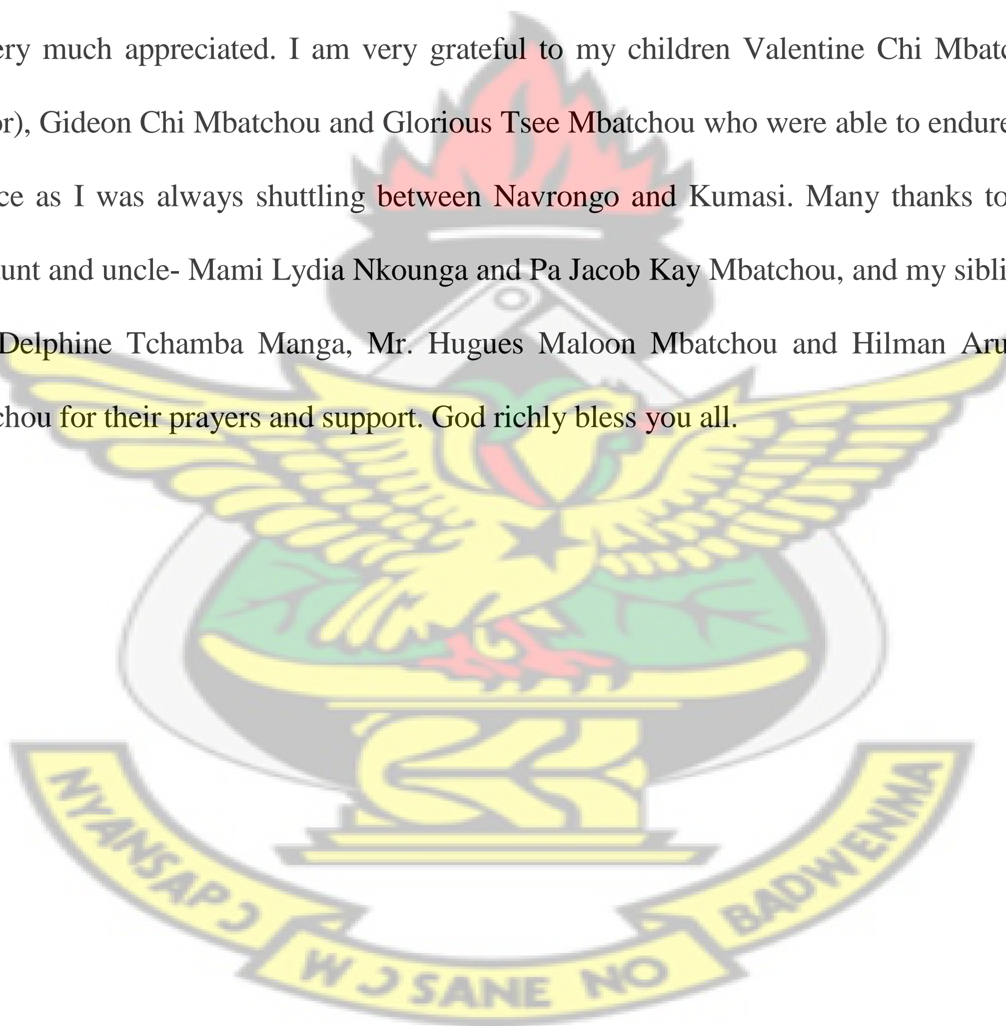

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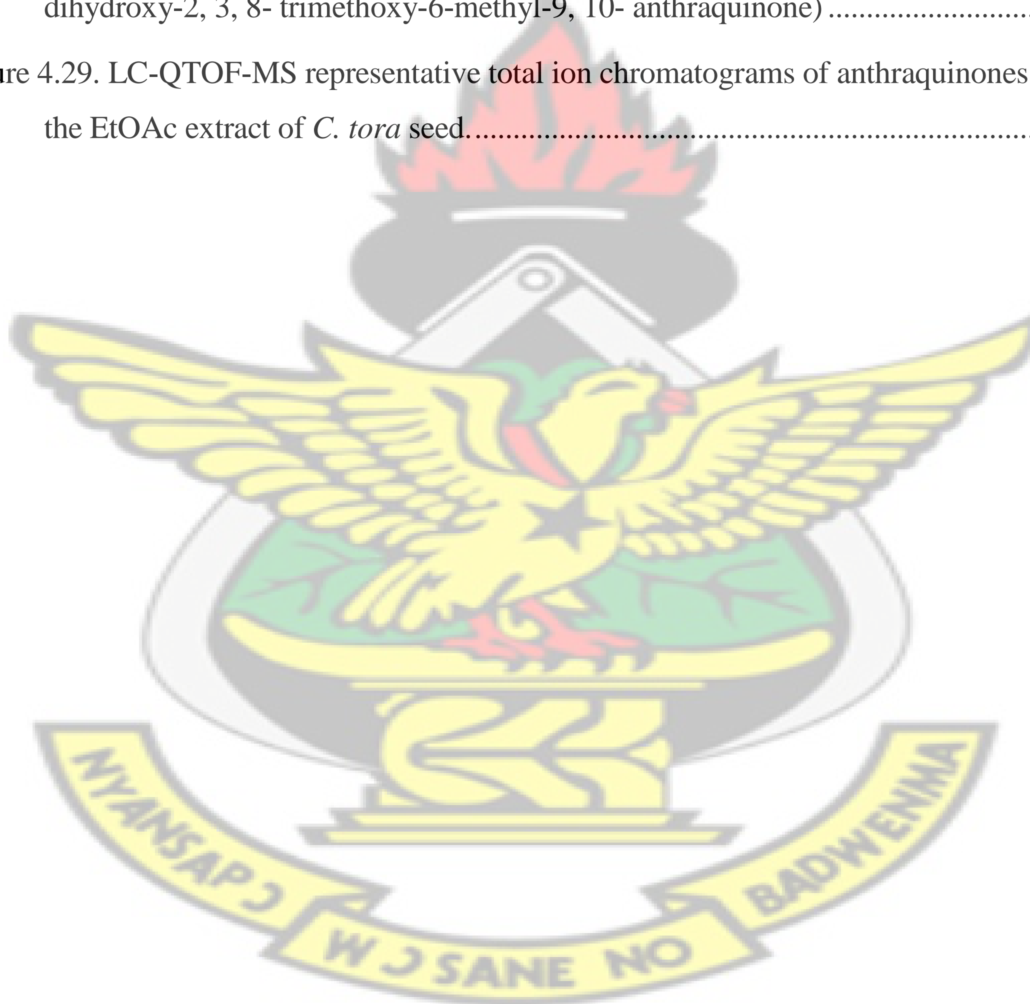
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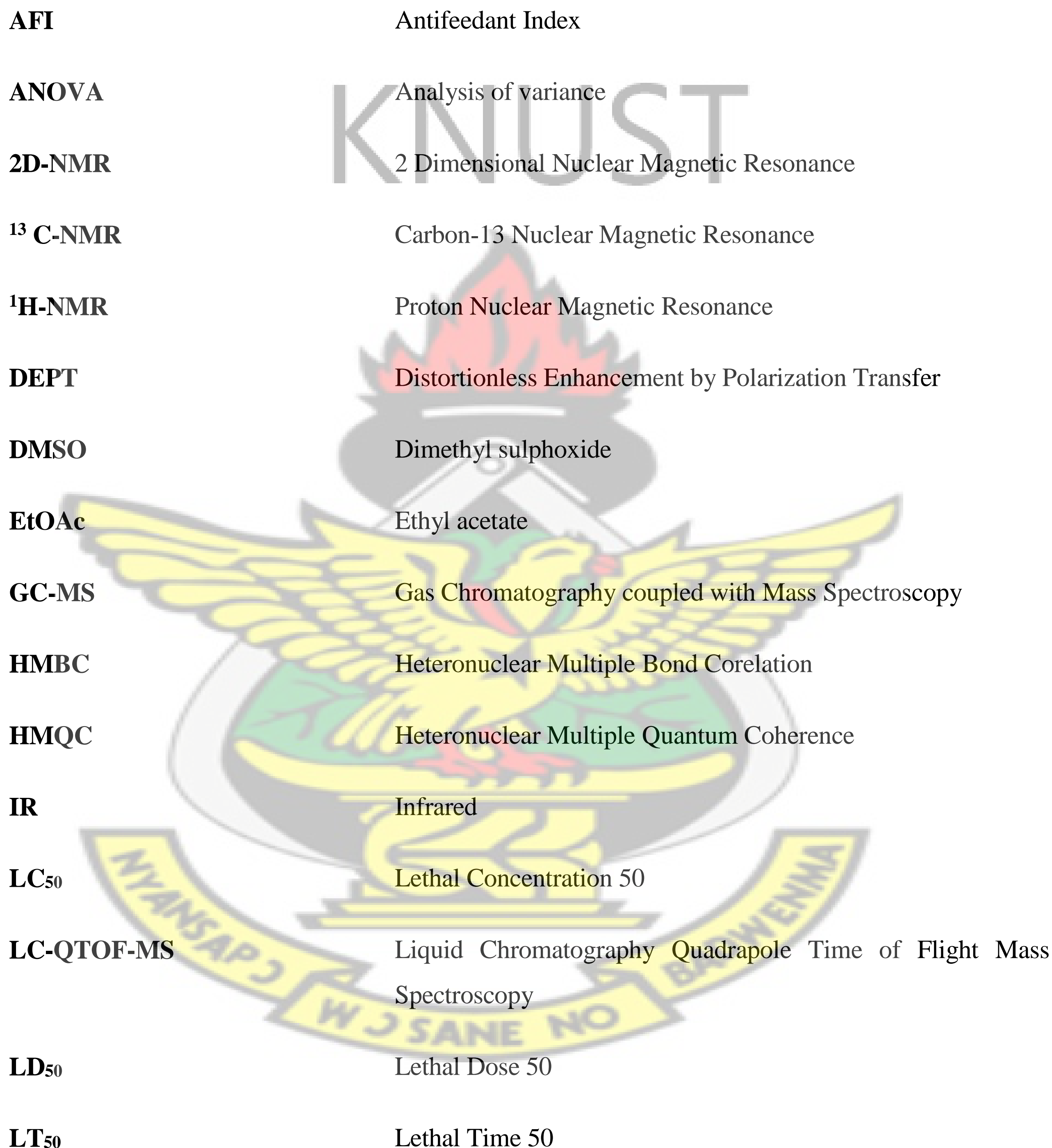
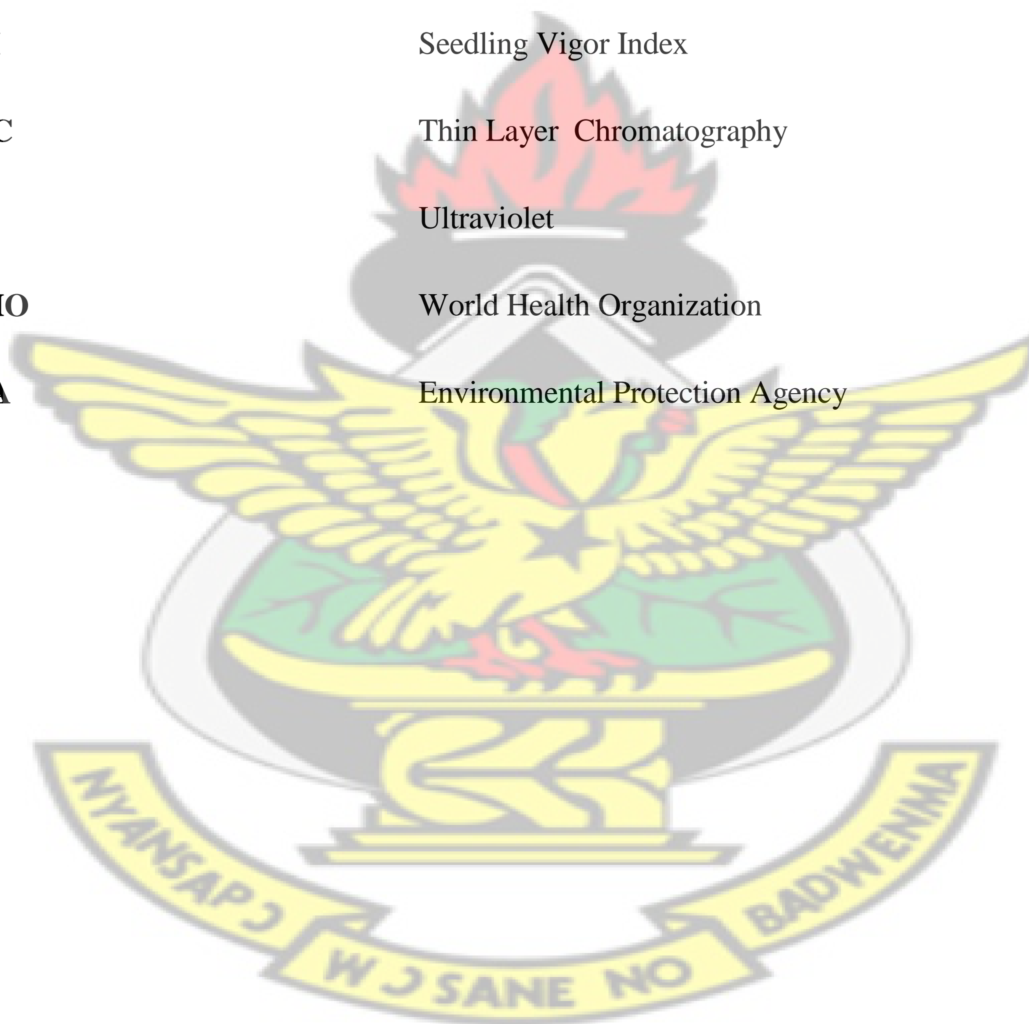


AFI	Antifeedant Index
ANOVA	Analysis of variance
2D-NMR	2 Dimensional Nuclear Magnetic Resonance
^{13} C-NMR	Carbon-13 Nuclear Magnetic Resonance
^{1}H-NMR	Proton Nuclear Magnetic Resonance
DEPT	Distortionless Enhancement by Polarization Transfer
DMSO	Dimethyl sulphoxide
EtOAc	Ethyl acetate
GC-MS	Gas Chromatography coupled with Mass Spectroscopy
HMBC	Heteronuclear Multiple Bond Corelation
HMQC	Heteronuclear Multiple Quantum Coherence
IR	Infrared
LC$_{50}$	Lethal Concentration 50
LC-QTOF-MS	Liquid Chromatography Quadrapole Time of Flight Mass Spectroscopy
LD$_{50}$	Lethal Dose 50
LT$_{50}$	Lethal Time 50

MeOH	Methanol
NMR	Nuclear Magnetic Resonance
ODI	Oviposition Deterrence Index
Rf	Retardation factor
Rt	Retention time
SVI	Seedling Vigor Index
TLC	Thin Layer Chromatography
UV	Ultraviolet
WHO	World Health Organization
EPA	Environmental Protection Agency



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Chapter 1

INTRODUCTION

1.1 NATURAL PRODUCTS AS SOURCES OF DRUGS AND PESTICIDES

Natural products have been used as effective source of drugs and pesticides in virtually all cultures since time immemorial. Their varied applications against diseases and pests originated from folklore medicine and can be linked to their phytochemical principles (Zaker, 2016; Seabrooks and Hu, 2017).

Interest in the use and development of plant natural products for pest management started in the early 1930s and continued to the 1950s, but was phased out when synthetic pesticides were discovered (Dubey *et al*., 2010). The negative effects of synthetic pesticides on plants, animals and the environment necessitated phyto-pesticides to resurface. This resurgence resulted from effective use against pests with lesser side effects on non-targeted plants, animals and the environment compared to synthetic pesticides (Ahmed *et al*., 1984). More than 2,000 plant species have been reported to possess chemicals with pest control properties and close to 500,000 secondary metabolites or phytochemicals are estimated to have been isolated from plants (Ascher, 1993; Ahmed *et al*., 1984).

The chances of finding new molecules from plant products are immeasurable. For this reason, plant products and their derivatives constitutes a significant source of currently available therapeutic and pesticidal agents (Kirkpatrick, 2002). They contribute about 30 % of the drugs in the pharmaceutical market. It is reported that of about 877 new molecules introduced into the market between the 1981 and 2002, 49 % of them were isolated compounds from natural

sources and their semi-synthetic derivatives or synthesized molecules resembling the structures of molecules of natural origin (Newman *et al*., 2003).

Plants synthesize different types of chemicals which interact with the environment. The compounds enhance their communication with microbes, animals and other plants. Natural product molecules may serve as attractants to beneficial insects or microbes, whereas others serve as insecticides or repellents (Theis and Lerdau, 2003).

These secondary metabolites are therefore perfect substitutes to synthetic pesticides which cause severe harm to humans and the environment (Ramechwar, 2010). The move towards green chemistry processes and the need for finding new crop grain protection agents make discovery and marketing of natural products as green pesticides attractive, profitable and therefore commands attention (Isman and Machial, 2006). The concept of "Green Pesticides" entails different types of nature-oriented and beneficial pest control ingredients that can reduce pest population and maximize food production (Ramechwar, 2010). Green pesticides are safe, ecofriendly and more compatible with the environment than synthetic pesticides (Isman and Machial, 2006). Thus, in the present concept of green pesticides, some attempts have been made to include plant extracts, hormones, pheromones and toxins of organic origin, aspects of pest control such as microbial, entomophagous nematodes, plant derived pesticides and secondary metabolites from microorganisms that transform crops to resist pests. The use of ingredients from natural resources and the extremely biodegradable synthetic and semisynthetic products in pest management, is considered under the umbrella of green pesticides (Dhaliwal and Koul, 2007; Koul, 2008).

Several plants and their extracts have been used locally to protect stored commodities, especially in the Mediterranean Region and Southern Asia. Plant oils have particularly been of interest, in this regard, due to their emerging fumigant and contact insecticidal activities to a wide range of pests in the 1990s (Isman, 2000). The immediate action of the plant oils against some pests has been reported to be as a result of their interference with the neuromodulator octopamine and with γ-aminobutyric acid-gated chloride channels (Kostyukovsky *et al*., 2002; Priestley *et al*., 2003). Eugenol and thymol, found in some oils, have been reported to be effective as a feeding deterrent against Asian armyworm (*Spodoptera litura* Fabricius) (Hummelbrunner and Isman, 2001). Menthone, trans-anethole and cinnamaldehyde are well known insecticidal compounds that have shown fumigant and antitermitic activities against a wide range of insects (Chang and Ahn, 2001; Chang and Cheng, 2002).

A number of plants have also been identified as potential sources of insect repellents. However, only a few of these and their derived substances have demonstrated the qualities of good repellents (Cockcroft *et al*., 1998). Some of these plants include basil, citronella, cedar, cinnamon, geranium, lavender, pennyroyal, peppermint, pine, rosemary, thyme and verbena (Choi *et al*., 2002; Traboulsi *et al*., 2002). Lemon, eucalyptus, geranium and lavender oils have exhibited repellent activity against *Ixodes ricinus,* a castor bean pest in the field and laboratory (Jaenson *et al*., 2006).

Plants and their compounds have also demonstrated ability to hinder or obstruct egg laying (oviposition deterrents) and as ovisides. For example, *Acorus calamus* oil oviposition deterrent effects was reported against female *Callosobruchus maculatus* (Dimetry *et al*., 2003). Garlic oil has shown oviposition deterrent and ovicidal effects against *Plutella xylostella* females and

eggs respectively (Govindaraddi, 2005). Essential oils from *Aegle marmelo* have been reported to have caused reduction in egg hatching by *Spilosoma obliqua* insects (Tripathi *et al*., 2003). 1-Carvone has also demonstrated reduction in egg hatching by *Tribolium castaneum* insects (Tripathi *et al*., 2003). Carvacrol, carveol, carvones, cinnamaldehyde, citral, citronellal, cinnamic acid, fenchone, geraniol, linalool, menthone, menthol, pulegone, terpineol, thujone, thymol, verbenol, and verbenone demonstrated ovicidal activity against *Musca domestica* eggs (Rice and Coats, 1994).

Despite the considerable research works carried out all over the world and the large volumes of scientific data on pesticidal properties of essential oils and their constituents, very few pest control products containing plant essential oils have been commercialized. This may be due to the very expensive nature of toxicological and environmental assessements or the fact that essential oils used against pests and diseases are not as active as currently available products. In the United States of America, pesticide production for commercialization using plant essential oils has been greatly encouraged by exempting such oils from registration (Quarles, 1996). This has greatly enhanced the production of essential oil-based insecticides, fungicides and herbicides for agricultural and industrial purposes and for the consumer market. Rosemary, clove, and thyme oils are often used as active ingredients in these products to control greenhouse gases, domestic and veterinary pests and diseases. The United State based company, EcoSMART Technologies has developed insecticides containing eugenol and 2-phenethyl propionate to control crawling and flying insects, under the trade name EcoPCO® for pest control. An insecticide/miticide made of rosemary oil with the name EcoTrolTM has also been recently commercialized for use on horticultural crops (Koul *et al*., 2008; Dubey *et*

al., 2010), while a fungicide made from rosemary oil is marketed with the name SporanTM. Numerous smaller companies in the U.S.A. and the U.K. have commercialized pest control products prepared from garlic-oil (Koul *et al*., 2008).

1.2 PROBLEM STATEMENT AND JUSTIFICATION OF RESEARCH

Forecasts prediction have stated the need for sub-Saharan African countries to increase grain import to 50 million tonnes in the highly competitive world market to match the food demand of its population that has risen by 53 %. Also reports have revealed that food production has risen only by 45 %, and over 800 million people in developing countries are starving and depended on food aid (Agada and Igbokwe, 2015). These are issues of concern associated with agricultural production. Research efforts to ensure enough food crop cultivation to meet the demand of increasing human population has led to the revelation that losses in food crop yields is perhaps one of the major problems confronting food production in sub-Saharan African countries. To minimize post-harvest losses, harvesting, handling and storage methods must be improved to match increase crop production (Oyewole and Oloko, 2006; Muhammad *et al* 2012).

Food and Agriculture Organization reported that 23 % of the population of developing countries consume 20 % below the level of food intake required to sustain life (FAO 2001). Protein, a major class of food, is reportedly not always present in the diets of people in developing countries and its deficiency causes malnutrition and promotes spread of human disease. Nigeria, Niger, Ghana, Burkina Faso, Senegal and Cameroon not being exceptions are into cowpea cultivation, a good source of plant proteins to meet the nutritional need of their populations which has been increasing to the present day (FAO 2001; Okorie, 2005). Despite

ongoing investment in agriculture, *Callosobruchus maculatus* (cowpea weevil) has been a serious threat to boosting of cowpea seed production.

The cowpea weevil is a cosmopolitan pest whose larvae consume substantial quantity of cowpea (*Vigna unguiculata*) seeds in a year, although accurate values of cowpea seed consumed are not available. A researcher has documented 50 % loss in weight of cowpea seeds at Ibadan and slightly above 30 % at Zaria in 1976. All over tropical Africa, *C. maculatus* larvae consume 50-90 % of cowpea seeds in storage each year (IITA, 1989). Cowpea seeds stored for six months have shown a damage of 70 % as a result of seed infestation, about 30 % weight loss to be virtually unfit for consumption (Singh and Jackai, 1985). Infestations by weevil can cause up to 60 % loss in seed weight and up to 66 % loss in protein content. Eggs of weevil glued on the surface of seeds, frass deposits and larval emergence holes in seeds are evidence of infestations in storage. Slight weevil feeding damage to the embryo of seeds inhibits germination. Consumption of seed cotyledons by weevil's larvae does not affect seed germination but reduces the vigor of seedlings (Talekar, 1988). The amount of cowpea seed fed on by larvae is very crucial because of altered nutritional and economic qualities. Heat, moisture and waste products generated from the activities of the weevils and its larvae also cause deterioration, growth of moulds and development of aflatoxins. This makes the cowpea seeds unfit for consumption and not marketable (Shiaza *et al*., 2006). Hence, cowpea weevil larvae in stored seeds are a major problem and affect the food basket of most African countries. Thus one of the greatest challenges encountered in agriculture in developing countries is post-harvest losses which often take place during storage (Adedire *et al*., 2011).

The cowpea weevil has been identified as the major post-harvest insect pest of cowpea seeds. Infestation of cowpea seeds by weevils starts in the field just before harvest and is later carried into the store where the insect population increases rapidly. The high post-harvest losses and poor quality of cowpea seeds caused by this weevil is a major hindrance to achieving food security in developing countries. Annual production loss of 5 %, equivalent to 40,000 tonnes of cowpea seeds caused by *C. maculatus* in Nigeria alone amounted to about $100 million (Singh and Ntare, 1985). The larval stages are the most harmful because adult insects lack snout (feeding mouth path) and do not feed (Ofuya, 2001; Gbaye and Holloway, 2011).

The use of synthetic insecticides and antifeedants on farmlands and stored grains to counter cowpea losses has been difficult because of their non-availability and costs, and in some instances caused food poisoning in human and aquatic organisms, and other environmental hazards as a result of their non-degradable nature. Synthetic insecticides have not solved the problem of cowpea seed infestation by insect pests. Murdock (2002) stated in his report that synthetic insecticides have failed to reduce insect pests of cowpea, and that cowpea yields in Africa are far below the expected yields. Besides being expensive, they require costly equipment to manage their use, train users and are potentially harmful (Murdock, 2002). Several attempts made in the past to minimize pest infestations on crops by the use of botanicals have been in favour of storage pests' control. Some of these attempts have revealed the long-term assessment of the potential use of plant extracts for integrated management of the storage pests of cowpea. Bamaiyi *et al.*, (2006) have reported that *Khaya senegalensis* seed oil significantly minimized the emergence of progenies and lowered the damage to cowpea seeds by *C. maculatus*. Swella and Mushobosy, (2007) reported that black pepper (*Piper nigrum*)

powder and coconut oil, having similar activity as the synthetic Actellic dust, protect cowpea grains in storage against *C. maculatus* damage on timely applications. Cowpea seeds treated with the powders of black pepper fruits, leaves of *Eucalyptus*, *Croton gratissilum*, *Spirostychus africana*, *Ochna pulera*, *Solanum nigrum* and *Syzygium aromaticum* flowers were better protected from *C. maculatus* infestations than untreated seeds. Thus, more research is required to explore natural pesticides available in the local settings for a more sustainable approach in reducing the population of storage pests (Shiaza *et al*., 2006). Neem, African curry, lemongrass, tomato, Eucalyptus, African bush tea and bitter leaf extracts mixtures have greatly reduced pod damage per cowpea plant and ensured higher grain productivity in a two year investigation (Oparaeke *et al*., 2005). There is the necessity to investigate insecticides and antifeedants of natural origin to replace harmful ones.

The Southwest Region of Cameroon falls within an evergreen tropical rainforest, characterized by tall trees, with very rich biodiversity and volcanic soils which harbour pests, weeds and pollinators to food and cash crops grown on farms. The presence of *Zonocerus variegatus L.* (Green locust), commonly known as 'smelling grasshopper' or 'rainbow grasshopper' is a major concern in this area. It has a dormant life cycle in the rainy season of which it exists in the form of an egg and resurfaces after hatching in the dry season to invade the ecosystem and newly cultivated farms. The destruction caused by *Zonocerus variegatus* L. at this time is always severe (Sevilor *et al.,* 2006a). It destroys over 50 % of crops cultivated in the rainy season. It feeds on all fresh green leafy plant materials, including crops like egusi (60-70 %) being its delicacy, young maize (50-60 %), sweet potatoes (55-65 %), oranges (20-40 %), cassava (50-60 %), banana (45-55 %), plantain (40-55 %), oil palms (40-55 %), groundnuts

(55-65 %), garden eggs (55-65 %), coffee (45-55 %), cocoa (45-55 %) and okro (50-60 %). Farmers and agric extension workers in the region have observed that *Zonocerus variegatus* L. is a threat and it is the highest crop pest which is difficult to control because it is highly mobile and has caused food shortages and famine in years of extended dry seasons (Suka, 2011). Little or no success has been achieved by the application of different methods including manual trapping and chemicals through the use of insecticides. Farmers have highlighted low usage of chemical methods and a complete absence of biological and ecological methods (Sevilor *et al.*, 2006a). The practice of indigenous agro-forestry using the *Cassia tora* system on the farmlands of the South-West Region of Cameroon offers a natural environment which prevents *Zonocerus variegatus* L. from causing severe damage to both mixed cropping and mono-cropping (Sevilor *et al*., 2006b). In a study, maize and other plants intercropped with *Cassia tora* L.in South-West Cameroon, revealed toxicant and antifeedant activities of *Cassia tora* L. on *Zonocerus variegatus* (Suka, 2011). *Cassia tora* L. is cheap and environmentally safe to be grown, has increased benefits, maintain ecology and biodiversity. It also eliminates invasive weeds and pests such as *Centrosema pubescens* and *Zonocerus variegatus* L. (Suka, 2011). From the foregoing, it is imperative that identification of the chemical components or compounds in *Cassia tora* L., responsible for the observed activities be carried out. It is also necessary to assess *Cassia tora* seed extract for possible insecticides, ovipositor deterrents and antifeedants using cowpea weevils on cowpea seeds in storage. Again, *Vigna unguiculata* (cowpea) seeds were investigated for enhancement of germination and vigor of seedlings after treatment with *Cassia tora* seed extracts and subjection to pesticide bioassay experiments.

1.3 AIM OF THE STUDY

This study seeks to investigate extracts of *C. tora* seeds for insecticidal, oviposition deterrent and feeding inhibition properties against *C. maculatus* on *Vigna unguiculata* (Cowpea) seeds and evaluate the viability of cowpea seeds after treatment with *C. tora* seed extracts and constituents. The study also aims to explore the most potent seed extract of the plant for larvicidal potential against third-fourth instar larvae of the vector *Anopheles gambiae*.

1.4 SPECIFIC OBJECTIVES OF RESEARCH

The research work is undertaken to:

1. investigate *Cassia tora* seeds for insecticidal, oviposition deterrent, feeding inhibition, germination enhancement and seedling vigor effects in a single application on cowpea seeds.
2. assess the most potent seed extract for larvicidal potential against *A. gambiae* third and fourth-instar larvae.
3. isolate compounds from the most active seed extract using a number of chromatographic techniques.
4. investigate constituents of the most active seed extract for insecticidal, oviposition deterrent, feeding inhibition, germination enhancement and seedling vigor effects in a single application on cowpea seeds, and larvicidal potential against *A. gambiae* third and fourth instar larvae.
5. characterize isolated compounds using spectroscopic methods

Chapter 2

LITERATURE REVIEW

2.1 PESTICIDES

Pesticides are a group of chemical agents that kill, destroy or reduce numbers of pests, and cause nuisance to pests (James, 2012; Chandra, 2014). They include rodenticides, piscicides, bactericides, miticides, fungicides, herbicides, insecticides, molluscicides, nematicides, acaricides, algaecides, ovipositor deterrents, feeding inhibitors, growth retardants and repellents. They are introduced to the environment with the aim to reduce plant and animal pest population, protect agricultural and industrial products (Chandra, 2014).

Most pesticides do not target pests only but may also affect non-target plants and animals. Depending on their chemical properties, they penetrate organisms, accumulate in food chains and consequently affect human health. Many pesticides do not easily degrade and their routine application may lead to loss of biodiversity. They remain non degradable in the soil, leach to ground and surface water, and pollute the environment. Thus, the negative effects of pesticides on living organisms and the environment cannot be ignored (Bakkali *et al*., 2008; James, 2012).

2.1.1 Synthetic pesticides

Synthetic pesticides usage in pest control programmes around the world has yielded toxic effects on non-targeted organisms (Abdulai *et al*., 2001). They become harmful if food contaminated by them is ingested. They include the organochlorines, organophosphates, carbamates and pyrethroids (Finlay, 2006: James 2012).

Organochlorines persist in living systems and the environment due to their high stability to heat, partial solubility in either water or organic solvents and low vapour pressure. In humans, they act on the central nervous system altering electrophysiological properties and enzymatic neuronal membranes resulting in seizures, acute poisoning, respiratory arrest and even death (James, 2012). Their presence in food chains has made species at the top of chains to be adversely affected, resulting in ecological imbalance (Dent, 1991). Diphenyl dichloro trichloroethane (DDT) was widely used in the past to control malaria. Its acute toxicity led to decline in the population of birds, fishes and some lower aquatic organisms after the consumption of foods contaminated by it (Hill and Waller, 1982; Pimentel, 1971). Ingestion of aldrin, dieldrin, heptachlor and chlordane resulted in symptoms such as headache, dizziness, nausea, vomiting and convulsion. Dibromochloropropane and ethylene dibromide usage for the control of nematodes caused pulmonary congestion and mild depression of the central nervous system when inhaled. The bipyridyl herbicide, paraquat, is toxic to the lungs and kidneys. Its mechanism of action in relation to toxicity is focused on its ability to produce superoxide anions (Bus *et al*., 1976).

Organophosphates are sulphur and phosphorus compounds which function by inhibiting the respiratory enzyme acetylcholinesterase. They are more poisonous to mammals and are degraded in 2-4 weeks. Their systemic action is mostly against phloem feeding insects. Malathion, parathion, dimethoate, acephate, phorate, chlorphyrifos and dichlorvos have encountered resistance from strains of target insects (Schaafsma, 1990; Taylor, 1994; Ahmed and Naqvi, 2011). Carbamates operate in a similar manner as organophosphates by inhibiting the activity of acetylcholinesterase enzyme. Carbamates are esters of carbonic acid and usually

act topically or systematically. These pesticides are less persistent than organochlorines and organophosphates, and degrade in about 4 weeks (Ahmed and Naqvi, 2011).

Another group of pestcides are the synthetic pyrethroids which originated from natural pyrethroids present in flowers of *Pyrethrum cinearaefolium*. Currently, they are the most widely used synthetic pesticides since they are less toxic to mammals and more toxic to insects (Ahmed and Naqvi, 2011). The naturally occuring pyrethrins have quick knockdown effect on insects, but are less poisonous than synthetic pyrethroids which take 2-4 weeks to degrade to half their initial composition. They affect the central nervous system, altering the dynamics of Na^+ channels in the membrane of nerve cells, extending the time of creation of sodium current across the membrane by increasing its opening time in both insects and vertebrates and can lead to neuronal hyper-excitation. Synthetic pyrethroids have a low persistence and are effective at very low doses (Ahmed and Naqvi, 2011). They inhibit cholesterase activity and negatively affect other enzymes as well. Their high contact activity has made them to be very effective against lepidopterous larvae. They include cypermethin, bioallethrin, permethrin, resmethrin fenvalerate, deltamethrin, and have demonstrated low mammalian toxicity (King and Saunders, 1984; Barlow, 1985; Elliot *et al*., 1978).

On the other hand, synthetic pestcides have a high persistance in the environment. For example, the time it takes DDT to decompose to half of its concentration in the soil ranges from 22 to 30 years, dieldrin around 7 years, and chlordecone up to 30 years. Due to their poor degradable nature and ability to be transported across boundaries, these pestcides are found around the globe. Significant quantities have been detected in fishes, birds and bears in the polar regions of the world where they have never been used (Saldanha *et al*., 2010). Pesticide

use has caused disastrous environmental and health problems in two French Caribbean islands, Martinique and Guadeloupe. Today, these islands are highly polluted by the pesticide chlordecone after many years of its application on banana farms have ellapsed. The two islands with a total population of about 800,000 people currently face serious human health problems. For example, they have recorded one of the highest prostate cancer cases globally. Additionally, congenital malformation and birth defects in children are on the rise (Bocquené and Franco, 2005; Coat *et al*., 2006; Belpomme' *et al*., 2009).

All these disastrous and health threatening situations faced when synthetic pesticides are used have drawn the attention of researchers in favor of non-chemical methods and biopesticides for pest management.

2.1.2 Biopesticides

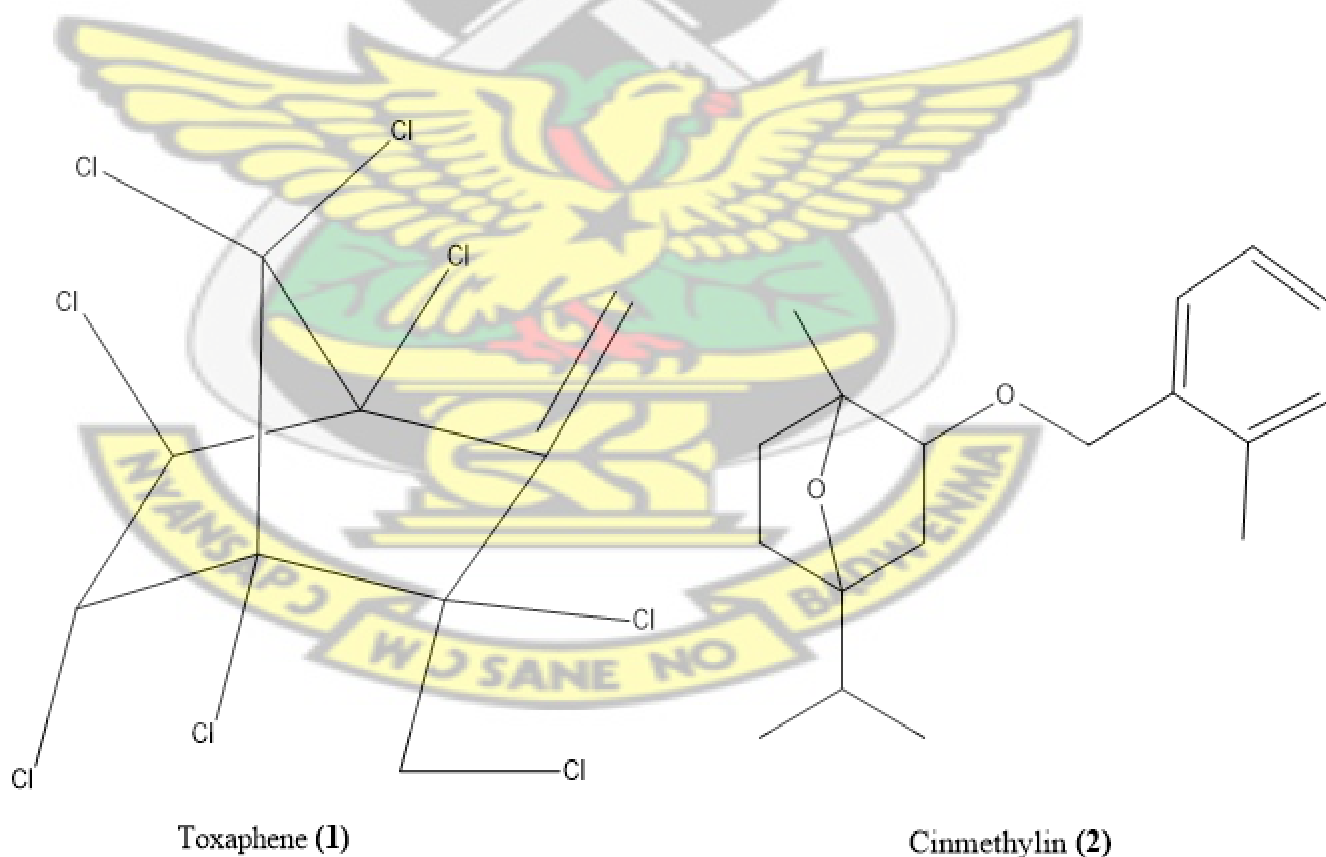
Biopesticides encompass a wide range of compounds which are ecologically safe and friendly to man and animals. They possess short shelf lives, narrow target ranges, specific and slow modes of action, and some suppress pest population rather than elimination. They act simultaneously on both behavioral and physiological processes, and their chances to be resisted by pests are low (Amer and Mehlhom, 2006a, b and c).

Biopesticides belonging to steroidal terpenoids, terpenes, flavonoids and acetogenins are known to protect crops from insects and other pest infestations (Ananthakrishnan, 1999; Anuradha *et al*., 2010a). Toxaphene (1) and cinmethylin (2), are naturally occurring terpenoids found in plant species of the genus, Artemisia (Compositae) which functions as fungicides, herbicides, insecticides and insect feeding inhibitors. The terpenoids azadirachtin (3), 3-O-acetyl salannol (4), salannol (5) and salannin (6) isolated from the seeds of *Azadirachta indica*

have shown growth inhibition and antifeedant activity against *Helicoverpa armigera* and *Spodoptera litura* larvae (Mordue and Blackwell, 1993; Koul *et al*., 2004). Other triterpenoids such as kokosanolide A (7), kokosanolide B (8), kokosanolide C (9) and 8, 14-secogammacera-7, 14(27)-diene-3, 21-dione (10) isolated from *Lansium domesticum* seeds and stem-bark have demonstrated antifeedant activity against *Epilacha vigintiotopunctata* larvae (Tri *et al*., 2011). Limonoids from *Khaya senegalensis* including khayanolide A, khayanolide B, 1-O-acetyl khayanolide B have inhibited the feeding habit and growth of the cotton leaf-worm, *Spodoptera littoralis* (Aswad *et al*., 2003). Two limonoids, zumsin (11) and dumnin (12) isolated from methanolic extract of *Croton jatrophoides* whole plant, have exhibited potent antifeedant activity against the pink bollworm, *Pectinophora gossipiella* and fall armyworm, *Spodoptera frigiperla* larvae (Nihei *et al*., 2002; Nehei *et al*., 2004). Parthenin and sesquiterpene lactone derivatives of parthenin isolated from the wild feverfew, *Parthenium hysterophorus* have demonstrated antifeedant activity against *Spodoptera litura* larvae, insecticidal activity against *Callosobrucus maculates* adult insects, and herbicidal activity against *Cassia tora* L. plants (Subhendu and Dinesh, 2001). Antifeedant coumarins, imperatorin (13) and xanthoxyletin (14) are active against the African armyworm (*Spodoptera exempta*) larvae, and have been obtained from *Clausena anisata* whole plant petroleum ether extract (Gebreyesus and Chapya, 1983).

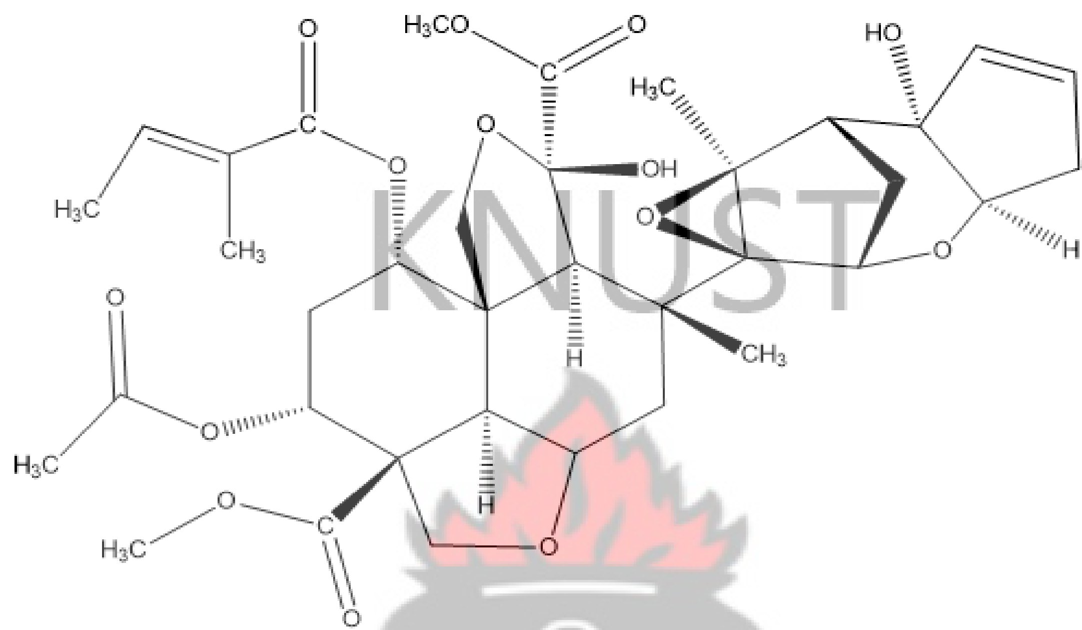
Similarly, flavonoids have shown antifeedant and larvicidal activity *in vitro* against different phytophagous organisms. Luteolin from *Cassia nigricans* leaves, a plant commonly used in West Africa to protect grains from insects during storage, exhibited mortalities against the larvae of *Anopheles gambiaea* (Kambou *et al*., 2008). The flavonoids (-)-homopterocarpin

(15), (-)-pterocarpin (16) and (-) – hydroxyhomopterocarpin (17) and the sesquiterpene alcohol (+) pterocarpol (18) isolated from the heartwood of *Pterocarpus macrocarpus* showed antifeedant activity against cutworm, *Spodoptera litura* and subterranean termite, *Reticultermes speratus* (Morimoto *et al*., 2006). Other flavonoids such as Schinifoline (19) and skimmianine (20) from *Zanthoxylum schinifolium* fruit pericarp have demonstrated feeding deterrent activity against *Tribolium castaneum* and *Sitophilus zeamais* adult insects (Liu *et al*., 2009). The lignan, sesamin (21) isolated from the fruits of *Piper mullesua* has shown significant antifeedant activity and moderate growth inhibition against the larvae of *Spilarctia oblique* (Srivastava *et al*., 2001). Their benzopyranone moiety and loss of a hydroxyl group appear to be responsible for their antifeedant activity (Morimoto *et al*., 2000).

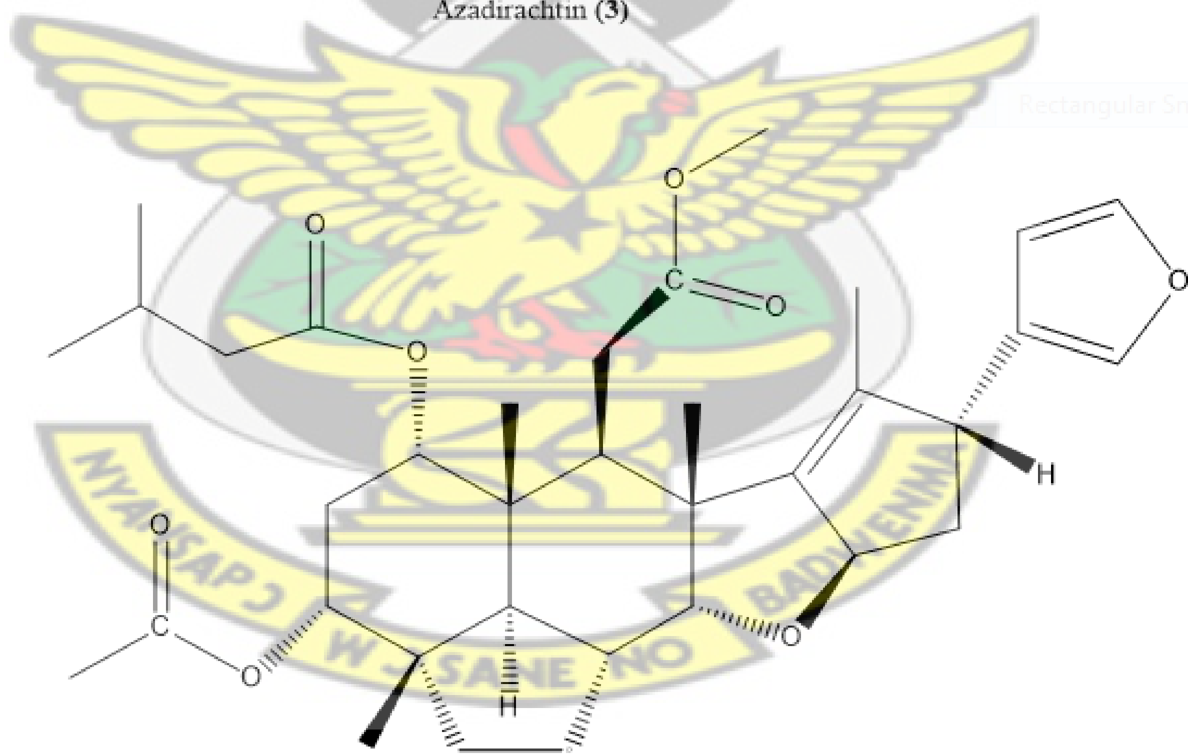


Toxaphene **(1)**

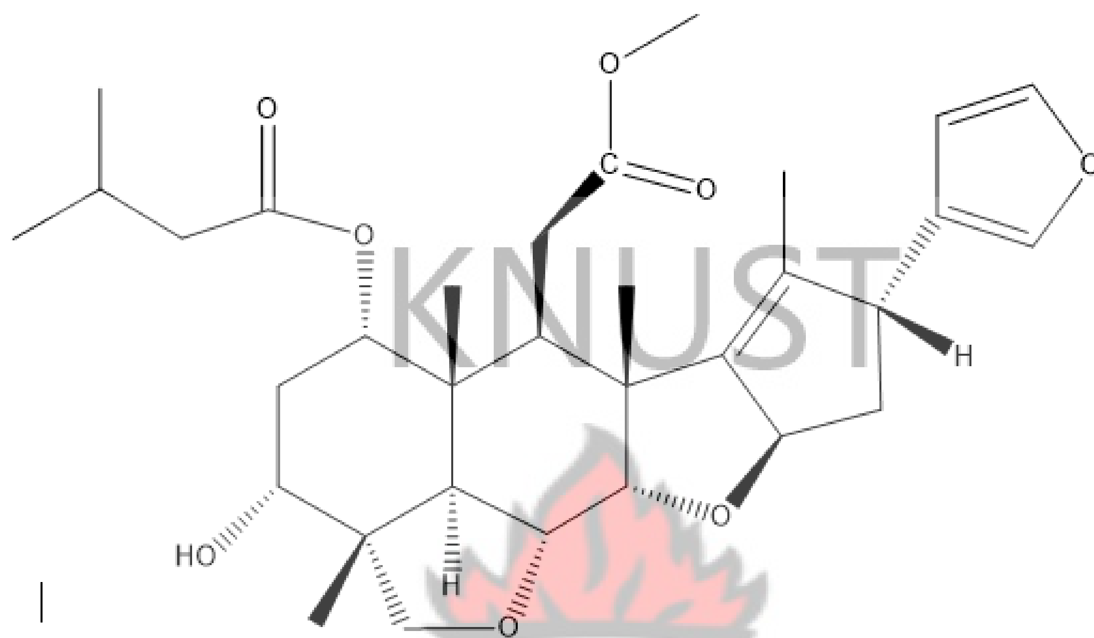
Cinmethylin **(2)**



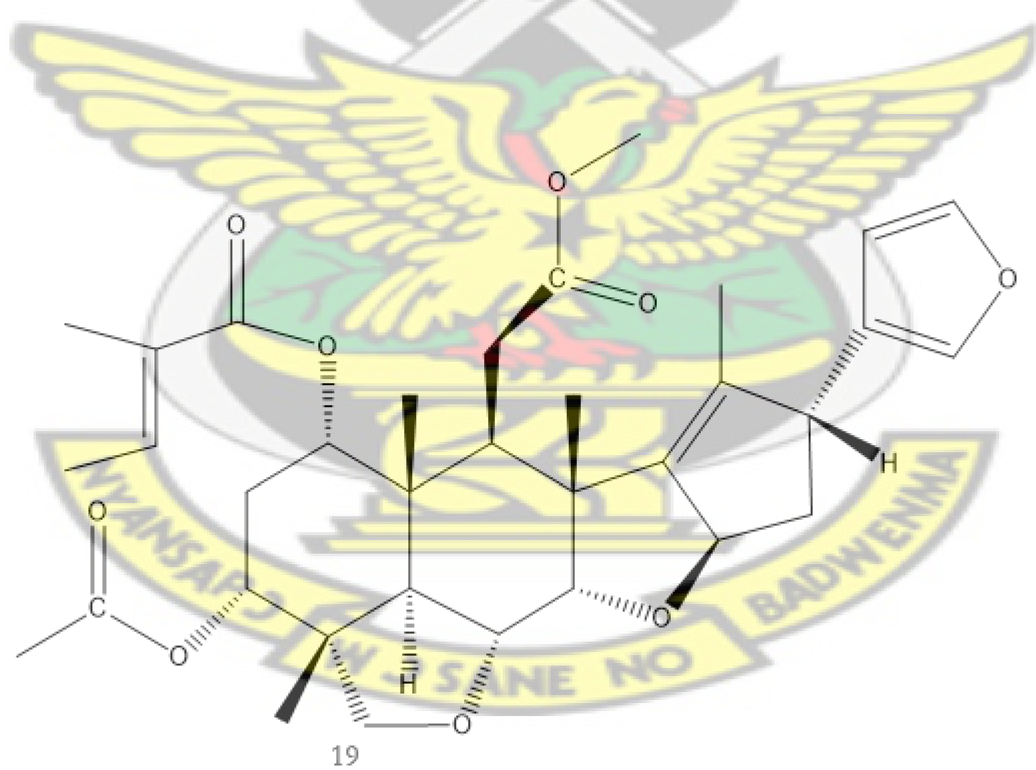
Azadirachtin (**3**)



3-O- acetyl salannol (**4**)



Salannol (**5**)



Salannin (**6**)

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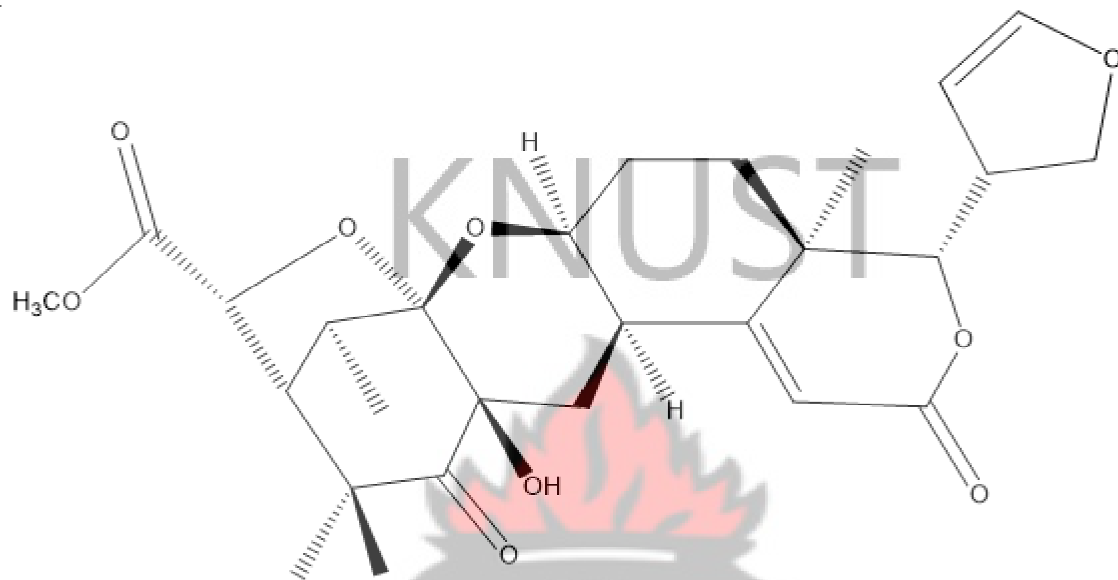
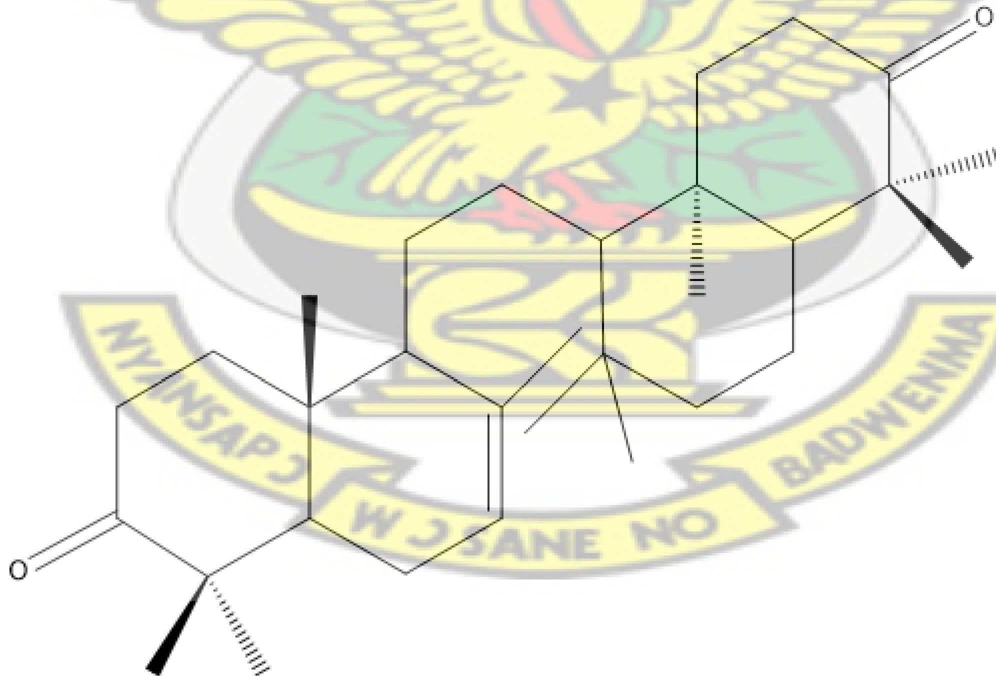


Kokosanolide A **(7)**



Kokosanolide B **(8)**

Kokosanolide C (**9**)

8, 14-secogammacera -7, 14 (27) - diene-3, 21-dione (**10**)

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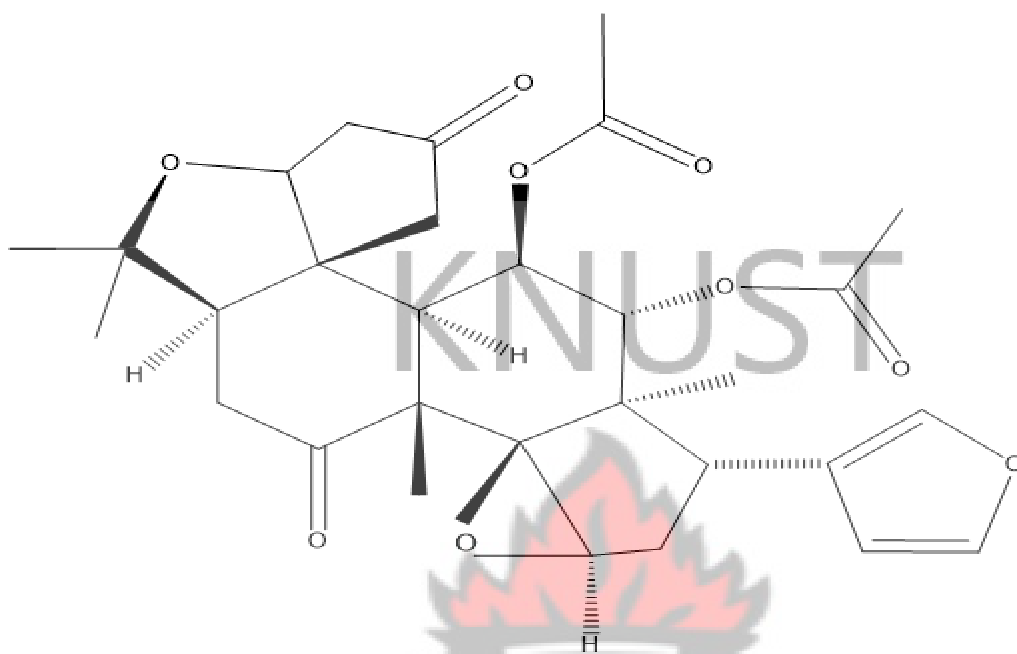
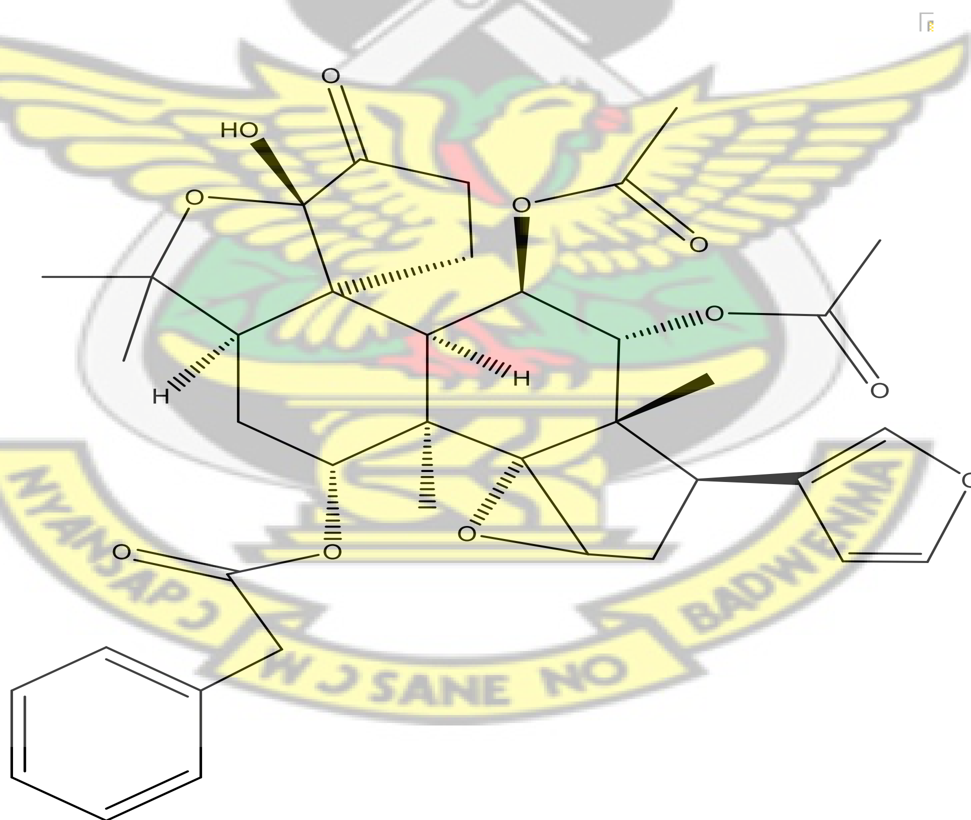
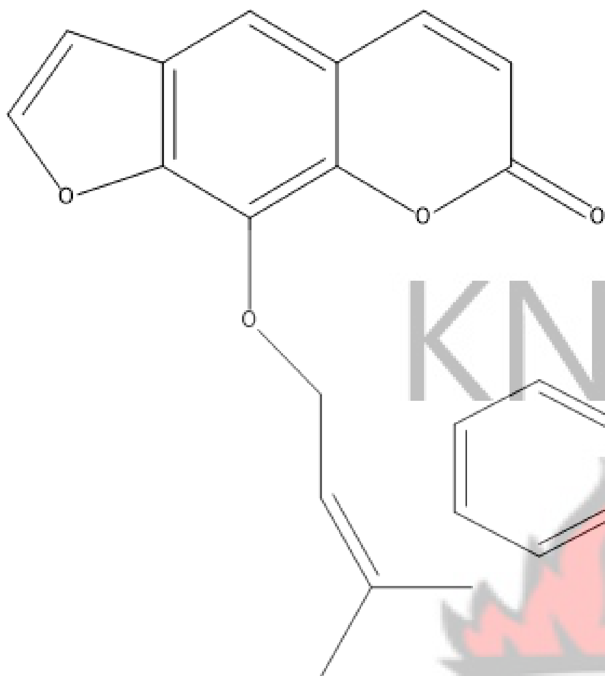


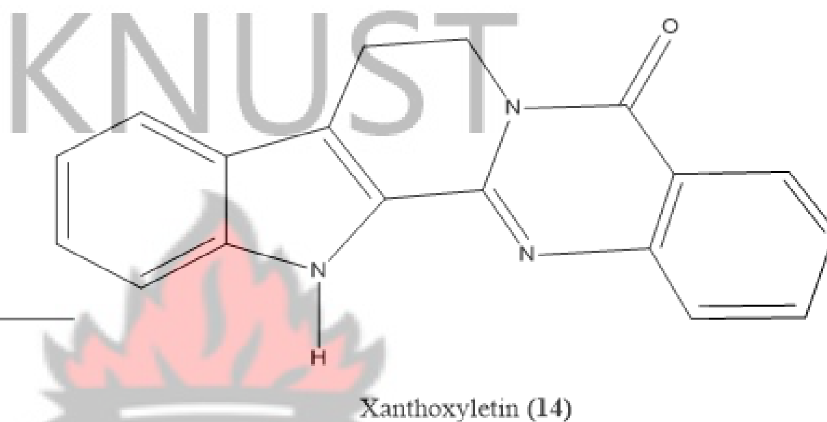
Zumsin (11)



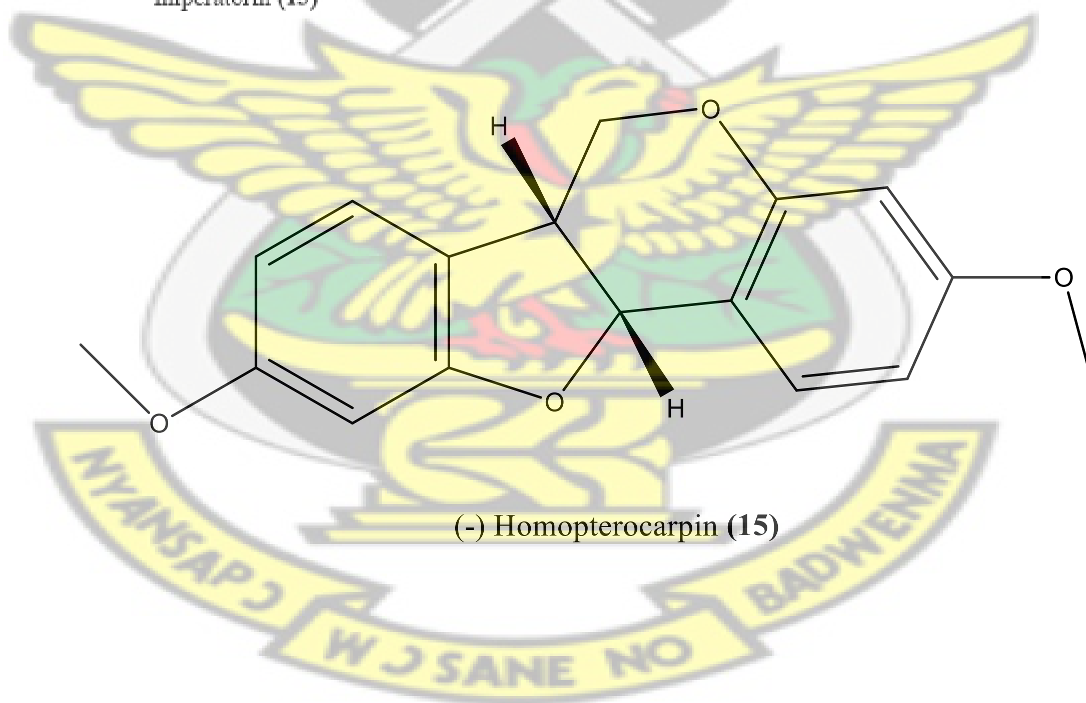
Dumnin (**12**)



Imperatorin (**13**)



Xanthoxyletin (**14**)



(-) Homopterocarpin (**15**)

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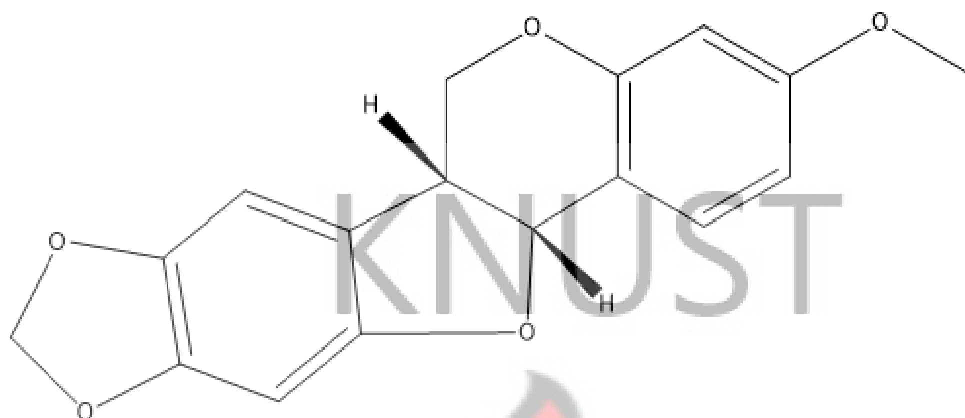
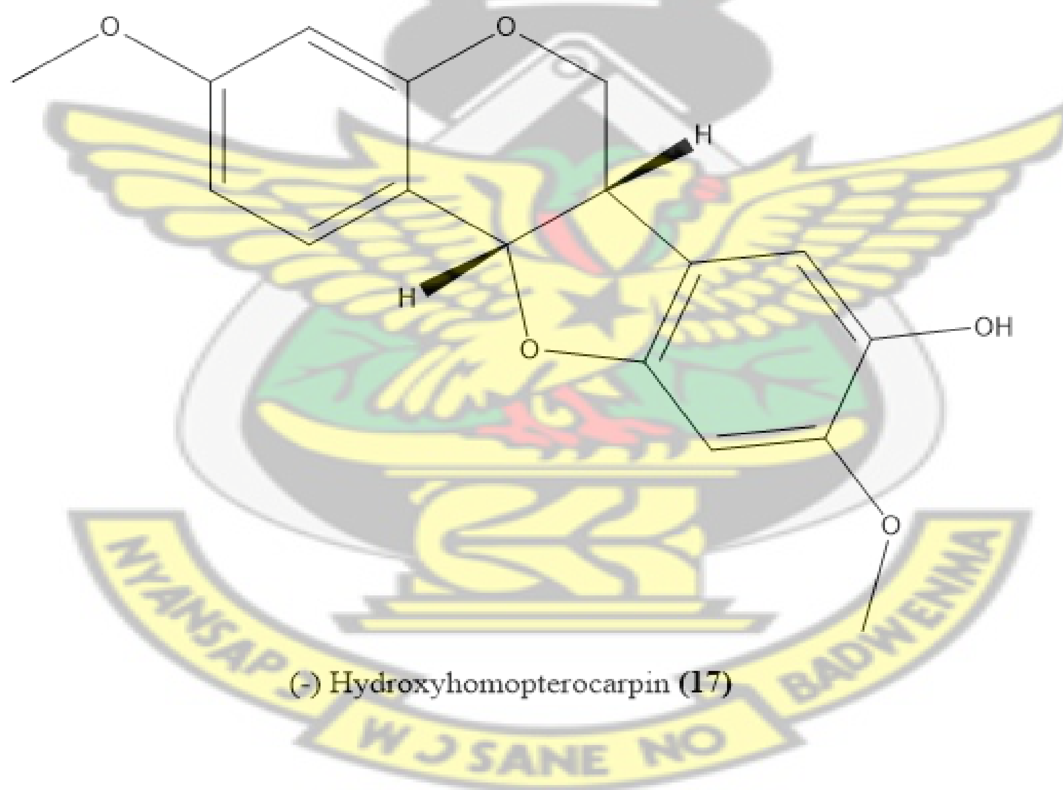
Rectangular Snip



(-) Pterocarpin **(16)**



(-) Hydroxyhomopterocarpin **(17)**

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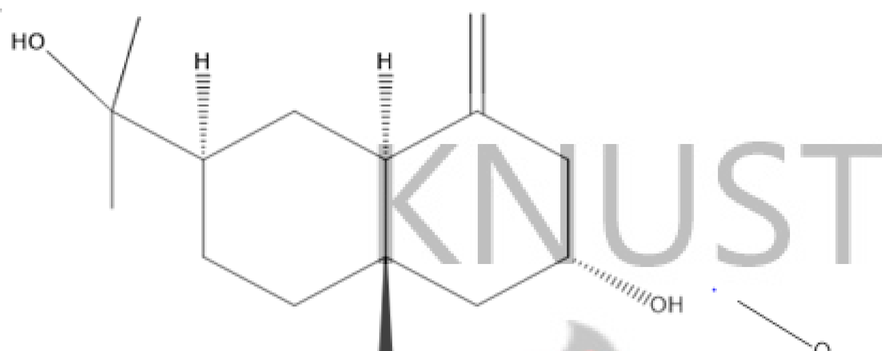
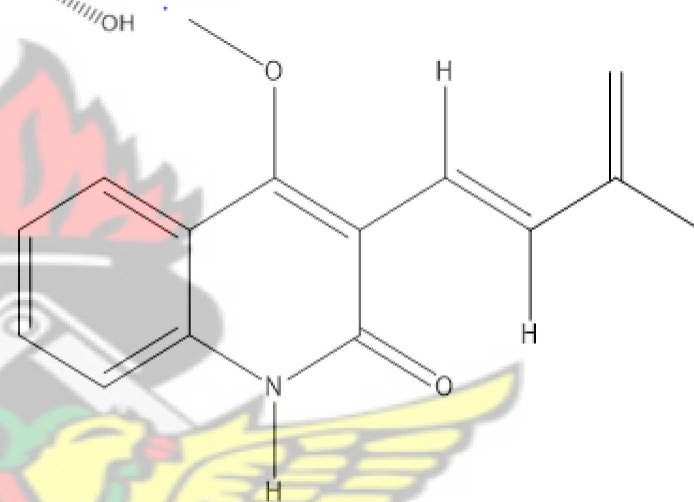
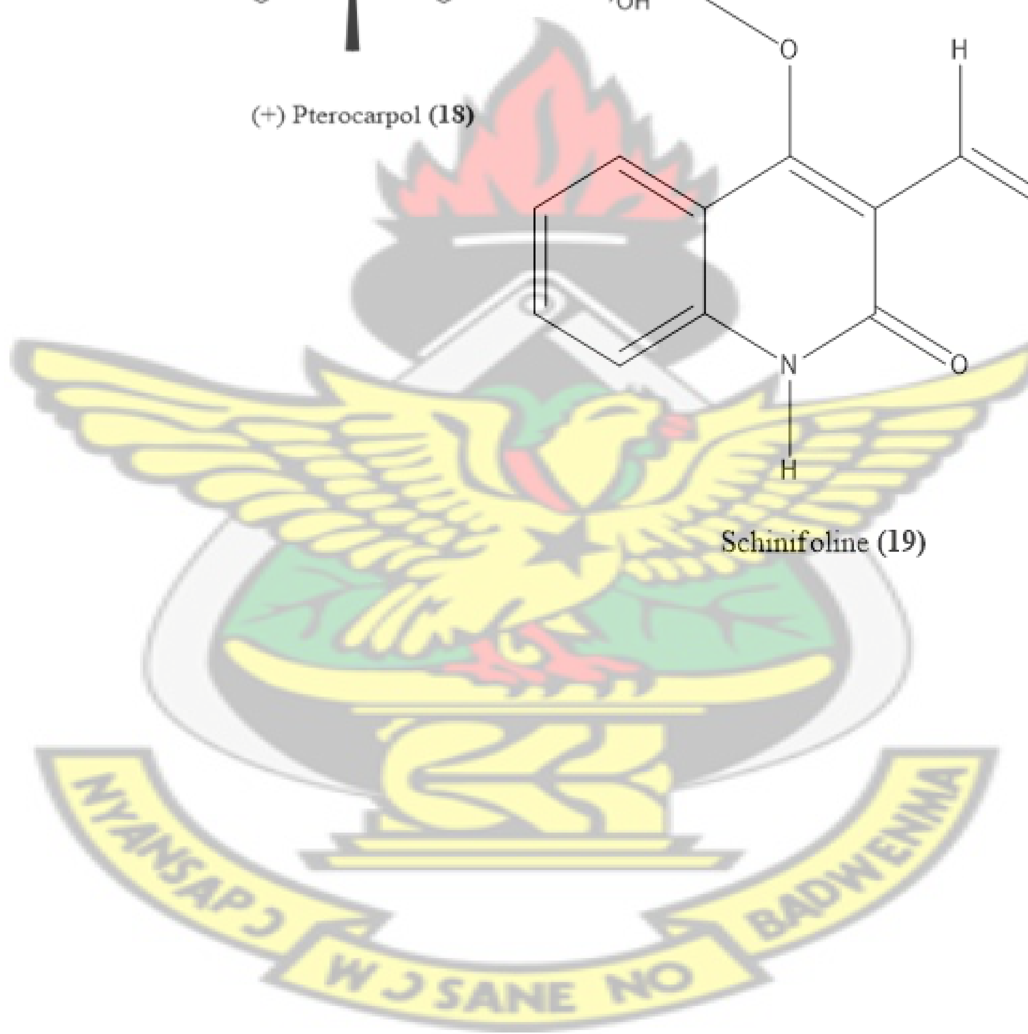


(+) Pterocarpol **(18)**



Schinifoline **(19)**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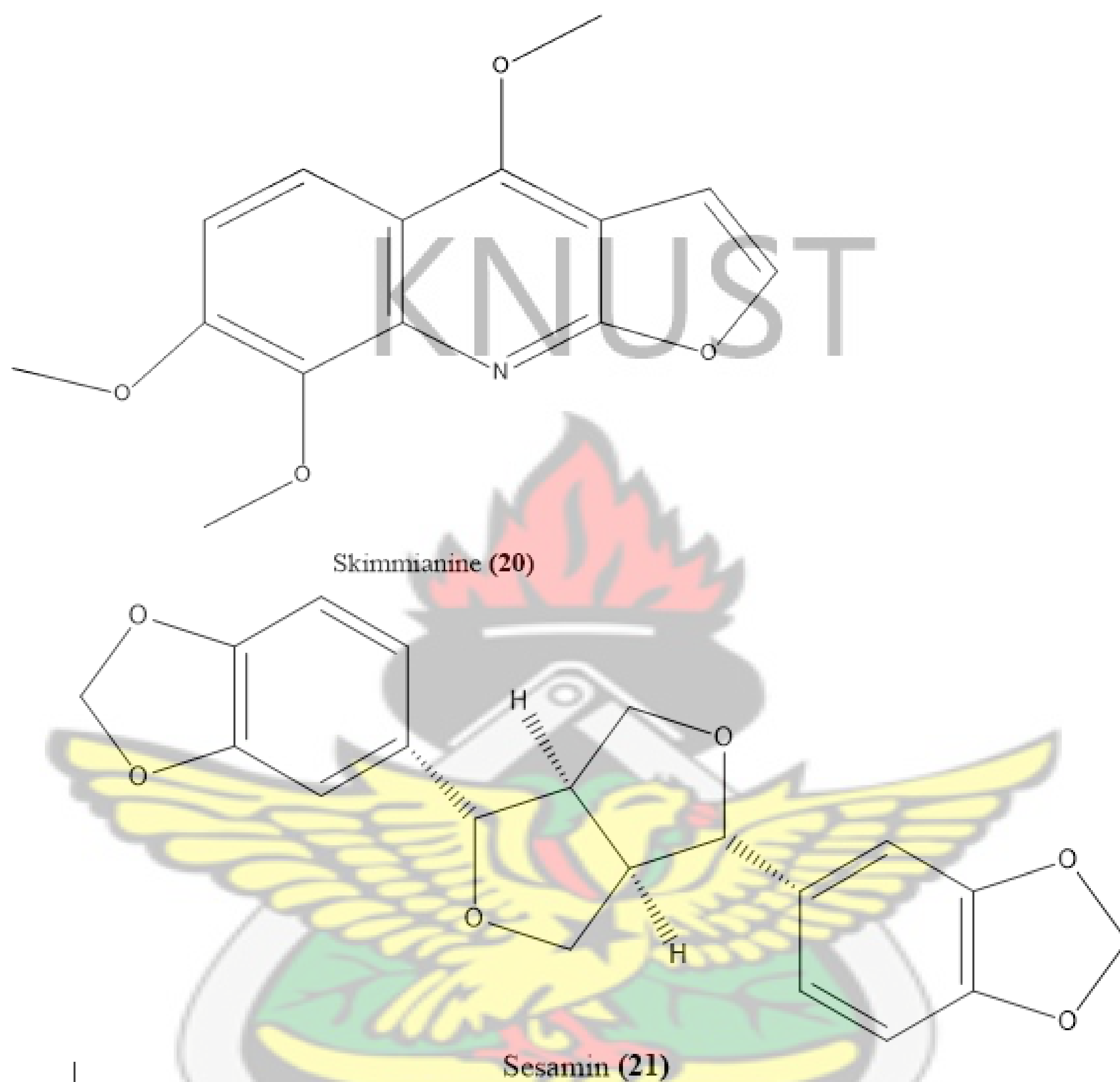

Skimmianine (20)

Sesamin (21)

2.2 PLANT SELECTION

Cassia tora plant is widely grown on farm-lands in the South-west region of Cameroon to manage pests on cultivated food crops and sometimes storage grain pests. Evidence has shown that the plant posseses pesticidal and crop yield enhancement properties. Three plots of 1 hectare each having assorted crops, hedged round with *Cassia tora L.* were set up for

experimentation. Physical observation during 5 years period revealed that *Zonocerus variegatus* (insect pest) fed on all other plants and avoided *Cassia tora*. *Cassia tora* was observed to contain toxic components that demonstrated antifeedant activity on *Zonocerus variegatus* as insects that ate it died. Crops cultivated in a mixed cropping fashion with existing economic trees hindered the spread of disease and pest epidemics in plant communities (Suka, 2011). Thus there is the need to substantiate this claim with scientific data. Therefore the identification of ecologically-friendly phyto-pesticides in *Cassia tora* seeds for sustainability of the plant and its usage against storage grain pests is long overdue.

2.3 THE FAMILY FABACEAE (LEGUMINOSAE)

Fabaceae (Leguminosae) is the third largest family of flowering plants. It is next in rank to Orchidaceae and Asteraceae (Ghosh and Keshri, 2007). The name is derived from "faba" the Latin word for broad bean. It consists of 720-730 genera and 19, 500 species (Michael, 2010). The Fabaceae is traditionally sub-classified into Caesalpinioideae. Members are known to be sources of gums, dyes, oils, insecticides, fibre, fuel, medicinals and pulses (Mabberley, 1997; Wojciechowski, 2003). Almost all species in this family produce root-nodules which harbor symbiotic bacteria that fix atmospheric nitrogen to the soil, thereby adding to soil nutrients (Sprent and McKey, 1994; Sprent, 2001). The woody members of Caesalpiniaceae are important for providing timber (Ghanzanfar, 1989).

2.3.1 The genus Cassia

Cassia is a genus that belongs to the family Caesalpiniaceae. Members of this genus are widely distributed predominantly in tropical and warm temperate regions. It consists of almost 500 species of flowering plants and contains mostly ornamental herbs, shrubs, under-shrubs and

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trees (Chandra *et al*., 2012; Rani and Satish, 2014). The species have bright yellow flowers, consisting of five similar sepals and petals. Plants belonging to *Cassia* species are widely used in different parts of the world to treat different ailments. In folklore, they are well known for their uses as laxatives and purgatives (Hennebelle *et al*., 2009; Verma *et al*., 2010). Members of this genus are also widely used to treat wounds, gastro-intestinal and uterus disorders, rheumatism, anorexia, jaundice and skin diseases such as ringworm, scabies and eczema (Joshi, 2000; Elujoba, 1999; Pieme *et al*., 2006). Cassia species have been proven to possess anti-inflammatory, antioxidant, hypoglycemic, hyperglycemic, antiplasmodial, larvicidal, antimutagenic and anticancer activities (Silva *et al*., 2008; Prasanna *et al*., 2009). Most members of the genus are rich in polyphenols, anthraquinone derivatives, flavonoids and polysaccharides (Bahorun *et al*., 2005; Ayo, 2010).

2.4 *CASSIA TORA* LINN

2.4.1 Botanical description

Cassia tora Linn is a legume which belongs to the family *Fabaceae* and sub-family *Caesalpinioideae* (Figure 2.1). It is an annual herbaceous herb, almost an under shrub of 30-90 cm in height, considered as a weed in many places or as a waste land rainy season plant and grows in dry soils in the world's tropical zones. It is found growing in the tropical parts of India, China, Sri Lanka and West tropics. The plant is often confused with Chinese senna or Sicklepod, *Senna obtusifolia.* Its distinct common name is sickle wild sensitive-plant. The name *Senna tora* is sometimes used as its botanical name (Rani and Satish, 2014). The plant has green, pinnate leaves with leaflets in pairs of three, opposite, obovate and oblong. Flowers are yellow, often in pairs in axil of leaves having five petals. Pods are four angled or somewhat

flattened, 10-15 cm long and sickle shaped. Each pod contains 30-50 seeds. Seeds have smooth surfaces, and range in colour from greenish-brown to dark-brown (Figure 2.1) (Rani and Satish, 2014).

Figure 2.1. *C. tora* L. whole plant (A), and seeds (B)

2.4.2 Ethnobotanical uses

Traditionally, *C. tora* is used as bitter tonic, mild laxative, anthelmintic, antidiabetic, and to treat liver disorders, skin and eye diseases (Rani and Satish, 2014). In Chinese medicine, seeds of *Cassia tora* are known to function as antiasthenic, aperient, diuretic agents, lowering of cholesterol and blood pressure levels in the body (Foster and Chongxi, 1992). It is employed in different Ghanaian medicines, mainly as a purgative. Decoction of the leaves of *Cassia tora* is used as a laxative. The seeds of *Cassia tora* are prepared as a remedy for headache, vertigo, constipation, high blood pressure, hepatitis, liver cirrhosis and ascites. In China, seeds are externally used for different eye diseases; preparations are given for liver complaints and boils.

In Madagascar, the root is considered bitter, tonic and stomachic (Kirtikar and Basu, 2005; Das *et al*., 2011).

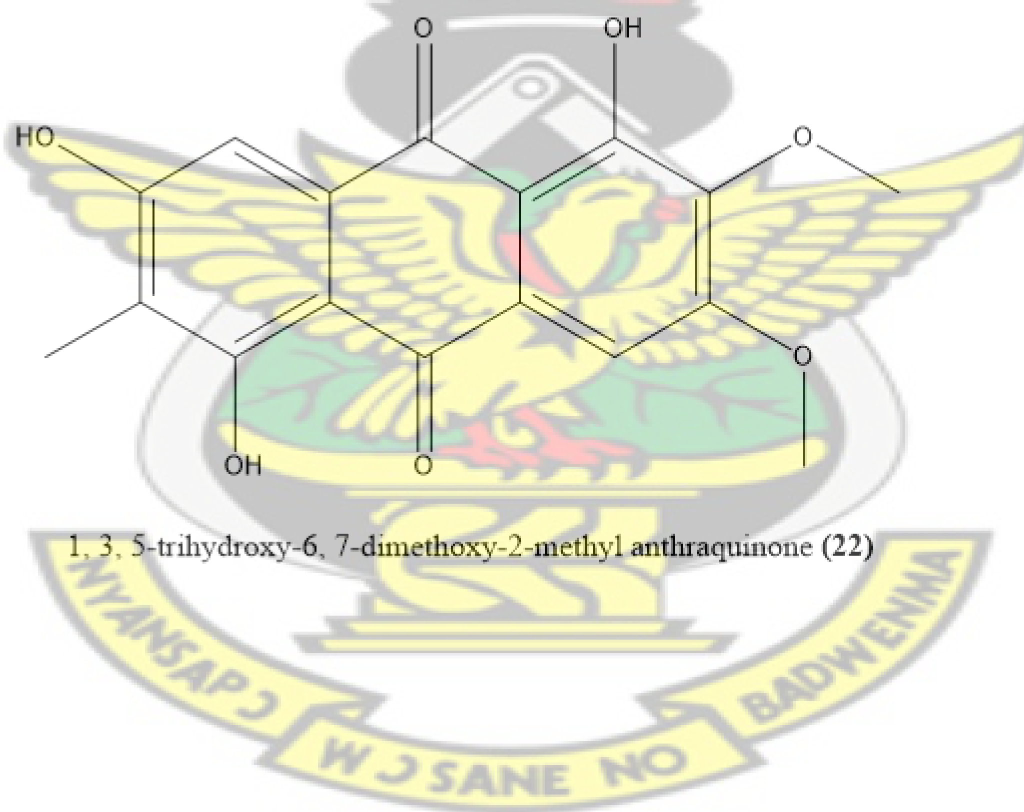
2.4.3 Pharmacological activities

Pharmacological investigations have demonstrated antifungal, hepatoprotective, spasmogenic, wound healing, anti-asthmatic, purgative, anti-arthritic and anti-inflamatory activities of *C. tora* leaf extracts (Ingle *et al*., 2012; Choudhary and Gulial, 2011; Jayasutha and Monic, 2011), antiplasmodial, anthelmintic antibacterial, cardiotonic, oxytocic and hypotensive activities of *C. tora* seed extracts (Jain and Patil, 2010; Choudhary *et al*., 2011; Sathya and Ambikapathy, 2012; Janardan *et al*., 2011). Seed extracts have exhibited inhibitory activity against advanced glycation end products formation (AGEs) (Lee *et al*., 2006). Raw and roasted seeds extracts have shown inhibitory properties against angiotensin converting enzyme (ACE). Glucoaurantioobtusin, an anthraquinone glycoside from *Cassia tora* seeds has shown inhibitory properties against angiotensin converting enzymes (Hyun *et al*., 2009). Ononitol monohydrate, obtained from leaves of *Cassia tora* has shown antihepatoprotective activity in Wistar rats by reducing levels of serum transaminase (Dhanasekaran *et al*., 2009).

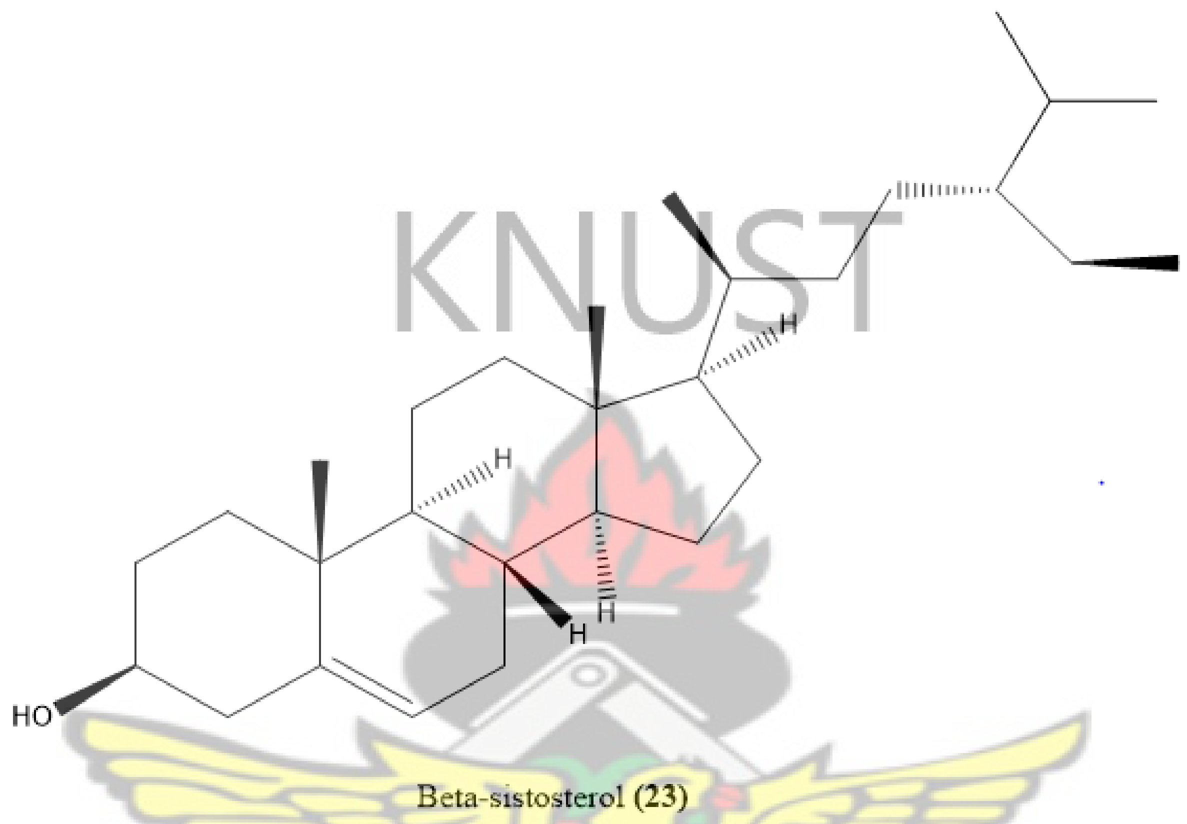
2.4.4 Phytochemistry

Some compounds isolated from *Cassia tora* include 1, 3, 5-trihydroxy-6-7- dimethoxy-2-methyl anthraquinone (22) and β-sistosterol (23) from the roots, kaempferol (24) and leucopelargonidin (25) from the flowers, chyrsophanol (26), physcion (27), emodin (28) and rubrofusarin (29) from the seeds. The presence of palmitic (30), stearic (31), lignoceric (32), oleic (33) and linoleic (34) acids has been reported in the seed oil (Jain and Patil, 2010). The leaves are a rich source of stigmasterol (35), β-sistosterol-β-D-glucoside (36), succinic (37) and

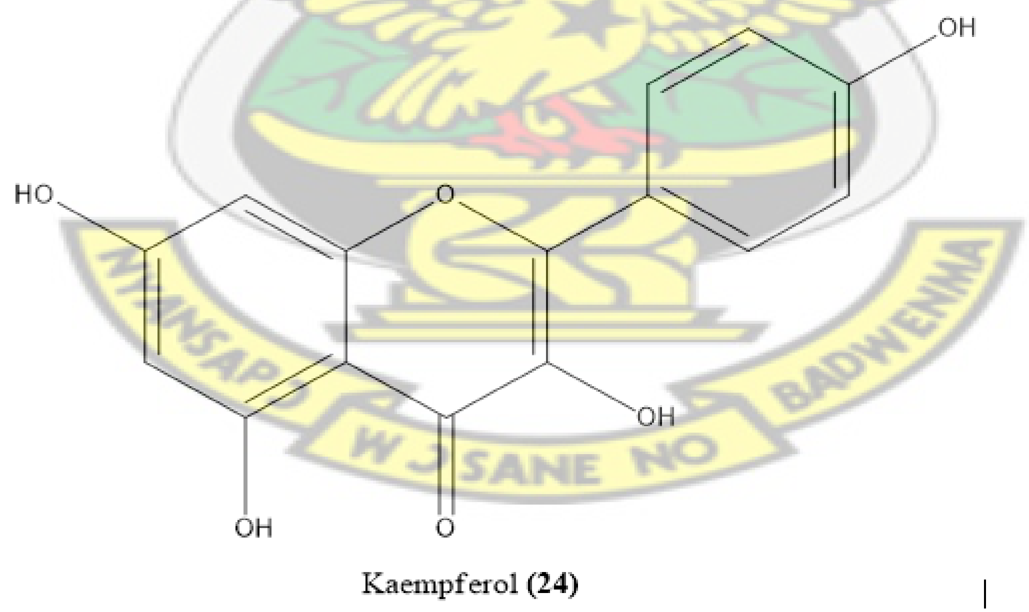
d-tartaric (38) acids, uridine (39), quercitrin (40), and iso-quercitrin (41) (Yun Choi, 1990; Mukherjee, 2002; Soumyanath, 2005). The seeds contain toralactone (42), chrysophanol (43) and chrysophonic acid-9-anthrone (44), cassiaside (45), rubrofusarin-6-O-β-D-gentiobioside (46) and toralactone-9-O-β-D-gentiobioside (47) (Trease and Evans, 1996; Mukherjee, 2002; Lee *et al*., 2006; Jain and Patil, 2010). The anthraquinones, aurantio-obtusin (48), chryso-obtusin (49), obtusin (50), chryso-obtusin-2-O-β-D-glucoside (51), obtusifolin (52), obtusifolin-2-O-β-D-glucoside (53) have been isolated from *Cassia tora* seeds (Jang *et al*., 2007).



1, 3, 5-trihydroxy-6, 7-dimethoxy-2-methyl anthraquinone **(22)**



Beta-sistosterol **(23)**



Kaempferol **(24)**

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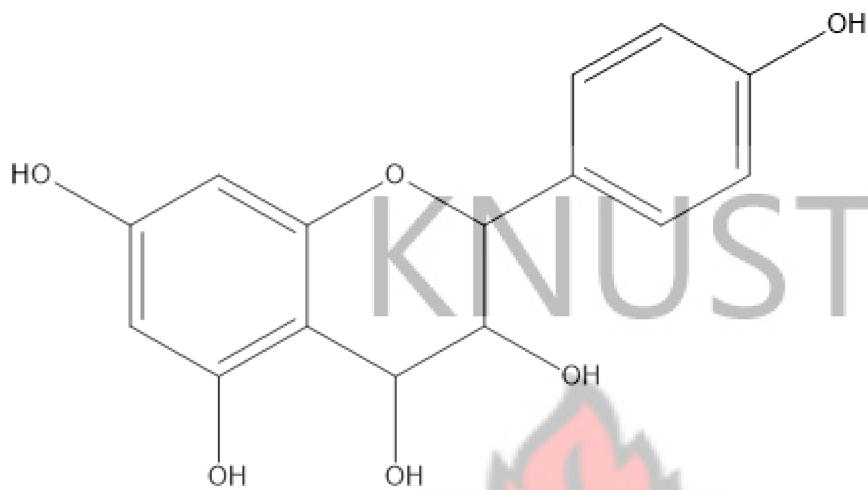
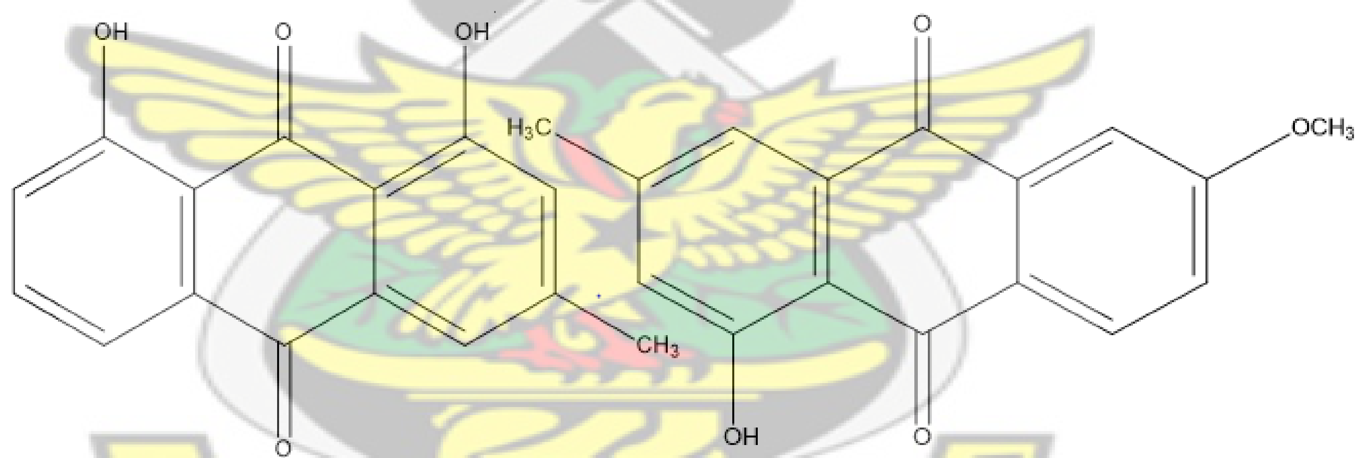


Leucopelargonidin **(25)**



Chrysophanol **(26)** Physcion **(27)**

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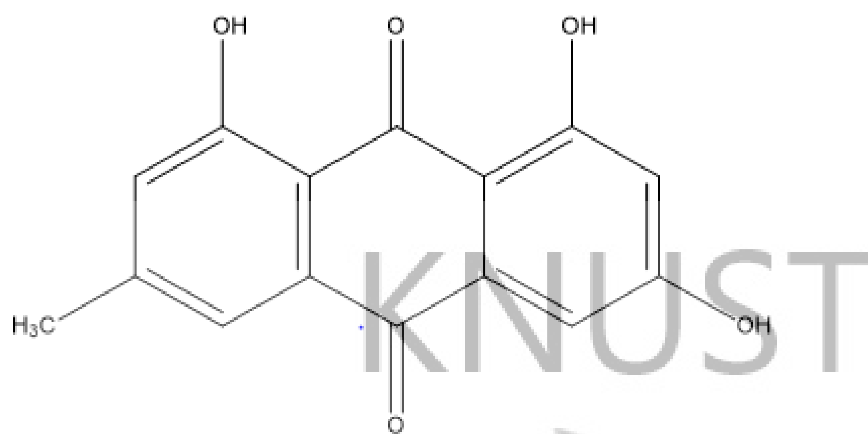
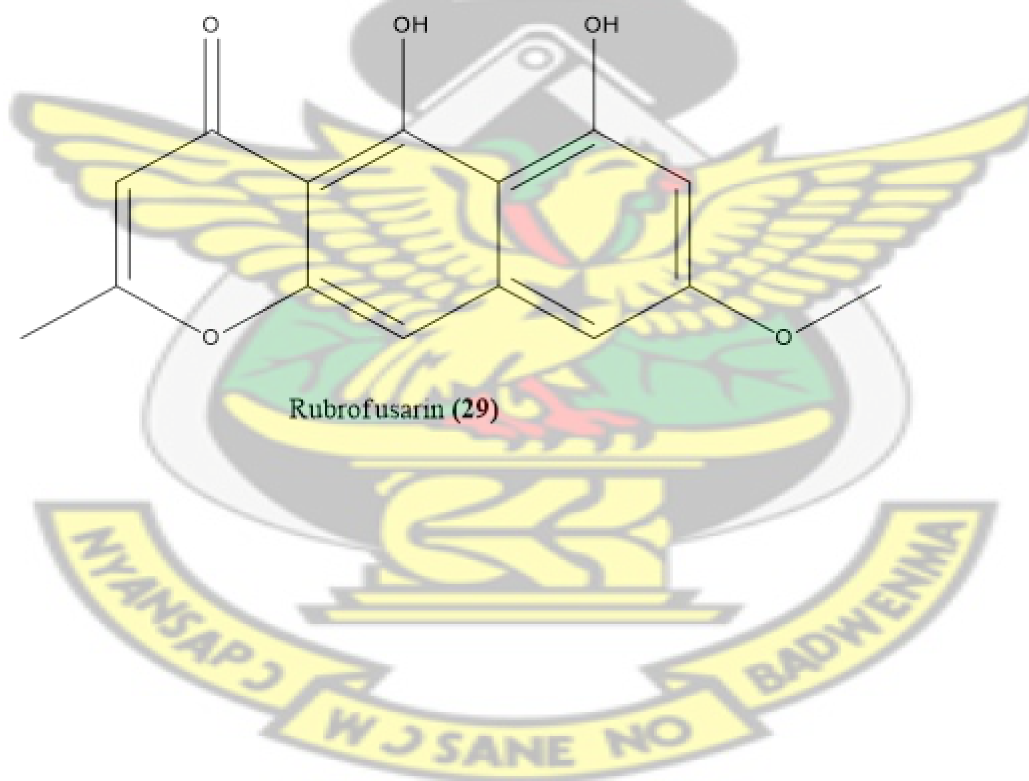


Emodin (**28**)



Rubrofusarin (**29**)

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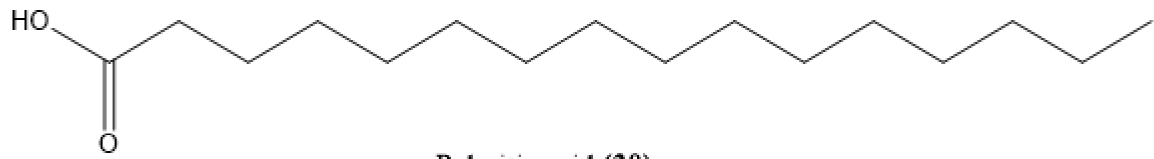

Palmitic acid **(30)**

Stearic acid **(31)**

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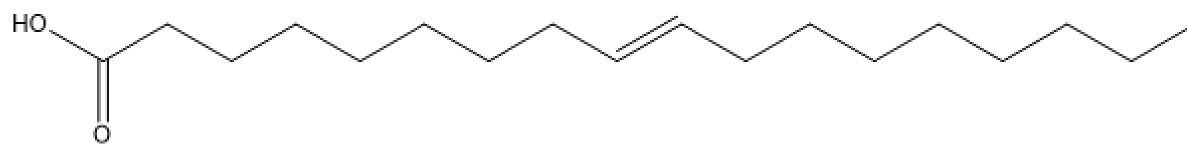
Lignoceric acid **(32)**



Oleic acid **(33)**

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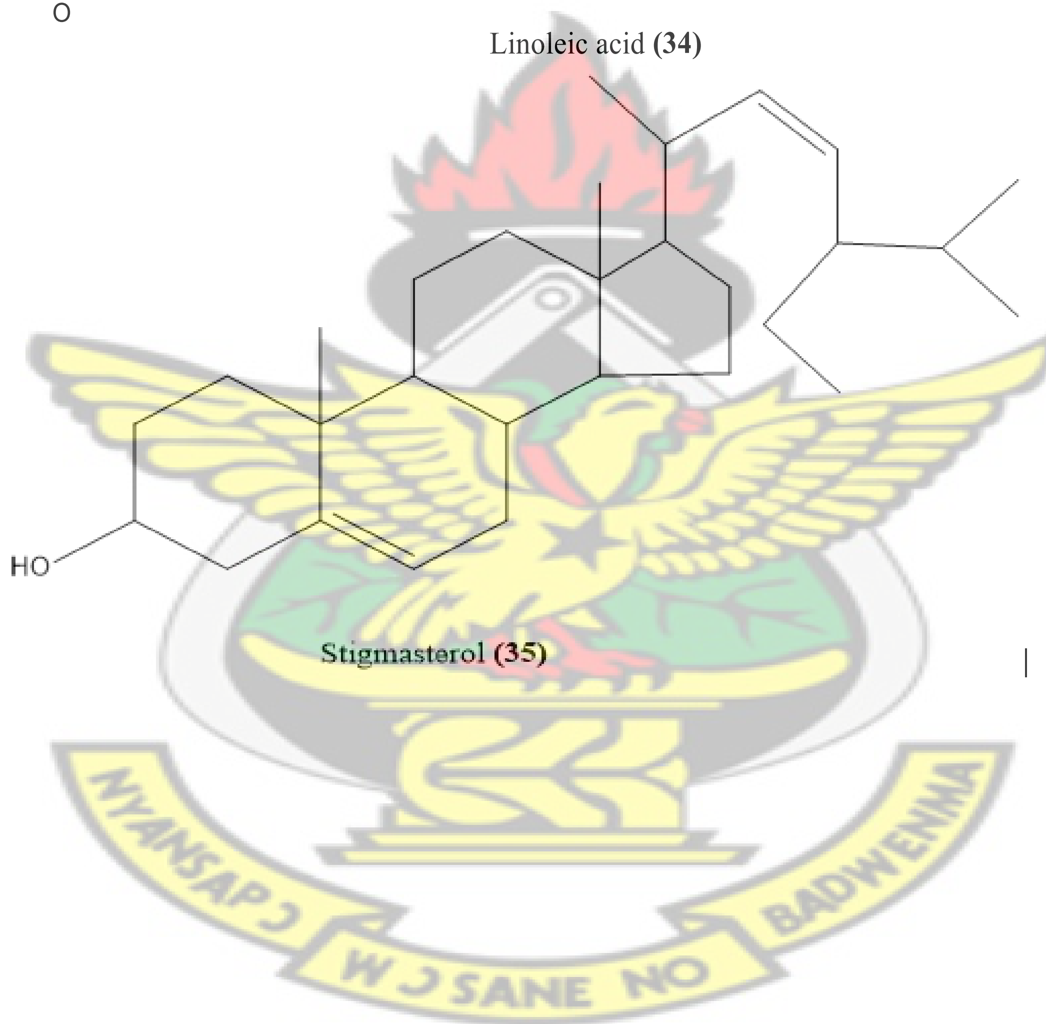
Linoleic acid **(34)**



Stigmasterol **(35)**

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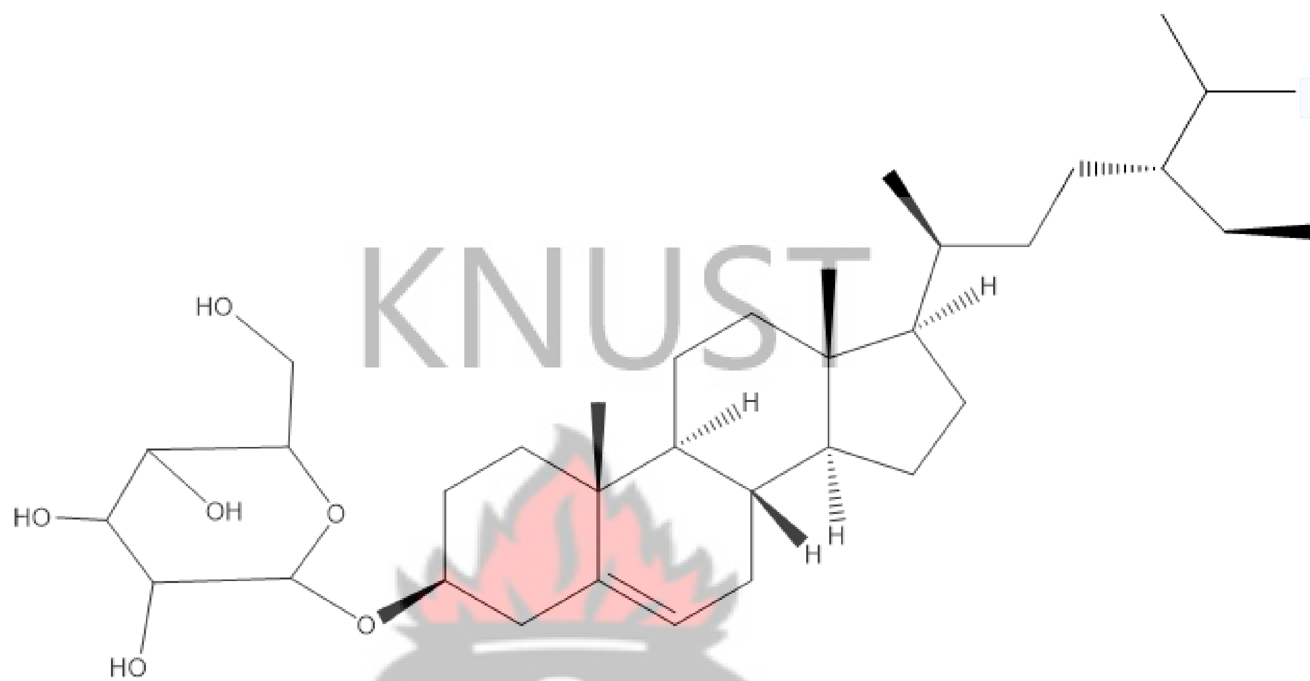
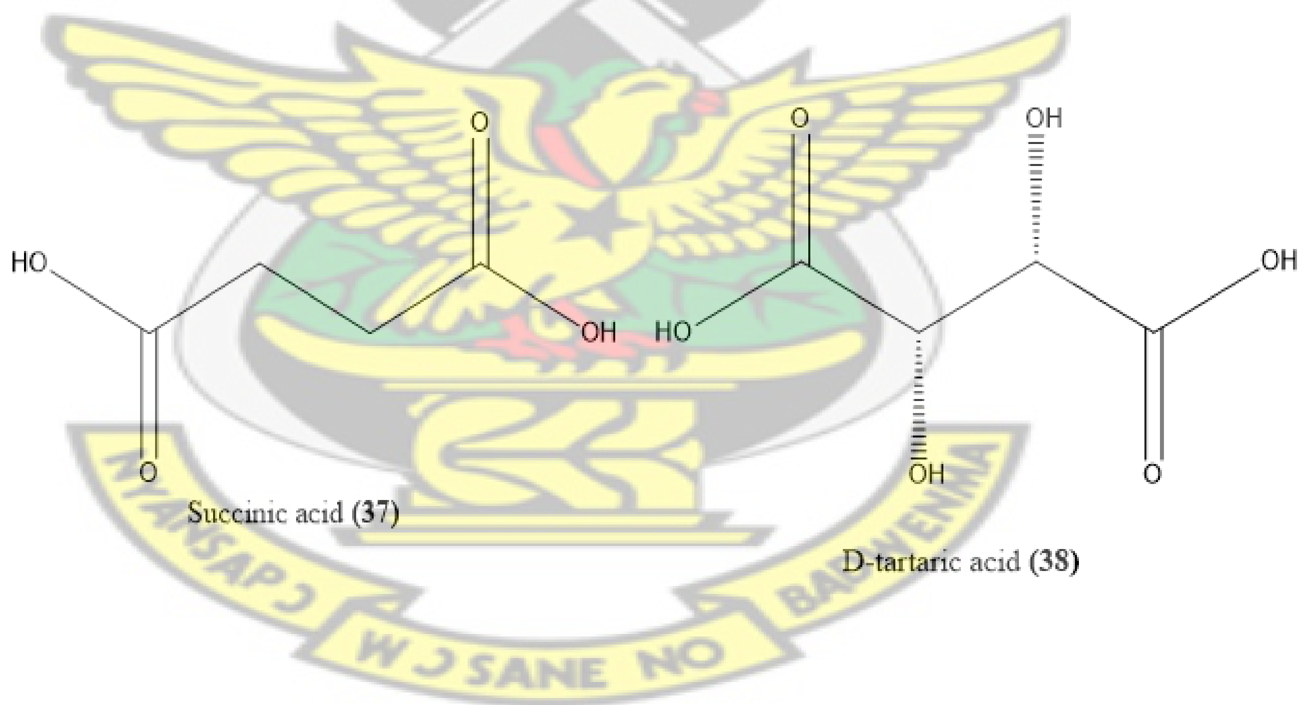


Beta-sistosterol-beta-D-glucoside (**36**)



Succinic acid (**37**)

D-tartaric acid (**38**)

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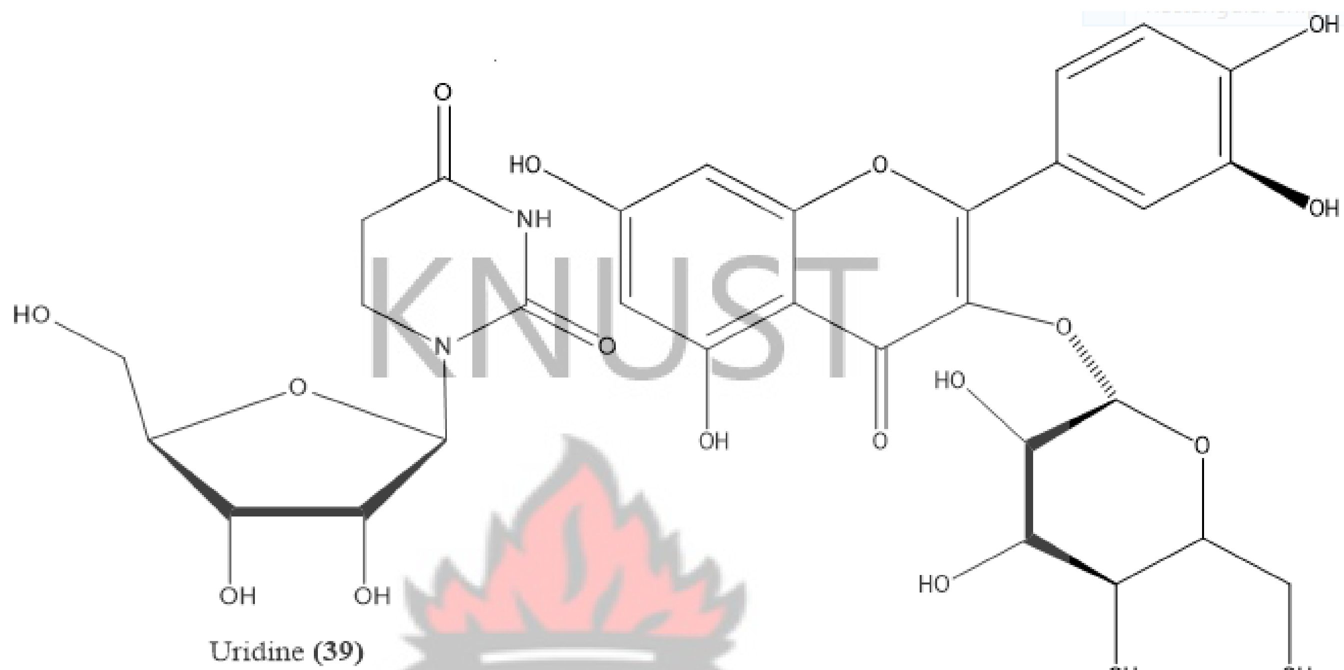
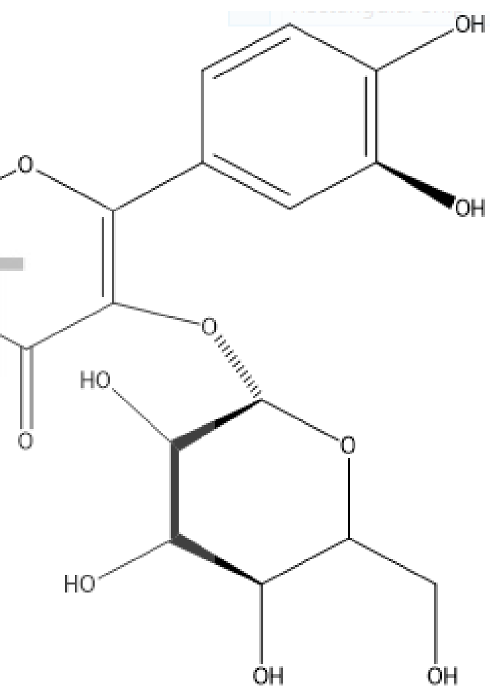
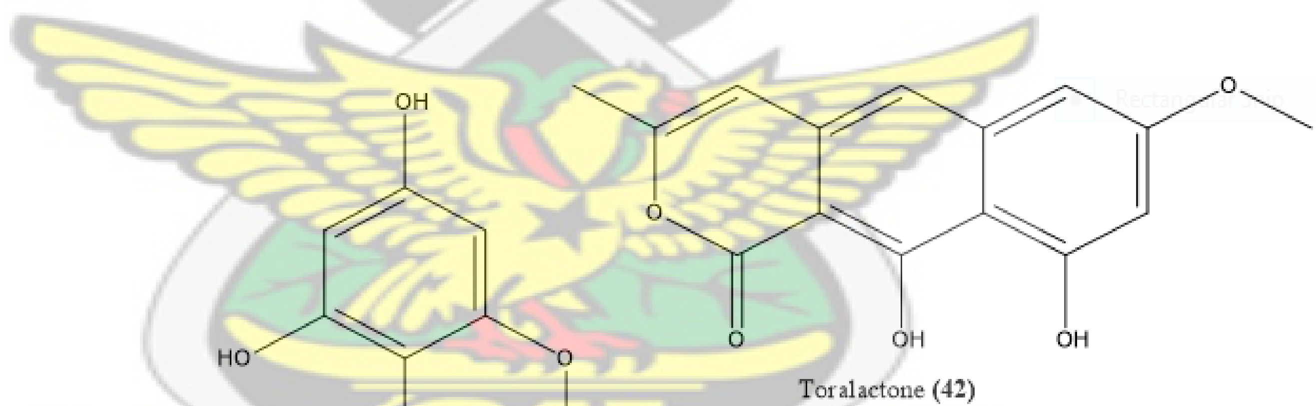


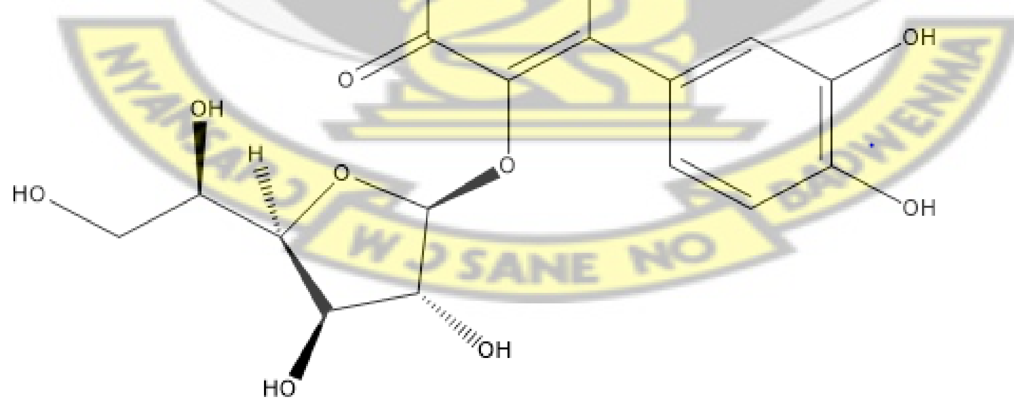
Uridine **(39)**



Quercitrin **(40)**



Toralactone **(42)**



Isoquercitrin **(41)**

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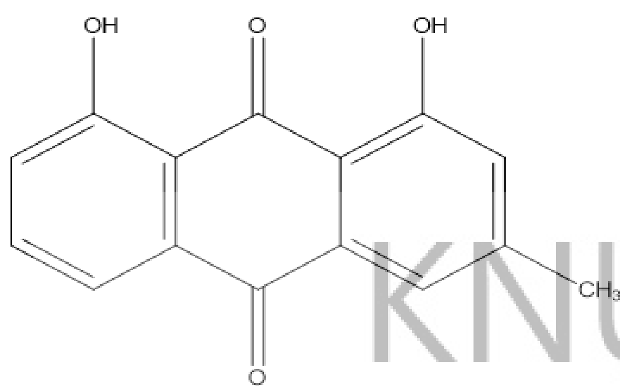
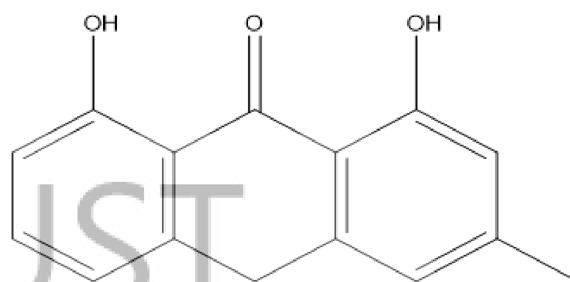
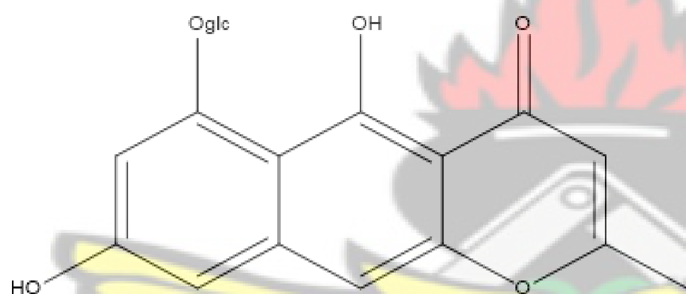


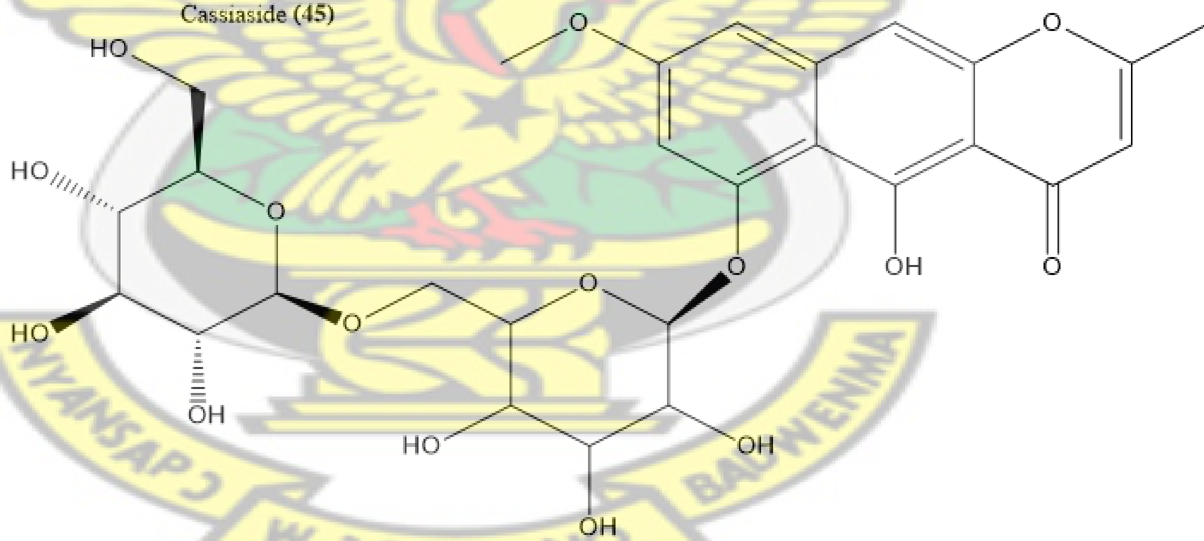
Chrysophanol **(43)**



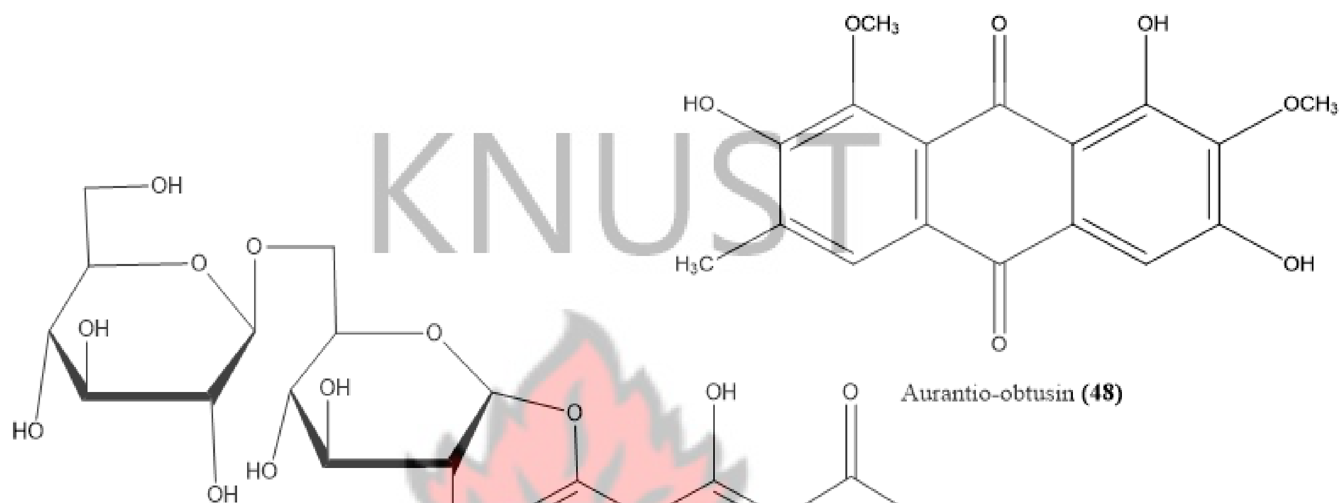
Chrysophanic acid-9-anthrone **(44)**



Cassiaside **(45)**

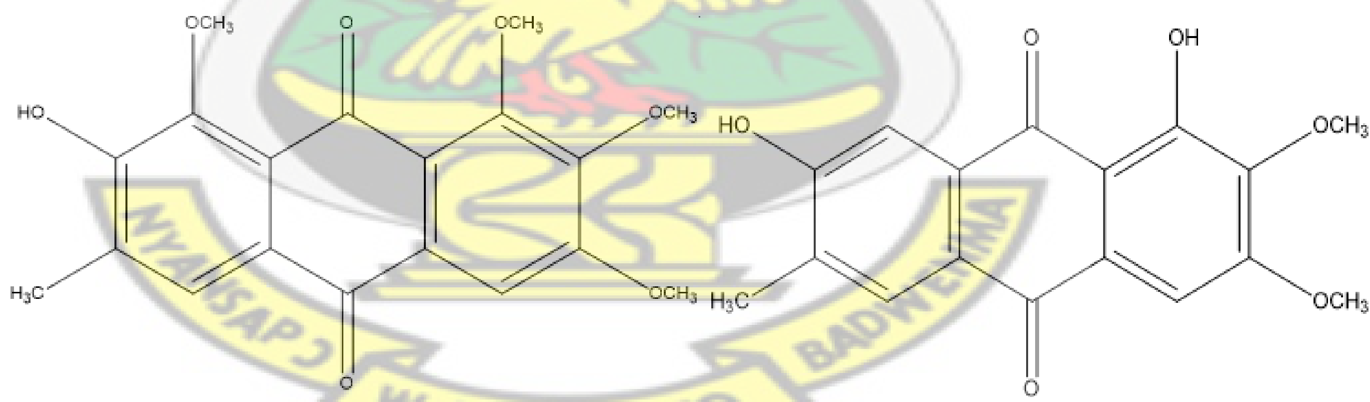


Rubrofusarin-6-O-beta-D-gentiobioside **(46)**



Aurantio-obtusin (**48**)

Toralactone-9-O-beta-D-gentiobioside (**47**)



Chryso-obtusin (**49**)

Obtusin (**50**)

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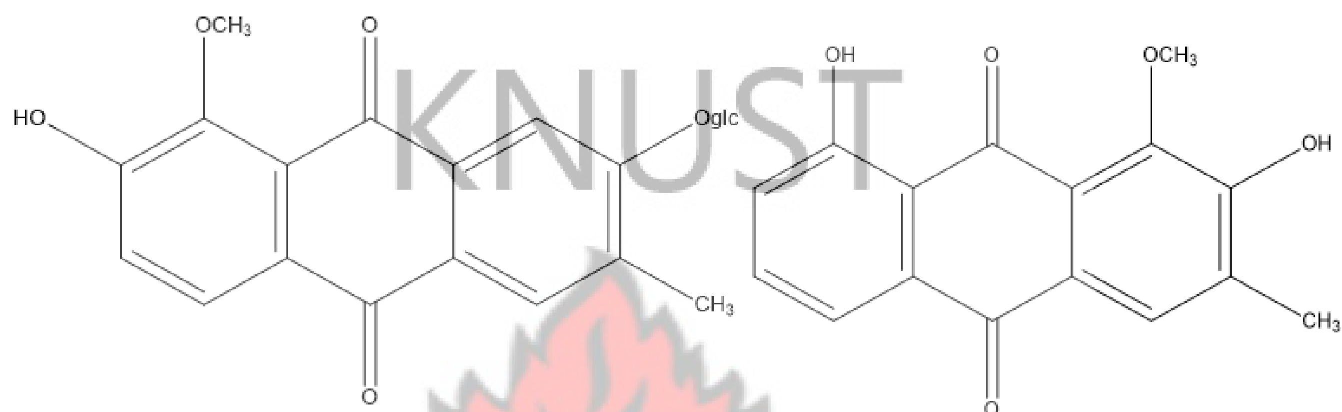
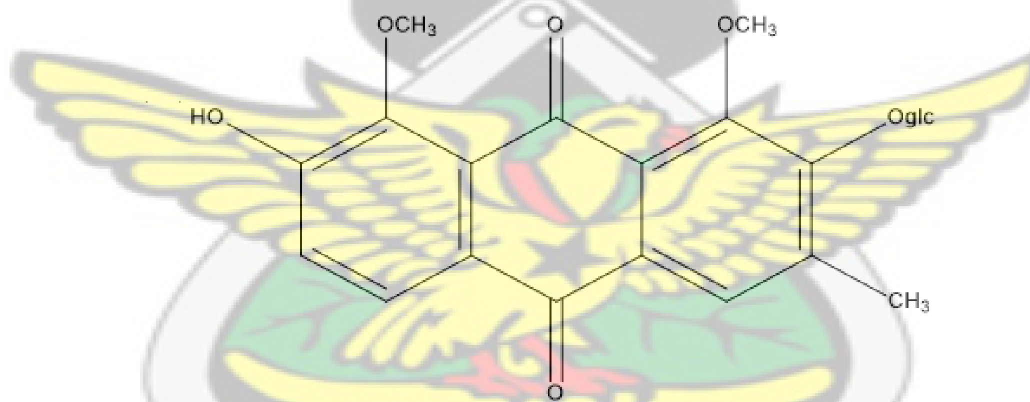


Chryso-obtusin-2-O-beta-D-glucoside **(51)**

Obtusifolin **(52)**



Obtusifolin-2-O-beta-D-glucoside **(53)**

2.5 *VIGNA UNGUICULATA* (COWPEA) LAM

Vigna unguiculata (Cowpea) also called black-eye pea and southern pea is a legume which originates from Africa. The plant has alternate and trifoliate leaves which are usually dark green in colour, and flowers may appear white, dirty yellow, pink, pale blue or purple. The flowers fall off the plant the same day after opening early in the morning and closing by noon. The plant is almost entirely self-pollinated but with its sticky and heavy pollen, extra-floral nectaries on its petioles and leaflets, cross pollination (10–15%) is sometimes carried out by beneficial insects like honey bees, lady beetles, predatory wasps, ants and soft-winged beetles (Aveling, 1999). Fruits are produced as pods which differ in size, shape, colour, texture and may appear erect, crescent shaped or coiled. The pods are always yellow when ripen, and may also appear brown or purple in some rare instances. Each pod usually contains 8-20 seeds which differ widely in size, shape and color. The globular shaped seeds are relatively large, long (2-12 mm), and 100 seeds weigh between 5-30 g (Figure 2.2). The testa of the seed could be smooth or wrinkled; white, green, red, brown, black, speckled, blotched, eyed (the hilum - central line - is white surrounded by a dark ring) or mottled in colour (Aveling, 1999).

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Figure 2.2. *V. unguiculata* seeds (White variety A. and red variety B.)

2.5.1 Economic importance and other uses of cowpea

Cowpea, the major essential legumimous crop cultivated in Sub-Saharan Africa occupies a greater area of land than any other grain legumes, of about 12 million hectares. Besides it being widely grown in West Africa, substantial quantities are also grown in East, Central and Southern Africa. In fact the leaves, green pods, green peas and dry grains of cowpea are eaten all over the continent in different dishes for being nutritious, providing proteins, vitamins and minerals especially micronutrients to humans and animals (Boukar *et al*., 2011). Although it is deficient in methionine and cystine in comparison with animal protein, it is rich in amino acids lysine and tryptophan, making it a good supplementary protein source for cereals and root-tuber based diets which are characteristic foods of many coastal and forest communities. The coinage of '*naman talaka'* meaning poor man's meat by Hausas of Central and West Africa reveals the nutritional attributes of cowpea grains. Comparatively, it is the second cheapest protein food source in Nigeria after soybean with a kilogram sold at US$ 2.13 (Langyintuo *et*

al., 2003). In Ghana, Nigeria and Cameroon, cowpea seed in its grain, split and powder forms is cooked for consumption as food in dishes commonly referred to as “waakye,’’ “red-red’’, “moin-moin alele’’or “nkoki”, “akara” or “ kosae’’. The rural households in Northern Ghana often prefer the ground form of cowpea seed because in this form it is not easily infested by post-harvest pests, and thus promote food protection between harvests (Nyankori, 2002). Green peas are sometimes roasted like peanuts. Seeds dried under the sun are also sometimes used in place of coffee. The whole plant is mixed with crops like corn or sorghum to form good quality hay or silage which provides fodder that massively supports the livestock industry of the dry savannas of West and Central Africa sub-regions (Hector and Jody, 2002). It is used to control weed in forestry plantations (Imrie, 2000). At least, 285, 000 tonnes of cowpea are shipped among countries in the West and Central African Regions year in and out. This quantity is probably below the actual quantity of cowpea seeds because records are not taken or kept on all flows. In 1998 Burkina Faso imported about 8, 000 tonnes of cowpea seeds from Niger and exported a total of 5, 500 tonnes to Togo, Côte d'Ivoire, Ghana and Benin (Langyintuo, 2000; Langyintuo *et al*., 2003).

2.5.2 Physical properties of cowpea

One hundred grammes (100 g) of cowpea seeds generate 338 calories of energy and consist of moisture 11.4 %, protein 22.5 g, fat 1.4 g, carbohydrates 61.0 g, fiber 5.4 g, ash 3.7 g, Ca 104 mg, P 416 g, thiamine 0.08 mg, riboflavin 0.09 mg, niacin 4.0 mg, and ascorbic acid 2.0 mg. Data from International Institute of Tropical Agriculture (IITA) on several thousand distinct cultivars have shown that cowpea seeds' proteins contain 90 % water-insoluble globulins and 10 % water-soluble albumins, amino acids isoleucine 239 mg/g N, leucine 440 mg/g N, lysine

427 mg/g N, methionine 73 mg/g N, cystine 68 mg/g N, phenylalanine 323 mg/g N, tyrosine 163 mg/g N, threonine 225 mg/g N, tryptophan 68 mg/g N, valine 283 mg/g N, arginine 400 mg/g N, histidine 204 mg/g N, alanine 257 mg/g N, aspartic acid 689 mg/g N, glutamic acid 1027 mg/g N, glycine 234 mg/g N, proline 244 mg/g N, and serine 268 mg/g N. Total sugars present in the cowpea seeds vary from13.7 to 19.7 %, and consist of 1.5 % sucrose, 0.4 % raffinose, 2.0 % stachyose, 3.1 % verbascose. Starch content may range from 50.6 to 67.0 %, consisting of 20.9–48.7 % amylase and 11.4–36.6 % amylopectin. Trypsin, chymotrypsin and cyanogen are present in seeds in concentrations of 2 mg/100 ml extract. Cooking of cowpea seeds increases its nutritive value inhibiting the activity of trypsin and other toxins as a result of heating (Duke, 1981b). Obviously, the positive health implications of consuming cowpeas are enormous and all must be done to safeguard its protection.

2.6 *CALLOSOBRUCHUS MACULATUS* (COWPEA WEEVIL)

Callosobruchus maculatus (Cowpea weevils) are important storage pests of cowpea seeds in the tropics (NRI, 1996). They are small insects with lengths of three millimeters, typically orange-brown or reddish-brown colored bodies with dark markings, and bodies taper towards their heads, but lack the extended snout of true weevils, which feed on cereal grains, and are often confused with other related bruchid species (Figure 2.3) (Wackers *et al*., 2006). Their slightly shorter wing (elytra) covers their abdomens which are marked with black and grey, with two black spots near their centers, and expose the last segment of their abdomens. Terminal abdominal segments also have two visible black spots. Limbs and antennae of insects are lengthy, and hind femurs are always large.

Transfer of the insects into storage is via seeds infested by the insects, either from the paddock into storage, or in infested seeds within and between storage facilities. The good insulating nature of seeds permits harvested seeds in bulk storage to maintain their at-harvest temperature for an extended time interval to include the rainy season. This therefore causes lesser development time for cowpea weevils in seeds than when seeds are kept to gain outside air temperatures (Zannou *et al*., 2003). The oval or spindle shaped eggs of 0.75 mm length with clear, shiny or white appearances, are firmly attached to the cowpea seed surface (Beck and Blumer, 2009).

Despite the small nature of their eggs, they are readily visible on the surfaces of cowpea seeds. In the field, female insects lay eggs on the pods of legumes as plants get to maturity though emergence of young adults often takes place after harvest. While in storage, they lay eggs on surfaces of the seeds (Ileke and Bulus, 2012a; Ileke *et al*., 2012). The rate at which female *C. maculatus* insects lay eggs depends on the number of hosts present. They lay less number of eggs when limited numbers of host are available which minmizes larval competition. In contrast, females will on rare situations continue to lay eggs onto unsuitable substrates when limited numbers of host are available. This egg laying attitude causes high mortality, and could be a way of varying the host if preferred hosts are not present or found (Wang and Horng, 2004; Cheng *et al*. 2003). Ovipositing females lay eggs on the largest seed first, and each egg laid is firmly attached to the seed coat. They prefer laying eggs on seeds that do not have any eggs than those that already bear eggs, and also prefer the seeds that have few eggs than the ones with many eggs. This behaviour demonstrated by the ovipositing females promotes a non-random, uniform distribution of eggs on seeds and decreases the level or extent of larval

competition (Messina, 1993). Eggs laid by adult females are usually hatched into larvae in four days. These larvae are whitish in colour or appear like short cream-coloured maggot, almost C-shaped possessing a small head, and are not often seen as they penetrate the seeds to feed on the endosperm. The second, third and fourth larval instars can be distinguished from the first instars larvae in that they all have well-developed thoracic legs, an H-shaped plate on the prothorax and a hatching spine on each lateral side of their first abdominal segment. As the egg hatches, the larva that comes out burrows into the seed, whilst the upper surface of the egg chorion remains attached to the seed coat or surface. Frass is tranferred into the egg charion vacated by the larva as feeding proceeds. Larvae excavate underlying seed tissues to form small openings directly beneath the seed testa, seen as tiny transparent windows in the seed coat, within which larvae pupate and enclose. Inside each cowpea seed, 8 to 10 or more larvae can undergo complete growth to form adult insects. If larval burrows meet at a point, the larvae return to where they have previously burrowed, put together frass, transport it to the intersection, and construct a wall to close up the meeting point between the burrows. Grains dissected after the meeting points between burrows are sealed with frass often illustrate larvae feeding alongside each other in the same direction. This proper use of space by larvae as they feed within a cowpea seed increases larval survival than when burrows intersect each other in a haphazard manner. The wall construction attitude shown by larvae could be linked to the mechanism by which scramble-type larvae of *C. maculatus* avoid larval interference (Mano and Toquenaga, 2008). Multiple *C. maculatus* larvae scramble for nutrients in just one cowpea seed because it is impossible for larvae to move from one grain to another. To complete the first, second, third and fourth instars larval stages, it takes twenty two days (Pajni, 1987). All

these four larval stages and pupation occur inside the seeds. Pupation takes three-four days to get to completion. Adults *C. maculatus* emerge through circular holes in the 'windows' and can mate within an hour. Severely damaged seeds are always having openings as windows, and egg charions or shells (Ofuya and Agele, 1990). Total insect development into adults takes approximately thirty days.

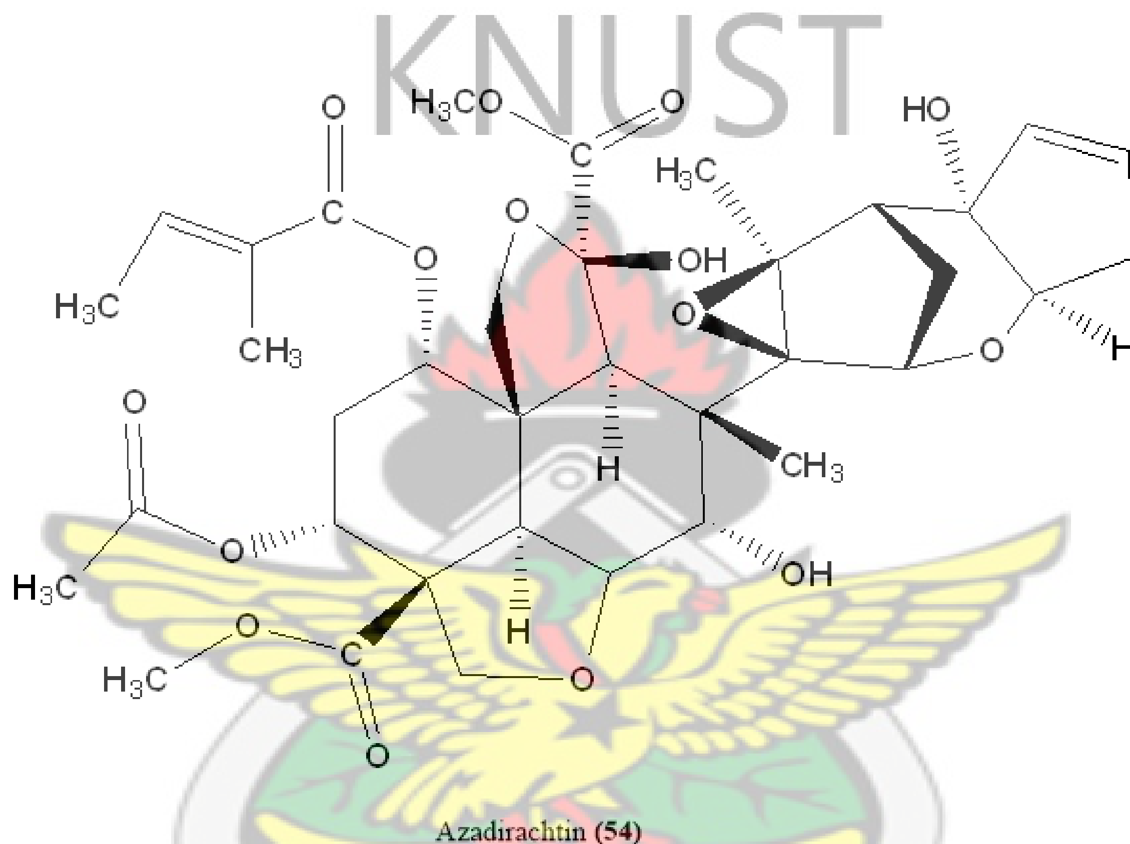
Figure 2.3. *C. maculatus* (Adult insect)

2.7 NEEM OIL

Neem oil is a natural plant based broad spectrum botanical pesticide extracted from seeds of *Azadirachta indica* and contains mainly azadirachtin (54) as its active ingredient. It exhibits multiple modes of action and acts as an insecticide, antifeedant, ovipositing deterrent, repellent, insect growth regulator and sterilant. Azadirachtin has properties that can be compared with insecticidal soap, superior horticultural oil and *Bacillus thuringiensis* used against harmful insects and microbes (SaiRam *et al.*, 2000). It can act as a substance which prevents a number of insect pests including beetles from feeding. The growth regulating property of azadirachtin

has made it to be very toxic to the immature stages of insects. It lowers the amount of ecdysome secreted halting the insect's molting process for larvae not to develop into adult insects. The larvae of most members in the lepidoptera family (moths and butterflies) are negatively affected by azadirachtin. After treatments with neem oil may show insects with crippled or distorted wings, larvae and nymphs remaining in their immature stages and dead insects or larvae (Dubey, 2010). Most soft-skinned insect larvae are easily killed as they come into direct contact with neem oil. Adult insects are not usually killed as they come into contact with azadirachtin, instead mating and sexual communication could be hindered causing reduced fecundity (Schmutter, 1990). Neem oil forms an oily layer on the body of the insect, blocking the breathing outlets and inlets and suffocating the adult insect to death. The oil can be applied indoor or outdoor on plants, vegetables, fruits, homes and residential landscapes to control fungal diseases including black spot, mildews, rusts, scabs, and kill insects including whiteflies, aphids, thrips, lace bugs, psyllids, mealy bugs, leafhopper, catepillars and scales. It prevents the germination and penetration of some fungal spores. In one study, researchers discovered that a one percent neem oil treatment effectively inhibited growth of powdery mildew on hydrangeas, lilacs and phlox (Quarles, 1994; Locke, 1994). Neem oil has limited persistence and is easily degraded by temperature, ultraviolet light, rainfall and other environmental factors. Since it is easily degraded, its negative effect on non-targeted organisms could be lesser than some traditional pesticides. Neem oil has shown low toxicity to mammals (Dubey, 2010). Since immediate knockdown effect cannot be seen, and insects may continue to feed, repeated applications of neem oil is required to obtain an excellent result. In contrast, its repellant effects can prevent or reduce feeding by the insects. Phytotoxicity of some

formulations of neem oil could be a call for concern. Plants wilted or under stress, and transplanted having limited root development should not be subjected to neem oil treatment (Naik and Dumbre, 1985; Lai *et al*., 1990).

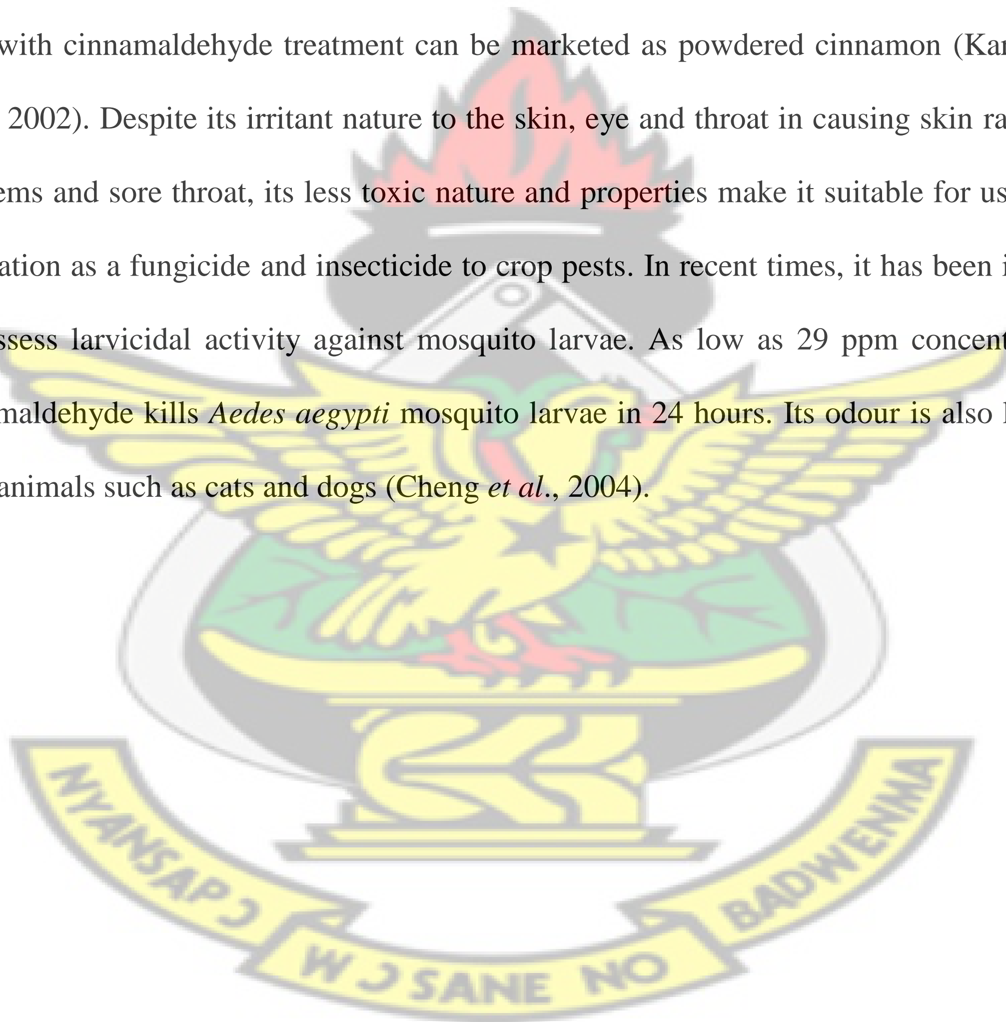


Azadirachtin (**54**)

2.8 CINNAMALDEHYDE

Cinnamaldehyde (55) synonymously referred to as benzylideneactaldehyde, cinnamic aldehyde, 3-phenylacrolein and 3-phenylpropenal is a viscous yellow oil with a sweet taste, and becomes viscous when exposed to air. It is one of the main constituents of *Cinnamomum cassia,* a Chinese herbal medicine and is known to inhibit the formation of ATP in bacterial cells (Gill and Hooley, 2004). Recent reports have revealed that it has demonstrated growth inhibitory effect on cancer cells. Reproduction, invasion, and cell growth were hindered in a

murine A375 model of human melanoma, though only at high doses, not achievable through dietary intake (Cabello *et al*., 2009). It is applied as flavoring in beverages, candy, chewing gum and ice cream; use concentrations range from 9 to 4900 parts per million. It is used in the production of perfumes with natural, sweet or fruity scents. Almond, apricot, butterscotch, and other aromas can be produced by partially employing the compound for their pleasant smells. Cinnamaldehyde may be used as a food adulterant; powdered beech nut husk with cinnamaldehyde treatment can be marketed as powdered cinnamon (Karl-George *et al*., 2002). Despite its irritant nature to the skin, eye and throat in causing skin rashes, eye problems and sore throat, its less toxic nature and properties make it suitable for use in crop cultivation as a fungicide and insecticide to crop pests. In recent times, it has been identified to possess larvicidal activity against mosquito larvae. As low as 29 ppm concentration of cinnamaldehyde kills *Aedes aegypti* mosquito larvae in 24 hours. Its odour is also known to repel animals such as cats and dogs (Cheng *et al*., 2004).



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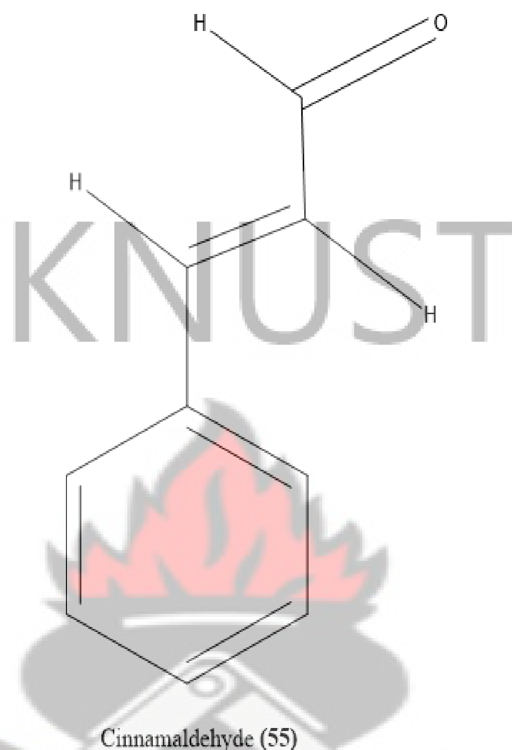

Cinnamaldehyde (55)

2.9 ENHANCERS AND INHIBITORS OF SEED GERMINATION

The application of organic ingredients from plants on seeds with the aid of organic solvents referred to as organopriming to serve as pesticides during storage, can enhance or inhibit germination and seedling vigor. Reduced time for germination and high vigor of seeded crops are essential for their final yields and quality, whereas increased time for germination and low vigor expose seeds and seedlings to deterioration by biotic and abiotic factors.

Currently, there are different seed priming methods which include thermopriming (seed treatment at low or high temperatures), halopriming (seed treatment with solutions of halogen salts), solid matrix priming (seed treatment with a solid matrix instead of osmotic solution to promote germination), hydropriming (seed treatment with water), biopriming (seed treatment

with biological agents that induce hydration) (Pandita *et al*., 2007). Biopriming of corn seed involves its treatment with a bacterium and soaking in warm water for moisture content of seed to increase. Organopriming of cowpea seed involves its treatment with blends of organic compounds of biological origin (plants) in an organic solvent (acetone). These priming methods have benefits and drawbacks which may depend on seed types, amount of priming agent, period of treatment, etc (Salwa and Samia, 2013).

2.10 PESTICIDAL AND SEED GERMINATION BIOASSAYS

2.10.1 Insecticidal (Adulticidal) activity bioassay

The experimental set up for insecticidal activity bioassays requires topical or non-topical application of treatments such as plant extracts, compounds, hormones or enzymes on adult insects to the extents that could cause biochemical reactions resulting in morphological or physiological changes and dead of insects (Prajapati *et al*., 2005; Upadhyay, 2012).

In the choice method, insects are given the opportunity to make a choice when kept in a set up consisting of different treatments, where they most often chose the treatment with no or the least toxicity, whereas in the no choice method, insects are introduced to just one treatment in a set up and do not have the chance to make a choice (Prajapati *et al*., 2005; Upadhyay, 2012).

Essential oils from ten medicinal plant species have demonstrated insecticidal effects on adult *Aedes stephensi*, *Aedes aegypti* and *Culex quinquefasciatus* respectively by the choice bioassay technique. Oils of concentrations 50.00-300.00 mg/mL were tested alongside acetone as a vehicle. 0.50 mL of each concentration was non-topically applied on adult mosquitoes by dispensing over a card board sheet. Solvent used to prepare different concentrations of oil was kept to evaporate at room temperature and card board sheet placed on a mosquito mat machine

and machine kept on for 15 minutes. The card board sheet treated with acetone only served as a control. Vapour toxicity of each oil was assessed in a special designer apparatus as 50 female adults of each insect species were introduced. Mortality of adult mosquito was recorded at the different dosages of oils one hour after treatment. The experiment carried out at each dose of oil was triplicated and data of dead insects recorded were statistically analyzed for LC_{50} values (Tripathi *et al*., 2004; Prajapati *et al*., 2005).

Contrary to the choice method of insecticidal activity bioassays, the no choice method of insecticidal activity test considers application of a treatment/ control either topically on the insects or non-topically on a host of the insects (Seeds or leaves of plants) or any substance, kept in a cage or confined set up and then monitored with time for death of insects. This bioassay method has an advantage over the choice method and does give a true picture of effectiveness of treatments since insects are not given the chance of preference (Upadhyay, 2012).

Considering the advantage that the no choice method of insecticidal activity assays has over the choice method, it was then selected and employed as an assay of choice in this research.

2.10.2 Larvicidal activity bioassay

Unlike the insecticidal activity bioassays which focus on the choice and no choice methods, larvicidal activity bioassays mostly deals with the no choice method since larvae of most insects often survive for a longer period only within their hosts or habitats. Outside and away from their hosts or habitats conditions of surviving become unfavourable and chances of living also become very slim. This explains why most research works reported are focused on the no choice bioassay method of larvicidal activity. Also, like the choice method of insecticidal

activity test which mimics the natural environment, the no choice method of larvicidal activity bioassays gives room for the natural host or habitat of the insect larvae to be used (Bhattacharya and Chandra, 2014).

Each gradient of extractive is tested thrice and the control consists of distilled water without any extractive. Larvae of specific instar stage (1st, 2nd, 3rd and 4th instar stages) are placed in Petri dishes of 9 cm diameter and 150 mL volume capacity, and then top up with 100 mL of tap water. Each Petri dish is kept at room temperature 27±1 °C and relative humidity 80±2 % for a total period of 72 hours. Larvae are pricked by a sharp needle in the siphon or cervical region to confirm death when there are no movement. The number of dead larvae are recorded after 24 hour intervals and percentage mortality calculated from average death of three replicates. The mortalities after 48 and 72 hours are obtained by adding up the mortalities after 24 and 48 hours, 24, 48 and 72 hours respectively (Bhattacharya and Chandra, 2014).

For the no choice bioassay method, extracts at varied concentrations are tested alongside distilled water (control) on mosquito larvae. Experiments are replicated for each test concentration at temperatures 25-28 °C, and dead larvae are counted after 24 hours (WHO, 2005b; Eliman *et al*., 2009).

2.10.3 Oviposition deterrence activity bioassay

Using the choice bioassay technique, extractives at varying concentrations in plastic containers alongside distilled water in containers were kept in separate cages to serve as treatments and control respectively. The female mosquitoes are introduced into the cage. At the end of 48 hours, eggs laid in the extracts and control containers by the mosquitoes were separately counted.

Effective oviposition deterrence was calculated as follows:

Effective deterrence (%) = [(NC-NT)/NC] * 100

Where NC= number of eggs laid in distilled water only, NT= number of eggs laid in aqueous extract solution (Bhattacharya and Chandra, 2014).

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2.10.4 Antifeedant activity bioassay

Using the non-choice method assay, leaf disks of the plant is treated with solutions of extracts/ compounds alongside distilled water treated leaf disks as a control. These were introduced to 3rd instar larvae of the organism. Results are obtained virtually by comparing treated and untreated leaf disks eaten by larvae (Huang *et al*., 2008).

Three khayanoside compounds isolated from *Khaya senegalensis* have shown antifeedant activity on larvae of *Spodoptera littoralis* in a two choices assay. Results are also obtained by comparing treated and untreated leaf disks eaten by larvae (Munehiro *et al*., 2002).

2.10.5 Seed germination bioassay

Seeds or grains of plants are treated with leaf samples of plant species at varying concentrations alongside actellic dust as a positive control. The treated seeds are then introduced to adult insect pests by the no-choice method assay alongside untreated seeds as a negative control. Results are obtained by comparing number of dead adult insects, oviposited eggs by female insects and weight loss of treated and untreated seeds. Seeds are randomly chosen from the treatments and control and then subjected to germination experiment to assess for percentage germination (Mundi *et al*., 2012).

Seeds of two cultivars of the plant (*Pennisetum glaucum* L. or millet) are treated with sodium chloride solution alongside seeds treated with distilled water only as a control. These seeds

were introduced on filter papers moistened with distilled water to undergo germination. Germinated seeds were counted, percentage germination and seedling vigor index (SVI) were calculated according to the International Seed Testing Association (ISTA) method.

GP= Number of germinated seeds / Total number of seeds planted $\times$100 (Ilori *et al*., 2012).

SVI was determined by the following formula:

SVI = (seedling length (cm) $\times$ germination percentage) $\div$100 (Siddig and Abdellatif, 2015).

To serve the need of farmers, seeds/grains are often protected from insect infestations in non-planting seasons by the use of botanical or synthetic pesticides so as to maintain/enhance viability of seeds/grains which are to be sown in planting seasons. Most often the botanical or synthetic pesticides used to protect seeds/grains from insect infestations turn to have negative impact on viability of seeds/grains (Naik and Dumbre, 1985).

In our search for botanical or synthetic pesticides, there is the need to identify agents/ ingredients that could give a true representation of what farmers encounter from when they apply pesticide to their seeds/grains to when they are sown to germinate. In other words, there is the need to identify agents/ ingredients that can function as both pesticides and seed germination enhancers. This then explains why seed germination experiments that are carried out after seeds have been treated with extracts/ compounds and subjected to biopesticide activity test are preferred to seed germination experiments conducted for seeds treated with extracts/ compounds and have not gone through pesticide activity evaluation.

Chapter 3

MATERIALS AND METHODS

3.1 COLLECTION AND AUTHENTICATION OF PLANT MATERIAL

Pods containing *Cassia tora* Linn (Fabaceae) seeds were randomly harvested from trees in the vicinity of Kramah quarters, Kumba, South-West Region, Cameroon on 2nd March, 2013. The plant was authenticated by Dr. Isaac Sackey and Dr. Walters Kpikpi, botanists at the Department of Applied Biology, University for Development Studies, Ghana where a voucher specimen has been deposited (AB/4/160/13).

3.2 PROCESSING AND EXTRACTION

Seed-pods of *Cassia tora* plant were opened with the hand. They were air dried at room temperature (25 °C) for one week, and pulverized into fine powder with the aid of mortar and pestle. Five kilograms (5 kg) of the powdered seed sample of *C. tora* was soxhlet-extracted sequentially by using pet ether (5 L), ethyl acetate (5 L) and methanol (5 L) solvents over 72 hours until material was completely exhausted. Each extract was concentrated under reduced pressure on a rotary evaporator at 40 °C to small volumes and subsequently evaporated to dryness on a water bath to afford pet ether (2.88 %w/w), ethyl acetate (0.51 %w/w) and methanol (0.21 %w/w) extracts of *C. tora* seeds. They were then kept in a desiccator until required for use.

3.3 COMMERCIAL COWPEA SEEDS

Cowpea seeds (*Vigna unguiculata*), not previously subjected to any pesticide treatments, were bought from Gitarhai grain market, Nairobi-Kenya to obtain infested seeds without perforations but with eggs glued on their surfaces for rearing of *Callosobrucus maculatus*

(cowpea weevils). Two to three days old adult weevils were collected from selected infested seeds after incubation period of 30-34 days and introduced into good viable and non-infected grains collected from farmers, disinfested and treated with *C. tora* seed extracts.

The infested seeds from grains bought at the market were kept in plastic containers, covered with muslin cloth as lids aided by rubber bands. They were taken to the insectary of the International Center for Insect Physiology and Ecology (ICIPE), and kept at humidity and temperature of 78±2 % and 28±3 °C respectively in the room maintained at 12 hours day and 12 hours night cycle. The incubation was extended for 30 - 34 days until emergence of young adult cowpea weevils.

3.4 NON-INFECTED COWPEA SEEDS

Cowpea seeds harvested from fields not subjected to any pesticide treatments were collected from farmers who work under the supervision of International Center of Insect Physiology and Ecology (ICIPE), Kenya. These seeds were used for pesticide activity, seed germination and seedling vigor enhancement ability experiments after disinfestation to obtain good viable, non-infected seeds and treatment with *C. tora* seed extract.

Before cowpea seeds collected from the farmers were subjected to biological activity experiments, they were checked for not having holes and eggs on their surfaces, and then kept in an air tight glass jar in a freezer at -20 °C for five days to kill all hidden infestations. This is because all the life stages, particularly the eggs are very sensitive to cold. The disinfested cowpea seeds inside the air tight glass jar were removed from the freezer kept on the laboratory bench for three hours at room temperature (25 °C). They were then placed inside a WT- Binder

oven at 40 ^{o}C for 2 hours and later spread on clean sheets of paper in the laboratory at room temperature to air dry and prevent mouldiness before being stored in plastic containers with tight lids (Jambere *et al*., 1995; Koehler, 2003).

3.5 PREPARATION OF EXTRACT CONCENTRATIONS

Acetone (Purity ≥ 99.9 %, Molecular Weight 58.08, Analytical standard from Sigma-Aldrich Chemicals Ltd.) as a vehicle, clarified hydrophorbic extract of neem oil (Neem oil 70 %, Inert material 30 %, Broad Spectrum Fungicide, Insecticide and Miticide of botanical origin, Palmetto FL 34220 B1, EPA Reg.70051-2-829) and cinnamaldehyde (Assay 98 %, Molecular Weight 132.16, Repellent and insecticide of botanical origin, BDH Chemicals Ltd., Poole England) were used to prepare concentrations for negative and positive controls respectively. The extracts and standard drugs were dissolved in acetone to prepare concentrations of 100.00, 200.00 and 300.00 μg/mL used for the study.

3.6 PREPARATION OF TREATMENT GROUPS

The experimental groups consist of (i) cowpea seeds treated with extracts of *Cassia tora* (Pet ether, EtOAc and MeOH extracts) (ii) seeds treated with positive controls (Neem oil -control 1 and cinnamaldehyde -control 2) (iii) seeds treated with acetone (negative control or control 3) (iv) untreated seeds introduced to weevils (control 4) (v) untreated seeds not introduced to weevils (control 5). Control 5 was used to check weight loss or gain of cowpea seeds as a result of either dehydration or hydration that could affect the exact quantity of cowpea seeds consumption by weevil larvae in control 4. All crude extracts and positive control treatment groups were tested at three different concentrations (100, 200 and 300 μg/ml). For the application of crude extracts and positive controls/vehicle, 2.5 g disinfected cowpea seeds (non-

infected seeds) in sample vials were separately immersed in 1.0 mL each of the crude extracts and positive control (prepared in acetone) stirred and left in open air to dry overnight. Experimental group (v), untreated seeds (2.5 g) not subjected to cowpea weevils in the course of experimentation served as control 5 for correction of factors which include dehydration and hydration that can affect weight loss of cowpea seeds. All experiments were set up in triplicate. Before commencement of the bioassay experiments, insects were separated into males and females according to standard methods (Halstead, 1963; Odeyemi and Daramola, 2000). The males of *C. maculatus* have comparatively shorter abdomens and the dorsal sides of their terminal segments are sharply curved downward and inward. In contrast, the females have comparatively longer abdomens and the dorsal sides of their terminal segments are only slightly bent downward. The females also have two dark visible spots on their elytra (a modified hardened forewing).

3.7 BIOPESTICIDE AND COWPEA SEED-GERMINATION EVALUATION ASSAYS

3.7.1 Insecticidal assay

Five pairs of adult cowpea weevils (2 to 3 days old) were introduced into each treatment (Pet ether, EtOAc and MeOH extracts) or control groups in plastic containers covered with muslin cloth as lids aided by rubber bands (Figure 3.1), except group (v). The plastic containers were all kept at humidity and temperature of 78±2 % and 28±3 ^{o}C respectively, 12 hours day and 12 hours night cycle. Weevils were observed for deaths, and were assumed dead when probed with a brush having a sharp edge and made no movement (Figure 3.1). Insecticidal effects were assessed every 24 hours for 7 days and expressed as percent mortality as follows:

% Mortality of weevils = (number of dead weevils)/(total number of weevils) ×100

The time taken for 50 % of the adult population to die (LT_{50}) and concentration at which 50 % of adult insects were killed (LC_{50}), were also calculated by subjecting mortalities caused by test extracts and time or concentration of mortalities to probit analysis (Finney and Stevens, 1948; Hayes and Kruger, 2014).

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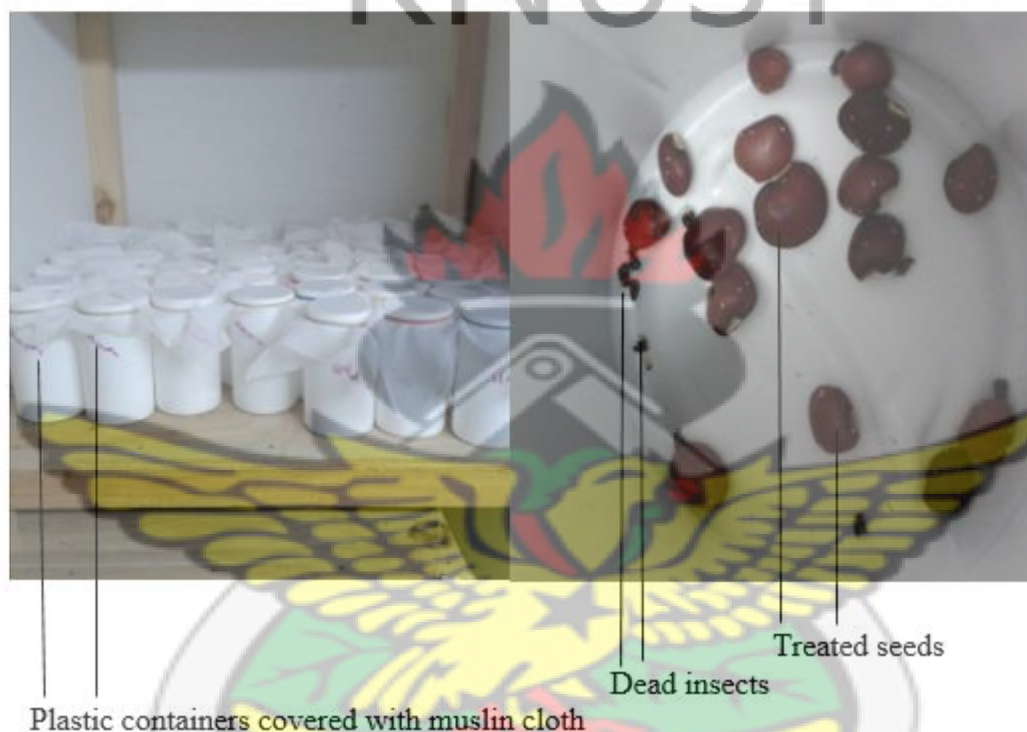


Figure 3.1. Insecticidal activity setups

The experimental setup for evaluation of bulked fractions F_1, F_2, F_3, F_4 and F_5 (BKF-1, BKF-2, BKF-3, BKF-4 and BKF-5), isolates/compounds A and B_1 for insecticidal activity was the same as that described for the seed extracts (Pet ether, EtOAc and MeOH extracts). They were also evaluated at concentrations of 100.0, 200.0 and 300.0 μg/mL. Insecticidal effects were

assessed every 24 hours for 7 days and expressed as percent mortality as described in section 3.8.1.

LT_{50} and LC_{50} values of bulked fractions, positive controls and isolated compounds were calculated by subjecting mortalities and time or concentration of mortalities to probit analysis.

3.7.2 Oviposition deterrence assay

The experimental set up for oviposition deterrence activity evaluation of crude seed extracts of *C. tora* (Pet ether, EtOAc and MeOH extracts) was the same as the insecticidal activity assay (Section 3.8.1). Cowpea seeds treated or primed with the crude extracts, the positive and negative controls in plastic containers having adult insects covered with muslin cloth were observed for eggs laid alongside dead weevils. The number of eggs laid on the surface of cowpea seeds by female cowpea weevils were counted every 24 hours for 7 days (Figure 3.2). The modified method of oviposition deterrence index initially described by Kramer and Mulla, (1979) and later reported by Eliman *et al*., 2009 was employed to assess the comparative effectiveness of test and positive control samples as follows:

$$\%\ oviposition\ deterrence\ index\ (\%ODI) = (1 - [NT \div NC]) \times 100$$

Where NT is the number of eggs laid on surface of treated cowpea seeds (Treatments = pet ether, EtOAc and MeOH extracts, neem oil, cinnamaldehyde and acetone) and NC is the number of eggs laid on the surface of untreated cowpea seeds exposed to weevils - control 4 (Eliman *et al*., 2009). Similarly, bulked fractions F_1, F_2 and F_3, F_4 and F_5, isolates/compounds A and B_1 were tested in the same way for oviposition deterrence activity as the crude seed extracts.

Figure 3.2. Eggs laid on the surface of cowpea seeds

3.7.3 Antifeedant assay

The antifeedant activity followed the same process as the insecticidal activity assay. At the end of day-7, after dead weevils and eggs laid by females have been counted, all insects both dead and alive were removed from each plastic container and the treated cowpea seeds were further kept inside the insect cage for another 27 days to allow for emergence of the first filial generation. Treatments and control seeds were weighed to assess antifeedant effect, which was done by calculating percent antifeedant index (% AFI) as follows:

$$\% AFI = (1 - [Treatment\ consumption \div Control\ consumption]) \times 100$$

Where treatment consumption is the weight loss of cowpea seeds treated with extract, positive controls and vehicle as a result of feeding insect larvae, and control consumption is the weight loss of untreated cowpea seeds as a result of feeding insect larvae (Tri *et al*., 2011). The bulked fractions and isolated compounds were tested in the same way.

3.7.4 Cowpea seed germination and seedling vigor assays

Viability test was performed on cowpea seeds after they had been treated with *C. tora* seed extracts at concentrations of 100, 200 and 300 µg/mL. This was done to assess the effect of *C. tora* seed extracts on germination of cowpea seeds and growth strength of young cowpea seedlings. Each concentration was tested in triplicate in the laboratory at the temperature 25±2.0 ^{0}C. Each replicate experiment consisted of ten cowpea seeds selected randomly from disinfected cowpea seeds previously treated with *C. tora* seed extracts. Cowpea seeds treated with acetone served as control 3, untreated seeds that were introduced to weevils as control 4, untreated cowpea seeds that were not introduced to weevils but kept under same conditions as control 4 as control 5, alongside cowpea seeds separately treated with neem oil and cinnamaldehyde pesticides as control 1 and 2 respectively. Treatments and controls were separately soaked in 0.50 L distilled water for six hours, removed and placed on moist cotton wool of 9 cm diameter and 0.50 cm thickness, inside non-disposable Petri dishes. Each Petri dish containing cowpea seeds was irrigated with 3 mL distilled water daily for the 15 days of experimentation. Germinated seeds were counted on daily basis, lengths of radicle and plumule were measured, and emerged seedlings were counted at the end of day 15. Percentage viability and vigor index (% SVI) of seeds and seedlings were calculated as follows:

$$\%\,Viability = \frac{Number\ of\ germinated\ seeds}{Total\ number\ of\ seeds} X\ 100$$

$$\%\,SVI = \%\,viability \times [length\ of\ radicle\ (cm) + length\ of\ plumule\ (cm)]$$

(Ilori *et al*., 2012; Siddig. and Abdellatif, 2015)

Bulked fractions and isolates were tested at 100, 200 and 300 µg/ml for enhancing power on cowpea seed germination and seedling vigor in the same way as the crude seed extracts.

3.8 MOSQUITO LARVICIDE EVALUATION ASSAY

Third-fourth instar larvae of *Anopheles gambiae* (Mbita strain) were obtained from a colony kept at the insectary of the Duduville Campus of the International Centre of Insect Physiology and Ecology (ICIPE), Nairobi. The strain was originally obtained from Mbita Point, Western Kenya, where colonies have been maintained and reared under laboratory conditions for over 20 years. The larvae were fed with Tetramin fish food and reared at mean temperatures of 31 and 24 °C, relative humidities of 52 and 72 % respectively in a day and night cycle (12 hours: 12 hours).

Mosquito larvicidal assay was carried out according to the standard procedure described by WHO (2005b) and Ndung'u *et al* (2004), except with the use of DMSO solvent in place of acetone to prepare concentrations of 100, 10, 5, 2.5 and 1 ppm respectively for the most potent crude seed extract (EtOAc extract of *C. tora* seeds) in the biopesticide and cowpea seed germination evaluation assays. Each test treatment (1.0 mL, w/v) in DMSO was made up to 20 mL with distilled water in 100 mL beakers in three replicates. A potent naturally occurring limonoid, azadirachtin previously isolated and characterized at ICIPE from *Azadirachta indica* (Ndung'u *et al.,* 2004) was used as positive control. It was similarly prepared in DMSO (1.0 mL) as the crude seed extract. Twenty late third-fourth instar larvae each were introduced into the beakers of test and control solutions, and larval mortality was monitored every 24 hours and recorded for up to 96 hours. Dead larvae were removed from each treatment every 24 hours after counting. The temperature of the room where the experiment was conducted was maintained at 25 – 27 °C and larvae in each beaker of the test and control solutions were supplied daily with approximately 1 mg of Tetramin fish food (Tetra, Spectrum Brands–Pet,

Melle, Germany). The bulked fractions (BKF-3 and BKF-4) and isolated compounds (A and B_2) were similarly tested for mosquito larvicidal activity at concentrations of 100, 10, 5, 2.5 and 1 ppm using DMSO as the vehicle**.**

3.9 CHROMATOGRAPHIC TECHNIQUES

3.9.1 Choice of EtOAc extract of *C. tora* for chromatographic fractionation

The bioassay results for pesticidal activity (Sections 4.1.1, 4.2.1 and 4.3.1), cowpea seed-germination and seedling-vigor enhancing abilities of seeds of the plant studied (Section 4.4.1) revealed the EtOAc extract to be the most active of the seed extracts. Thus, the EtOAc extract of *C. tora* seeds was subjected to different chromatographic techniques to isolate its bioactive molecules/compounds responsible for the observed activities.

3.9.2 Chromatographic materials

The stationary phase material used for the column chromatographic technique was silica gel 60 (70-150 mesh ASTM, Merck Germany), while aluminium pre-coated silica gel plates 60 F_{254} (0.25 mm thick) were used for the analytical thin layer chromatography (TLC).

3.9.3 Solvents and reagents

The solvents used for extraction, column chromatographic separation, TLC analysis and recrystallization of compounds were of analytical grade and included petroleum ether, n-hexane, trichloromethane (chloroform), ethanol, ethyl acetate and methanol. The reagents sulphuric acid, glacial acetic acid, sodium hydroxide and p-anisaldehyde were purchased from BDH laboratory supplies, England.

3.9.4 Development of thin layer chromatogram

The one way ascending thin layer chromatographic technique was employed. Samples to be analysed by TLC were dissolved in appropriate organic solvents. They were spotted with the aid of capillary tubes on the baseline at one end of the TLC plate, about 2 cm above the edge and 1.5 cm away from the margin.

The spots were air-dried and the plates placed inside a chromatographic tank containing the mobile phase with the baseline slightly above the mobile phase. The development of TLC plates was based on the principle of capillary action which permitted movement of the mobile phase along plates in an ascending manner, carrying with it the components of the solutes. When the mobile phase had reached a reasonable height on the plates, the operation was stopped and the solvent front marked (Brain and Turner, 1975).

3.9.5 Detection for analytical thin layer chromatography

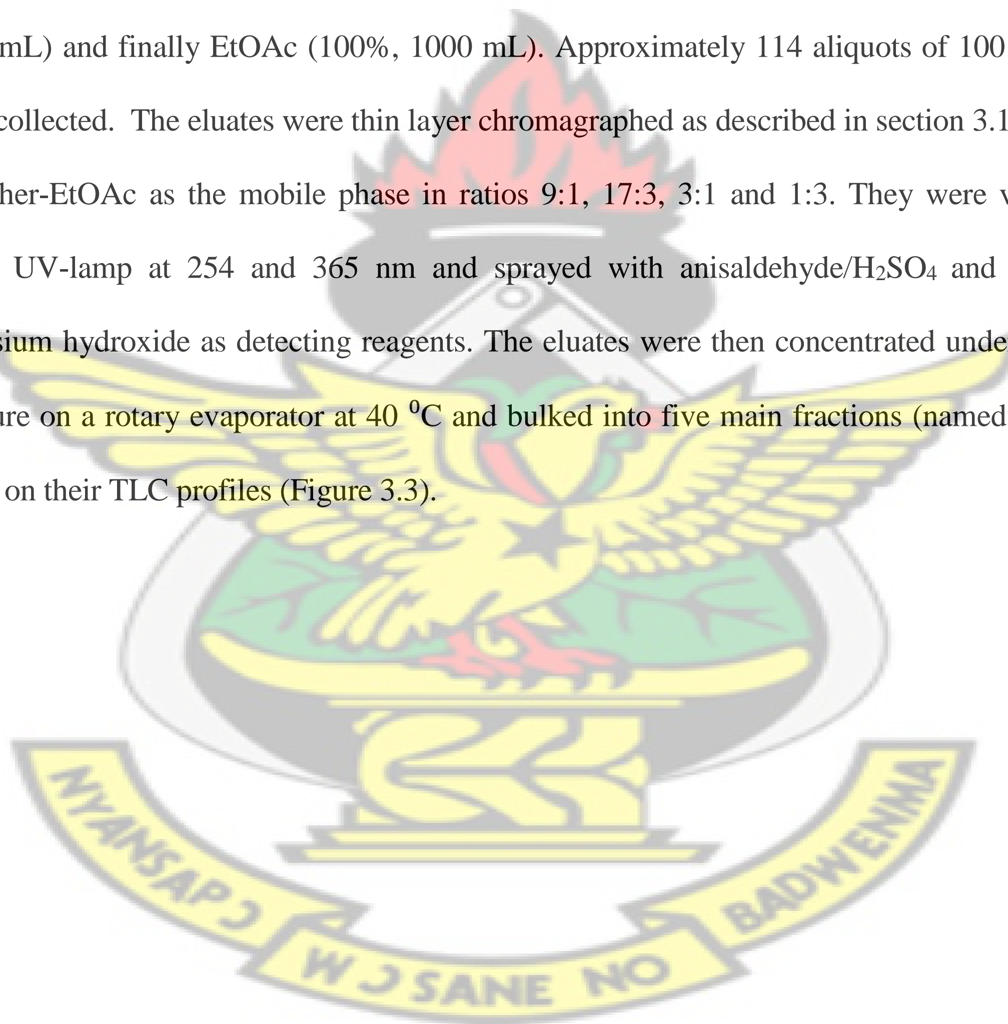
The area on TLC plates corresponding to separated compounds were detected by spraying with anisaldehyde/H_2SO_4 and ethanolic potassium hydroxide. TLC chromatograms were visualized under UV-lamp at 254 and 365 nm respectively. The anisaldehyde/H_2SO_4 reagent was prepared by mixing p-anisaldehyde, glacial acetic acid, methanol and concentrated sulphuric acid in volume ratio 1: 20: 170: 10 or 0.1: 2: 17: 1, while the ethanolic solution of potassium hydroxide was prepared by dissolving 0.5 g of potassium hydroxide in 10 mL of ethanol.

3.10 ISOLATION OF COMPOUNDS FROM ETHYL ACETATE EXTRACT

3.10.1 Column chromatographic fractionation of EtOAc extract

Silica gel 60 (300 g, 70-150 Mesh ASTM) was packed into a glass column (400 mm x 35 mm) by the dry method. The EtOAc extract (22.5 g) was dissolved in 45 mL of EtOAc, and mixed

with 55 g of silica gel, allowed to dry, and was loaded on to the column by spreading gently on top of the packed silica gel. A wad of cotton wool was placed on top of the EtOAc extract loaded on the packed column in order not to disturb the surface of the packing. The column was then eluted with solvents of increasing polarity: pet ether (100%, 1000 mL), pet ether/EtOAc (95%: 5%, 1500ml; 90%: 10%, 1000 mL; 85%: 15%, 1500 mL; 75 %: 25 %, 1000 mL; 65% : 35%, 1000 mL; 50%: 50%, 1000 mL; 30% : 70%, 2500 mL; 20 %: 80 %, 1000 mL) and finally EtOAc (100%, 1000 mL). Approximately 114 aliquots of 100 mL each were collected. The eluates were thin layer chromagraphed as described in section 3.10.4 using pet ether-EtOAc as the mobile phase in ratios 9:1, 17:3, 3:1 and 1:3. They were visualized under UV-lamp at 254 and 365 nm and sprayed with anisaldehyde/H_2SO_4 and ethanolic potassium hydroxide as detecting reagents. The eluates were then concentrated under reduced pressure on a rotary evaporator at 40 ^{0}C and bulked into five main fractions (named F_1 to F_5) based on their TLC profiles (Figure 3.3).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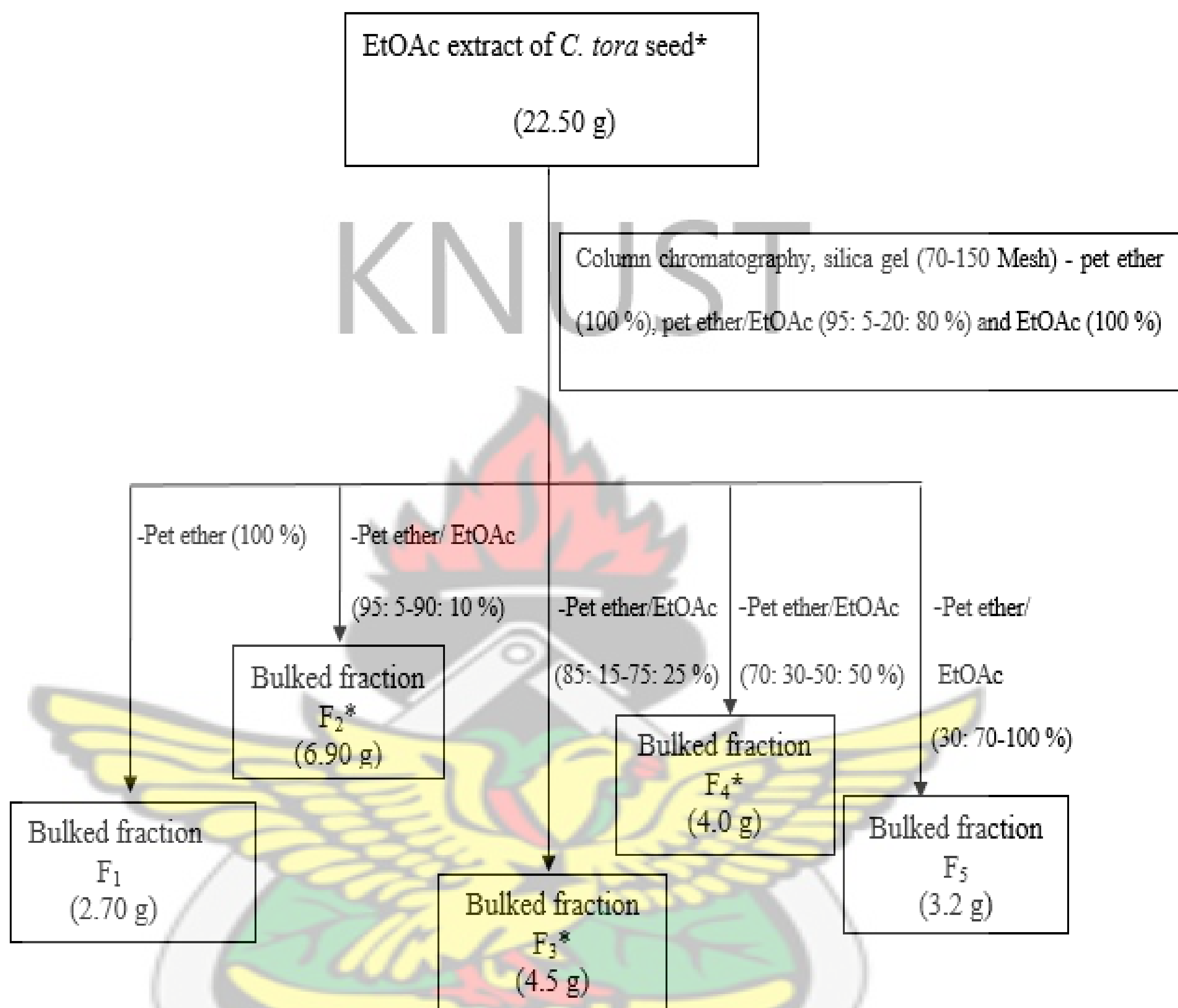

Figure 3.3. Scheme for the fractionation of EtOAc extract

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3.10.2 Bulked fraction F_2

The bulked fraction F_2 was obtained as a yellow oily mass (6.9 g), and when 0. 5 g of it was analysed by the TLC technique described in section 3.9.4 using pet ether (100%) and pet ether/EtOAc gradient mixtures as the mobile phase, appeared as a number of unresolved spots and was not followed up for isolation of compounds but was subjected to GC-MS analysis for compound identification.

3.10.3 Isolation of compound A from bulked fraction F_4

About 3.0 g of bulked fraction F_4 was weighed, reconstituted in ethyl acetate and subsequently adsorbed unto silica gel (25 g). It was loaded unto the column as described previously and then eluted isocratically with pet-ether/EtOAC (50 %: 50 %, 1200 mL). A total of 112 aliquots of 10.0 mL each were collected. The eluates were thin layer chromagraphed using pet ether/EtOAc as the mobile phase in the ratio 7:1. The plates were visualized under UV-lamp at 254 and 365 nm and sprayed with anisaldehyde/H_2SO_4 and ethanolic potassium hydroxide as detecting reagents. The eluates were then concentrated under reduced pressure on a rotary evaporator at 40 ^{0}C and bulked into two small fractions (F_{4a} [0.51 g] and F_{4b} [2.31g]) based on their TLC profiles (Figure 3.4). Fraction F_{4b} obtained as a semi-solid mass was subsequently washed repeatedly with $CHCl_3$: MeOH (1:1) to afford compound A (1.95 g) as a yellow powder (Figure 3.4).

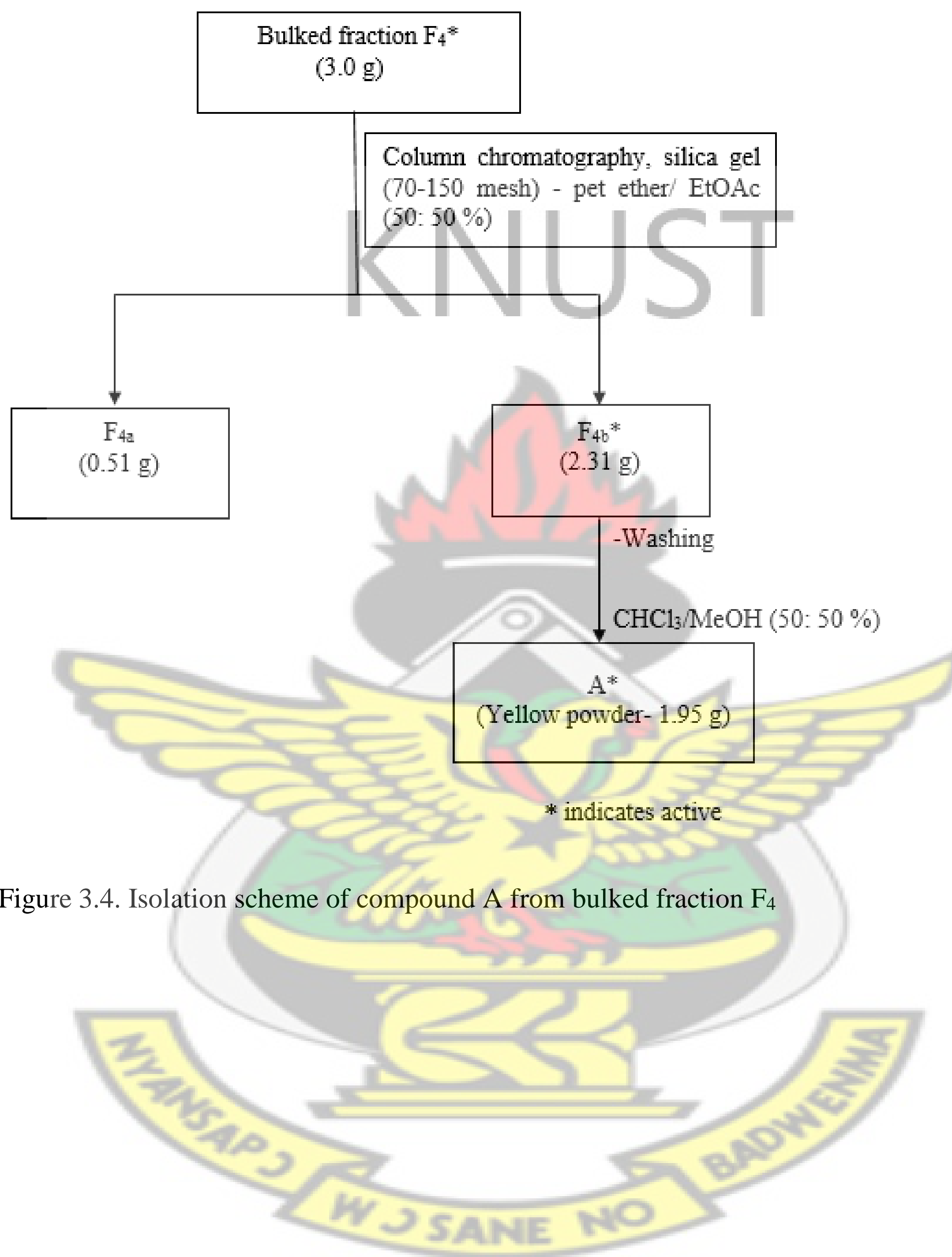
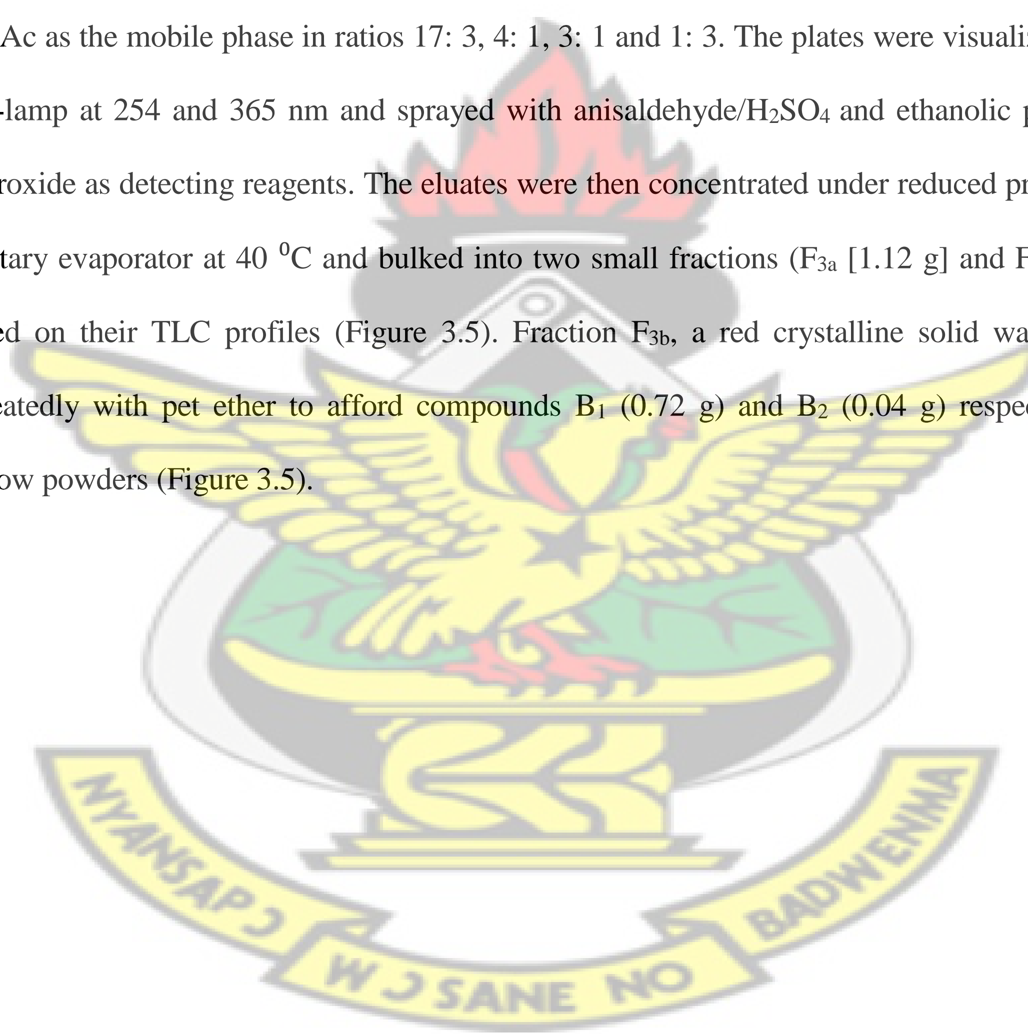


Figure 3.4. Isolation scheme of compound A from bulked fraction F_4

3.10.4 Isolation of compound B_1 and B_2 from bulked fraction F_3

Bulked fraction F_3 (3.5 g) was dissolved in a minimum amount of EtOAc (40 mL), and mixed with 50 g of silica gel, allowed to dry to attain the state of the silica gel that was used, and was loaded on to the column. It was then eluted with gradient mixtures of pet-ether/ethyl acetate (85 %: 15 %, 800 mL; 80 %: 20 %, 800 mL and 75 %: 25 %, 1000 mL). A total of 150 aliquots of 10.0 mL each were collected. The eluates were thin layer chromagraphed using pet ether-EtOAc as the mobile phase in ratios 17: 3, 4: 1, 3: 1 and 1: 3. The plates were visualized under UV-lamp at 254 and 365 nm and sprayed with anisaldehyde/H_2SO_4 and ethanolic potassium hydroxide as detecting reagents. The eluates were then concentrated under reduced pressure on a rotary evaporator at 40 ^{0}C and bulked into two small fractions (F_{3a} [1.12 g] and F_{3b} [2.11]) based on their TLC profiles (Figure 3.5). Fraction F_{3b}, a red crystalline solid was washed repeatedly with pet ether to afford compounds B_1 (0.72 g) and B_2 (0.04 g) respectively as yellow powders (Figure 3.5).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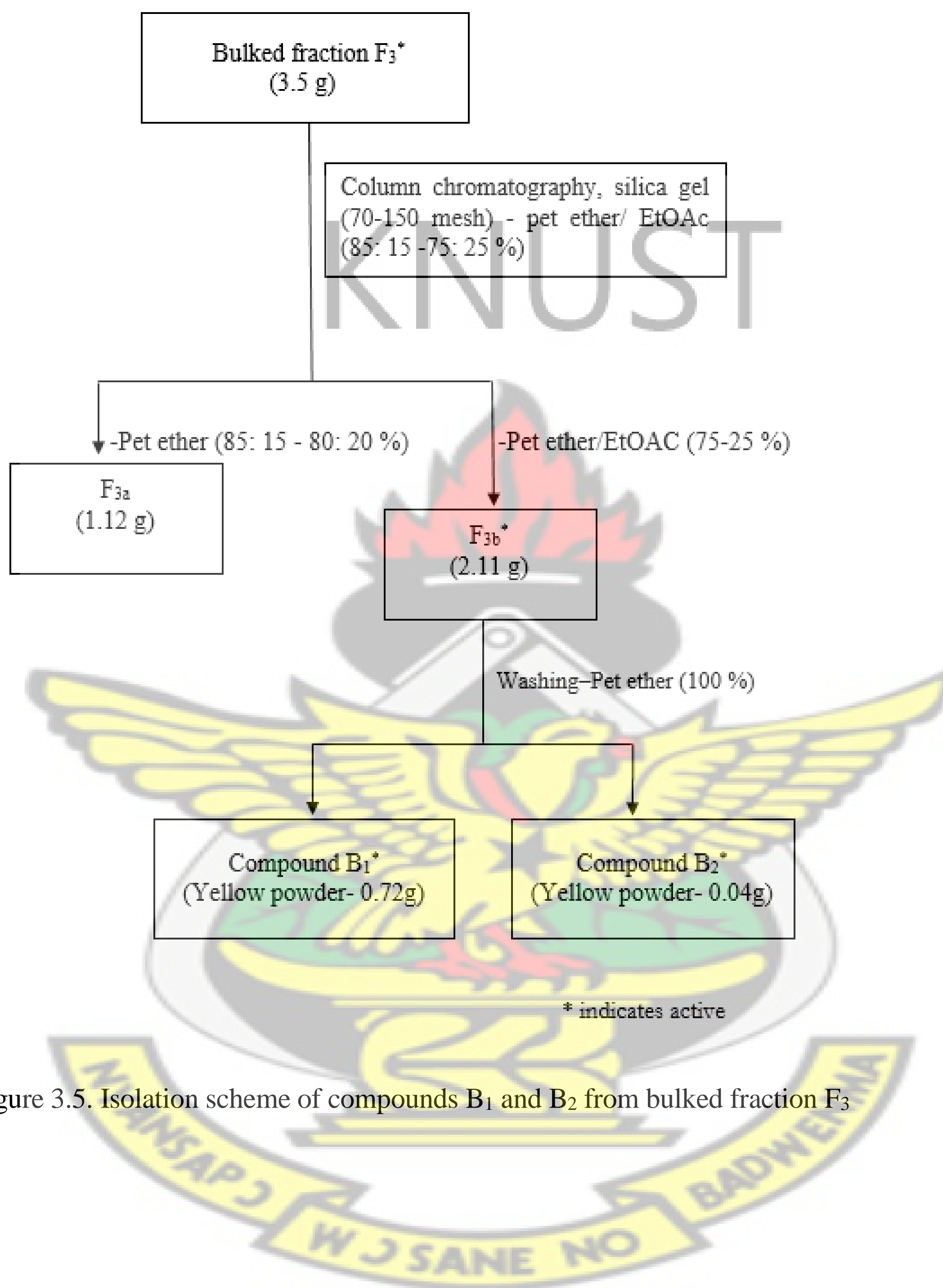

Figure 3.5. Isolation scheme of compounds B_1 and B_2 from bulked fraction F_3

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3.11 SPECTROSCOPIC ANALYSIS OF ISOLATES/COMPOUNDS

3.11.1 Experimental procedure for oily fraction F_2

The analyte (100 ng/µL of oily fraction F_2), prepared in dichloromethane solvent (Analytical grade, Aldrich Chemical Co Ltd., England), kept in vials sealed with teflon, on an auto-sampler was subjected to GC-MS analysis which was performed on a gas chromatograph model 7890A (Agilent Technologies, Inc., Beijing, China) coupled to a mass selective detector model 5975 C (Agilent Technologies, Inc., Santa Clara, CA, USA). One microliter of the analyte was injected into an HP-5 MS capillary column (30 m×0.25 mm i.d., 0.25 µm film thickness) with helium as the carrier gas at 100 kpa and flow rate of 1.25 mL/min, inlet temperature 270 °C, transfer line temperature 280 °C, column oven temperature programmed from 35 to 280 °C with the initial temperature maintained for 5 minutes, then increased by 10 °C/min to 280 °C, and held at this temperature for 10 minutes. Spectra were recorded at 70eV in the electron impact (EI) ionization mode. Fragment ions were analyzed over 40–550 m/z mass range in the full scan. Mass spectra and retention time values obtained were compared with those of authentic compounds for compound identification. Also, structures were proposed on the basis of MS fragmentation pattern using MS databases (NIST 05a.L and NIST08).

The fore-mentioned experimental procedure was also used for the GC-MS analysis of the pet ether extract of *C. tora* seeds.

3.11.2 General experimental procedures for compounds A and B_1

A JEOL 500 MHz instrument was used to obtain ^{1}H NMR, ^{13}C NMR and 2D-NMR (HMQC and HMBC) spectra. Homonuclear ^{1}H connectivities were achieved by the use of COSY experiment. One bond ^{1}H–^{13}C connectivities were obtained with HMQC while two- and three-bond ^{1}H–^{13}C connectivities were obtained by HMBC experiments. Chemical shift values were

reported in δ (ppm) with the use of Dimethyl sulfoxide-D_6 solvent. The melting points of compounds A and B_1 were determined using Stuart Digital Melting Point SMP10 instrument (Bibby Scientific Limited, Staffordshire, UK). Fourier transform-infra-red and ultra-violet spectrometry were employed in the analyses. Perkin Elmer Spectrum two FTIR (Coventry, UK) and Hitachi U-2900 spectrophotometers were used to obtain the infrared and ultra violet spectra respectively. For accurate mass determination over full mass range of m/z 50–2000, the high resolution mass spectroscopy instrument, Thermofisher LTQ Orbitrap XL (Thermofisher Scientific, UK), with an electrospray ionisation probe was employed. NanoMate was employed to deliver samples diluted into MeOH + 10% NH_4OAc in the Nano-electrospray analyses carried out in the positive ionization mode. The sheath gas flow rate was set at 2 units, capillary (ionizing) voltage at 1.4 kV and temperatureat 200 °C. The accurate mass measurements generated from this system were far better than 3 ppm.

3.11.3 Experimental procedures for compounds A and B_2

The identities of compound A and B_2 were confirmed by co-injecting isolated samples with the crude seed extract (EtOAc extract of *C. tora* seeds) from which they were obtained and analysed by LC-QtoF-MS. The extract (10 mg) was dissolved in 1 mL water (0.01 % formic acid): acetonitrile mixture of 5: 95 (LC-MS grade CHROMASOLV, Sigma-Aldrich, St. Luis, MO, US), vortexed for 30 s, and centrifuged at 14,000 rpm for 5 min, after which 0.2 µL of the supernatant was injected into a Waters ACQUITY UPLC I-class system (Waters Corp., Milford, MA, USA) fitted with an ACE C18 column, 4.6 x 250 mm x 4.6 µm (Scotland, UK) with a heater turned off and an autosampler tray cooled to 5 °C. Mobile phases of water A and acetonitrile B, each with 0.01 % formic acid were kept or programed at time/ gradient 0

minute, 5 % B; 0−3 minutes, 5-30 % B; 3−6 minutes, 30 % B; 6−7.5 min, 30−80 % B; 7.5-10.5 min, 80 % B; 10.5-13.0 min, 80-100 % B, 13-18 min, 100 % B; 18-20 min, 100-5 % B; 20-22 min, 5 % B. The flow rate was held constant at 0.3 mL/min.

The UPLC system was connected by electrospray ionization to a Waters Xevo QTOF-MS operated in full scan MSE in positive mode. Data were obtained in resolution mode over the m/z range 100–1500 with a scan time of 1 second using a capillary voltage of 0.5 kV, sampling cone voltage of 40 V, source temperature of 100 °C and desolvation temperature of 350 °C. The nitrogen desolvation flow rate was 500 L/h. For the high-energy scan function, a collision energy ramp of 25−45 eV was applied in the T-wave collision cell using ultra high purity argon (≥99.999%) as the collision gas. A continuous lock spray reference compound (leucine enkephalin; [M+H] + = 556.2766) was sampled at 10 s intervals for centroid data mass correction. The mass spectrometer was calibrated across the 50- to 2000-Da mass range using a 0.5 mM sodium formate solution prepared in 90:10 propan-2-ol: water. MassLynx version 4.1 SCN 712 (Waters Corp., Milford, MA, USA) was used for data acquisition and processing. The elemental composition was obtained for every analyte. Potential assignments were calculated using the monoisotopic masses with specifications of a tolerance of 10 ppm deviation and both odd- and even-electron states possible. The types of expected atoms and their numbers were set as follows: carbons ≤ 50; hydrogens ≤ 100; oxygens ≤ 50; nitrogens ≤ 10; chlorines ≤ 10; sulfurs ≤ 10. The empirical formula obtained was used to predict structures that were proposed based on the online database (METLIN, ChemSpider and ChemCalc, CSI: Fingerid), fragmentation pattern and literature (Lambert *et al*., 2015; Zheng *et al*., 2011; Kim *et al*., 2015).

Chapter 4

RESULTS

4.1 INSECTICIDAL ACTIVITIES

4.1.1 Insecticidal activities

The insecticidal activity of petroleum ether, ethyl acetate and methanol extracts of *C. tora* seed alongside standard controls (Neem oil and cinnamaldehyde) was evaluated. The cowpea seeds were treated with *C. tora* seed extracts at concentrations of 100 µg/mL, 200 µg/mL and 300 µg/mL. Acetone used as a vehicle for the dissolution of the extracts (control 3) did not exhibit any insecticidal activity. The different extracts of *C. tora* seeds exhibited varying degrees of insecticidal activities that may be either lesser or greater than, and even identical to those of the standard controls (Table 4.1).

The pet ether extract of *C. tora* seeds exhibited the highest insecticidal activity, followed by the EtOAc and MeOH extracts with neem oil (Reference sample) the least potent (Table 4.1).

Table 4.1. Insecticidal effect of seed extracts of *C. tora* on cowpea weevils

Extracts	**LC_{50} (µg/mL) Mean±S.E.M**
Pet ether (*C. tora*)	8.33±0.6
EtOAc (*C. tora*)	58.89±0.2
MeOH (*C. tora*)	139.69±0.1
Neem oil	>300
Cinnamaldehyde	258.51±0.1

Pet ether = petroleum ether, EtOAc = ethyl acetate, MeOH = methanol, LC_{50} = lethal concentration-50.

Moreover, each of the three crude extracts of *C. tora* seed (Pet ether, EtOAc and MeOH extracts) showed a quick onset of time- and concentration-dependent lethality to cowpea weevils with over 50 % mortality recorded within 2 days (Figures 4.1 and 4.2). The extracts

had identical insecticidal activity profile (no significant difference at $p > 0.001$) but were remarkably superior to either the neem oil or cinnamaldehyde (Figure 4.2).

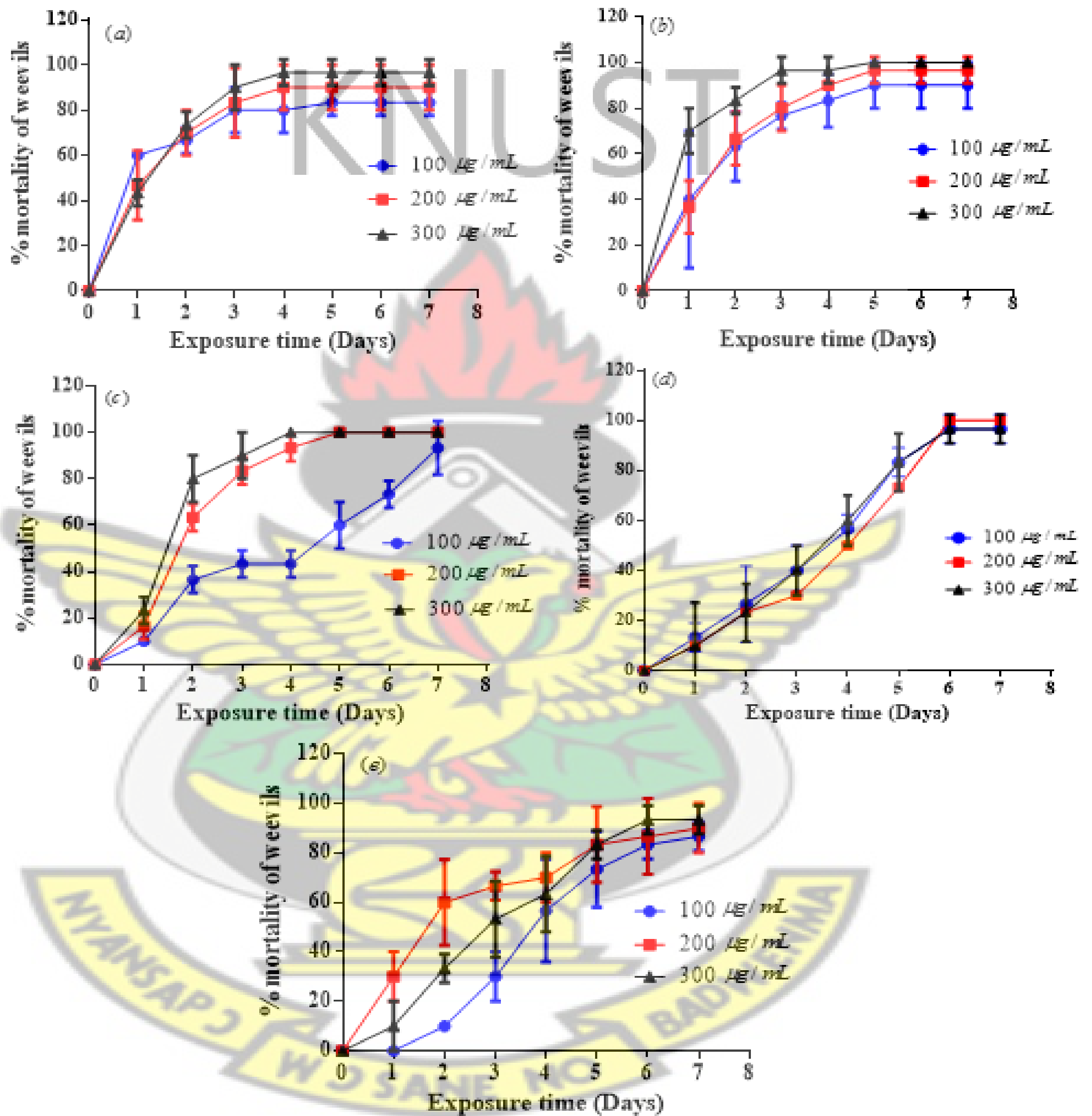

Figure 4.1. Time-dependent insecticidal effect of (a) pet ether, (b) EtOAc and (c) MeOH extracts of *C. tora s*eed, and reference samples (d) neem oil and (e) cinnamaldehyde

The pet ether extract of *C. tora* seeds exhibited the highest insecticidal activity, followed by the EtOAc and MeOH extracts with the neem oil the least potent. Statistically the activity of the three *C. tora* seed extracts were comparable but higher than those of the reference samples (Figure 4.2).

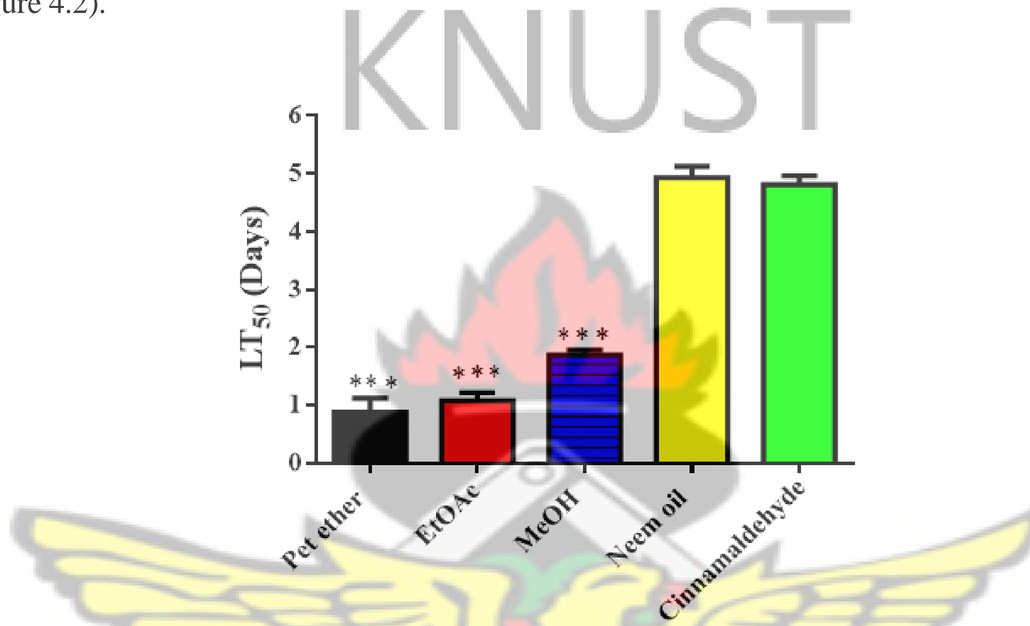

Figure 4.2. Overall lethal time-50 (LT50) for cowpea weevils exposed to extracts of *C. tora* seed. ***p< 0.001 vs the neem oil and cinnamaldehyde groups respectively (one way ANOVA followed by Dunnett multiple comparison test).

4.1.1.1 Insecticidal activity of EtOAc fractions

The three bulked fractions (BKF 2-4) from the EtOAc extract demonstrated dose and time dependent mortality of insects. BKF-2 showed the highest insecticidal activity (Table 4.2). The activity was four times higher than positive controls. This was followed by cinnamaldehyde, BKF-3 and BKF-4, with neem oil the least potent (Table 4.2).

The insecticidal acitivities of the bulked fractions was lower than the ethyl acetate extract suggesting a possible synergistic action of fraction-2-4.

Table 4.2. Insecticidal effect of bulked fractions-2-4 (BKF-2-4) on weevils

Bulk fractions-2-4	LC_{50} (µg/mL), Mean±S.E.M.
BKF-2	69.53±0.43
BKF-3	282.54±0.13
BKF-4	286.44±0.09
Positive control 1	>300
Positive control 2	258.51±0.08

BKF-2 = bulked fraction-2, BKF-3 = bulked fraction-3, BKF-4 = bulked fraction-4, control 1 = neem oil, control 2 = cinnamaldehyde and LC_{50} = lethal concentration-50.

Comparative analysis of relative potency was made by calculating the overall LT_{50} for each bulked fraction. Their activity profiles were significantly ($p < 0.001$) superior to either the neem oil or cinnamaldehyde positive control (Figure 4.3). Overall order of insecticidal activity is BKF-2> BKF-3> BKF-4 > cinnamaldehyde > neem oil.

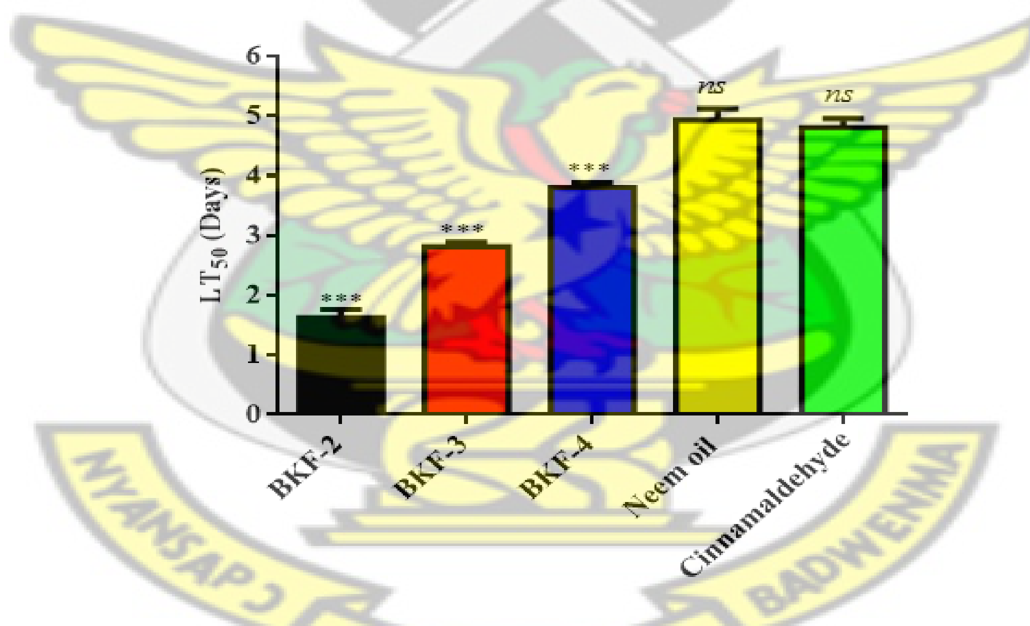

Figure 4.3. Overall lethal time-50 (LT50) for cowpea weevils exposed to BKF 2-4

Values are mean ± S.E.M (n=9). *** represent significant difference vs neem oil and cinnamaldehyde groups respectively at p< 0.001, ns = no significant difference between neem oil and cinnamaldehyde groups at p< 0.05 (one way ANOVA followed by Dunnett multiple comparison test).

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4.1.1.2 Insecticidal activity of compounds A and B_1

Bulked fraction 2, being oily, gave a number of unresolved spots on TLC chromatogram and thus was not probed further. BKF-3 and BKF-4 on the other hand were further column chromatographed which led to isolates/ compounds A, B_1 and B_2 (Section 3.11).

The compounds were evaluated for insecticidal activity against adult weevil survival. The compounds showed very close concentration-activity profiles. Concentration-dependent mortality effect was not so evident (Figure 4.4) and thus the lethal concentration-50 (LC_{50}) was calculated (Table 4.3). Isolates A and B_1 were about 1.8 times more potent than EtOAc crude extract of *C. tora* seeds (Tables 4.1 and 4.3). Thus further purification afforded more potent isolates than the ethyl acetate extract and the bulked fractions BKF-3 and BKF-4 (Tables 4.1, 4.2 and 4.3).

Table 4.3. Insecticidal effect of compounds A and B_1 on cowpea weevils

Isolates/ positive controls	**LC_{50} (μg/mL), Mean±S.E.M**
A	33.41±0.60
B_1	34.78±0.66
Positive control 1	>300
Positive control 2	258.51±0.08

Control 1 = neem oil, control 2 = cinnamaldehyde and LC_{50} = lethal concentration-50

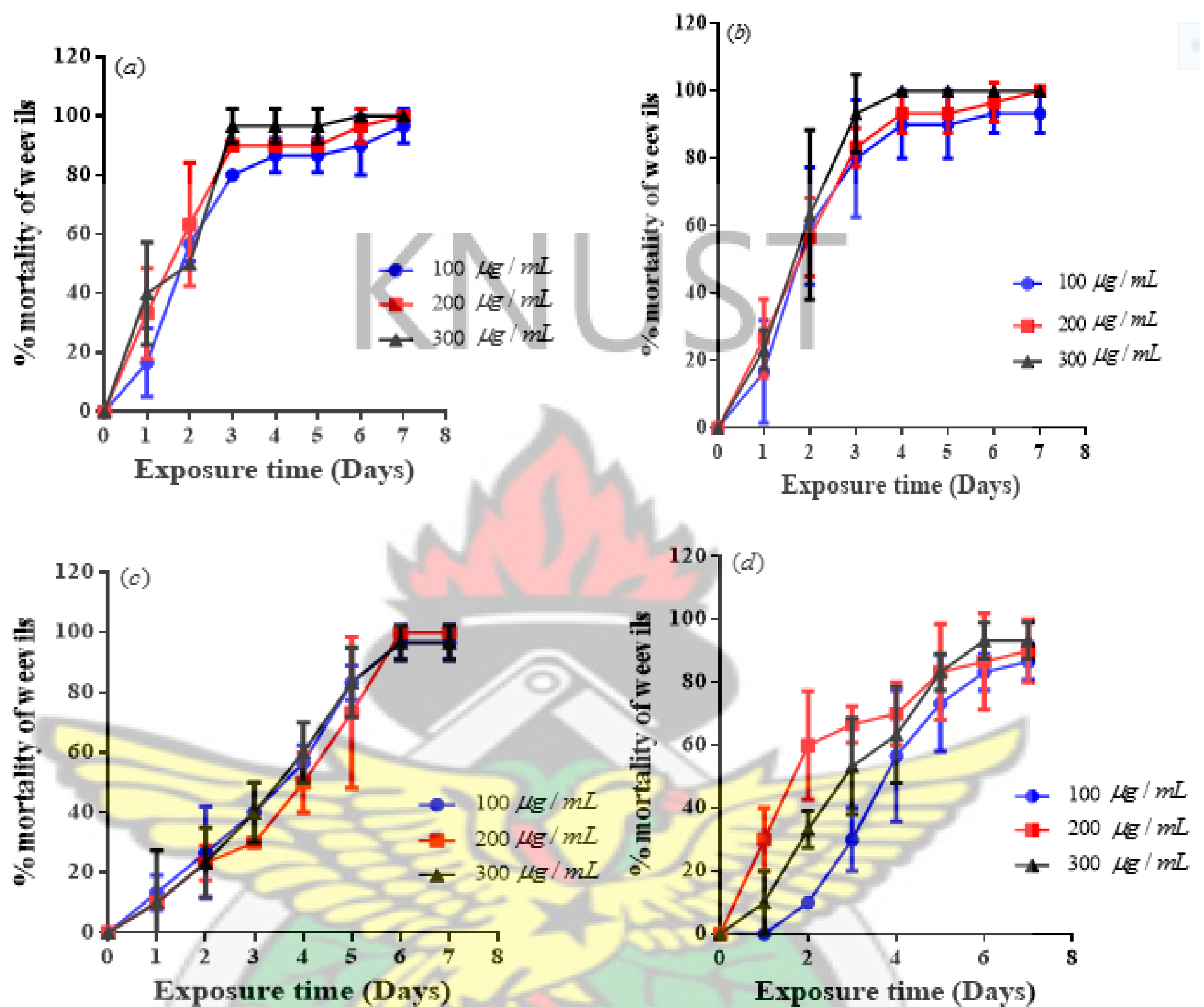

Figure 4.4. Time-dependent insecticidal effect of (a) compound A, (b) compound B_1, (c) neem oil and (d) cinnamaldehyde

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Comparative analysis of relative potency was made by calculating the overall LT_{50} for each tested sample. Compounds A and B_1 showed identical activity profile (no significant difference; $p < 0.05$) but were superior to either the neem oil or cinnamaldehyde. Compounds A and B_1 were about 2.76 fold more active insecticides than either the neem oil or cinnamaldehyde standard sample. Thus isolates A and B_1 have potential insecticidal properties that may even exceed those of the reference drugs (Table 4.3 and Figure 4.5).

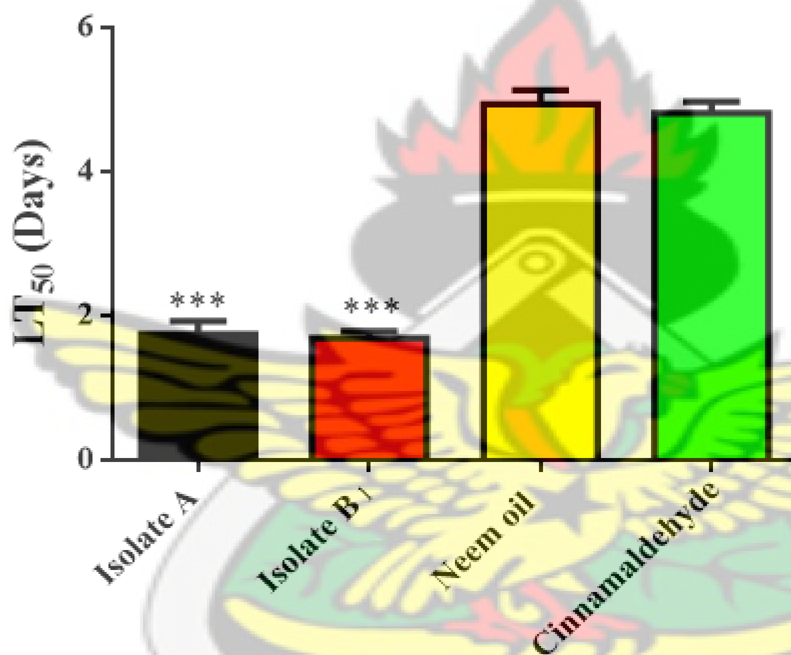

Figure 4.5. Overall lethal time-50 (LT50) for cowpea weevils exposed to isolates A and B_1 LT_{50} values calculated for each treatment group (mean and SEM from all the three concentrations). *** $p < 0.001$ *vs* the neem oil and cinnamaldehyde groups respectively (one way ANOVA followed by Dunnett multiple comparison test).

4.2 OVIPOSTION DETERRENT ACTIVITIES

4.2.1 Oviposition deterrence activity of *C. tora* seed extracts

The oviposition deterrent potentials of crude extracts (Pet ether, EtOAc and MeOH) of *C. tora* seeds on adult female weevils were assessed. Number of eggs laid by female weevils on

surfaces of cowpea seeds treated with different test substances were taken on daily basis for 7 days. The seed extracts of *C. tora* at different concentrations (100, 200 and 300 µg/mL) exhibited oviposition deterrence in the range of 0.00 – 90.28 % on the female weevils than cinnamaldehyde (13.33-70.28 %) (Figure 4.7). A minimum of 12 eggs were laid on cowpea seeds/grains treated with the highest concentration (300 µg/mL) of the *C. tora* seed extracts, while for the least concentration (100 µg/mL), 133 eggs were laid compared to untreated cowpea grains (control 4) with 128 eggs laid (Figures 4.6 and 4.7). Acetone vehicle (used to impregnate cowpea seeds with the test substances) did not prevent female weevils from laying eggs on surfaces of cowpea seeds (Control 3) just as untreated cowpea seeds (control 4) (Table 4.4). The oviposition deterrence was found to be time and concentration dependent (Figure 4.7)

Table 4.4. Oviposition deterrence indices (ODI) for cowpea seeds treated with controls

Extract	**Concentration (µg/mL)**	**% ODI (Mean±S.E.M)**			
		Day 1	**Day 3**	**Day 5**	**Day 7**
Control 1	100	19.48±11.22	19.14±4.98	19.14±4.98	19.14±4.98
	200	30.98±3.75	26.70±4.22	26.70±4.22	26.70±4.22
	300	31.90±4.48	28.60±3.62	28.60±3.62	28.60±3.62
Control 2	100	21.28±1.99	16.80±4.28	14.98±10.26	13.88±10.27
	200	66.16±3.88	64.16±2.99	66.08±1.76	65.89±0.42
	300	1.92±5.13	72.08±2.37	69.73±1.60	70.28±1.02
Control 3	1 (mL)	10.03±7.18	0.00±0.00	0.00±0.00	0.00±0.00

Control 1 = cowpea seeds treated with neem oil, control 2 = cowpea seeds treated with cinnamaldehyde and control 3 = cowpea seeds treated with acetone

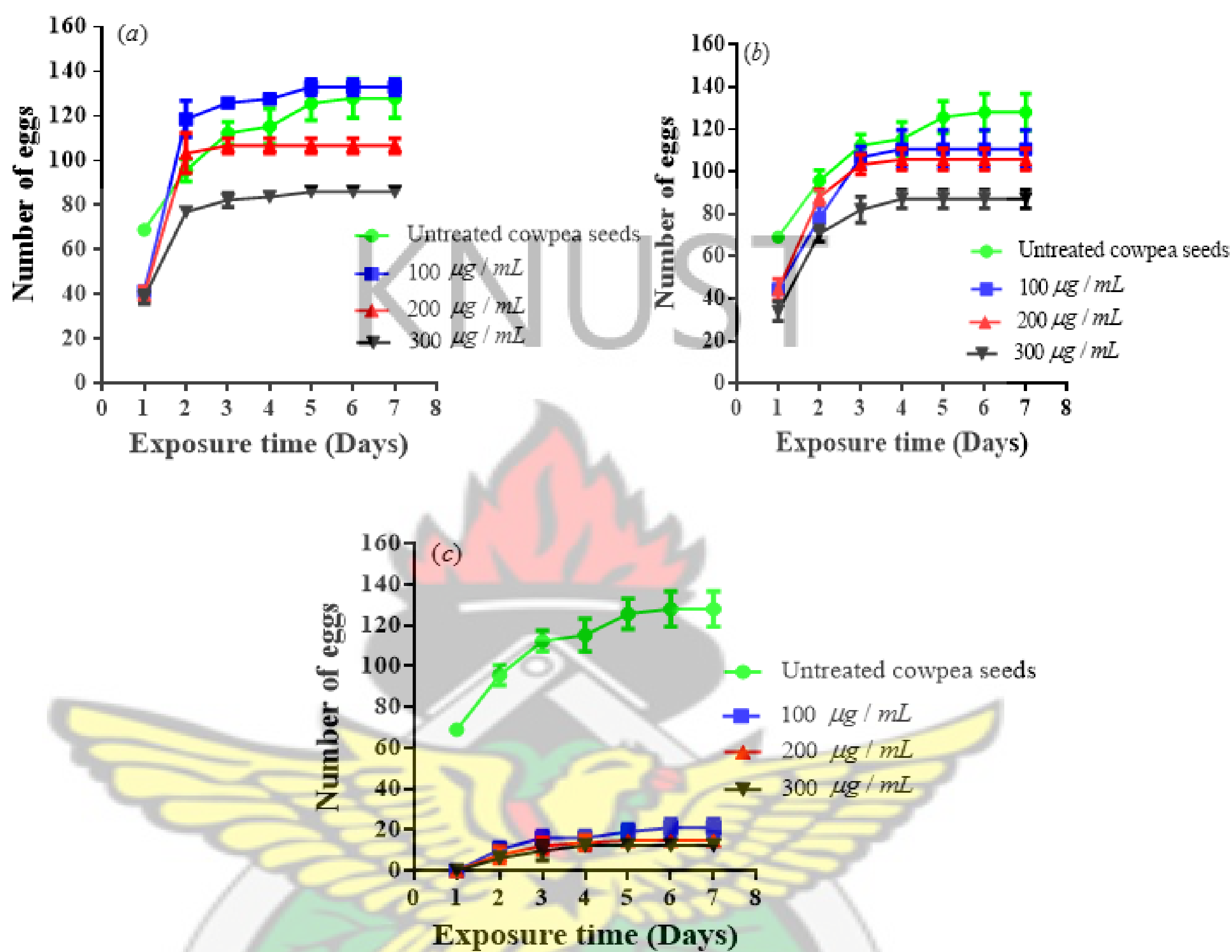

Figure 4.6. Oviposition deterrence activity of (a) pet ether, (b) EtOAc and (c) MeOH extracts of *C. tora* seeds. Data represent mean ± SEM values (n=3).

The methanol (MeOH) extract had a superior deterrence effect over neem oil and cinnamaldehyde (positive control) at $p< 0.01$. There was comparable deterrence effect by the pet ether, EtOAc and neem oil positive control at $p< 0.05$ (Figure 4.7).

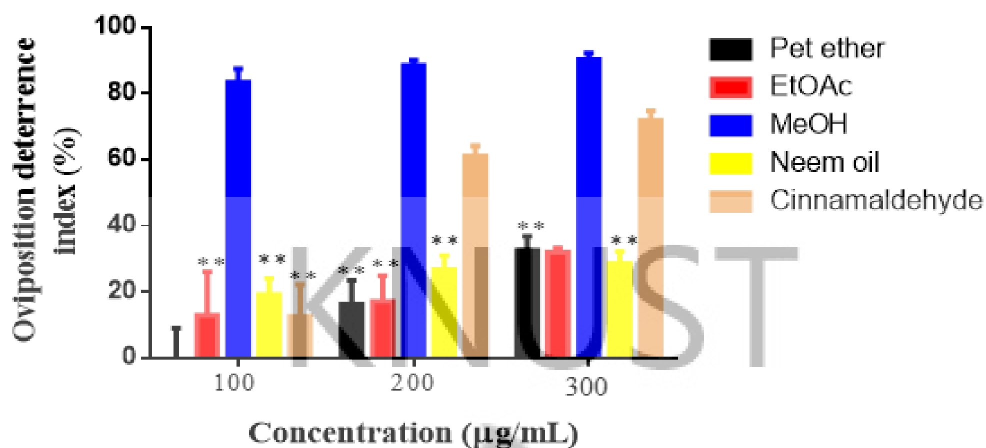

Figure 4.7. Oviposition deterrent index (% ODI) of *C. tora* seed exracts and positive controls. ** $p < 0.01$(one way ANOVA followed by Dunnett multiple comparison test). Data represent mean ± SEM values (n=3).

4.2.2 Oviposition deterrence activity of ethyl acetate bulked fractions

Oviposition deterrent effects demonstrated by bulked fractions-2-4 from the EtOAc extract of *C. tora* seeds on female cowpea weevils were also determined using neem oil and cinnamaldehyde as positive controls.

The time-dependency of the activity of the tested bulked fractions-2-4 and the positive controls was clearly observed but the close activity profiles of the three concentration range of the extracts and positive controls did not clearly demonstrate a concentration-dependency effect (Figure 4.8).

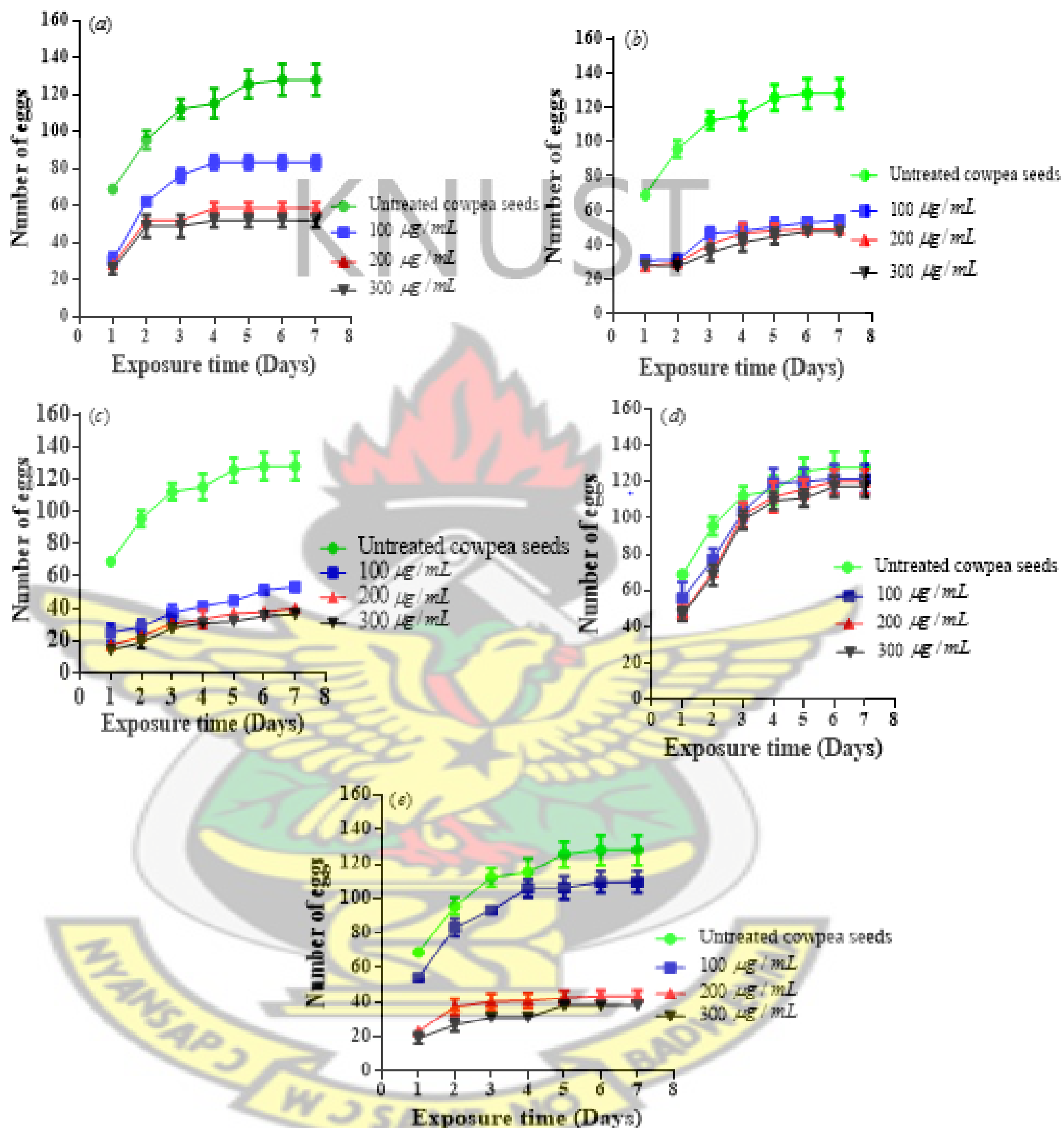

Figure 4.8. The oviposition deterrence activity of EtOAc fractions 2-4 and reference drugs. Bulked fractions 2, 3 and 4, and the reference drugs neem oil and cinnamaldehyde have been labelled as (a), (b), (c), (d) and (e) above. Values are Mean ± SEM from three determinations

The comparative oviposition deterrence activity parameter, oviposition deterrence index, was employed to demonstrate deterrence effect of the bulked fractions and the positive controls (Eliman *et al*., 2009) (Figure 4.9). The effect exhibited by each of the three bulked fractions on the female weevils was higher than that of pet ether and EtOAc (Figures 4.7 and 4.9).

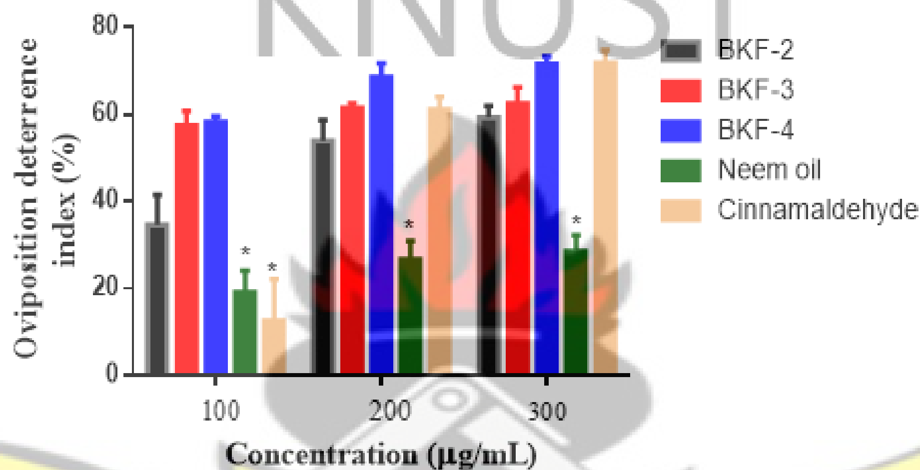

Figure 4.9. Oviposition deterrent index (% ODI) of EtOAc fractions 2-4 and reference drugs Mean and SEM values from three determinations are shown. * $p < 0.05$ (one way ANOVA followed by Dunnett multiple comparison test).

4.2.3 Oviposition deterrence activity of compounds A and B_1

Isolates A and B_1, from the EtOAc extract of *C. tora* seeds exhibited oviposition deterrent effect against female cowpea weevils that may either be greater than or compared to those of the reference controls (neem oil and cinnamaldehyde) (Figure 4.10).

With the exception of neem oil which apparently exhibited a weaker oviposition deterrence, the activity of isolates A and B_1, and cinnamaldehyde was identical to their insecticidal activity. The time-dependency of activity of the isolates and positive controls were clearly observed (Figure 4.10) but again there was no concentration-dependent effect (Figure 4.11).

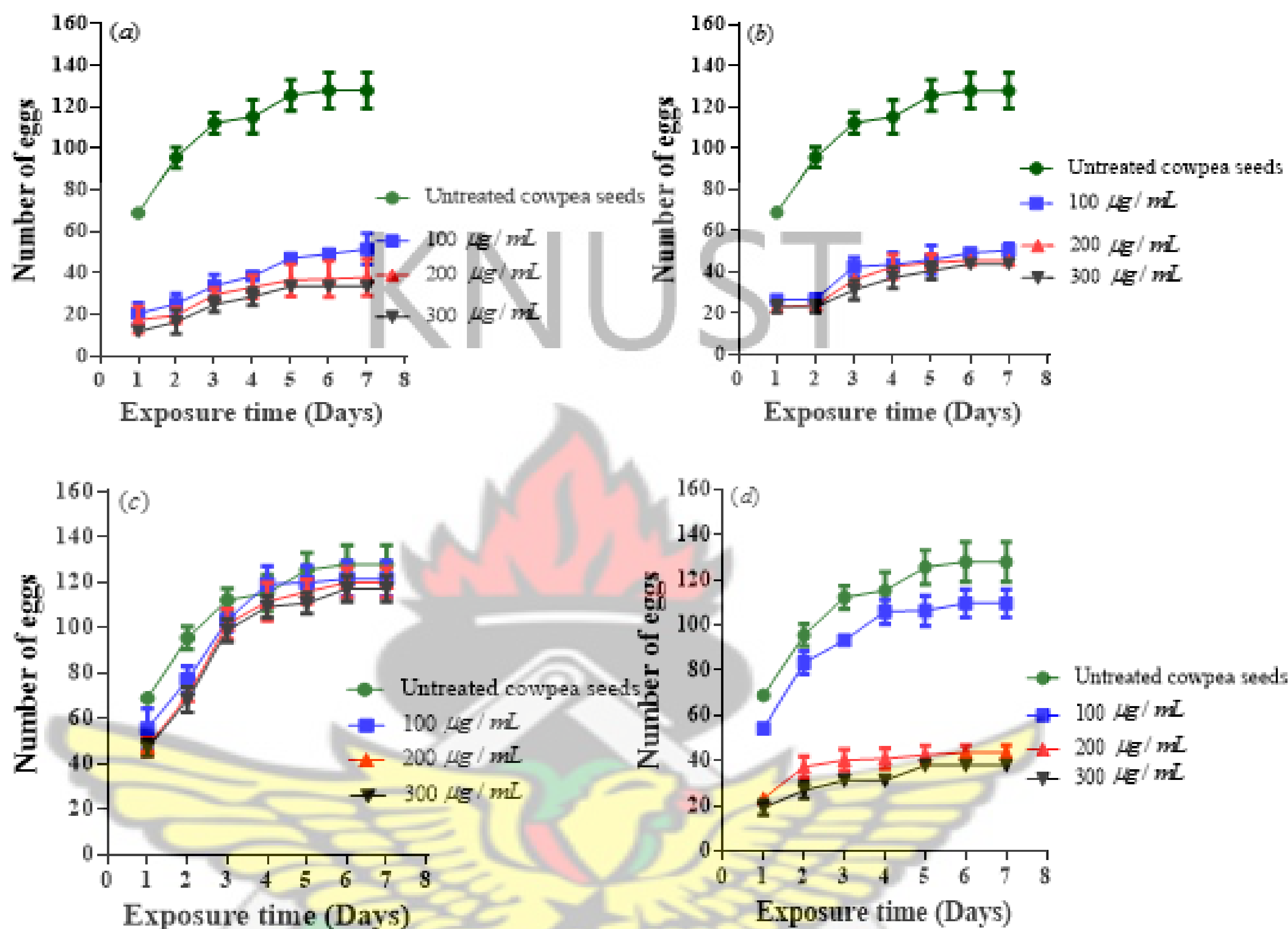

Figure 4.10. The oviposition deterrence activity of (a) isolate A, (b) isolate B_1, (c) neem oil and (d) cinnamaldehyde

The percentage oviposition deterrence index of compounds A and B_1 once again revealed a superior activity over the two positive controls. At all concentrations tested, compounds A and B_1 possess by order of magnitude more oviposition deterrent effect than neem oil, while about 3-fold activity over cinnamaldehyde was observed for the lowest concentration (Figure 4.11).

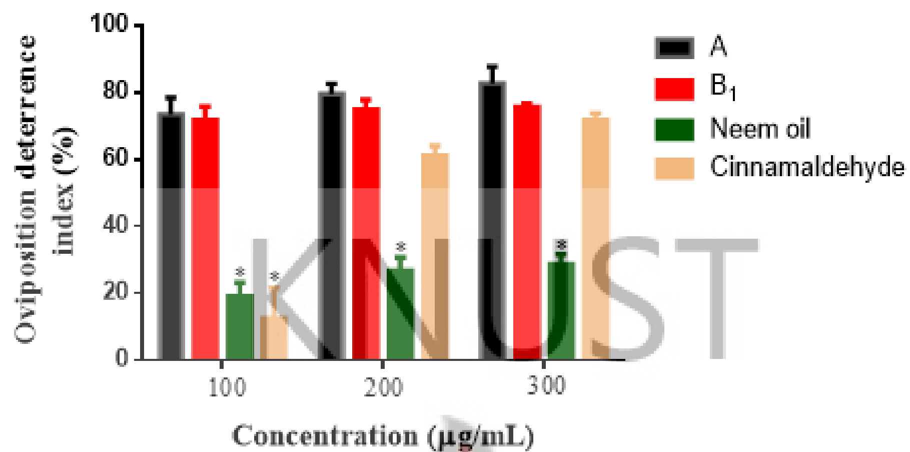

Figure 4.11**.** Oviposition deterrent index (% ODI) of isolates A and B_1
***p**< 0.05 (one way ANOVA followed by Dunnett multiple comparison test).

4.3 ANTIFEEDANT ACTIVITIES

4.3.1 Antifeedant activity of *C. tora* seed extract

An additional parameter commonly employed in crop/seed protection studies is feeding inhibition assay. Eggs laid by female adult weevils on the surface of cowpea seed hatch into larvae within 8 days. The larvae burrow into the bean to feed on its endosperm and only exit as adults after total insect development period of approximately 30 days, affecting seed viability (Fox, 1993).

The set-up for this assay was the same as the one described for oviposition deterrence effect of crude extracts of *C. tora* seed. Both living and dead weevils were removed from plastic containers at the end of day 7, and weights of treated cowpea seeds with the test substances (Crude extracts of *C. tora* seeds) were taken on daily basis for another 27 days.

Seed extracts (petroleum ether, ethyl acetate and methanol) of *C. tora* inhibited larval feeding on cowpea seeds more than the neem oil, and at a level comparable to cinnamaldehyde. The three extracts showed considerable feeding inhibition at all concentrations. They were about two fold more effective feeding inhibitors than neem oil, but with almost identical activity as cinnamaldehyde at $p < 0.01$ (Figure 4.12). Thus *C. tora* seed extracts proved to be more effective than neem oil and acetone, and reduced damage on cowpea seeds caused by larvae of the cowpea weevil.

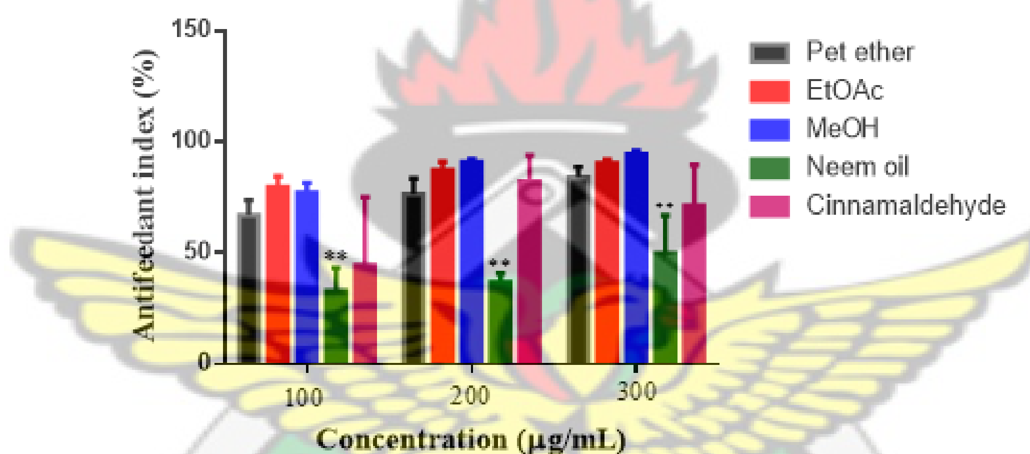

Figure 4.12. Antifeedant index for pet ether, EtOAc and MeOH extracts of C. tora seeds and positive controls. Mean and SEM values from three determinations are shown. ** $p < 0.01$ (one way ANOVA followed by Dunnett multiple comparison test).

4.3.2 Antifeedant activity of bulked fractions from EtOAc extract of *C. tora*

The results revealed that the bulked fractions-2-4 from the EtOAc crude extract of *C. tora* possessed feeding inhibition activity on larvae of weevils at extents greater than the standard controls (neem oil and cinnamaldehyde) (Figure 4.13).

Fraction BKF-2 had the highest feeding inhibition activity on larvae of weevils ($p< 0.01$), followed by BKF-3, BKF-4, cinnamaldehyde and neem oil (Figure 4.13).

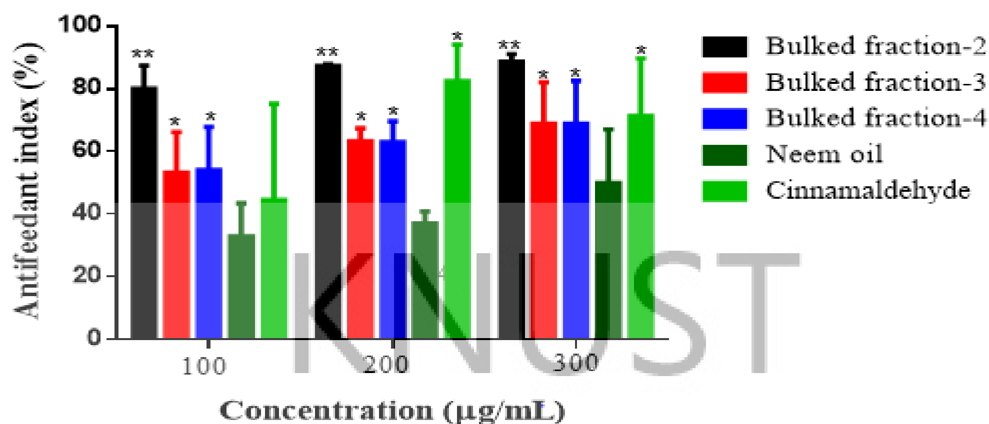

Figure 4.13. Antifeedant index for bulked fractions 2-4 and reference drugs. Mean and SEM values from three determinations are shown. ** and *indicate significant difference *vs* neem oil positive control at $p < 0.01$ and $p < 0.05$ respectively, and no significant difference between BKF-2-4 and cinnamaldehyde positive control at $p < 0.05$ (one way ANOVA followed by Dunnett multiple comparison test).

4.3.3 Antifeedant activity of isolated compounds A and B_1

Potency evaluation of compounds A and B_1, and the positive controls were determined as shown by their antifeedant indices (Figure 4.14). Compounds A and B_1 appeared to possess activity comparable to cinnamaldehyde ($p < 0.05$), and higher than neem oil ($p < 0.01$) (Figure 4.14).

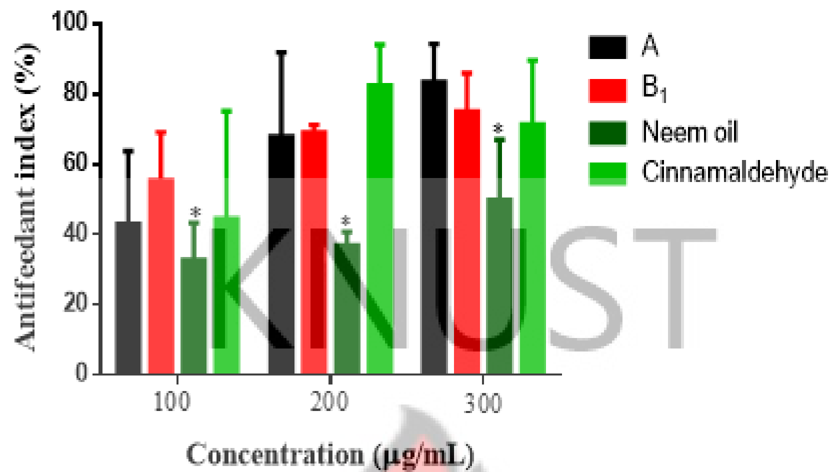

Figure 4.14. Antifeedant index for compounds A and B1.
Values are mean± SEM (n=3). No significant difference between isolate A or B_1 and the positive controls (Neem oil and cinnamaldehyde) at $p < 0.05$ (one way ANOVA followed by Dunnett multiple comparison test).

4.4 GERMINATION AND VIGOR ENHANCEMENT ACTIVITIES

4.4.1 Germination and vigor enhancement activities of *C. tora* seed extracts

Cowpea seeds treated with crude extracts of *C. tora* showed varying germination enhancement effect. Seeds treated with the methanol extract and the positive controls (control 1 and 2) showed 100 % reduction in germination similar to untreated seeds subjected to weevils (control 4) (Figure 4.15). This demonstrated phyto-toxic effect of treatments on cowpea seeds that made them non-viable.

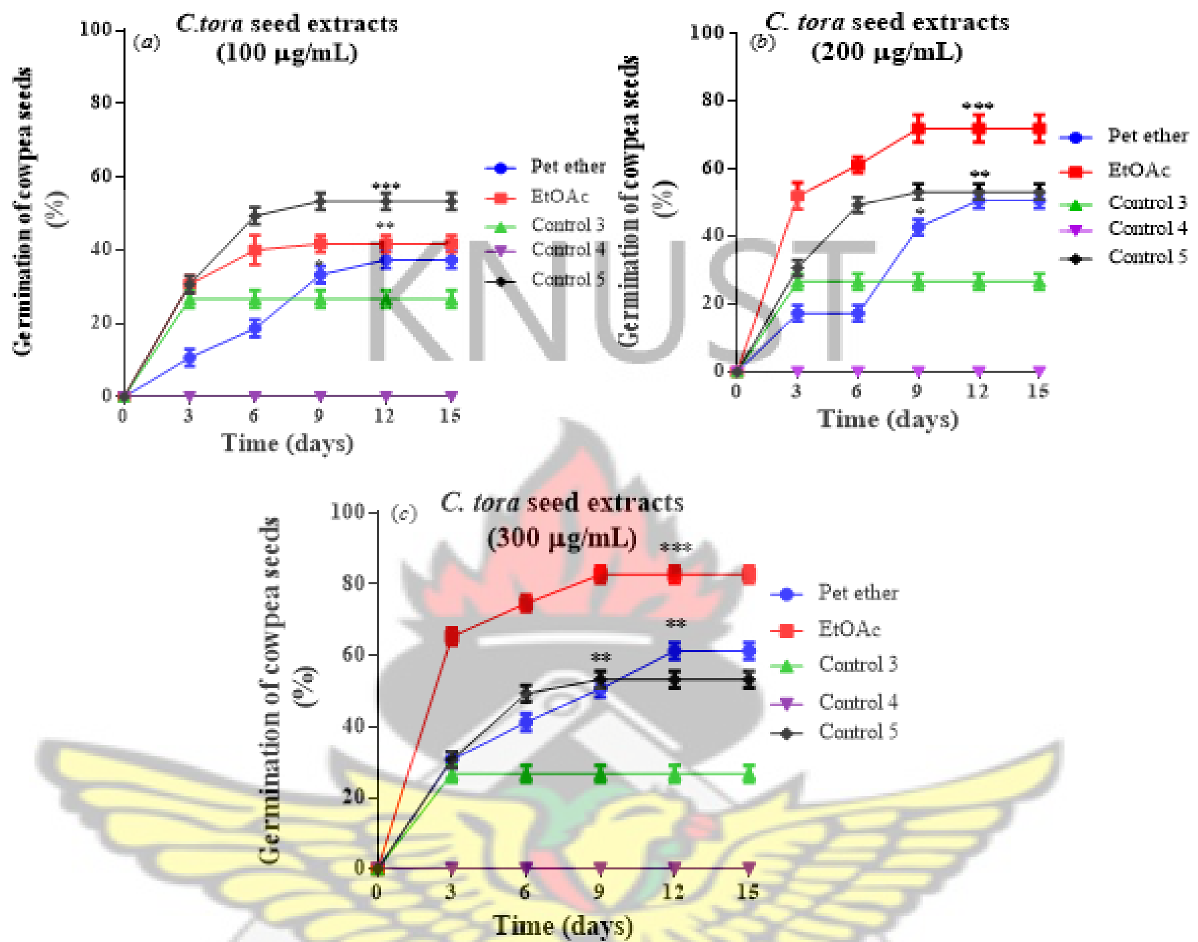

Figure 4.15. The germination enhancement profile of cowpea seeds (%) treated with *C. tora* seed extracts. Pet ether = cowpea seeds treated with petroleum ether extract of *C. tora* seeds, EtOAc = cowpea seeds treated with ethyl acetate extract of *C. tora* seeds, control 3= cowpea seeds treated with acetone, control 4 = untreated cowpea seeds with weevil larvae infestations and control 5 = untreated cowpea seeds. *, ** and *** indicate significant difference *vs* control 4 at $p < 0.001$, 0.01 and 0.05 respectively (one way ANOVA followed by Dunnett multiple comparison test).

Of the three crude seed extracts tested for viability of cowpea seeds, pet ether and EtOAc extracts had higher germination enhancement effect and seedling vigor than untreated cowpea seeds not introduced to weevils or infested by larvae (control 5) (Figure 4.15).

The enhancement ability of these crude extracts was effective from day 1-9 of the 15 days period of experimentation. They significantly increased the percentage germination of cowpea seeds compared to untreated cowpea seeds introduced to weevils or infested by weevils' larvae (control 4), which showed 0.00 % germination at $p < 0.05$ (Figure 4.15).

The ethyl acetate extract showed a higher germination enhancement ability than the vehicle treated seeds (control 3) (Figure 4.16a and b).

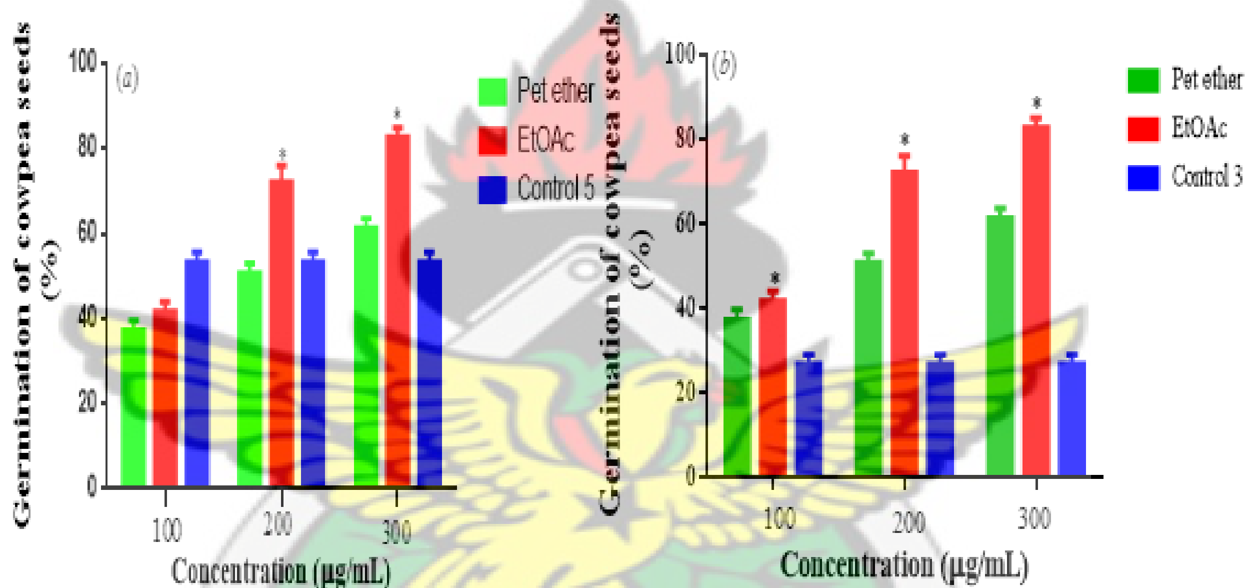

Figure 4.16a and b. Overall germination enhancement profile of cowpea seeds treated with *C. tora* seed extracts. Pet ether= petroleum ether extract, EtOAc= ethyl acetate extract, control 3= cowpea seeds treated with acetone and control 5= untreated cowpea seeds not introduced to weevils. * indicates significant difference *vs* control 3 and control 5, no significant difference between pet ether and controls or EtOAc at $p < 0.05$ (one way ANOVA followed by Dunnett multiple comparison test).

Similarly as in the germination enhancement of cowpea seeds, both pet ether and EtOAc extracts of *C. tora* seeds concentration-dependently enhanced vigor of cowpea seedlings. The

overall seedling vigor enhancement profile revealed EtOAc extract to have a higher activity than the vehicle treated seeds (control 3) (Figure 4.17a and b).

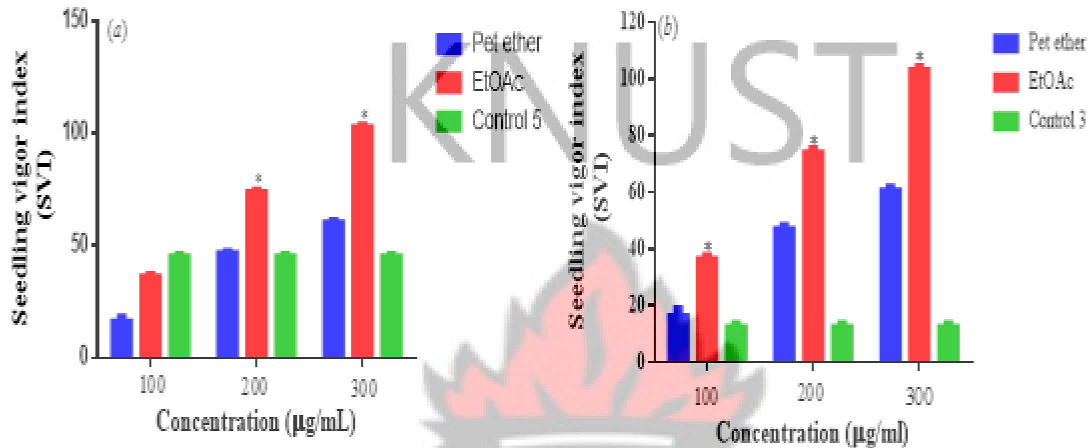

Figure 4.17a and b. Cowpea seedling Vigor index enhancement activity of *C. tora* extracts
Pet ether= seedlings from cowpea seeds treated with petroleum ether extract of *C. tora* seeds, EtOAc= seedlings from cowpea seeds treated with ethyl acetate extract of *C. tora* seeds and control 3= seedlings from cowpea seeds treated with acetone (1 mL). Mean and SEM values from three determinations are shown. * indicates significant difference *vs* control 3 and control 5, no significant difference between pet ether and controls or EtOAc at $p < 0.05$ (one way ANOVA followed by Dunnett multiple comparison test).

4.4.2 Germination and vigor enhancement activities of bulked fractions 2-4

BKF-2, BKF-3 and BKF-4 demonstrated time and concentration dependent germination enhancement abilities on cowpea seeds. They effectively enhanced germination of cowpea seeds from day 1 to day 9 of the entire period of the germination experiment just as the EtOAc extract of *C. tora* seeds from which they were originally obtained (Figures 4.15 and 4.18). The activity of these three bulked fractions was comparable to those of the pet ether and EtOAc extracts of *C. tora* and far higher than that of the MeOH extract which had 0.00% seed

germination just as the untreated seeds infested by larvae (Control 4) (Figures 4.15 and 4.18. $p< 0.001$, 0.01 and 0.05).

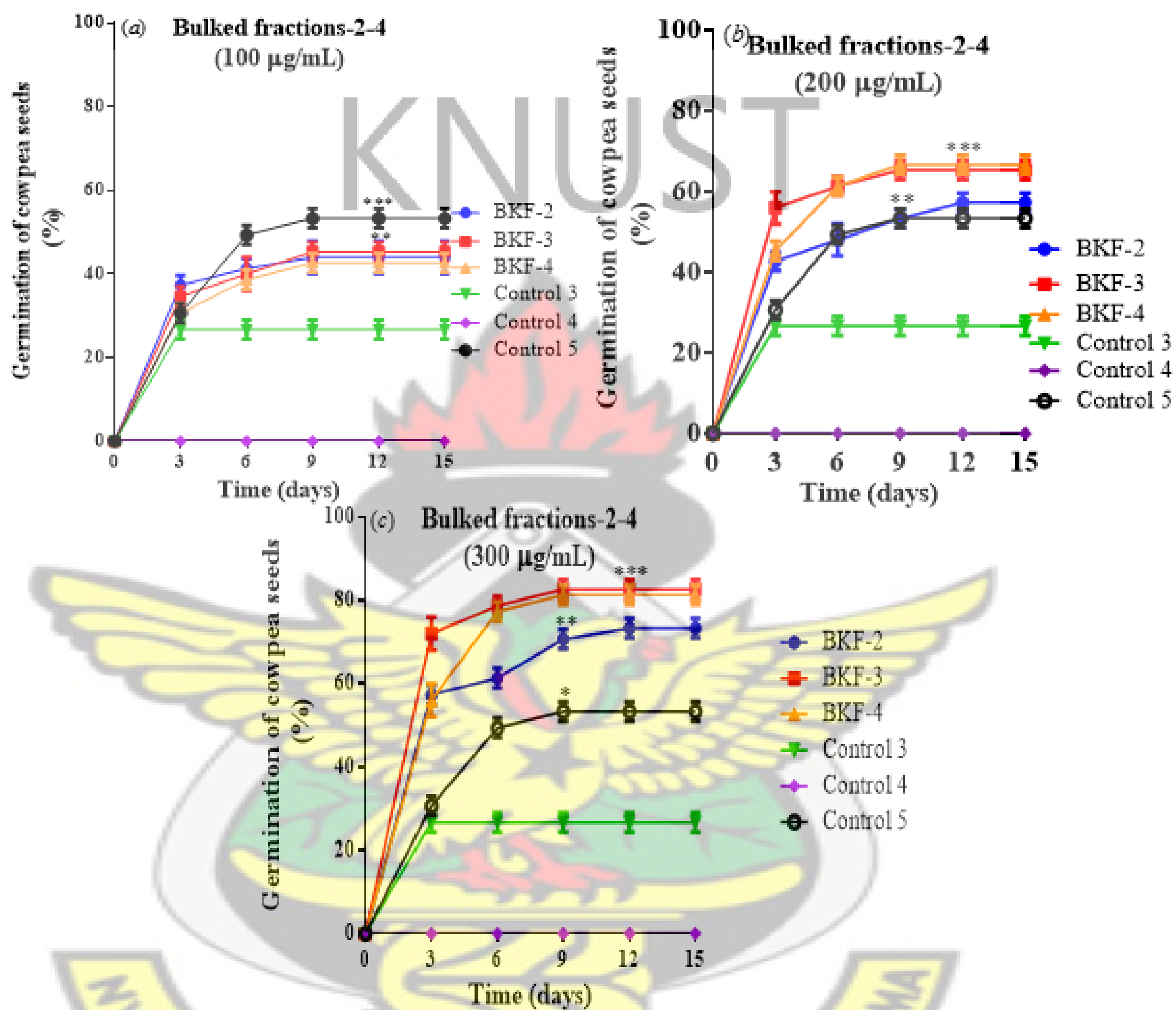

Figure 4.18. Germination enhancement activity of EtOAc fractions 2-4 on cowpea seeds

***, ** and * indicate significant difference *vs* control 4 at $p< 0.001$, 0.01 and 0.05 respectively, and no significant difference between control 3 and control 4 at $p<0.05$ (one way ANOVA followed by Dunnett multiple comparison test).

BKF-2, BKF-3 and BKF-4 demonstrated identical seed germination enhancement ability as untreated cowpea seeds not introduced to weevils (Control 5) at all concentrations (Figure 4.19;

at $p < 0.05$). Thus bulked fractions 2-4 may contain good seed germination enhancers (Figure 4.18).

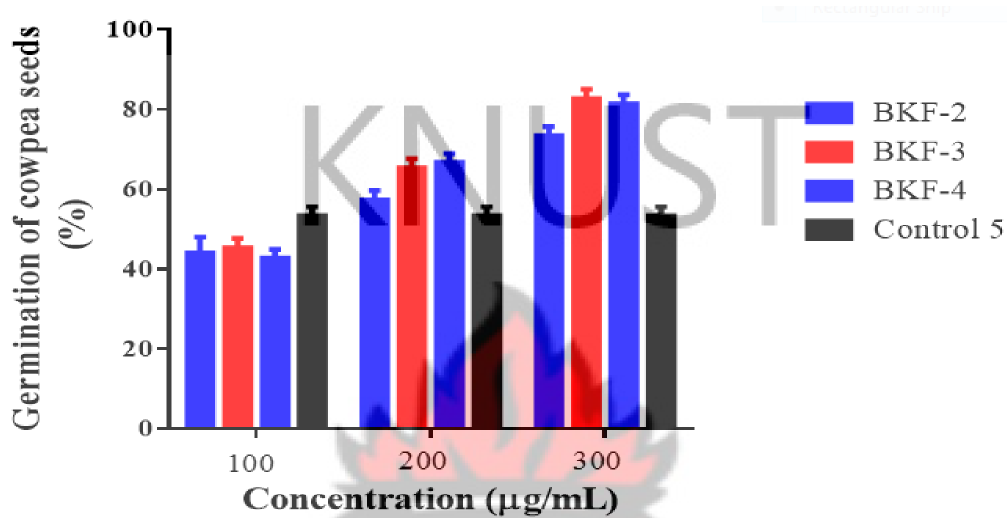

Figure 4.19. Overall germination enhancement activity of EtOAc fractions 2-4 on cowpea seeds No significant difference between control 5 and BKF-2-4 at $p < 0.05$ (one way ANOVA followed by Dunnett multiple comparison test).

Similarly, the fractions demonstrated seedling vigor enhancement abilities comparable to untreated cowpea seeds not introduced to weevils (Control 5) (Figure 4.20). The concentration-dependent vigor enhancement abilities exhibited on cowpea seeds/grains by the fractions again suggest that they could contain good seedling vigor enhancers (Figure 4.20a and b).

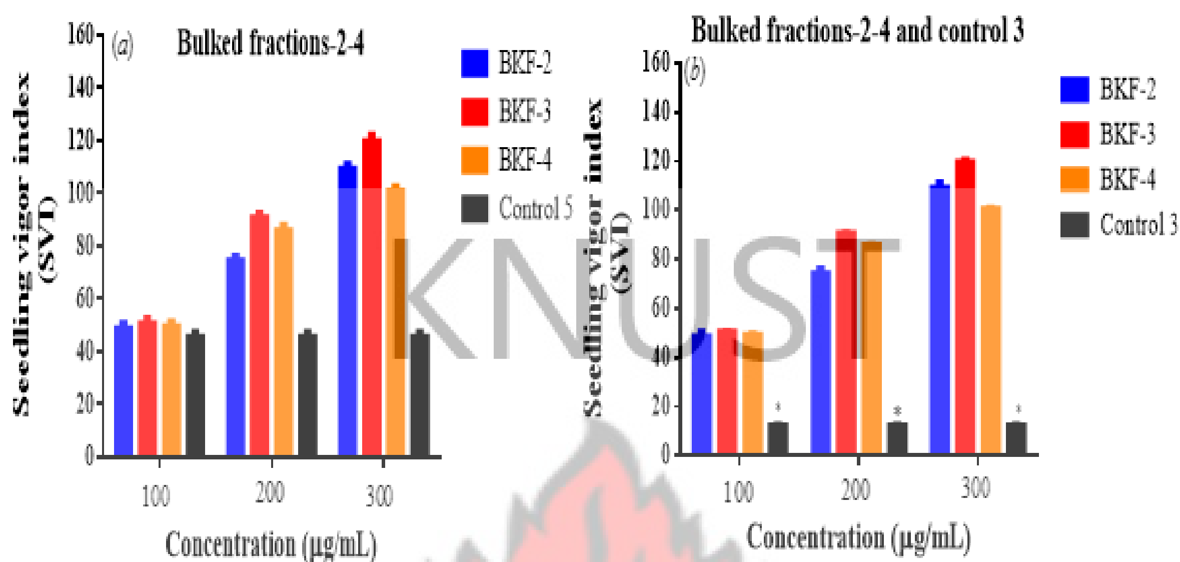

Figure 4.20a and b**.** Vigor index enhancement profile of seedlings from treated seeds Control 5 = seedlings from untreated cowpea seeds (0.00 µg/mL) and control 3= seedlings from cowpea seeds treated with acetone. Mean and SEM values from three determinations are shown. * $p<0.05$, and no significant difference between control 5 and bulked fractions-1-3 at $p< 0.05$ (One way ANOVA followed by Dunnett multiple comparison test).

4.4.3 Germination and vigor enhancement activities of isolated compounds A and B_1

Isolates A and B_1 demonstrated time and concentration dependent germination enhancement abilities on cowpea seeds. Both isolates were effective germination enhancers from day 1-9 of the 15 days period of experimentation just as the EtOAc extract of *C. tora* seeds (Figures 4.15 and 4.21). They were 1.3, 1.5 and 1.8 folds more potent than untreated cowpea seeds not introduced to weevils (Control 5) at the tested concentrations (100 µg/mL, 200 µg/mL and 300 µg/mL) (Figure 4.21). This also reflected in the overall germination enhancement profile of the isolate A and B_1 compared with untreated cowpea seeds not introduced to weevils (control 5) at $p< 0.05$ (Figure 4.22).

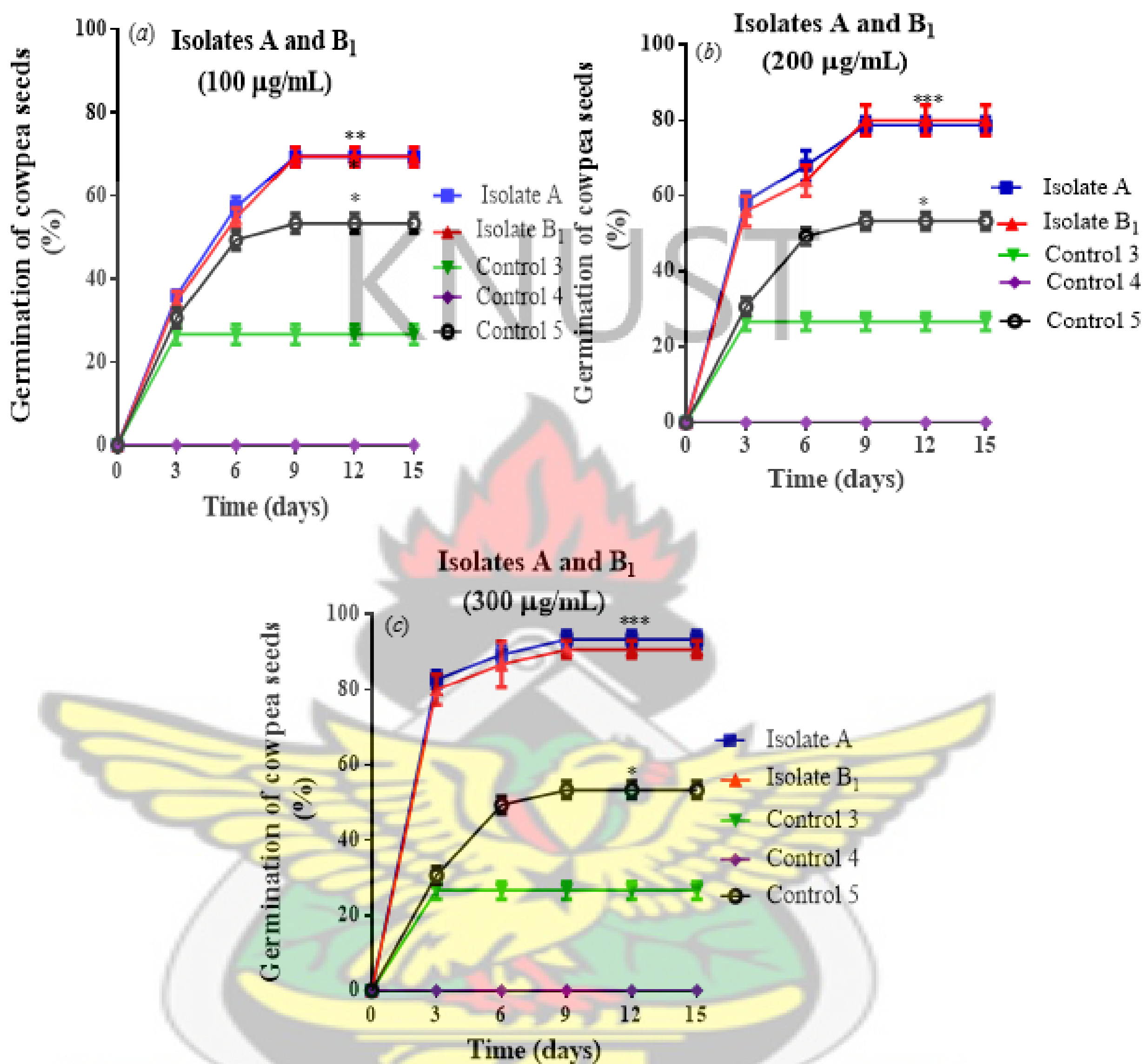

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Figure 4.21. Germination enhancement profile of cowpea seeds treated with compounds A and B_1. *and *** indicate significant difference *vs* control 4 at $p<0.05$, 0.01 and 0.001 respectively (one way ANOVA followed by Dunnett multiple comparison test).

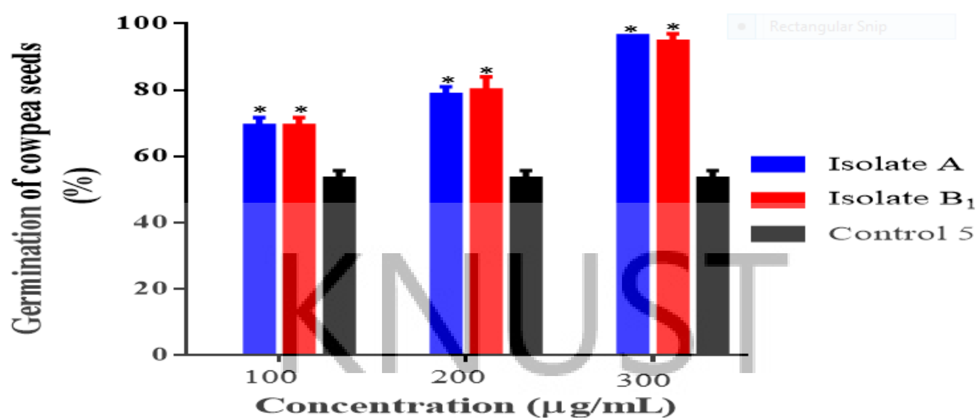


Figure 4.22. Overall germination enhancement profile of cowpea seeds treated with isolates A and B1 * $p < 0.05$ (one way ANOVA followed by Dunnett multiple comparison test).

Comparatively, compounds A and B_1 had identical activity as the bulked fractions 3 and 4, from which they were isolated respectively as well as the ethyl acetate extract (Figures 4.23 and 4.24).

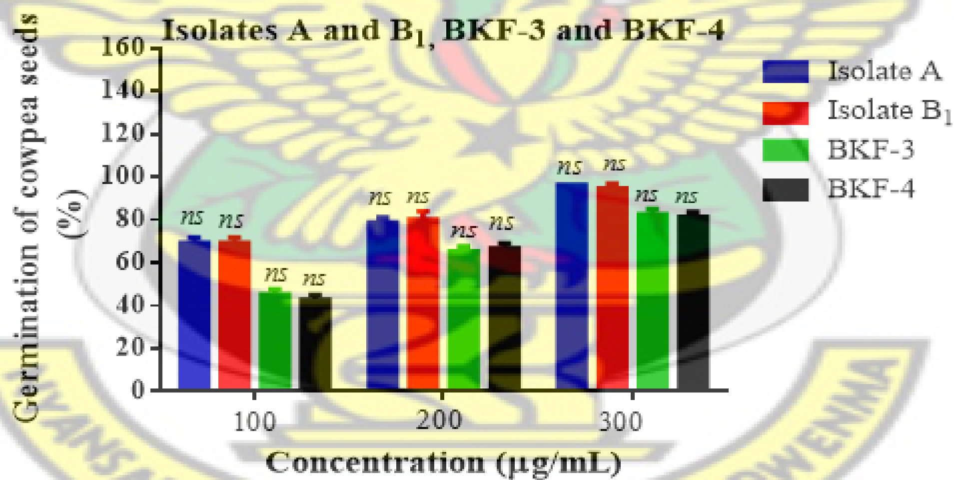


Figure 4.23. Comparative germination enhancement profile of cowpea seeds treated with isolates and fractions. *ns* indicates no significant difference at $p < 0.05$ (one way ANOVA followed by Dunnett multiple comparison test).

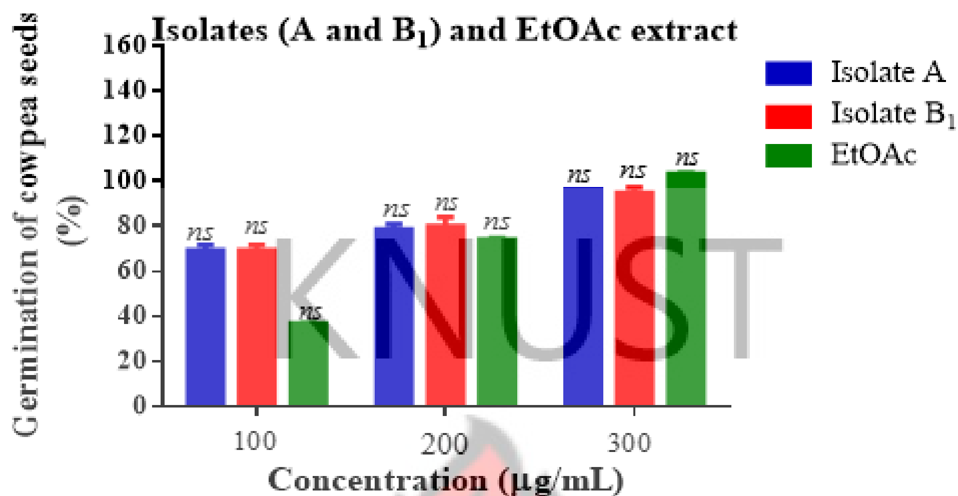


Figure 4.24. Comparative germination enhancement profile of cowpea seeds treated with isolates and the EtOAc extract. *ns* indicates no significant difference at $p< 0.05$ (one way ANOVA followed by Dunnett multiple comparison test).

In a similar manner, both isolates A and B_1 concentration-dependently enhanced vigor of cowpea seedlings. The overall seedling vigor enhancement profile revealed both isolates to have a higher activity than untreated cowpea seeds not introduced to weevils (Control 5) and the vehicle treated seeds (control 3) at $p< 0.05$ (Figure 4.25).

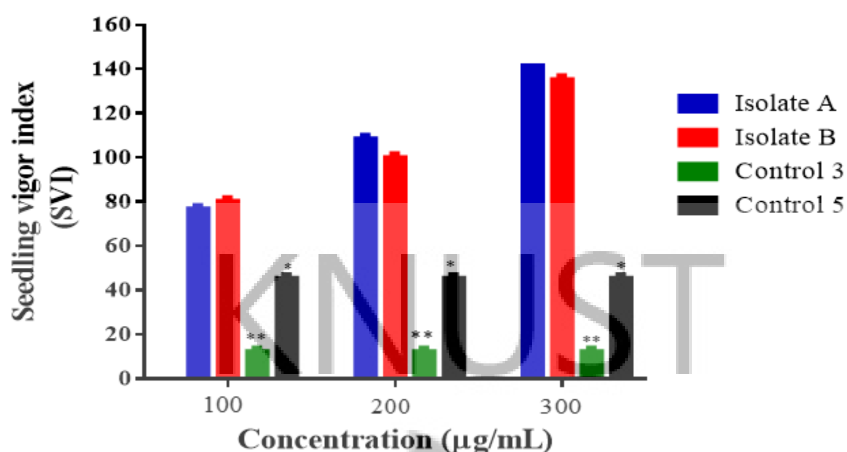


Figure 4.25 Vigor index enhancement activity of seedlings from cowpea seeds treated with isolates A and B1. Control 3 = seedlings from cowpea seeds treated with acetone (1 mL) and control 5 = seedlings from untreated cowpea seeds (0.00 µg/mL). * $p < 0.05$, ** $p < 0.01$ (one way ANOVA followed by Dunnett multiple comparison test).

4.5 MOSQUITO LARVICIDAL ACTIVITIES

4.5.1 Mosquito larvicidal activity of EtOAc extract, its fractions and compounds

The EtOAc extract exhibited a higher larvicidal activity than the bulked fractions (BKF-3 and BKF-4) and the compounds (A and B_2). BKF-3 and -4 were approximately two to four-fold less potent than the EtOAc extract and the positive control azadirachtin, as shown by their LD_{50} values (Table 4.5) and exposure time (Figuere 4.26). The larvicidal activity for all treatments except the negative control (Dimethyl sulphoxide, DMSO), increased with increasing concentration and time of exposure (Table 4.5, Figure 4.26). BKF-2 was not tested for larvicidal activity due to its insolubility in DMSO and the assay media.

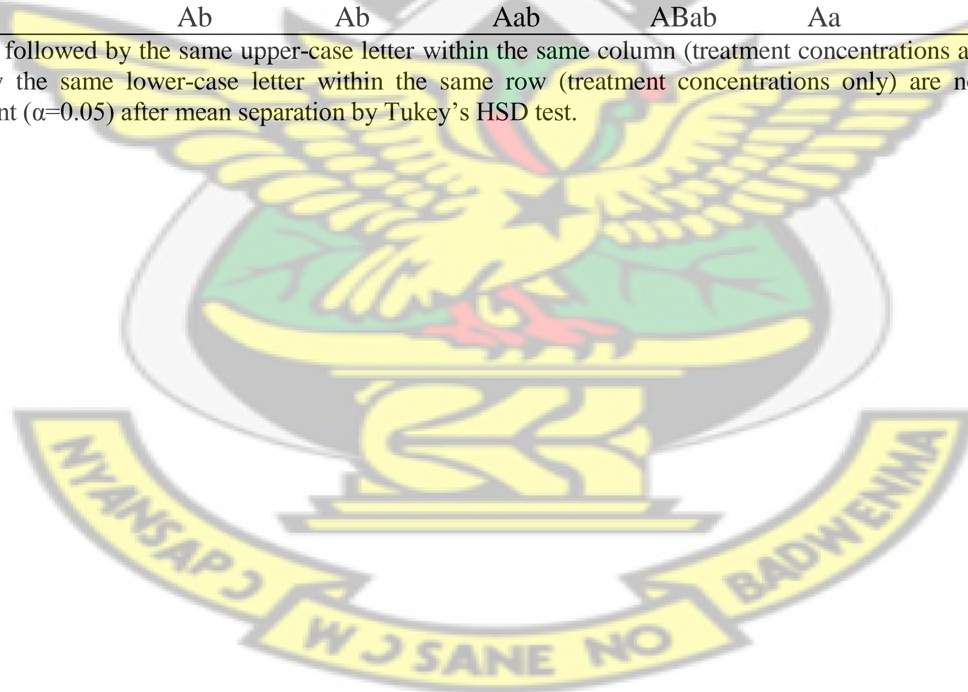
Similarly, compounds A and B_2, had LD_{50} values of 10 ppm (95% CI= 0.02-0.05) and 10.2 ppm (95% CI= 0.02-0.12), respectively, which were four- and about six-fold less potent than

the EtOAc extract and the positive control azadirachtin respectively (Table 4.5, Figures 4.26 and 4.27).

Table 4.5. Mosquito larvicidal ativity of the EtOAc extract and compounds against third-fourth instar larvae of *Anopheles gambiae*

Drug/Treatments	**Concentration Mortality (Mean±S.E) %**					
	1 ppm	**2.5 ppm**	**5 ppm**	**10 ppm**	**100ppm**	**LD_{50} (95 % CI)**
EtOAC extract	33.3±7.3 Ac	38.3±14.5 Ac	65±13.2 Abc	91.7±8.3 Aab	100±0 Aa	2.5 (0.001-0.003) A
BKF-3	11.7±7.3 Bc	18.3±4.4 Ac	53.3±6.0 ABcb	75±10 ABab	100±0 Aa	5 (0.004-0.006) B
BKF-4	18.3±6.0 Bb	20±2.9 Ab	43.3±8.3 ABb	66.7±3.3 ABab	88.34±11.6 Aa	7 (0.005-0.01) B
Compound A	6.7±4.4 Bc	6.7±4.4 Bc	3.3±3.3 Bc	58.3±8.8 ABb	100±0 Aa	10 (0.008-0.013) B
Compound B_2	1.7±1.7 Bb	8.6±5.0 Bb	-	13.2±76 Ba	-	10.2 (0.008-0.017) B
Azadirachtin	43±12.0 Ab	51.7±10.1 Ab	73.3±14.2 Aab	90±0 ABab	100±0 Aa	1.69 (0.001-0.002) A

Means followed by the same upper-case letter within the same column (treatment concentrations and LSD 50/90) and by the same lower-case letter within the same row (treatment concentrations only) are not significantly different (α=0.05) after mean separation by Tukey's HSD test.



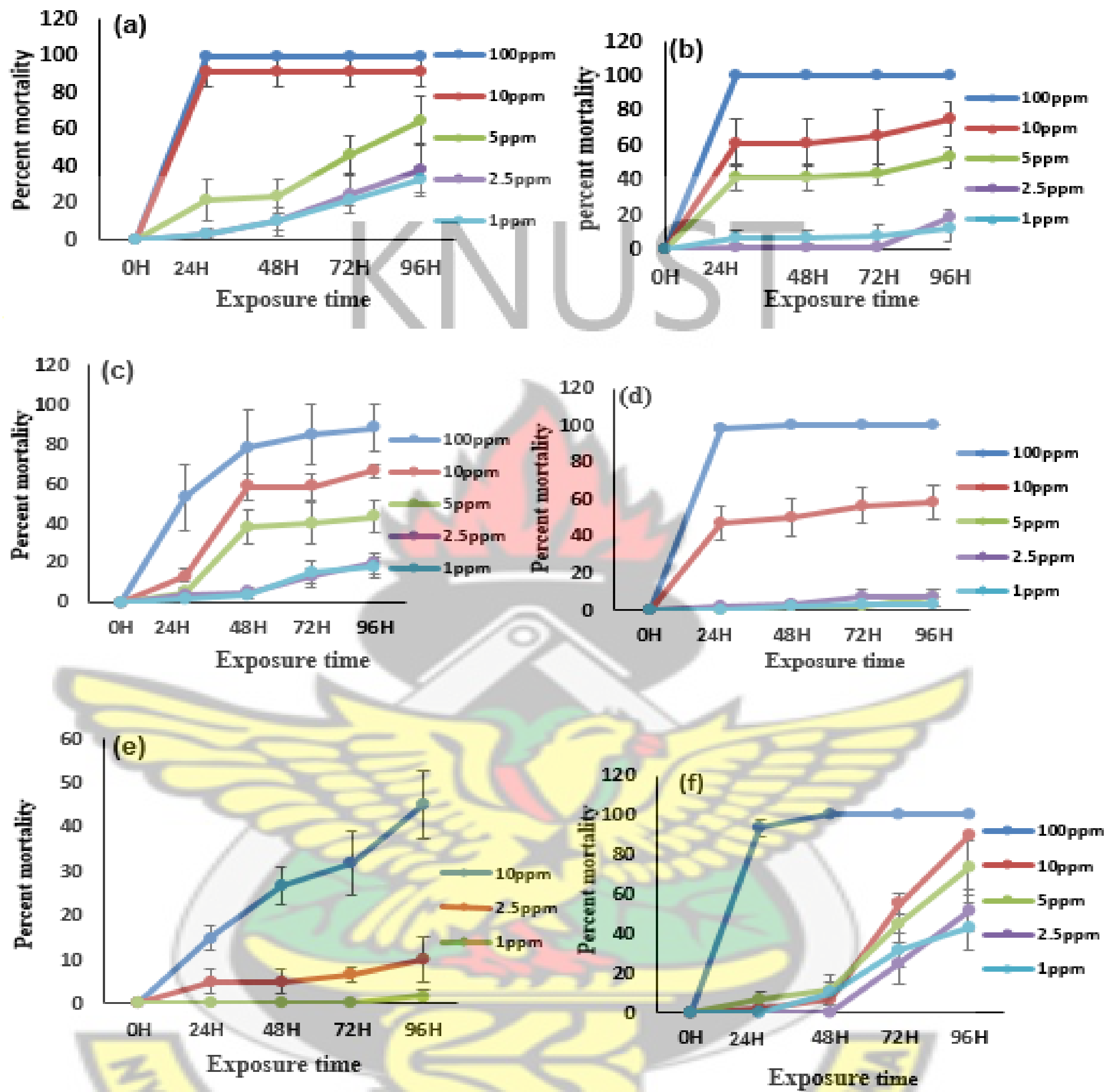


Figure 4.26. Mortality of larvae (%) by (a) EtOAc extract, (b) bulked fraction 3, (c) bulked fraction 4, (d) isolate A, (e) isolate B_1 and (e) azadirachtin

The EtOAc extract and azadirachtin solutions to which the larvae were exposed to at the highest concentration (100 ppm), were observed not to be metabolized (Figure 4.27), suggesting the two treatments share similar mode of action.

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Figure 4.27. Pictorial representation of dead *Anopheles gambiae* larvae
Appearance of larvae in (a) negative control (DMSO), (b) EtOAc extract of *C. tora*, and (c) azadirachtin (positive control)

4.6 SPECTROSCOPIC IDENTIFICATION OF COMPOUNDS

Results of pesticide activity, and seed-germination enhancement ability of the crude extracts and bulked fractions (Sections 4.1.1, 4.1.1.1, 4.2.1., 4.2.2, 4.3.1 and 4.3.2) revealed the EtOAc extract and its fractions BKF-3 and 4 to be the most potent followed by the pet ether extract and fraction BKF-2 respectively.

BKF-3 and 4 were further purified to afford compounds A and B_1 respectively which also showed higher pesticide activity profiles than the positive controls, and seed germination ability profiles superior to the untreated cowpea seeds (Control 5) (Sections 4.1.1.2, 4.2.3, 4.3.3 and 4.43).

The EtOAc extract of *C. tora* seed and the compounds A and B_2 from its bulked fractions (BKF-3 and BKF-4) exhibited high and moderate larvicidal activity against the *An. gambiae* third-fourth instar larvae (Section 4.5.1), and were therefore subjected to LC-QtoF-MS analysis for compound identification.

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4.6.1 Identification of compounds in pet ether extract of *C. tora*

The active oily pet ether extract of *C. tora* (Sections 4.1.1, 4.2.1, 4.3.1 and 4.4.1) was analysed by GC-MS to establish its phytochemical constituents.

GC-MS spectrum of pet ether extract of *C. tora* seeds showed 44 peaks corresponding to forty-four compounds (Appendix I). The spectrum of each peak was compared with the spectra in National Institute of Standards and Technology (NIST) data base and identified on the bases of retention time, fragmentation pattern and molecular weight of compounds (Table 4.6). GC-MS analysis revealed most of the compounds to contain mostly oxygenated and unsaturated hydrocarbons with carboxylic acid, alcohol, ester, alkanal, alkanone and ether functionalities.

Table 4.6. Compounds in pet ether extract of *C. tora* seed by GC-MS analysis

Number	Retention time(minutes)	Phytochemical compound	Molecular formula	Molecular weight(gmol^{-1})
1	11.04	3-heptyne	C_7H_{12}	96.17
2	11.37	3, 7-dimethyl-1,6-octadiene	$C_{10}H_{18}$	138.24
3	11.87	2-methoxy-5-(1-propenyl)-phenol	$C_{10}H_{12}O_2$	164.20
4	12.24	2-isopropenyl-5-isopropyl-7,7-dimethyl bicyclo(4.1.0)-3- heptane	$C_{15}H_{24}$	204.35
5	12.86	7, 11-dimethyl-3-methylene-1,6,10-dodecatriene	$C_{15}H_{24}$	204.35
6	13.32	3, 7-dimethyl-1, 3, 6-octatriene	$C_{10}H_{16}$	136.23
7	13.54	1a,2, 3,4,4a,5,6,7b-octahydro-1,1,4,7-tetramethyl-(1aR-(1a.α.4α, 4aα,7aα)-1H-cycloprop(e) azulene	$C_{15}H_{24}$	204.35
8	13.62	Octahydro-7-methyl-3-methylene-4-(1-methylethyl)-(3aS-3aα,3bα,4α)-1H-cyclopenta(1,3)-cyclopropa(1,2)-benzene	$C_{15}H_{24}$	204.35
9	13.89	2-methoxy-4-(2-propenyl) acetate phenol (eugenyl acetate)	$C_{12}H_{14}O_3$	206.23
10	14.04	2-isopropenyl-5-isopropyl-7,7-dimethyl-bicyclo(4.1.0)-3-heptene	$C_{15}H_{24}$	204.35
11	14.25	1-(1,4-dimethyl-3-cyclohexen-1-yl)-ethanone	$C_{10}H_{16}O$	152.23

12	14.52	2,4-octanedione	$C_8H_{14}O_2$	142.19
13	14.89	10,13-dioxo tricyclo(6.3.3.0)tetradec-4-ene	$C_{14}H_{18}O_2$	218.29
14	15.23	2-isopropyl-5-methyl-2-hexenal	$C_{10}H_{18}O$	154.25
15	15.38	1,4-diisopropylbenzene	$C_{12}H_{18}$	162.27
16	16.01	2,2,2,4-tetramethyl-pentane	C_9H_{20}	128.25
17	17.49	1,2-dibromo-2-methylundecane	$C_{12}H_{24}Br_2$	328.12
18	18.36	Methyl tridecanoate	$C_{14}H_{28}O_2$	228.37
19	18.75	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256.43
20	19.01	Ethyl hexadecanoate	$C_{18}H_{36}O_2$	284.48
21	19.97	Methyl octadeca-9,12-dienoate	$C_{19}H_{34}O_2$	294.47
22	20.03	Methyl octadec-6-enoate	$C_{19}H_{36}O_2$	296.48
23	20.27	Methyl octadecanoate	$C_{19}H_{38}O_2$	298.51
24	20.36	9-Octadecynoic acid	$C_{18}H_{32}O_2$	280.45
25	20.42	11-Tetradecenoic acid	$C_{14}H_{26}O_2$	226.35
26	20.60	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256.43
27	21.75	2-Methyl heptadecane	$C_{18}H_{38}$	254.50
28	21.99	Methyl tridecanoate	$C_{14}H_{28}O_2$	228.37
29	22.23	3-Methyl-4-octanolide(methyl octalactone)	$C_9H_{16}O_2$	156.22
30	22.51	Bis (2-ethylhexyl) adipate	$C_{22}H_{42}O_4$	370.57
31	23.18	7-Tetradecene	$C_{14}H_{28}$	196.37
32	23.38	2-Methyl tricosane	$C_{24}H_{50}$	338.66
33	23.68	Diisooctyl-1,2-benzene dicarboxylate (Diisooctyl phthalate)	$C_{24}H_{38}O_4$	390.56
34	24.14	Methyl-2-oxo-octadecanoate	$C_{19}H_{36}O_3$	312.49
35	24.70	1-Docosene	$C_{22}H_{44}$	308.59
36	24.87	2-Methyl heptadecane	$C_{18}H_{38}$	254.50
37	25.70	2,6,10,15,19,25-Hexamethyl-2,6,10,14,18,22-tetracosahexaene (squalene)	$C_{30}H_{50}$	410.71
38	26.11	1-Docosene	$C_{22}H_{44}$	308.59
39	26.26	2-Methyl tricosane	$C_{24}H_{50}$	338.66
40	27.66	17-Pentatriacontene	$C_{35}H_{70}$	490.94
41	28.28	α-Tocopherol (vitamin E)	$C_{29}H_{50}O_2$	430.71
42	29.55	3-Methoxy choles-5-ene	$C_{28}H_{48}O$	400.68
43	29.83	Stigmasta-5-22-dien-3-ol, acetate	$C_{31}H_{50}O_2$	454.73α
44	30.61	α-Sitosterol	$C_{30}H_{50}O_9$	426.72

4.6.2 Identification of compounds in bulked fraction 2 of *C. tora*

GC-MS chromatogram of bulked fraction-2 of *C. tora* seeds showed ten peaks (Appendix II). Each peak was compared with spectra in NIST library to confirm identity. Detailed analyses of bulked fraction-2 revealed it to contain mainly di-esters of benzoic acid, a dichloro-substituted mono ester and a dimethyl ethyl substituted phenol (Table 4. 7).

Table 4.7. Identified phytochemical compounds in bulked fraction-2

Number	Retention time(minutes)	Phytochemical compound	Molecular formula	Molecular weight (gmol^{-1})
1	13.79	2, 4 – bis(1, 1-dimethyl ethyl)-phenol	$C_{14}H_{22}O$	206.32
2	23.48	Diisooctyl-1, 2-benzene dicarboxylate	$C_{24}H_{38}O_4$	390.55
3	23.57	Decyl octyl-1, 2-benzene dicarboxylate	$C_{26}H_{42}O_4$	418.60
4	23.70	Diisooctyl-1, 2-benzene dicarboxylate	$C_{24}H_{38}O_4$	390.56
5	23.88	Diisooctyl-1, 2-benzene dicarboxylate	$C_{24}H_{38}O_4$	390.56
6	23.92	2,2-dimethylpropyl dichloroacetate	$C_7H_{12}C_{l2}O_2$	199.07
7	24.01	Diisooctyl-1, 2-benzene dicarboxylate	$C_{24}H_{38}O_4$	390.56
8	24.05	Diisooctyl-1, 2-benzene dicarboxylate	$C_{24}H_{38}O_4$	390.56
9	24.14	Dipentyl-1, 2-benzene dicarboxylate	$C_{18}H_{26}O_4$	306.40
10	24.48	Dipentyl-1, 2-benzene dicarboxylate	$C_{18}H_{26}O_4$	306.40

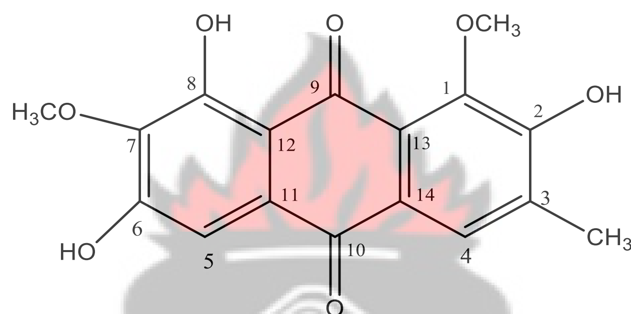
4.6.3 Identification of compound A as aurantio-obtusin

Compound A was isolated as a yellow powder with melting point 246-250 ^{o}C, and whose spectroscopic data including UV, IR, MS and 2D-NMR were similar with those reported for aurantio-obtusin previously isolated from *C. tora* (Hyun *et al*., 2009; Zhu *et al*., 2008). IR

spectrum showed a broad band of absorption at 3305 $cm^{-1}$of hydroxyl (OH) groups, C-H stretches at 2918 and 2850 cm^{-1} of hydrocarbons with corresponding CH_3 bending peaks at 1447 and 1392 cm^{-1}, strong absorption bands at 1732 and 1658 $cm^{-1}$of C=O bonds, a weak absorption of C=C bonds at 1624 cm^{-1} and a corresponding medium to strong absorption around 1580 cm^{-1} of aromatic rings, = C-H out of plane bendings of aromtics at 715 cm^{-1} indicative of tri-substituted rings, C-O-H bending peak at 1309 cm^{-1}, C-O stretches of phenols and ethers at 1176, 1262 and 1089 cm^{-1} respectively. High resolution mass spectral data ESI-MS revealed the molecular formula of isolate/compound A as $C_{17}H_{14}O_7$ from $[M + H]^+$ ion at m/z 331.0812 (expected/theoretical 331.0815). ^{13}C-NMR spectrum showed 17 carbon signals, and according to DEPT-135 two were carbonyls with chemical shifts at δ_C 187.1 and 180.1 assigned to C_9 and C_{10}, typical of a quinone ring system; ten quaternary and two methine (olefinic) carbons with signals at δ_C 147.2, 155.7, 132.0, 157.0, 139.4, 157.0, 128.6, 110.9,123.8, 124.8, 125.8 and 107.8, indicative of an aromatic ring sytem, assigned to C_1, C_2, C_3, C_6, C_7, C_8, C_{11}, C_{12}, C_{13}, C_{14}, C_4 and C_5, two methoxyl methyls and one deshielded methyl at δ_C 61.2, 60.0 and 16.5 typical of an aliphatic region assigned to $C_{1'}$, $C_{7'}$ and C_{15} respectively (Appendix III). The ^{1}H-NMR spectrum (Appendix IV) exhibited a broad singlet of 1H at δ_H 13.27 indicative of hydroxyl protons (One chelated hydroxyl), a singlet of 3H at δ_H 2.28 (H-15) which corresponded to protons of the deshielded methyl group with carbon C_{15} earlier identified in ^{13}C-NMR and DEPT-135 spectra, two singlets of 3H each at δ_H 3.80 and 3.83 which corresponded to protons of the two methoxyl methyls with carbons $C_{1'}$ and $C_{7'}$previously identified in ^{13}C-NMR and DEPT-135 spectra, two singlets of 1H each at δ_H 7.77 and 7.16 indicative of protons of an aromatic benzene ring system, corresponded to the two

methine groups with carbons C_4 and C_5 earlier shown in ^{13} C-NMR and DEPT-135 spectra. The occurrence of proton signals of the methyl and methoxyl methyl groups at shift positions lower than normal upfield positions suggested that the carbons bearing their protons are attached to unsaturated carbons of an aromatic compound and electronegative oxygen atoms. Signals at δ_C 155.7, 157.0, 157.0, 147.2, 139.4 and 132.0 are assigned to the hydroxyl, methoxyl and methyl substituted carbons C_2, C_6, C_8, C_1, C_7 and C_3 respectively. Assignment of the structure of isolate/ compound A as aurantio-obtusin came from the 2D-NMR data (HMQC and HMBC). The ^3J HMBC data showed connectivities between methoxyl-1 (OMe-1) protons and C_1 of the anthraquinone skeleton; methoxyl-7 (OMe-7) protons and C_7 of the anthraquinone skeleton; methyl (Me) protons and C_3 of the anthraquinone skeleton, which facilitated the identification of the methoxyls and methyl bearing carbons. There were ^3J HMBC connectivities between the methyl protons at C_{15} and C_2, methyl protons at C_{15} and C_4, C_3 and C_{10}, C_3 and C_{13}, methine proton at C_5 and C_7, methine proton at C_5 and C_{10}, methine proton at C_5 and C_{12}, C_6 and C_8. Moreover, the presence of one chelated hydroxyl signal in the ^{1}H NMR spectra suggested the presence of a free hydroxyl attached to C_8, and the methoxyl group attached to C_1 contributed to the chemical shift at δ_C 123.8 of C_{13} (Table 4.8). UV bands at 285 and 314 nm, and visible light absorption at 401 nm corresponded to $\pi \rightarrow \pi^*$ and $n \rightarrow \pi^*$ transitions of aromatic compounds with carbonyl (C=O) functionalities, and revealed a coloured polycyclic aromatic compound having chromophores and a quinoid basic structure whose features were identified in the IR and NMR spectra (Donald *et al*., 2009). Based on the above observation compound A was identified as aurantio-obtusin and according to the IUPAC nomenclature, it is named as 1, 3, 7-trihydroxy-2, 8-dimethoxy-6-methyl-9, 10-anthraquinone

(Numbering starts at carbon atom bearing the hydroxyl functional group of the anthraquinone basic structure and moves anticlockwise). This IUPAC numbering system is different from the common anthraquinone numbering system employed in the structure below (Numbering starts at carbon atom bearing the methoxyl functional group of the anthraquinone basic structure and moves clockwise).



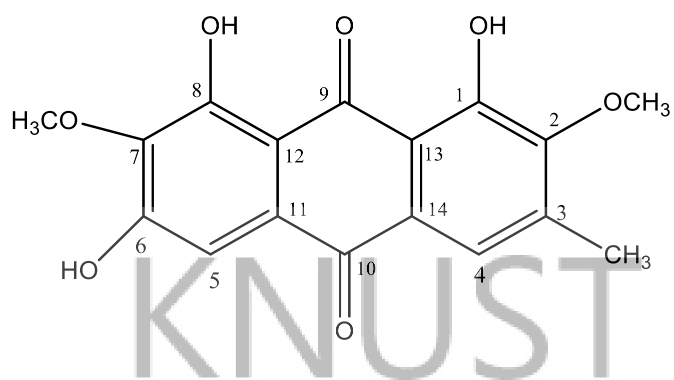
Aurantio-obtusin **(A)**

4.6.4 Identification of compound B_1 as cassiatorin

Chromatographic separation of bulked fraction-3 and further purification yielded another yellow powder isolate/compound $\mathbf{B_1}$ with melting point 262−268 ^oC.The MS data, UV and IR spectra of compound $\mathbf{B_1}$ showed similarities comparable to compound **A** (Aurantio-obtusin). Its molecular formula was revealed as $C_{17}H_{14}O_7$ from $[M + H]^+$ ion at m/z 331.0812 (expected/theoretical 331.0815). IR spectrum showed broad bands of absorption at 3305 cm^{-1} of hydroxyl (OH) groups, C-H stretches at 2918 and 2850 cm^{-1} of hydrocarbons with corresponding CH_3 bending peaks at 1447 and 1392 cm^{-1}, a strong band of absorption at 1656 $cm^{-1}$of C=O bonds, a weak absorption of C=C bonds at 1624 cm^{-1} and a corresponding

medium to strong absorption around 1580 cm^{-1} of aromatic rings, =C-H out of plane bendings of aromtics at 715 cm^{-1} indicative of tri-substituted rings, C-O-H bending peak at 1309 cm^{-1}, C-O stretches of phenols and ethers at 1176, 1262 and 1089cm^{-1} respectively. Thorough view of NMR data of compound B_1 indicated apparent differences in the chemical shift position values of some of its δ_H/δ_C signals compared to those of aurantio-obtusin. Inspection of ^{13}C and DEPT-135 NMR spectra of compound B_1 showed signals representing 17-carbons of which two were carbonyls with chemical shifts at δ_C 190.5 and 181.2 assigned to C_9 and C_{10}, typical of a quinone ring system; ten quaternary and two methine (olefinic) carbons with signals at δ_C 154.4, 151.4, 139.2, 158.2, 139.5, 156.7, 127.3, 109.6, 115.3, 129.2, 121.7 and 109.6, indicative of an aromatic ring sytem, assigned to $C_1, C_2, C_3, C_6, C_7, C_8, C_{11}, C_{12}, C_{13}, C_{14}, C_4$ and C_5, two methoxyl methyls and one deshielded methyl at δ_C 59.9, 60.0 and 16.8 typical of an aliphatic region assigned to $C_{2'}$, $C_{7'}$ and C_{15} respectively (Appendix V; Table 4.8). The ^1H-NMR spectrum (Appendix VI) exhibited two broad singlets of 1H each at δ_H 12.27 and 12.14 indicative of hydroxyl protons (Two chelated hydroxyls), a singlet of 3H at δ_H 2.32 (H-15) which corresponded to protons of the deshielded methyl group with carbon C_{15} earlier identified in ^{13}C-NMR and DEPT-135 spectra, two singlets of 3H each at δ_H 3.90 and 3.83 which corresponded to protons of the two methoxyl methyls with carbons $C_{2'}$ and $C_{7'}$ previously identified in ^{13}C-NMR and DEPT-135 spectra, two singlets of 1H each at δ_H 7.52 and 7.21 indicative of protons of an aromatic benzene ring system and corresponded to the two methine groups with carbons C_4 and C_5 earlier shown in ^{13}C-NMR and DEPT-135 spectra. The two chelated hydroxyl signals in the ^1H NMR spectra suggested a free hydroxyl is attached to C_8 just as in compound A, and unlike compound A another free hydroxyl is attached to C_1

instead of a methoxyl group, and contributed to the chemical shift of C_{13} more upfield at δ_C 115.3 (Table 4.8). Signals at δ_C 154.4, 158.2, 156.7, 151.4, 139.5 and 139.2 are assigned to the hydroxyl, methoxyl and methyl substituted carbons C_1, C_6, C_8, C_2, C_7, and C_3 respectively. Unambiguous assignment of the NMR signals and the structure as **B_1** came from comprehensive 2D-NMR studies (HMQC and HMBC). The ^3J HMBC data showed connectivities between methoxyl-2 (OMe-2) protons and C_2 of the anthraquinone skeleton; methoxyl-7 (OMe-7) protons and C_7 of the anthraquinone skeleton; methyl (Me) protons and C_3 of the anthraquinone skeleton, which revealed the identificaties of the methoxyls and methyl bearing carbons. There were ^3J HMBC connectivities between the methyl protons at C_{15} and C_2, methyl protons at C_{15} and C_4, methine proton at C_4 and C_2, methine proton at C_4 and C_{10}, methine proton at C_4 and C_{13}, methine ptoton at C_5 and C_7, methine proton at C_5 and C_{10}, methine proton at C_5 and C_{12}, C_5 and C_{14} (Table 4.8). UV (MeOH) bands at 285 and 314 nm, and visible light absorption at 401 nm corresponded to $\pi\rightarrow\pi^*$ and $n\rightarrow\pi^*$ transitions of aromatic compounds having carbonyl (C=O) functionalities, and revealed a coloured polycyclic aromatic compound with chromophores and a quinoid basic structure whose features were confirmed in the IR and NMR spectra (Donald *et al*., 2009). Hence, a novel structure **B_1** is herewith identified from *C. tora* that we named '*cassiatorin'*. Given that anthraquinones from *Cassia species* are widely known with their trivial name of botanical origin, we consider the naming of the compound as '*cassiatorin*' as most appropriate. According to the IUPAC nomenclature, cassiatorin (B_1) can also be named as 1, 3, 8-trihydroxy-2, 7-dimethoxy-6-methyl-9, 10-anthraquinone.



Cassiatorin (**B_1**)

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Table 4.8. ^{1}H, ^{13}C and ^3J HMBC NMR data for isolates A and B$_1$

Position	Aurantio-obtusin (A)			Cassiatorin (B$_1$)		
	δ_H	δ_C	Key ^3J HMBC Correlations	δ_H	δ_C	Key ^3J HMBC Correlations
1	-	147.3	-	-	154.4	-
2	-	155.7	-	-	151.3	-
3	-	132.0	180.4; 155.7; 123.8; 16.5	-	139.2	-
4	7.77 br s	125.9	-	7.52 br s	121.7	181.2; 151.3; 115.3
5	7.16 s	107.8	180.4; 157.0; 139.4; 128.6; 110.9	7.21 s	109.6	181.2; 158.2; 139.5; 129.2; 109.8
6	-	157.0	-	-	158.2	-
7	-	139.4	-	-	139.5	-
8	-	157.0	-	-	156.7	-
9	-	187.1	-	-	190.4	-
10	-	180.4	-	-	181.2	-
11	-	128.6	-	-	129.2	-
12	-	110.9	-	-	109.8	-
13	-	123.8	-	-	115.3	-
14	-	124.8	-	-	127.3	-
15	2.28 s	16.5	155.7; 132.0; 125.9	2.32 s	16.8	151.3; 139.2; 121.7
OH-1	-	-	-	12.27 br s*	-	-
OH-8	13.27 brs	-	-	12.14 br s*	-	-
OMe-1	3.80	61.2	147.3	-	-	-
OMe-2	-	-	-	3.90 s	59.9	151.3
OMe-7	3.83 s	60.0	139.4	3.83 s	60.0	139.5

* δ Values indicated by asterisk may be interchangeable

4.6.5 Identification of compound B$_2$ by LC-QtoF-MS

LC-QtoF-MS analysis of isolate B$_2$ revealed it to be the anthrraquinone obtusin. This was done by comparing the Rt and molecular weight with those of a reference library (METLIN, ChemSpider, ChemCalc, CSI: Fingerid). Obtusin was again identified in the ethyl acetate

extract of *C. tora* alongside the anthraquinones aurantio-obtusin, obtusiolin and chryso-obtusin by LC-QtoF-MS analysis (Figures 4.28 and 4.29, Table 4.9).

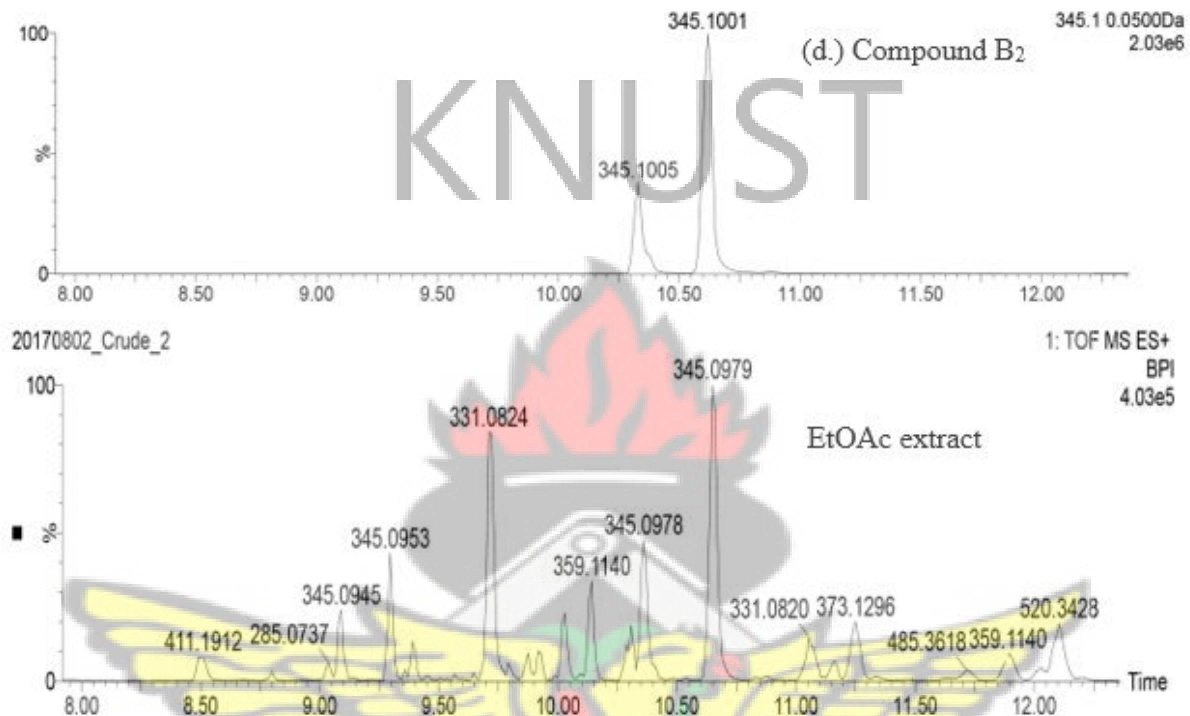


Figure 4.28. LC-QTOF-MS representative total ion chromatogram showing compounds identified in the EtOAc extract of *C. tora* seed and that of isolate B_2 (1, 7-dihydroxy-2, 3, 8-trimethoxy-6-methyl-9, 10- anthraquinone)

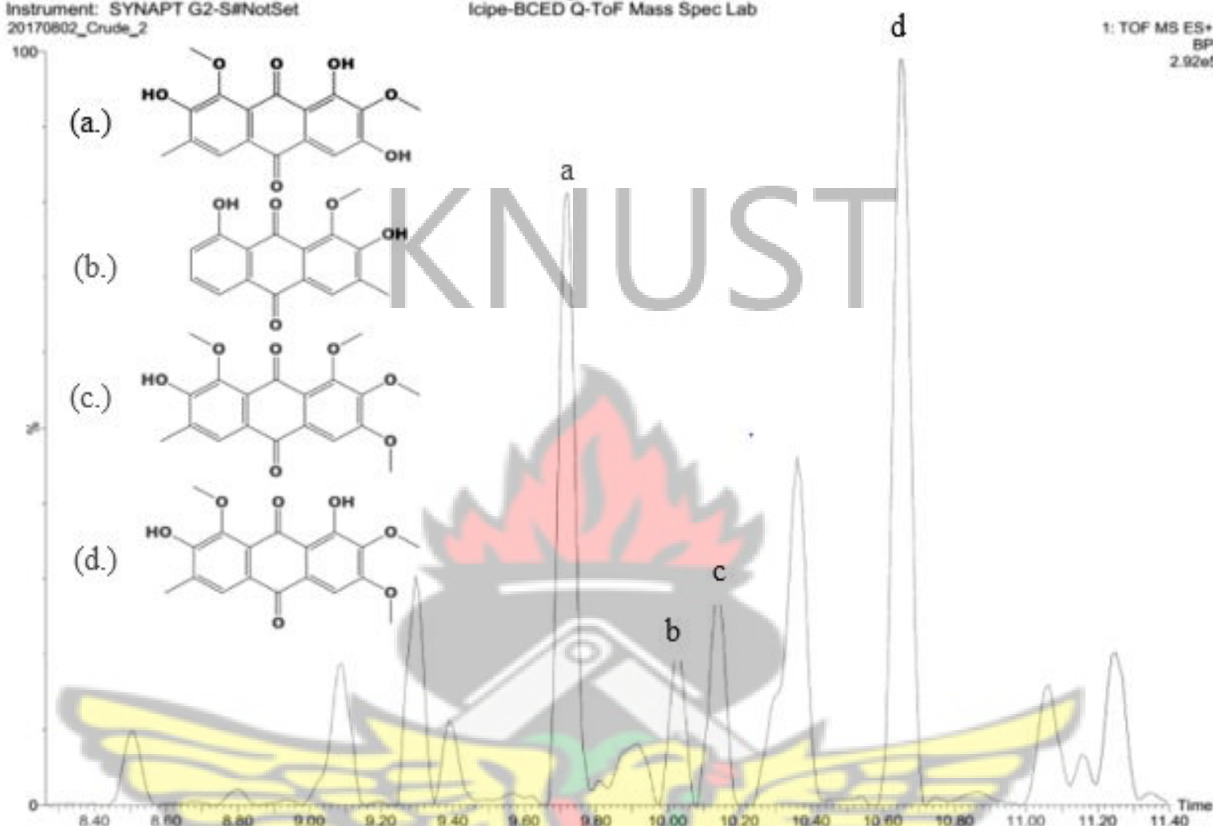
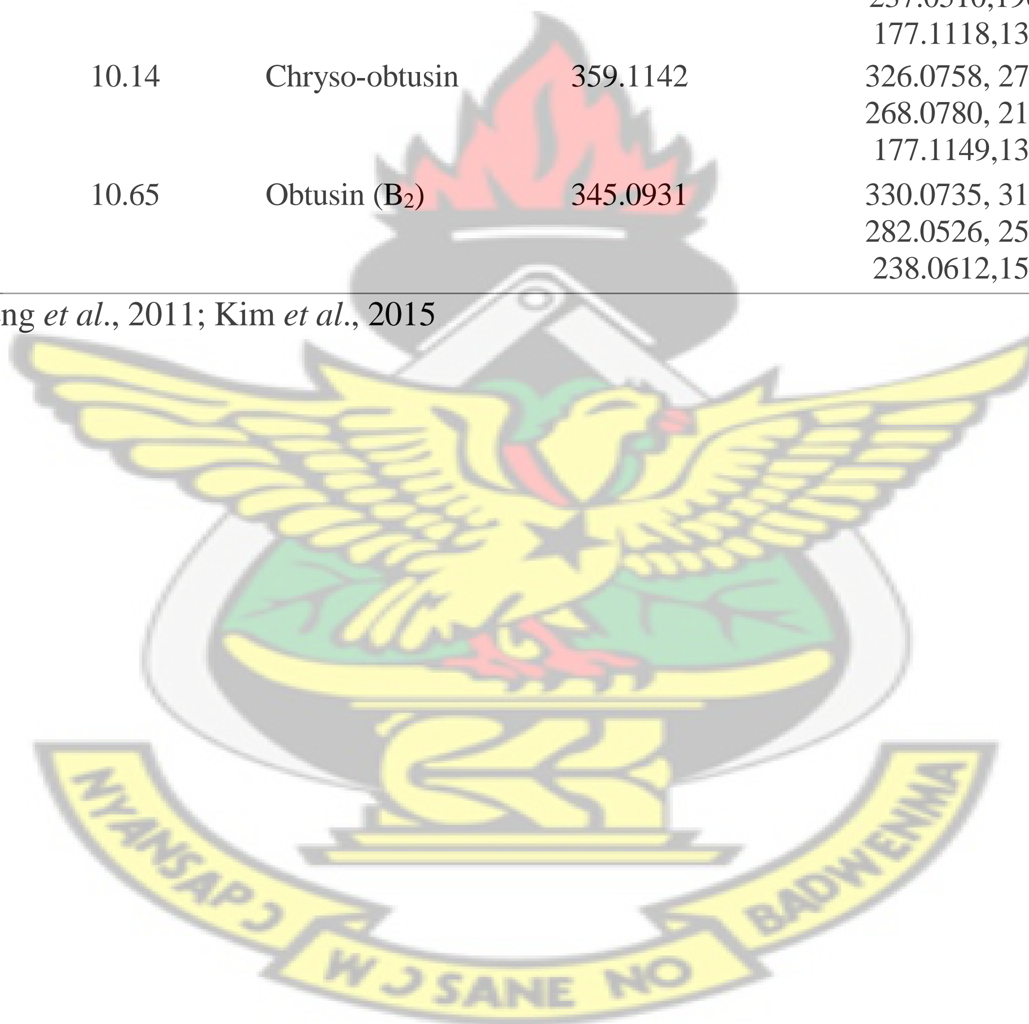


Figure 4.29. LC-QTOF-MS representative total ion chromatograms of anthraquinones in the EtOAc extract of *C. tora* seed.

Table 4.9. Retention time (Rt) and major fragment ions of compounds identified in the EtOAc extract

Peak no.	Rt (min)	Compound Name	$[M+H]^+$	Major fragments
a.	9.72	Aurantio-obtusin (A)	331.0824	316.0610, 298.0490, 288.0645, 270.0531, 253.0491, 242.0589
b.	10.03	Obtusiolin	285.0766	270.0522, 255.0648, 237.0510,196.0431, 177.1118,133.0861
c.	10.14	Chryso-obtusin	359.1142	326.0758, 270.3160, 268.0780, 211.0748, 177.1149,133.0853
d.	10.65	Obtusin (B_2)	345.0931	330.0735, 312.0622, 282.0526, 254.0513, 238.0612,154.0484

Zheng *et al*., 2011; Kim *et al*., 2015



Chapter 5

DISCUSSION

5.1 BIOPESTICIDES

Use of biopesticides is largely encouraged as opposed to synthetic ones due to their short shelf lives, narrow target ranges, specific and concerted actions on both behavioral and physiological processes that minimize negative impacts on the ecosystem and low chances of developing resistance by pests (Amer and Mehlhom, 2006a, b).

The present study investigated the *C. tora* seed extracts, bulked fractions and isolated compounds (A, B_1 and B_2) for pesticidal activity against cowpea weevils and mosquito larvae, cowpea seed germination and seedling vigor enhancement abilities.

5.1.1 Insecticidal effects

In the insecticidal assay the *C. tora* seed extracts (pet ether, EtOAc and MeOH) killed adult cowpea weevils to prevent mating of insects and ovipositing by females. Thus adequately protecting cowpea grains from weevil infestations. The three seed extracts exhibited higher mortalities on the weevils than the standard drugs (Table 4.1) which suggest the extracts could be more effective botanical insecticides against cowpea weevil infestations than the reference drugs cinnamaldehyde and neem oil. The pet ether extract exhibited the highest activity (LC_{50} =8.33±0.61 μg/mL), followed by the ethyl acetate and methanol extracts (Table 4.1). The activity of the pet-ether extract (in terms of the LC_{50}) was 30 times more potent than the reference insecticides neem oil and cinnamaldehyde. Similarly the ethyl acetate extract was about 4 times more potent than the reference drugs (Table 4.1). To the best of my knowledge, this is the first report of the insecticidal activity of the seed extracts of *C. tora*. The considerable insecticidal effect on cowpea weevils exhibited by *C. tora* seeds therefore justifies the local use of the entire plant and seeds to protect crops and

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cowpea seeds from insect pests. Other natural products with insecticidal activity against cowpea weevil include *Piper guineense* and *Azadirachta indica* seed extracts and oils (Ofuya and Dawodu, 2002). Once their toxicities are established, *Cassia tora* extracts may be clear candidates for the discovery of novel ecofriendly insecticides to migitate the infestations on grains by insect pests.

The oily pet-ether extract could potentially be a useful biopesticide for the protection of field crops and their stored products. GC-MS analysis afforded fatty acids (9-Octadecynoic acid, 11-tetradecenoic acid and n-hexadecanoic acid) and compounds bearing alcohol, alkanal, alkanone and carboxylic acid functionalities (Table 4.6). These compounds have been suggested to contribute to the insecticidal activity of the oily n-hexane extracts of *Cassia italica* seed-pods against *Callosobruchus analis* (Sakina *et al*., 2013) and thus may be responsible for the insecticidal activity of the pet-ether extract. Other natural products with insecticidal activity against cowpea weevil include the terpenes thymol, carvanol and α-pinene.

The next active ethyl acetate extract afforded three anthraquinones aurantio-obtusin, obtusin and a novel compound named cassiatorin (Figures 3.3, 3.5 and 3.6). Aurantio-obtusin (A) and obtusin (B_2) have been isolated from *C. tora* and other *Cassia species*. Obtusin was not tested for insecticidal activity due to paucity of material. Aurantio-obtusin and cassiatorin showed a concentration dependent comparable insecticidal activity but were approximately two times more potent than the ethyl acetate extract and seven times more active than the reference compounds used as positive control (Figure 4.4, Tables 4.1 and 4.3). Thus purification of the ethyl acetate extract resulted in greater activity. To the best of

my knowledge, this is the first report of the insecticidal activity of aurantio-obtusin and the novel compound cassiatorin against cowpea weevils.

Anthraquinones have been reported as repellents, insecticides and feeding deterrents in many wild birds, and in some mammals, insects and fishes (DeLiberto and Werner, 2016). As a biopesticide, they are preferred to other synthetic insecticides due to their efficacy and non-lethality in the management of agricultural predation. A number of anthraquinone based insecticides and repellents are used as preplant seed treatment. Natural sources of anthraquinones and their derivatives have also been identified as insecticides and insect repellents. As a natural or synthetic biopesticide, anthraquinone are considered as promising candidates in non-lethal and insecticidal pest management (DeLiberto and Werner, 2016). Similarly, the anthraquinone emodin isolated from *Cassia nigricans* leaf was found to show considerable insecticidal effect on adult white flies, *Bemisia tabaci* (Kambou *et al*., 2008).

Thus this study has highlighted the potential of extracts and anthraquinones from *C. tora* for uptake in the development of ecofriendly natural insecticides for a wide range of agricultural applications.

5.1.2 Oviposition deterrence activity

A number of chemicals have been used to control insect oviposition responses causing reduced levels of egg deposition on the surface of crops/seeds, minimizing crop/seed infestations (Upadhyay and Ahmad, 2011). Thus, drugs having both insecticidal and oviposition deterrent potencies are better crop protectants than those with just one of the two properties.

In the present study, oviposition deterrence potentials of the seed extracts (pet ether, EtOAc and MeOH), fractions and compounds of *C. tora* were investigated using the same no

choice cowpea weevil assay employed in the insecticidal activity test. Oviposition deterrence activity was found to increase with increasing exposure time to the seed extracts (Figure 4.6). The methanol extract of the *C. tora* seed extracts at the different concentrations of 100, 200 and 300 µg/mL exhibited higher oviposition deterrence activity in the range 83.48 - 90.28 % against the female weevils, compared to that of cinnamaldehyde (13.33 - 70.28 %) (Figures 4.6 and 4.7). The ethyl acetate and pet-ether extracts showed comparable oviposition deterrence effect as the neem oil but lower than cinnamaldehyde and the methanol extract (Figure 4.7). Thus the present investigation has revealed that *C. tora* seed extracts (pet ether, EtOAc and MeOH) possess significant oviposition deterrence effect on adult female cowpea weevils and this is the first report of this activity by the plant. This finding provides useful lead for future possible use of *C. tora* seed extracts to protect cowpea seeds against weevil infestations in storage.

The oviposition deterrence effect shown by the *C. tora* seed extracts against female cowpea weevils in this study is in agreement with some previous studies. The aqueous extract of *Ricinus communis* was found to show concentration dependent oviposition deterrence (90–100%) against gravid female *Anopheles arabiensis* and *Culex quinquefasciatus* (Abdalla *et al.*, 2009). Pongam oil from the seeds of *Pogamia pinnata* was reported to exhibit anti-oviposion activity on the greenhouse whitefly, *Trialeurodes vaporariorum* (Paveda and Herda, 2007). The Pongam oil is reported to contain fatty acids (Sarma *et al.*, 2005), just as the oily pet ether extract of *C. tora* seed in this research which exhibited oviposition deterrent activity against female weevils.

The isolated aurantio-obtusin and cassiatorin showed concentration dependent oviposition deterrence effect (Figures 4.10). The EtOAc extract was less active than the compounds

and again it appears purification of the ethyl acetate extract yielded more potent compounds (Figures 4.7 and 4.11). The activities of the compounds were comparable to cinnamaldehyde reference compound but superior to neem oil. Thus the present investigation has reported for the first time that aurantio-obtusin and cassiatorin from *C. tora* possess significant oviposition deterrence effect on adult female cowpea weevils. Extracts and compounds form *C. tora* again could be formulated as a more ecofriendly protectant of cowpea seeds against weevil infestations in storage.

The oviposition deterrence effect shown by the isolated compounds against the female cowpea weevil in this study is in agreement with a previous study. The carbonyl and hydroxy functional groups in the structure of the compound 7-hydroxy-undec-1-en-3-one isolated from *Syzigium lineare* leaves was found to be responsible for its oviposition deterrent effect on gravid females of the *Spodoptera litura* insect pest (Jeyasankar *et al*., 2013). Thus the presence of the carbonyl and hydroxy functionalities in the structure of aurantio-obtusin (A) and cassiatorin (B_1) in the present study is suggested to have deterred female cowpea weevil insects to lay less eggs on cowpea grains.

5.1.3 Antifeedant activity

Insect feeding activity on crops/grains has posed serious food shortage problems to the growing population of sub-Sahara Africa. There has been an intense search for antifeedants to combat insect infestation of crops/grains. The efficacies of plant based antifeedants used in indigenous practices are being investigated. The plants used include some members of the genus Cassia (Duraipandiyan *et al.,* 2011; Suka, 2011).

In the present study, the extracts of *C. tora* seeds (Pet ether, EtOAc and MeOH extracts) exhibited considerable antifeedant activity against the cowpea weevil larvae that was comparable to cinnamaldehyde but superior to neem oil used as reference drugs (Figure

4.12). Thus the present research work has shown that the *C. tora* seed extracts possess significant antifeedant activity on cowpea weevil's larvae and further supports indigenous use of the plant and seeds for crop/grain protection against insect pests. The findings in this report agrees with the reflections of Duraipandiyan and colleagues who documented that EtOAc extract of *Cassia fistula* flowers exhibit antifeedant activity against *Helocoverpa armigera* (Duraipandiyan *et al* 2011).

Weevils and larvae are known to destroy a number of seeds in storage. For example, the maize weevil and larvae is reported to damage about 22.7 % of untreated *Triticum aestivum* (wheat) grains in storage by creating holes and significantly causing weight reduction (Ileke and Oni, 2011). Thus the antifeedant activity exhibited by the extracts and compounds of *C. tora* demonstrated in this research is notably helpful in the quest for new safe crop/seed protectants as alternatives to synthetic ones whose use are limited by resistance and toxicity problems (Ileke and Oni, 2011).

The isolated compounds aurantio-obtusin and cassiatorin in the present study exhibited slightly lower antifeedant activities than the extracts of *C. tora* seeds (Pet ether, EtOAc and MeOH extracts) (Figures 4.12 and 4.14). The higher antifeedant activities of the EtOAc extract of *C. tora* seeds then the isolated aurantio-obtusin and cassiatorin may suggest a possible synergistic action. Thus the present research work has shown that the anthraquinones aurantio-obtusin and cassiatorin possess antifeedant activity on the cowpea weevil's larvae and contributed to the overall activity of *C. tora* seeds for use in crop/grain protection against insect pests. This is the first report of the antifeedant activity of extracts and compounds of *C. tora*, to the best of my knowledge.

Some anthraquinones have been reported to possess antifeedant activity against a number of grain pest. For example, the anthraquinone, rhein (1, 8-dihydroxyanthraquinone -3-carboxylic acid) from EtOAc extract of *Cassia fistula* flowers has shown antifeedant activity against the pest, *Helocoverpa armigera* (Duraipandiyan *et al* 2011). Similarly, a banana diet that contained > 0.001 % emodin exhibited feeding deterrent activity on yellow-vented bulbuls (*Pycnonotus xanthopygos*) and house sparrows (*Passer domesticus*) compared to an emodin-free banana diet (Tsahar, 2001).

5.2 GERMINATION AND VIGOR ENHANCEMENT ACTIVITIES

The use of some chemical substances to protect seeds/grains from insect pests in storage have produced non-viable seeds/grains after being sown by farmers (Naik and Dumbre, 1985). Seed priming or treatment is a cheap and almost risk free method employed to improve seed performance and germination (Bradford, 1986). It has been confirmed in many research works that pre and post storage priming treatments reduce chromosomal aberrations of seeds, repair DNA lesions occurring in storage, decrease morphologically abnormal seedlings and increase rate of root growth. During priming treatments, seeds enter the lag stage of germination where there is little or no fresh weight increase prior to radicle emergence, and upon subsequent rehydration, they show improved germination characteristics which include reduced time to radical emergence, synchronization of germination within a seed lot, greater percentage germination and improved seed vigor in deteriorated seed lots (Agrawal and Dadlani, 1995; Anuradha *et al*., 2010b).

In the present study, treated and untreated seeds used for the insecticidal assay, observed for oviposition and emergence of larvae were subjected to germination enhancement activity to assess for seed viability.

The methanol extract of *C. tora* exhibited 100% inhibition of cowpea seed germination (Figure 4.15). The pet ether and EtOAc extracts enhanced cowpea grain germination and seedling vigor (Figures 4.15, 4.16 and 4.17). The MeOH extract had the highest oviposition deterrence activity (Figure 4.7) but inhibited cowpea seed germination considerably when compared with the pet ether and EtOAc extracts (Figure 4.15). Similarly, the reference drugs neem oil and cinnamaldehyde also caused 100% inhibition of germination. This results confirms report that these reference compounds used on the field to protect cowpea seeds render them non- viable. Thus the methanol extract of *C. tora* should not be used for protecting cowpea grains intended to be used for planting. Instead the pet-ether and ethyl acetate extracts should be used.

The pet ether and EtOAc extracts showed concentration dependent increase in seed germination and seedling vigor (Figures 4.15, 4.16 and 4.17). The EtOAc extract showed a higher activity when compared to untreated cowpea seeds not introduced to weevils (Control 5), whereas the pet ether and bulked fractions 2-4 had a comparable activity to untreated cowpea seeds not introduced to weevils (Figures 4.16, 4.17, 4.19 and 4.20). Thus the present research work has shown that the EtOAc extract of *C. tora* seeds significantly enhanced germination and vigor of cowpea seeds and seedlings (Figures 4.16 and 4.17). To the best of my knowledge, this is the first report showing the germination and vigor enhancing activities of EtOAc extract of *C. tora* seeds on cowpea seeds and seedlings respectively. Hence the ethyl acetate extract could be considered for future application as effective cowpea seed-germination and seedling-vigor enhancer.

The isolated anthraquinones (aurantio-obtusin and cassiatorin) exhibited concentration dependent seed germination and seedling vigor activity (Figures 4.21 and 4.25). They

showed better activity than the pet ether and EtOAc extracts of *C. tora* seeds. Thus purification afforded more active compounds than the EtOAc extract and fractions from which they were isolated (Figures 4.17, 4.20 and 4.25). Thus the present research has shown that aurantio-obtusin and cassiatorin of *C. tora* seeds significantly enhanced germination and vigor of cowpea seeds and seedlings. To the best of our knowledge the activity of the anthraquinones of *C. tora* seeds shown in this study is reported for the first time. In other research, the anthraquinone emodin in the pulp of *Rhamnus alaternus* was shown to increase seed viability by repairing damaged DNA in the seed embryo; also protects it against insects and microorganisms (Ido, 2002). Hence these anthraquinones could be considered for future application as effective cowpea seed-germination and seedling-vigor enhancers than the extracts of *C. tora* seed (Figures 4.17 and 4.25).

5.3 MOSQUITO LARVICIDAL ACTIVITY

The ethyl acetate extract of *C. tora* showed considerable insecticidal, oviposition deterrent, antifeedant and exhibited the best germination and seedling vigor activities (Table 4.1, Figures 4.2, 4.7, 4.12, 4.15, 4.16 and 4.17). Therefore, it was tested for larvicidal activity against larvae of the malaria vector *Anopheles gambiae*. The EtOAc extract was toxic to the larvae. It exhibited larvicidal activity that was comparable to the positive control azadirachtin but higher than its bulked fractions (Table 4.5, Figure 4.26). These results suggest that the EtOAc extract of *C. tora* seed is a more preferred option for the management of *Anopheles gambiae* third-fourth instar larvae than use of any of its bulked fractions. This therefore suggests a possible alternative larvicide to those resisted by *A. gambiae* larvae or toxic to the environment. Ethyl acetate extract of *C. tora* may find

application locally to control mosquito population since the seeds of the plant are readily available from plants.

The isolated anthraquinones aurantio-obtusin (A) and obtusin (B_2) of *C. tora* seed were toxic to larvae of the malaria vector *Anopheles gambiae*. They exhibited lower activities than the EtOAc extract and the positive control azadirachtin against the larvae (Table 4.5, Figure 4.26). The mosquito larvicidal activity of the compounds aurantio-obtusin and obtusin against the larvae of *Anopheles gambiae* in this current work is reported for the first time. This agrees with report of the larvicidal activity of the anthraquinone tectoquinone (2-methyl anthraqinone) isolated from *Cryptomeria japonica* sapwood against *Anopheles aegypti* and *Anopheles albopictus* (Seng-Sung *et al*., 2008). Due to the need to reduce and eliminate vector-borne diseases, the WHO (2017) encourages a vector control system that supports locally tailored approaches. *C. tora* plant is readily available and can be used in a local scale for mosquito population control. This is very necessary because of the development of resistance to common larvicides or biological agents including temephos and *Bacillus thuringiensis* (Dua *et al*., 2009).

5.3.1 Structure activity relationship of the anthraquinones

Analysis of the bioactive EtOAc extract of *C. tora* seed by LCqToF-MS revealed predominantly the presence of anthraquinones. Purification of its bioactive bulked fractions afforded the major anthraquinones, aurantio-obtusin (Compound A), cassiatorin and obtusin (Compound B_2).

The identical insecticidal, oviposition deterrence, antifeedant, germination and seedling vigor-enhancing activities of the two anthraquinones, aurantio-obtusin (A) and cassiatorin (B_1) the novel compound is suggested to be due to similarities in their basic anthracene structures. Interchanging the hydroxy and methoxy functionalities at positions C-1 and C-2

of their structures did not show any significant difference in their biological activities, and suggest bioactivity of the two compounds is not dependent on the substituents located at these positions, but may be distributed across the basic structures of the anthraquinone molecules, as found for compounds in other studies. The same explanation is suggested for the comparable mosquito larvicidal activity demonstrated by aurantio-obtusin (A) and obtusin (B_2). The hydroxy group at position C-6 of aurantio-obtusin and the methoxy group at the same position in obtusin also suggest bioactivity is distributed across the basic structure of the two compounds. Mosquito larvicidal activity of the other structurally different anthraquinones identified in the EtOAc extract would help shed light on this suggestion (Table 4.9, Figure 4.29).

Both aurantio-obtusin and obtusin were moderately effective as mosquito larvicides when compared to the EtOAc extract and azadirachtin (Table 4.5). The larvae exposed to the EtOAc extract and azadirachtin at the highest concentration (100 ppm) were observed not to be metabolized by these specific treatments (Figure 4.31), suggesting the two treatments share similar mode of action.

5.4 CONCLUSIONS AND RECOMMENDATIONS

5.4.1 Conclusions

The present study has showed that the pet-ether, ethyl acetate and methanol extracts of *C. tora* exhibit insecticidal, oviposition deterrent, antifeedant activities on cowpea weevils. They also enhanced germination of cowpea seeds (Methanol extract did not) and exhibited mosquito larvicidal activities that were largely superior to the reference compounds. The pet-ether extract showed the highest insecticidal activity whereas the ethyl acetate extract showed considerable activities in all the assays and was also found to have the highest germination enhancement and seedling vigor. The ethyl acetate extract was also found to

possess larvicidal activity against the third-fourth instar larvae of the malaria vector *Anopheles gambiae.* The activities of the extracts are being reported for the first time.

The compounds aurantio-obtusin, obtusin and the novel compound cassiatorin were isolated from the EtOAc extract of *C. tora* seeds. Thus the investigation has been able to establish that the anthraquinones obtusin, cassiatorin and aurantio-obtusin possess insecticidal activity against adult weevils, oviposition deterrence effect against adult female weevils, feeding inhibition property against weevil's larvae, germination and vigor enhancing effects on cowpea seeds and seedlings respectively and could be referred to as "five in one" meaning one compound performed five different functions. These compounds therefore contributed significantly to the biological activities of *C. tora* seeds.

To the best of our knowledge, it is the first time these anthraquinones are reported to exhibit these bilogical activities. Cassiatorin is a novel anthraquinone reported in this study. This study has therefore given some evidences to support folkloric use of *C. tora* seeds or plants for crop/grain-protection from insect pest infestations and crop yield-enhancement.

5.4.2 Recommendations

Toxicity studies of the extracts of *C. tora* seed (pet ether and EtOAc) and isolated compounds should be considered in future works, though no report on toxicity has been given on consumption of seeds as food in India and other parts of the world.

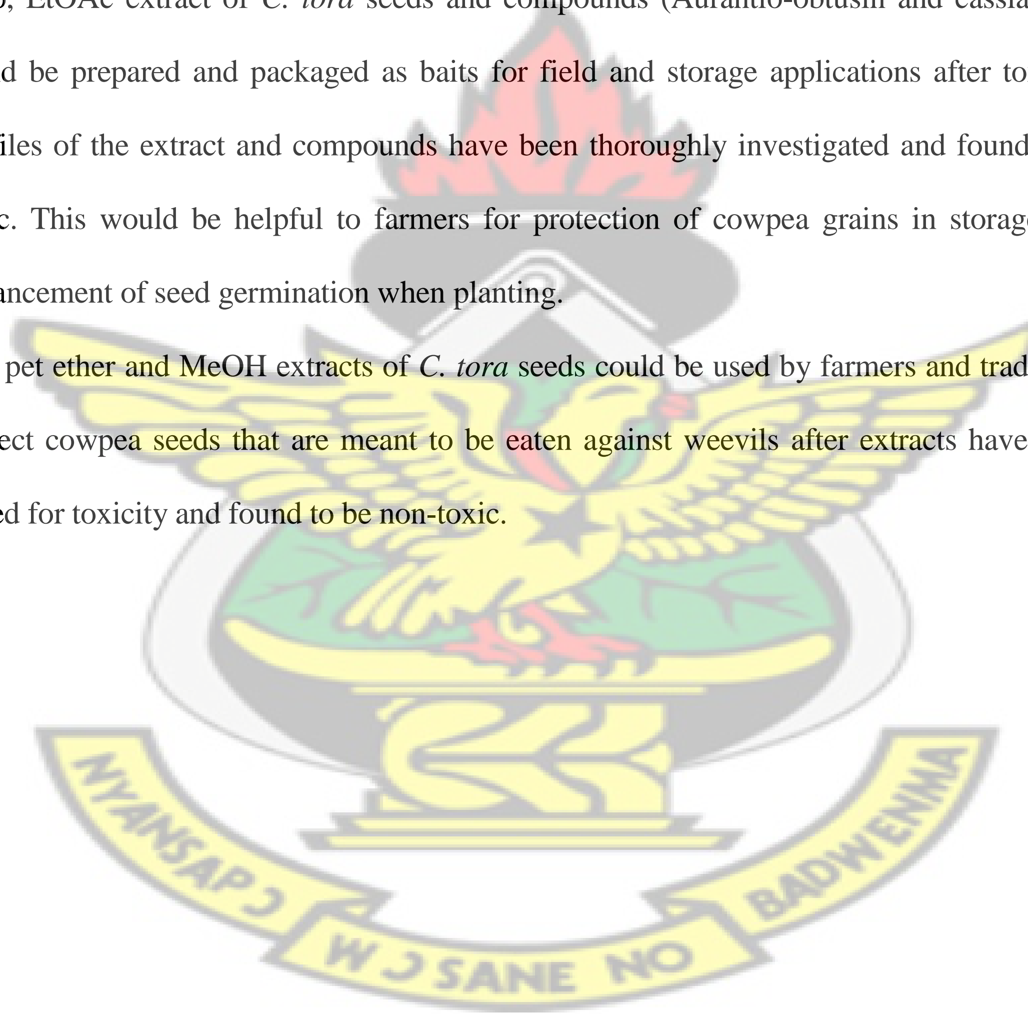
Considering the pesticidal activity, germination and vigor enhancement abilities demonstrated by the EtOAc extract of *C. tora* seed, aurantio-obtusin and cassiatorin in the present study, the extract and compounds should be investigated for pesticidal activity, germination and vigor enhancement abilities in the field in future research.

The EtOAc extract of *C. tora* seeds and compounds (aurantio-obtusin and cassiatorin) could be investigated for either synergistic or antagonistic effects on weevils, cowpea seeds and seedlings in the field and storage.

The mode of action of the individual anthraquinones (aurantio-obtusin, obtusin and cassiatorin) isolated from the EtOAc extract of *C. tora* seed may not be the same and worthy of further investigation.

Also, EtOAc extract of *C. tora* seeds and compounds (Aurantio-obtusin and cassiatorin) could be prepared and packaged as baits for field and storage applications after toxicity profiles of the extract and compounds have been thoroughly investigated and found non-toxic. This would be helpful to farmers for protection of cowpea grains in storage and enhancement of seed germination when planting.

The pet ether and MeOH extracts of *C. tora* seeds could be used by farmers and traders to protect cowpea seeds that are meant to be eaten against weevils after extracts have been tested for toxicity and found to be non-toxic.



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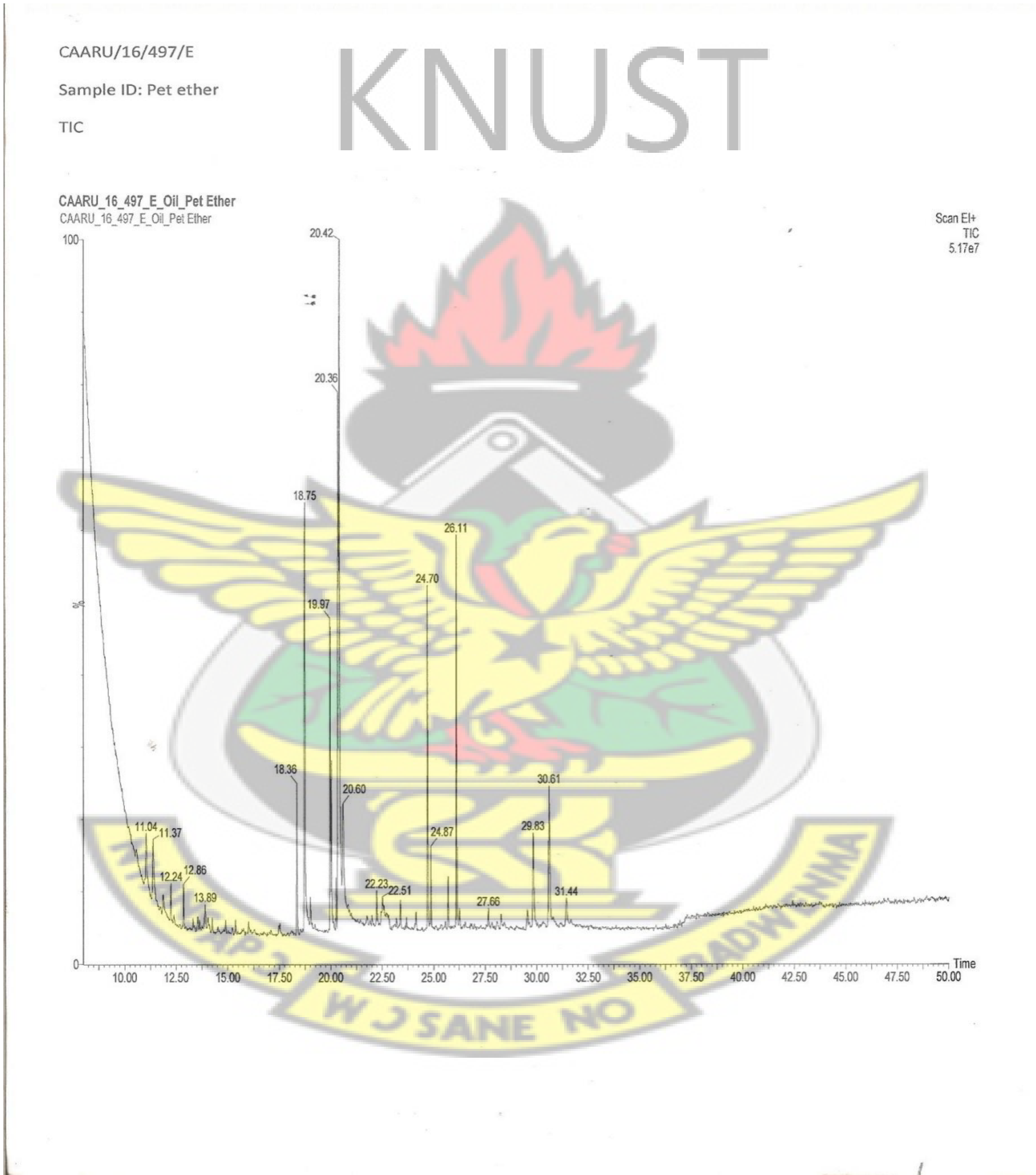
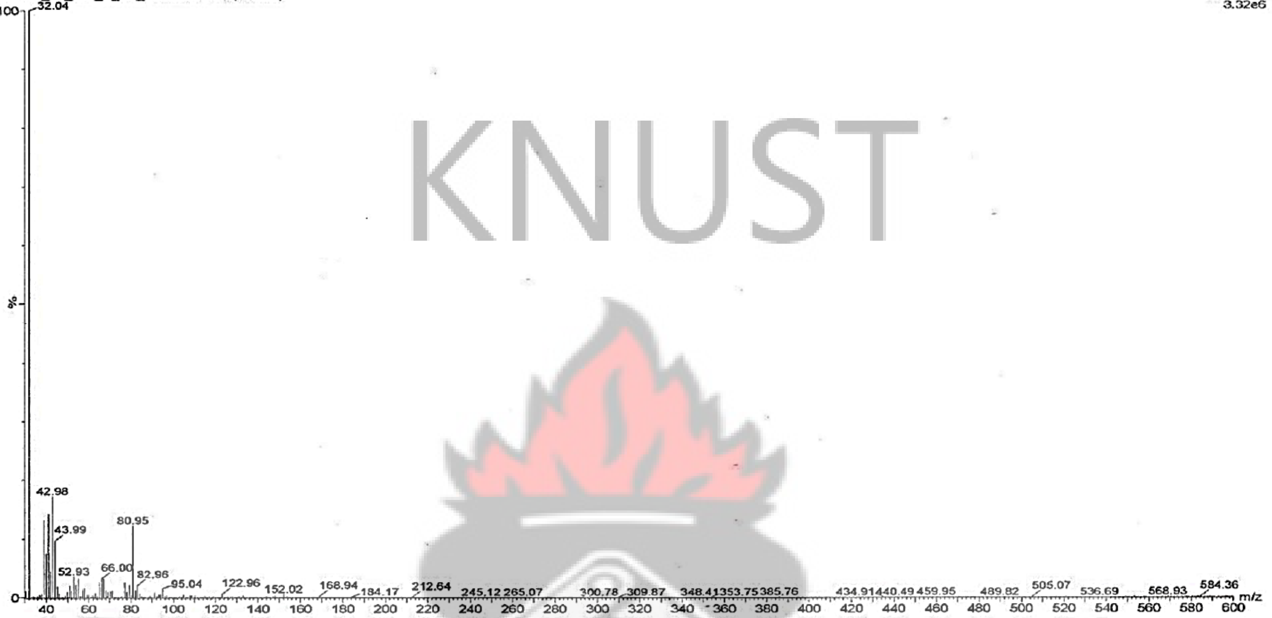
APPENDICES

APPENDIX I: SPECTRUM OF COMPOUNDS IN PETROLEUM ETHER EXTRACT OF *C. TORA* SEEDS



APPENDIX I (1): SPECTRUM OF 3-HEPTYNE

Mass Spectrum and Library Match of RT 11.04



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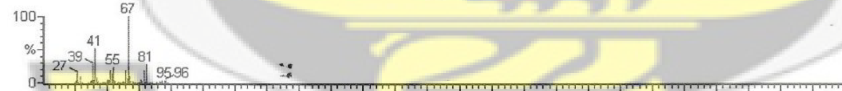
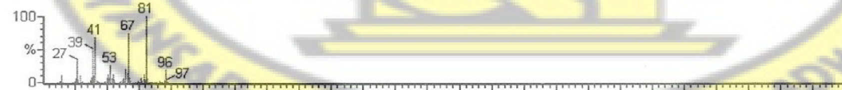
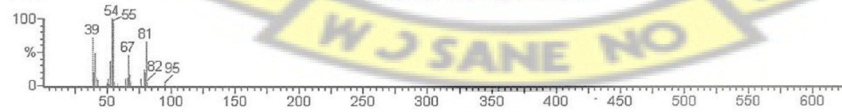


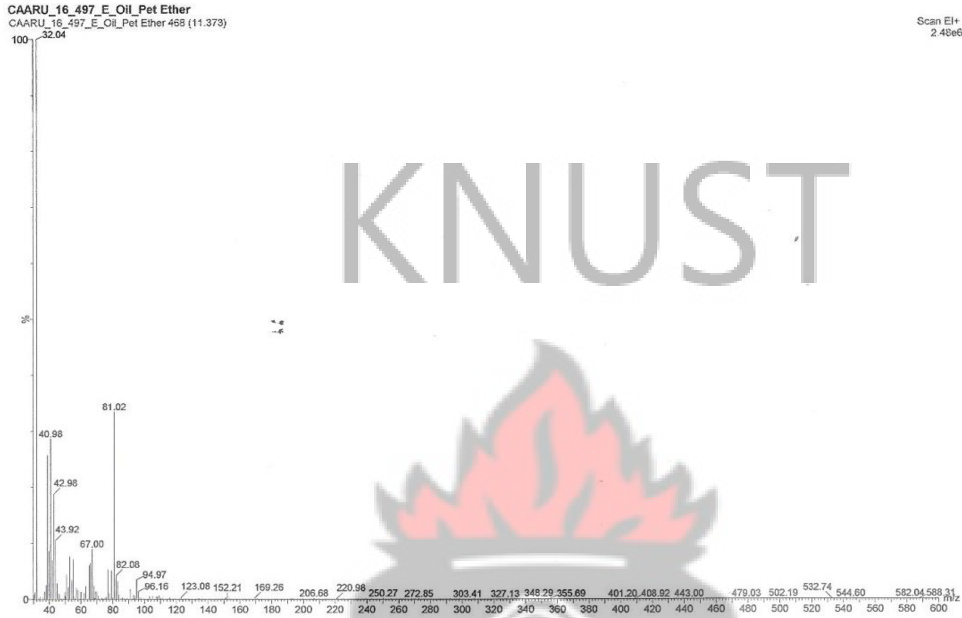


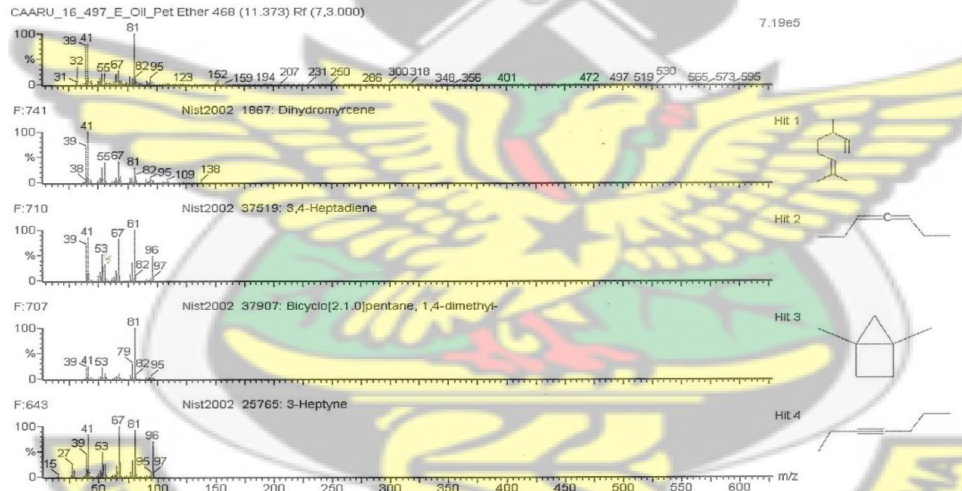


Mass Spectrum and Library Match of RT 11.37

W J SANE NO BADWENMA

APPENDIX I (2): SPECTRUM OF 3, 7-DIMETHYL-1, 6-OCTADIENE





Mass Spectrum and Library Match of RT 11.87

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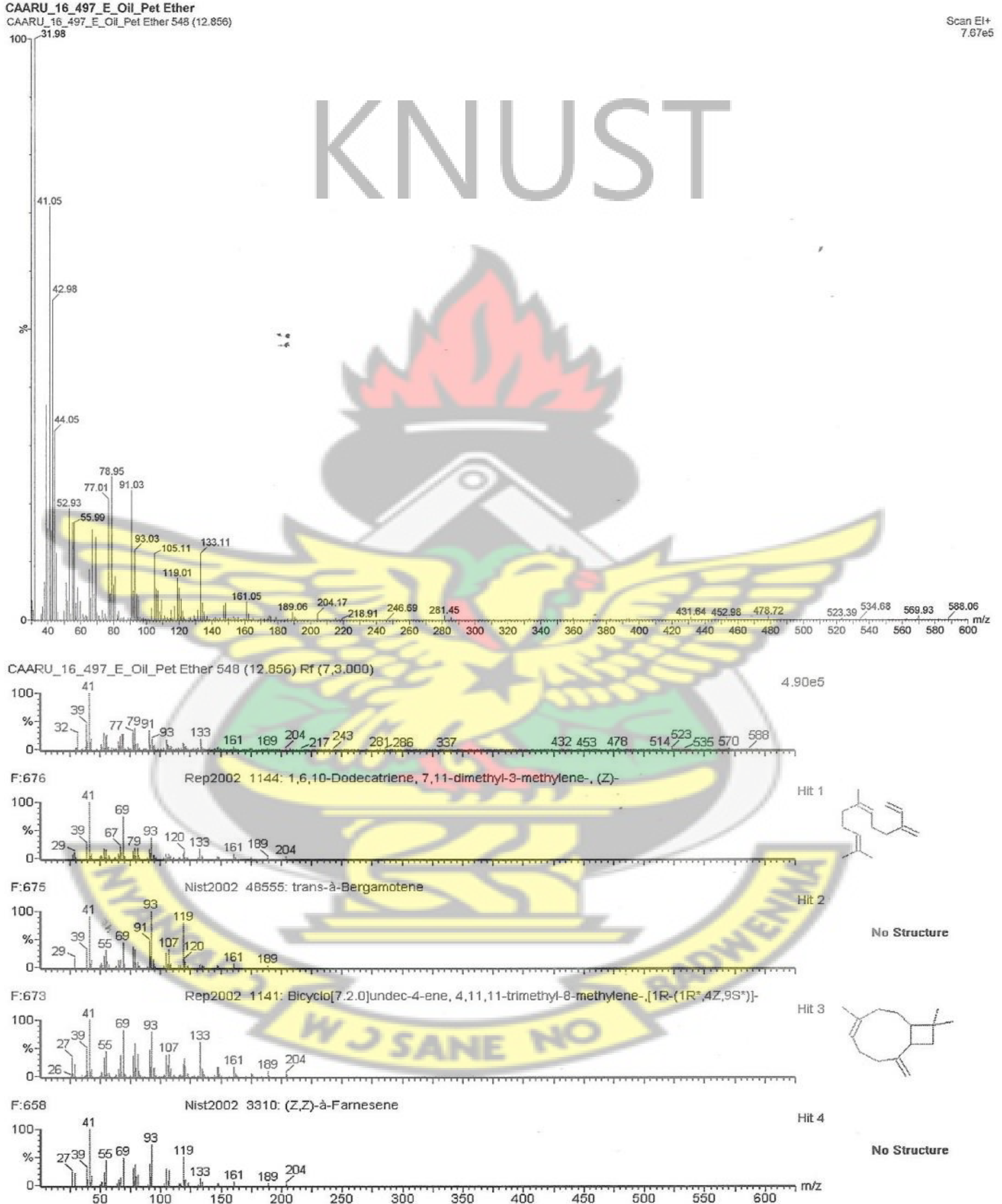
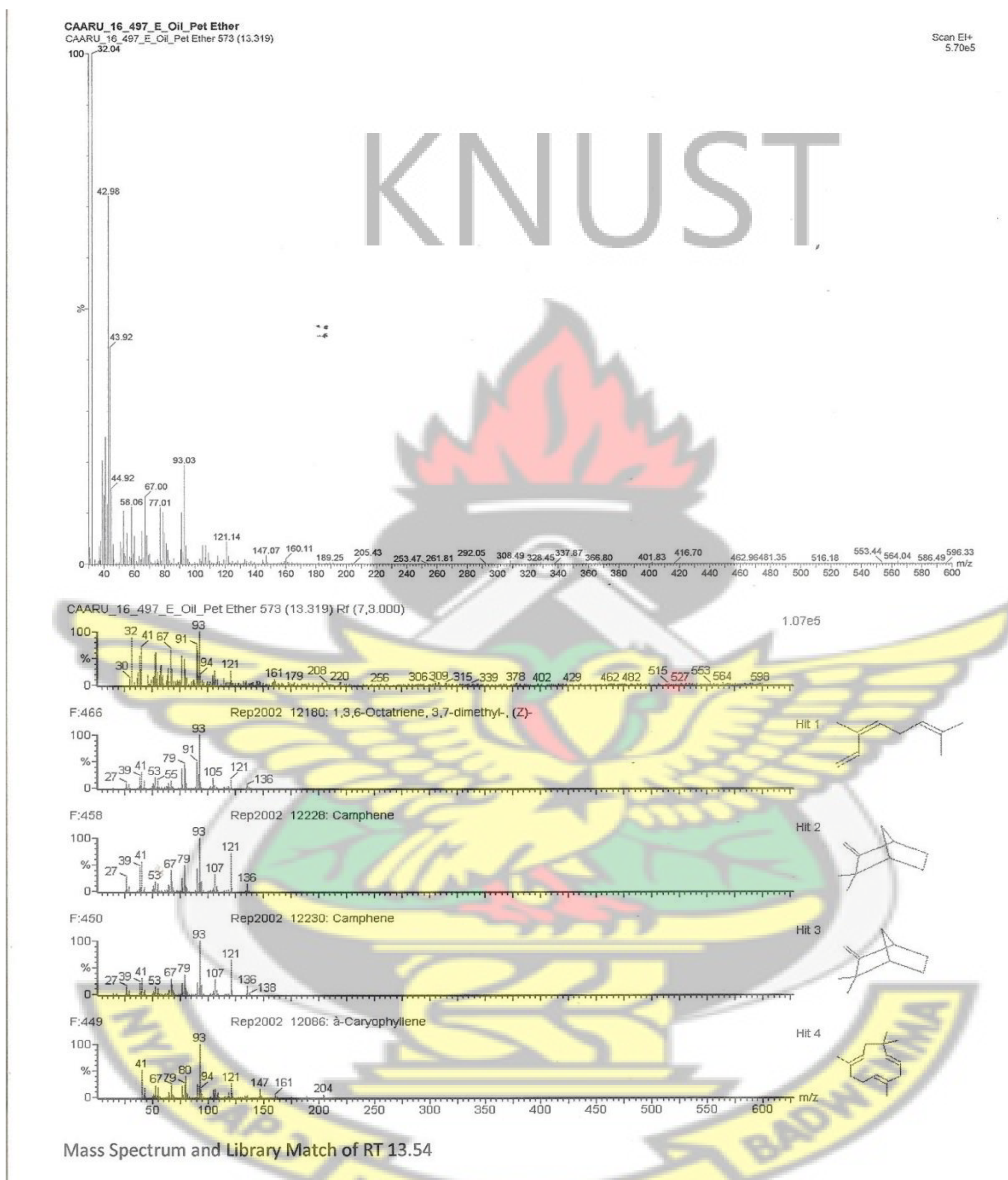
W J SANE NO BADWENMA

APPENDIX I (3): SPECTRUM OF 2-METHOXY-5(1-PRPPENYL) PHENOL



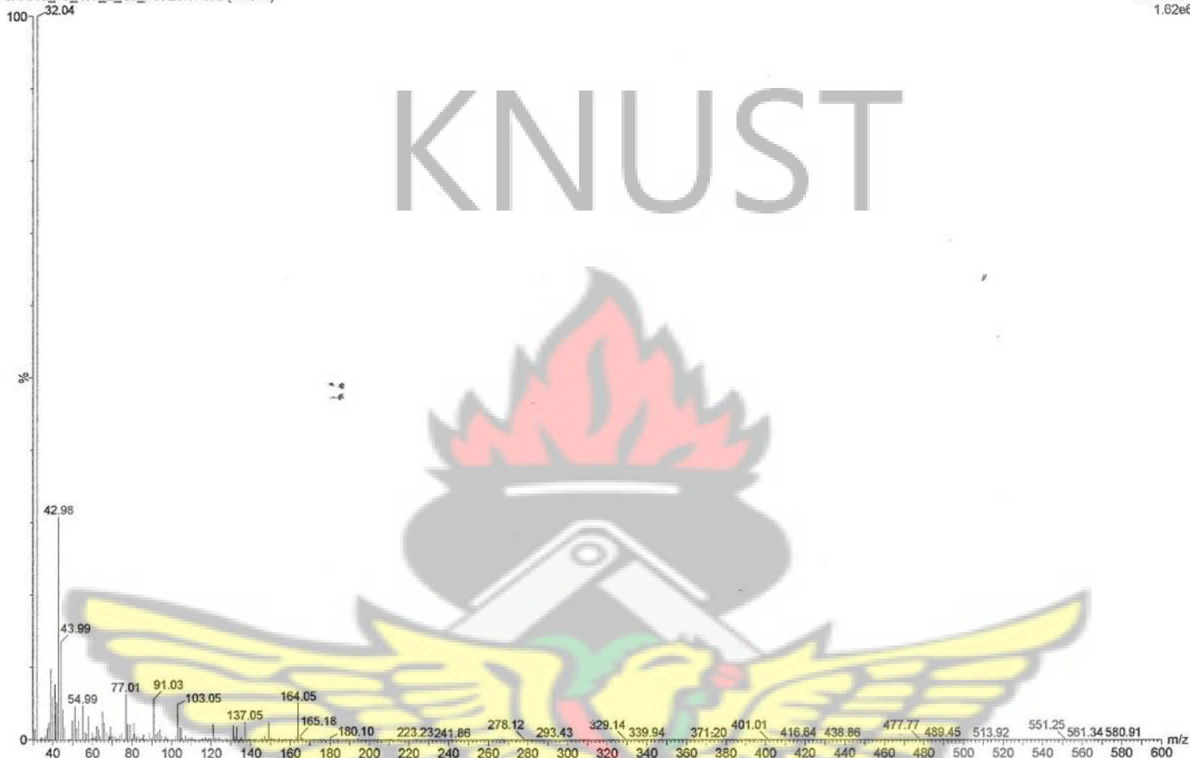
Mass Spectrum and Library Match of RT 13.32

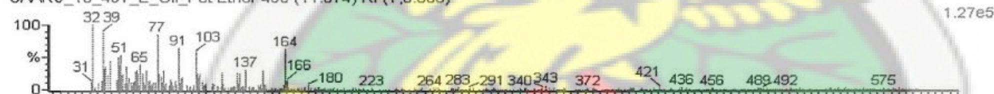
APPENDIX I (4): SPECTRUM OF 2-ISOPRPENYL-5-ISOPROPENYL-7, 7-DIMETHYL BICYCLO (4.1.0)-3-HEPTANE

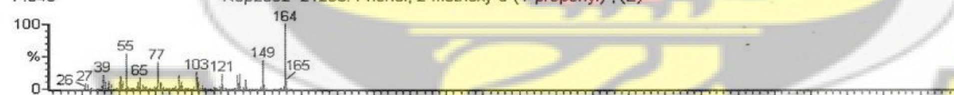


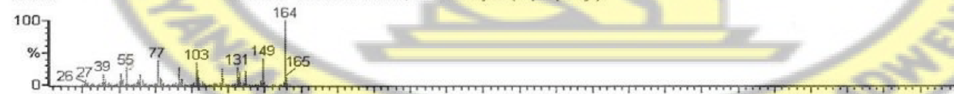
Mass Spectrum and Library Match of RT 13.54

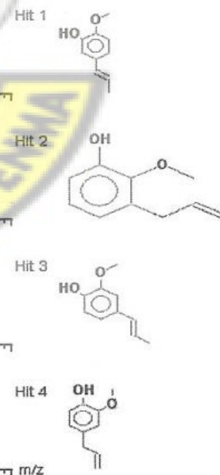
APPENDIX I (5): SPECTRUM OF 7, 11-DIMETHYL-METHYLENE-1, 6, 10-DODECATRIENE

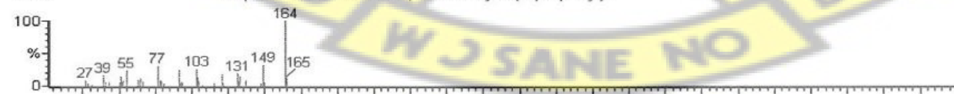


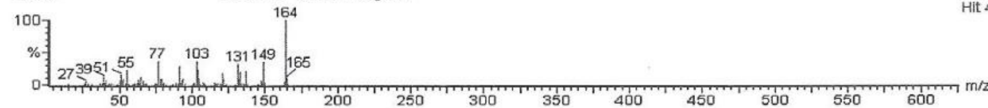






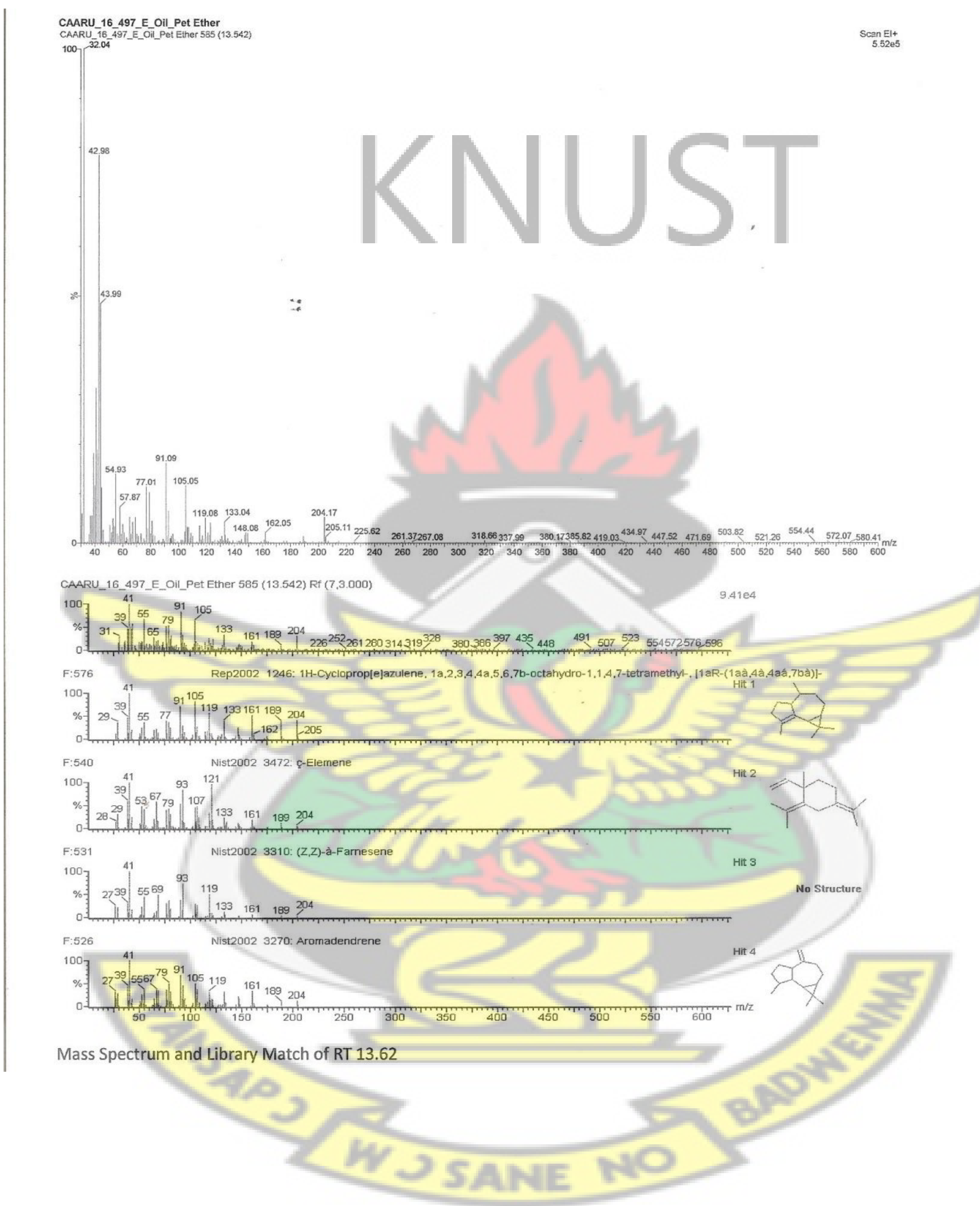






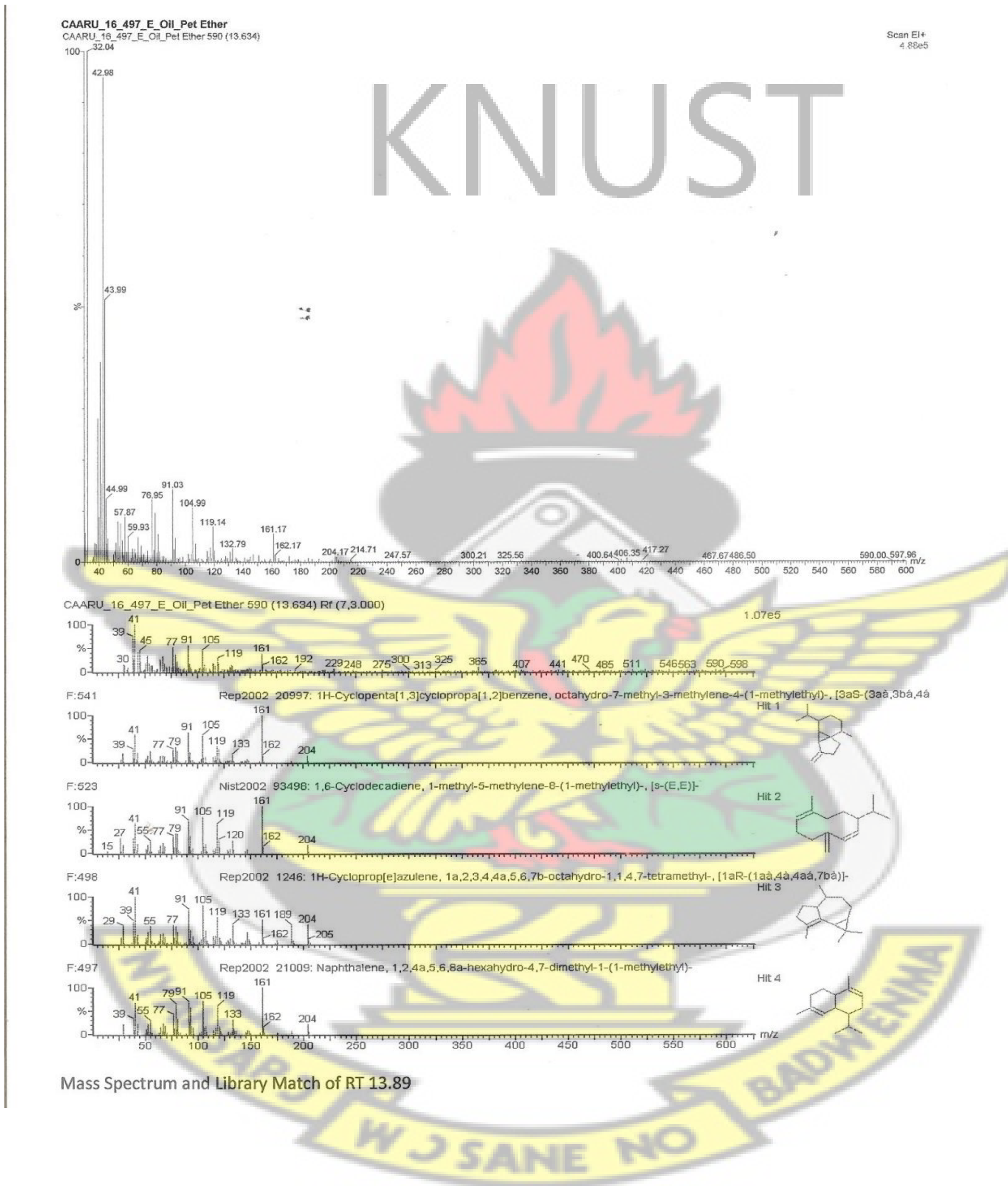
Mass Spectrum and Library Match of RT 12.24

APPENDIX I (7): SPECTRUM OF 1A, 2, 3, 4,4A, 5, 6, 7B-OCTAHYDRO-1, 1, 4, 7-TETRAMETHYL-(1AR-(1A.A.4A, 4AA, 7AA)-1H CYCLOPROP(E) AZULENE



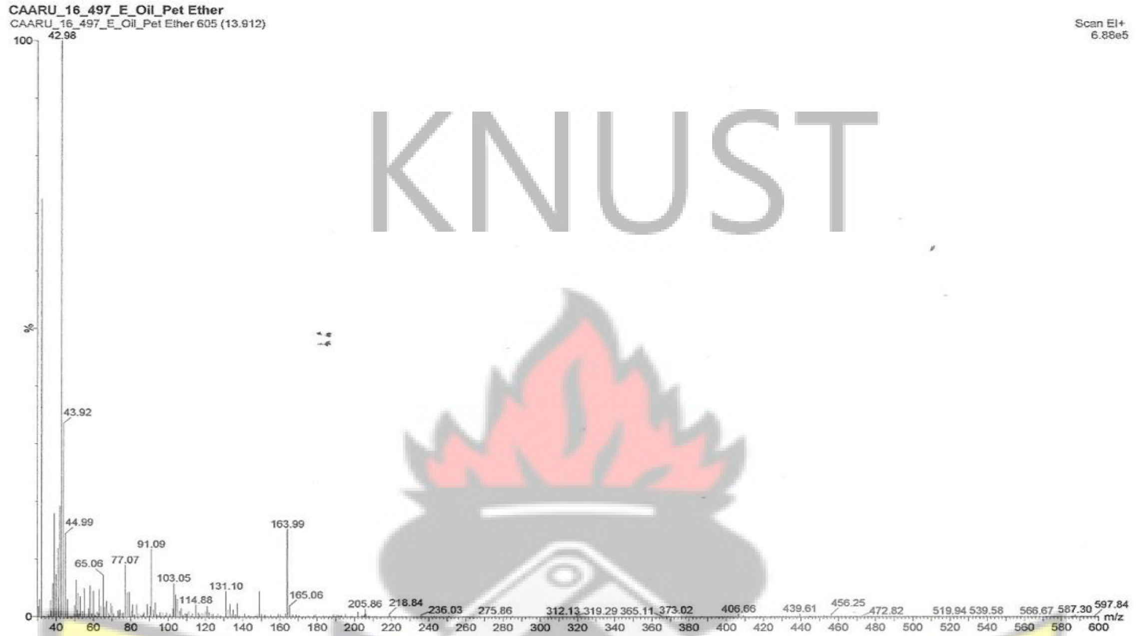
Mass Spectrum and Library Match of RT 13.62

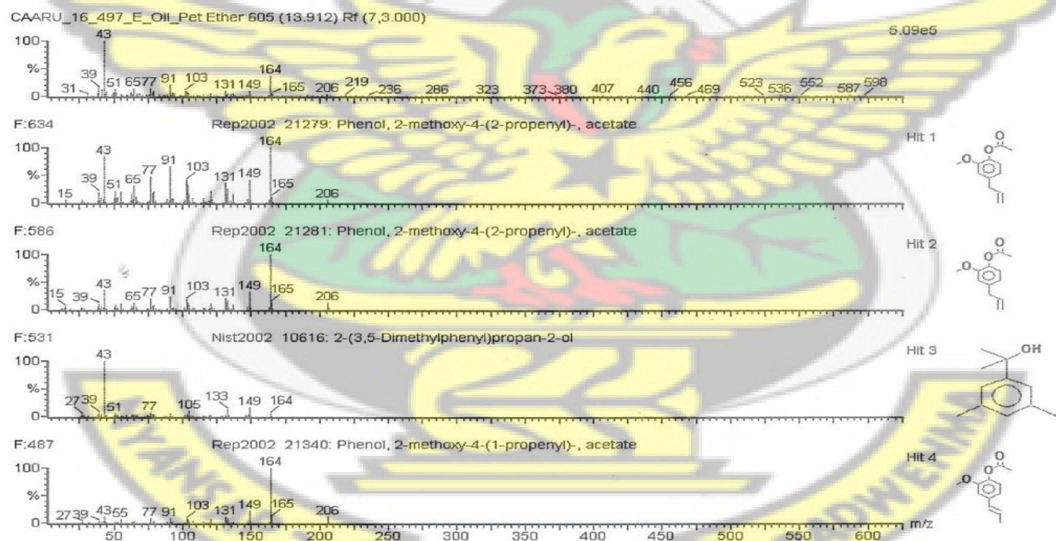
APPENDIX I(8): SSPECTRUM OF OCTAHYDRO-7-METHYL-3-METHYLENE-4-(1-METHYL ETHYL)-(3AS 3AA, 3BA, 4A)-1H-CYCLOPENTA(1, 3)- CYCLOPROPA(1, 2)-BENZENE



Mass Spectrum and Library Match of RT 13.89

APPENDIX I(9): SPECTRUM OF 2-METHOXY-4,2-(2-PROPENYL) ACETATE PHENOL (EUGENYL ACETATE)





Mass Spectrum and Library Match of RT 14.04

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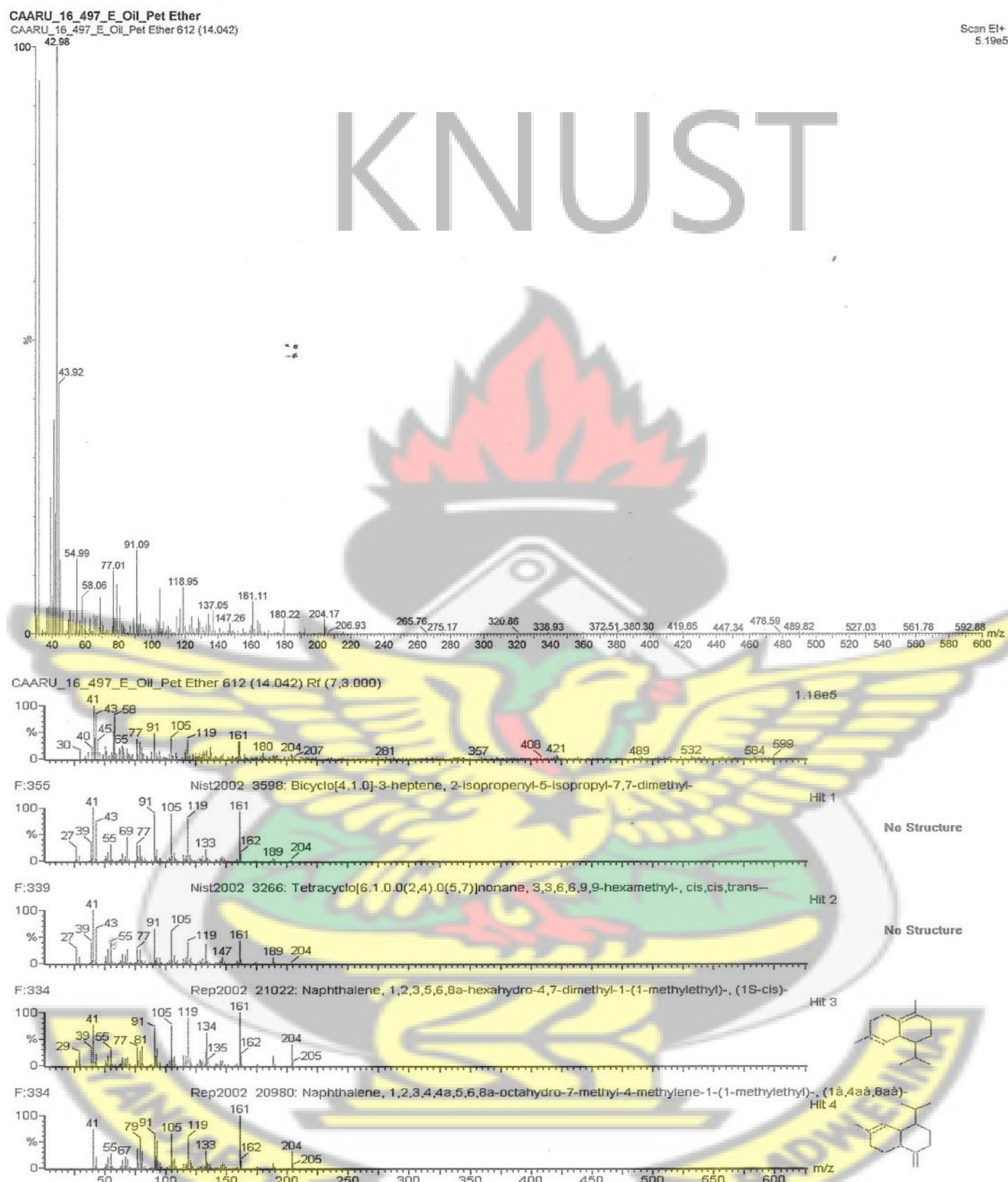
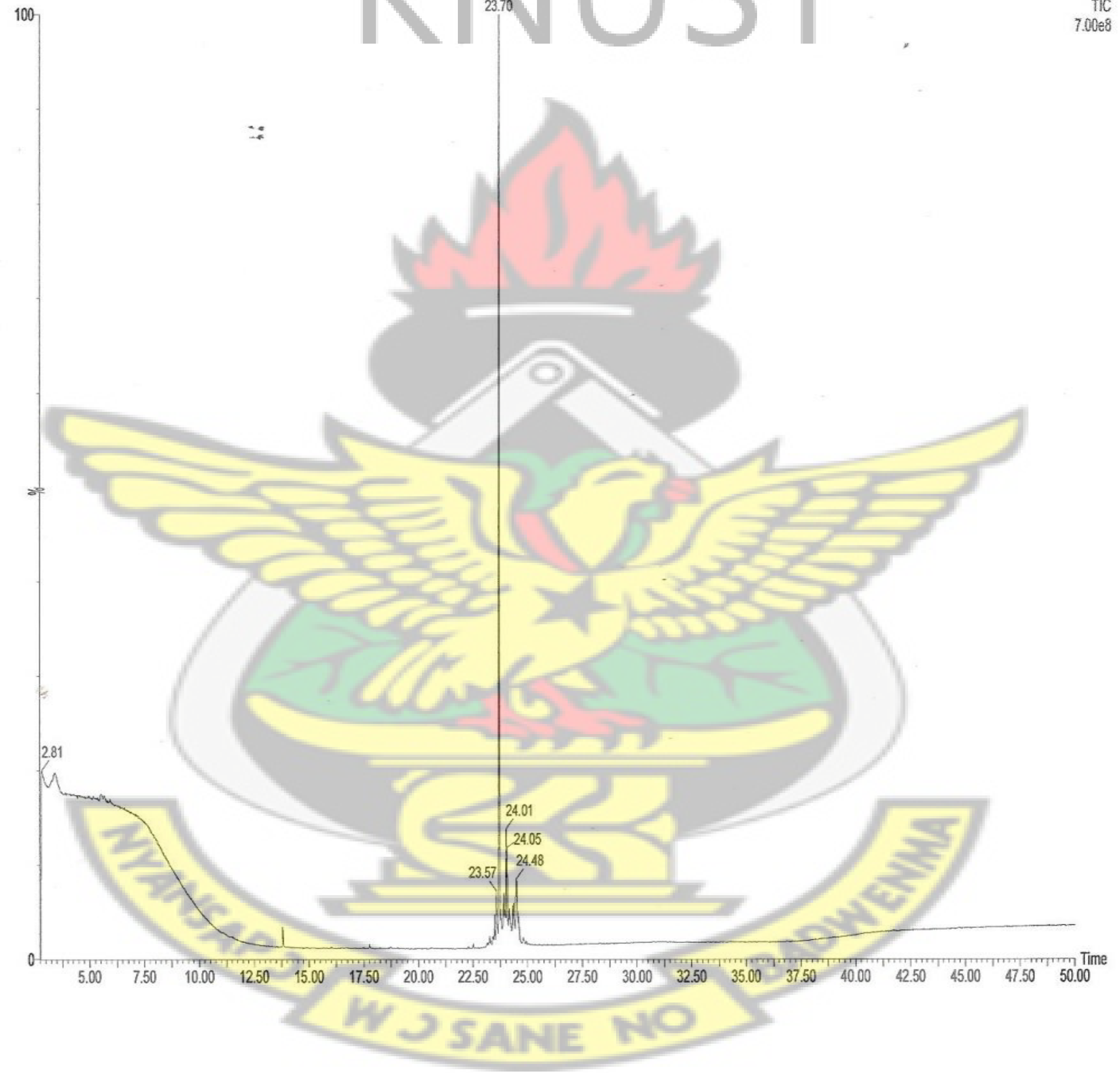
APPENDIX I(10): SPECTRUM OF 2-ISOPROPENYL-5-ISOPROPYL-7, 7-DIMETHYL-BICYCLO(4.1.0)-3-HEPTENE

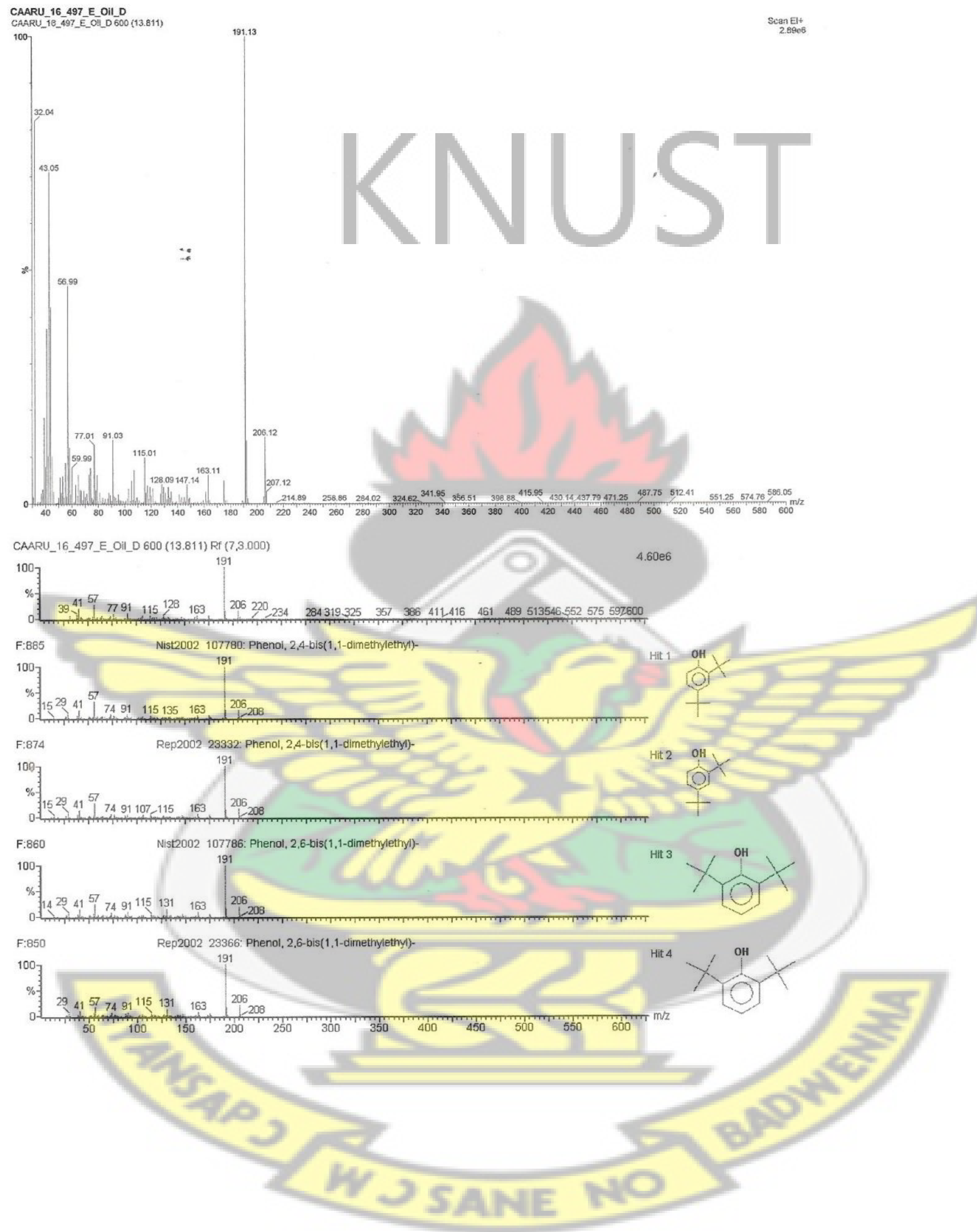


Mass Spectrum and Library Match of RT 14.25

APPENDIX II: SPECTRUM OF COMPOUNDS IN BULKED FRACTION 2 OF ETHYL ACETATE EXTRACT OF *C. TORA* SEEDS



APPENDIX II(1): SPECTRUM OF 2, 4-BIS(1, 1-DIMETHYL ETHYL)-PHENOL



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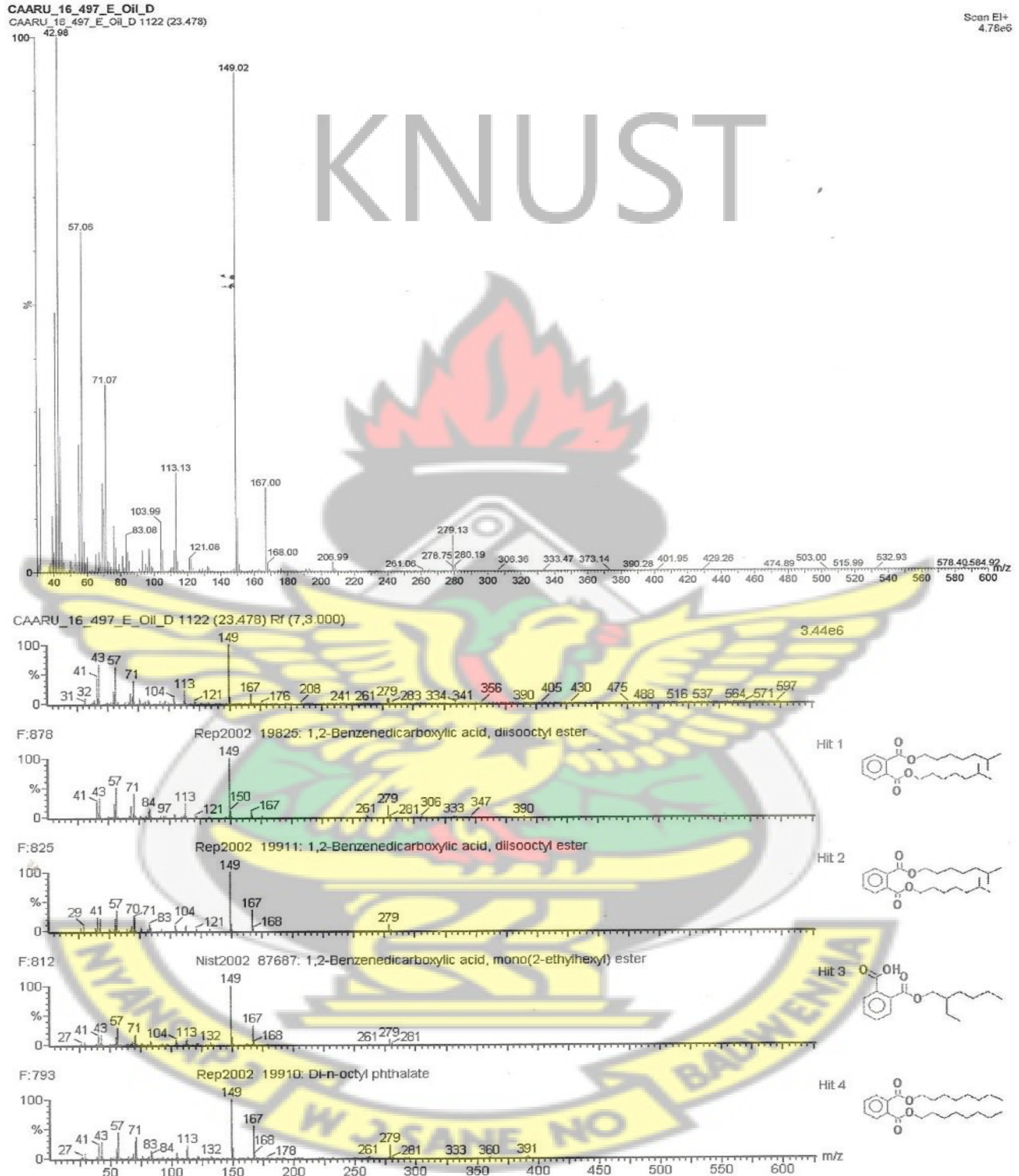
W J SANE NO BADWENMA

APPENDIX II(2): SPECTRUM OF DIISOOCTYL-1, 2-BENZENE DICARBOXYLATE



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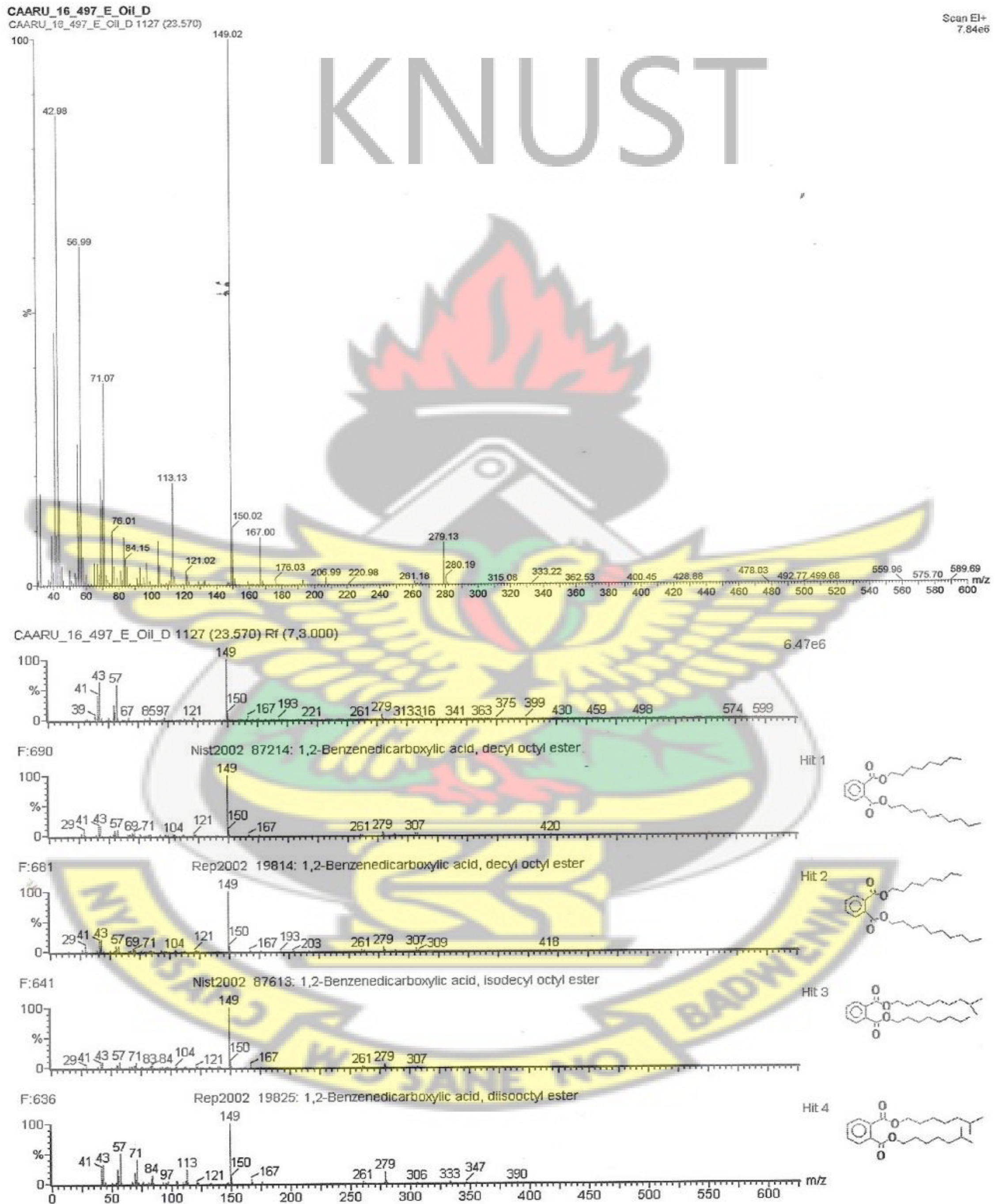
APPENDIX II(3): SPECTRUM OF DECYL OCTYL-1, 2- BENZENE DICARBOXYLATE



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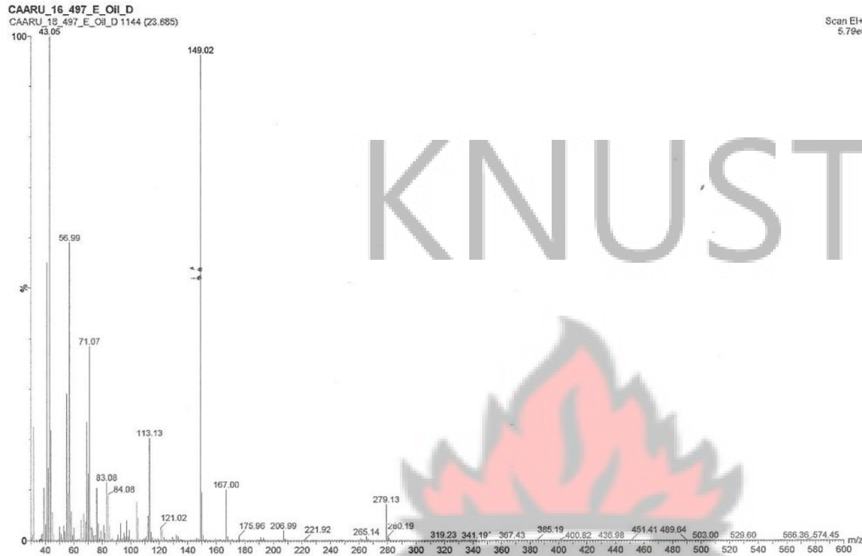
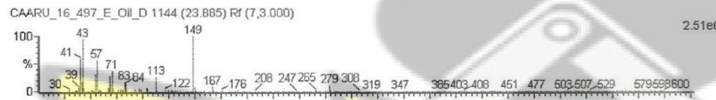
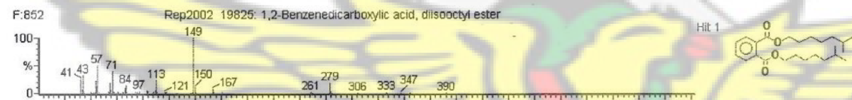
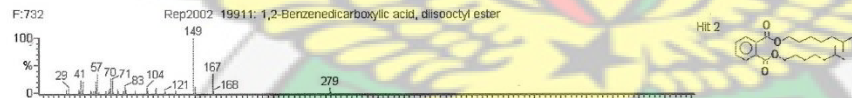
APPENDIX II(5): SPECTRUM OF DIISOOCTYL-1, 2-BENZENE DICARBOXYLATE

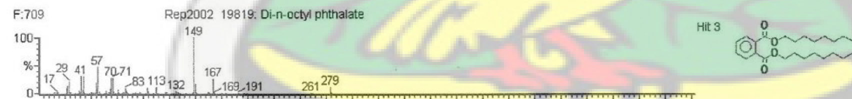
Mass Spectrum and Library Match of RT 23.88

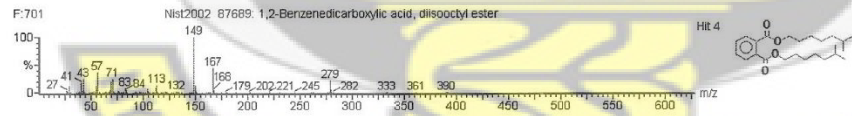












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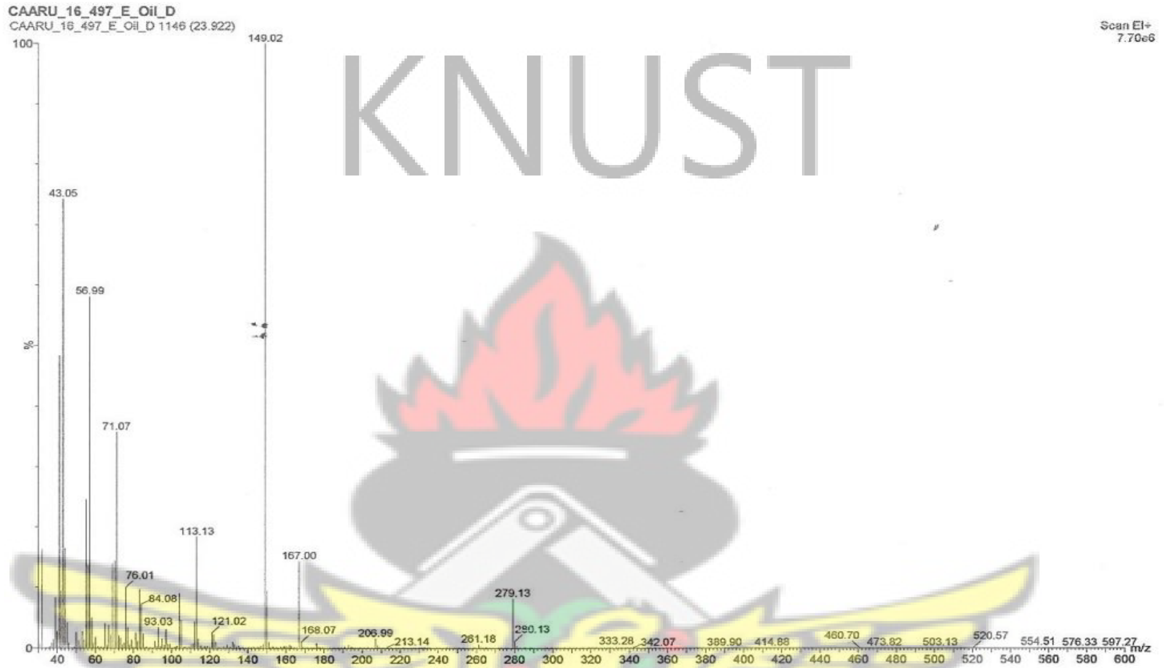
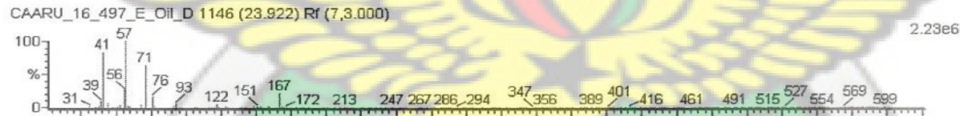
APPENDIX II(6); SPECTRUM OF 2, 2-DIMETHYL PROPYL DICHLOROACETATE

APPENDIX II (6): SPECTRUM OF 2, 2-DIMETHYLPROPYL DICHLOROACETATE

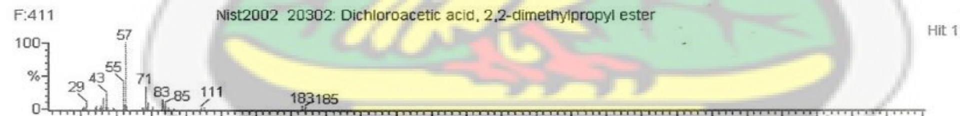
Mass Spectrum and Library Match of RT 23.92



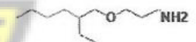
CAARU_16_497_E_Oil_D 1146 (23.922) Rf (7,3.000) 2.23e6

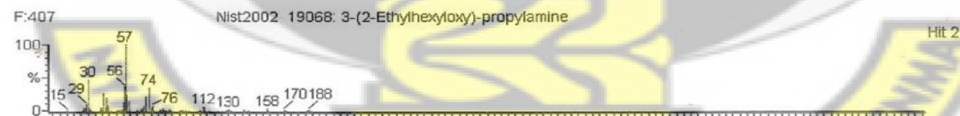


F:411 Nist2002 20302: Dichloroacetic acid, 2,2-dimethylpropyl ester Hit 1 No Structure

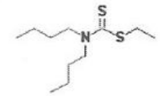


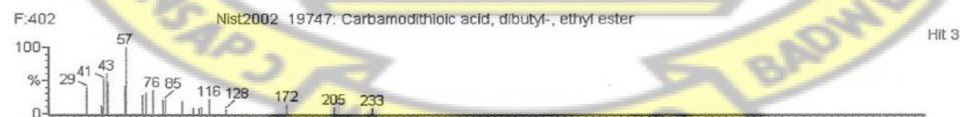
F:407 Nist2002 19068: 3-(2-Ethylhexyloxy)-propylamine Hit 2



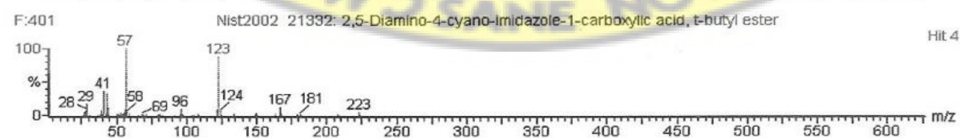


F:402 Nist2002 19747: Carbamodithioic acid, dibutyl-, ethyl ester Hit 3





F:401 Nist2002 21332: 2,5-Diamino-4-cyano-imidazole-1-carboxylic acid, t-butyl ester Hit 4 No Structure



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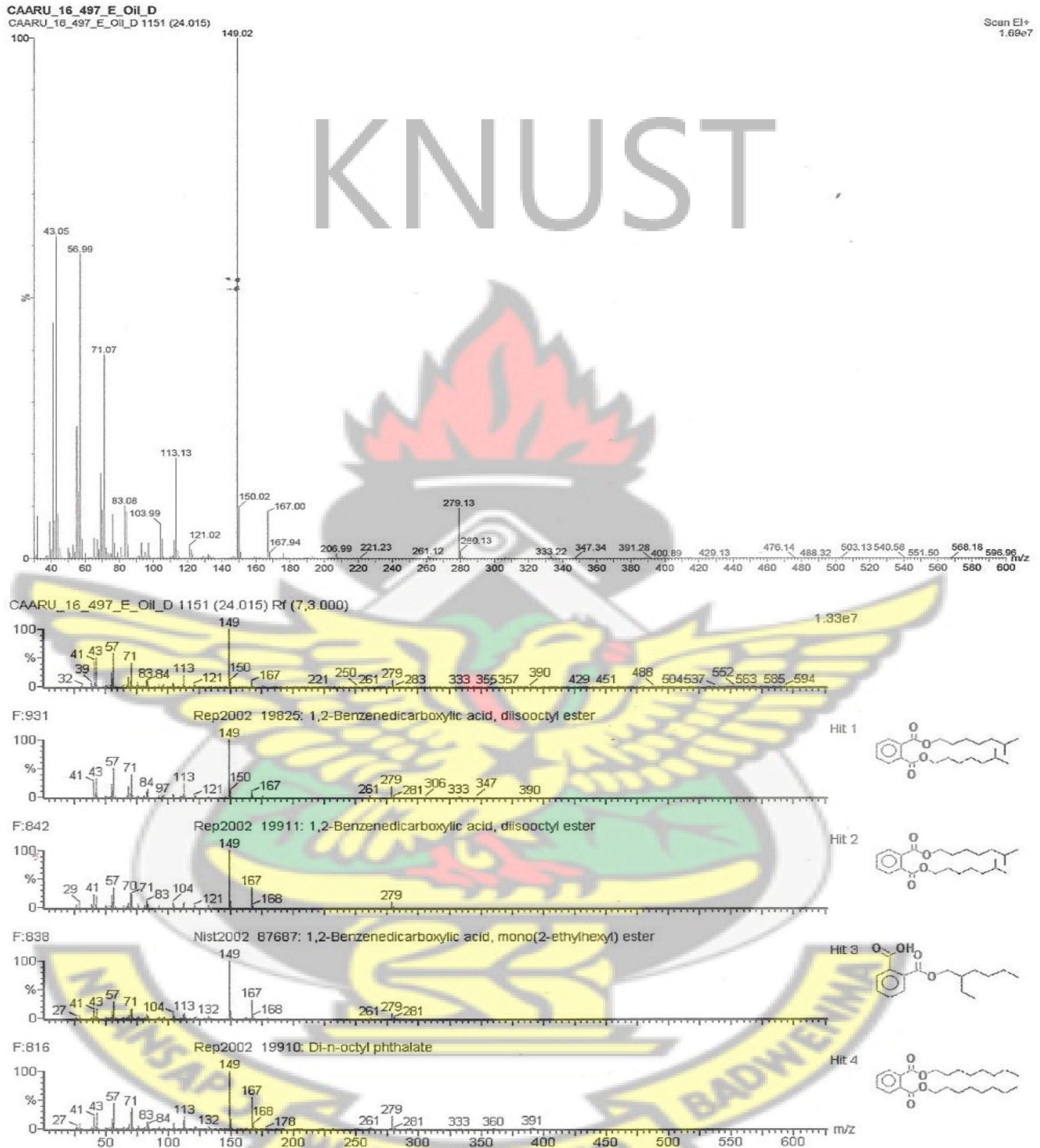
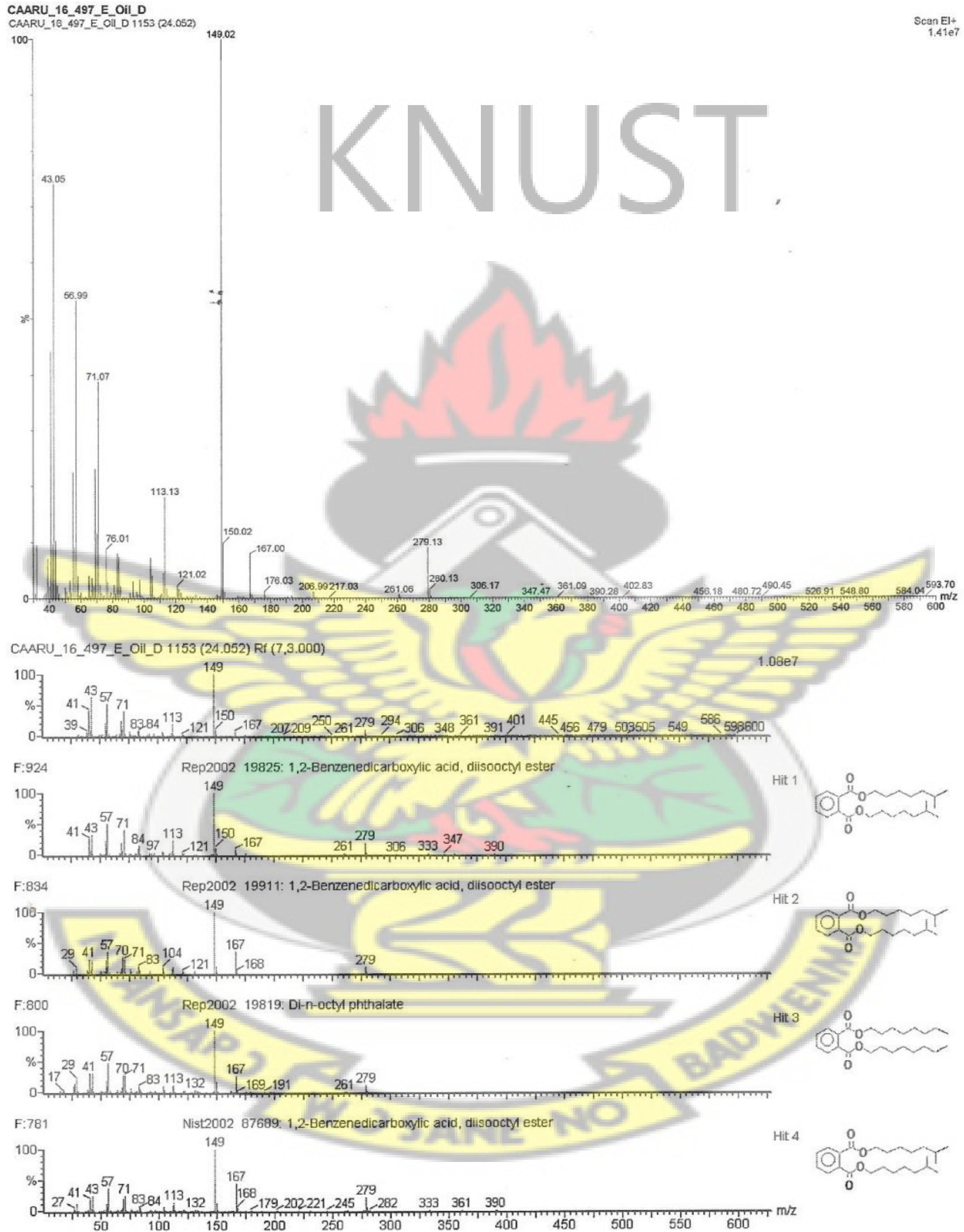
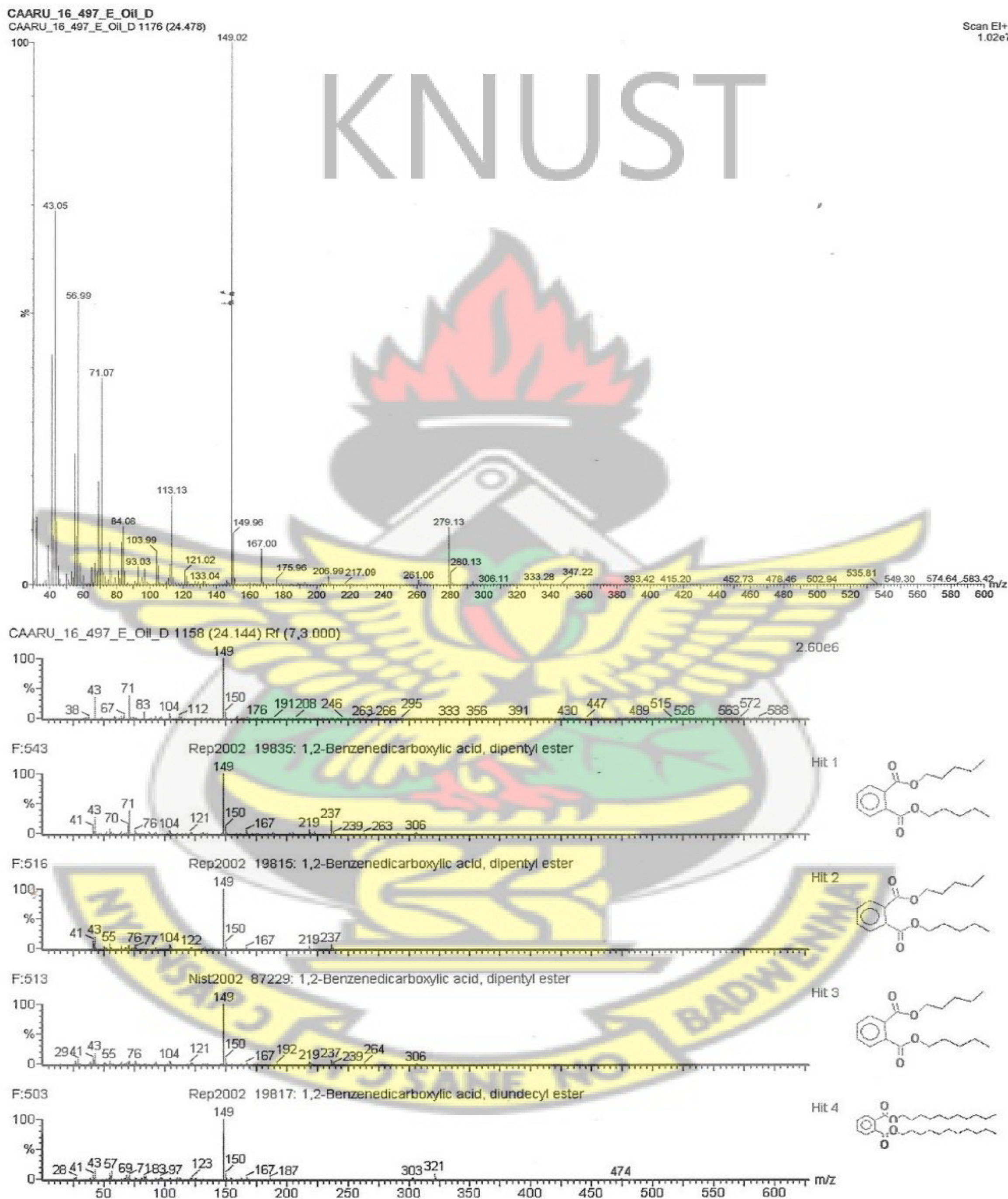
APPENDIX II(7): SPECTRUM OF DIISOOCTYL-1, 2-BENZENE DICARBOXYLATE



APPENDIX II(8): SPECTRUM OF DIISOOCTYL-1, 2-DENZENE DICARBOXYLATE

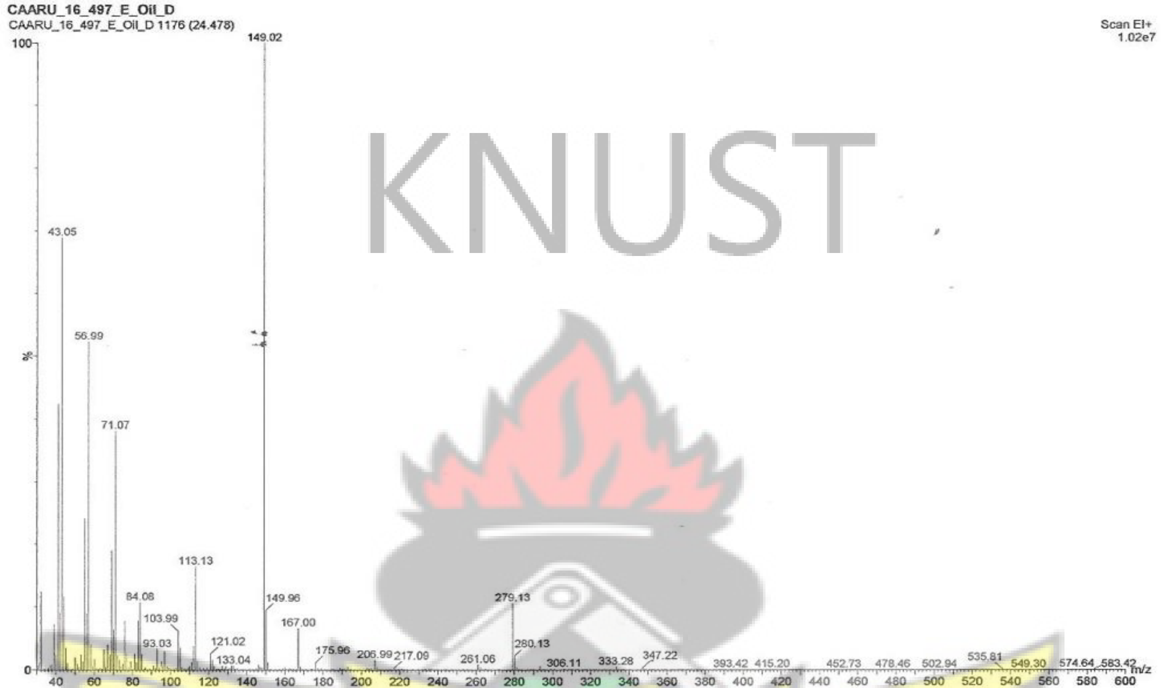


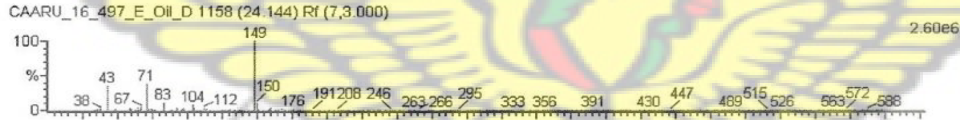
APPENDIX II(9): SPECTRUM OF SPECTRUM OF DIPENTYL-1, 2-BENZENE DICARBOXYLATE

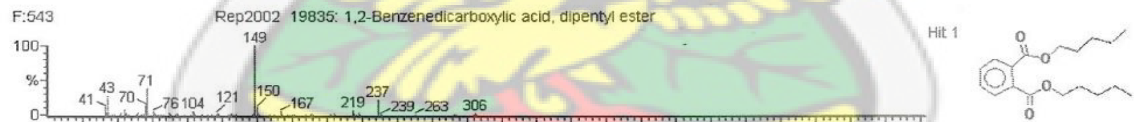


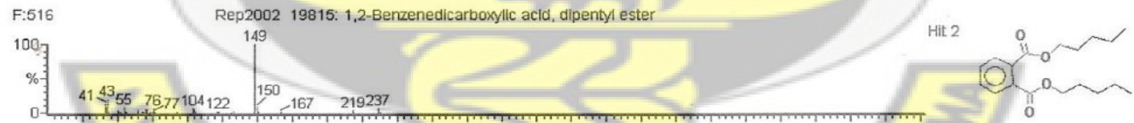
APPENDIX II(10): SPECTRUM OF DIPENTYL-1, 2-BENZENE DICARBOXYLATE

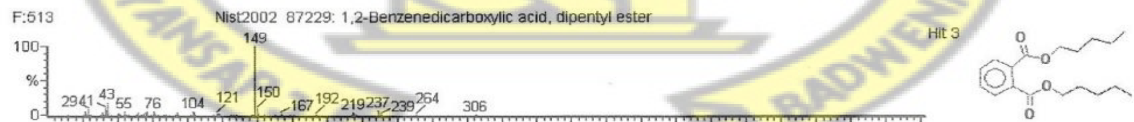
Mass Spectrum and Library Match of RT 24.48

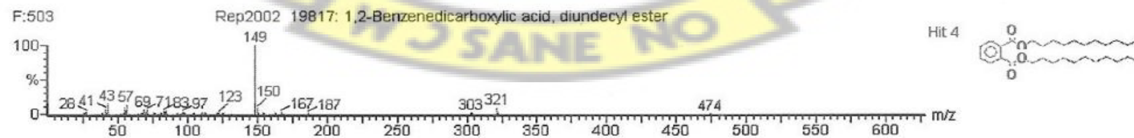




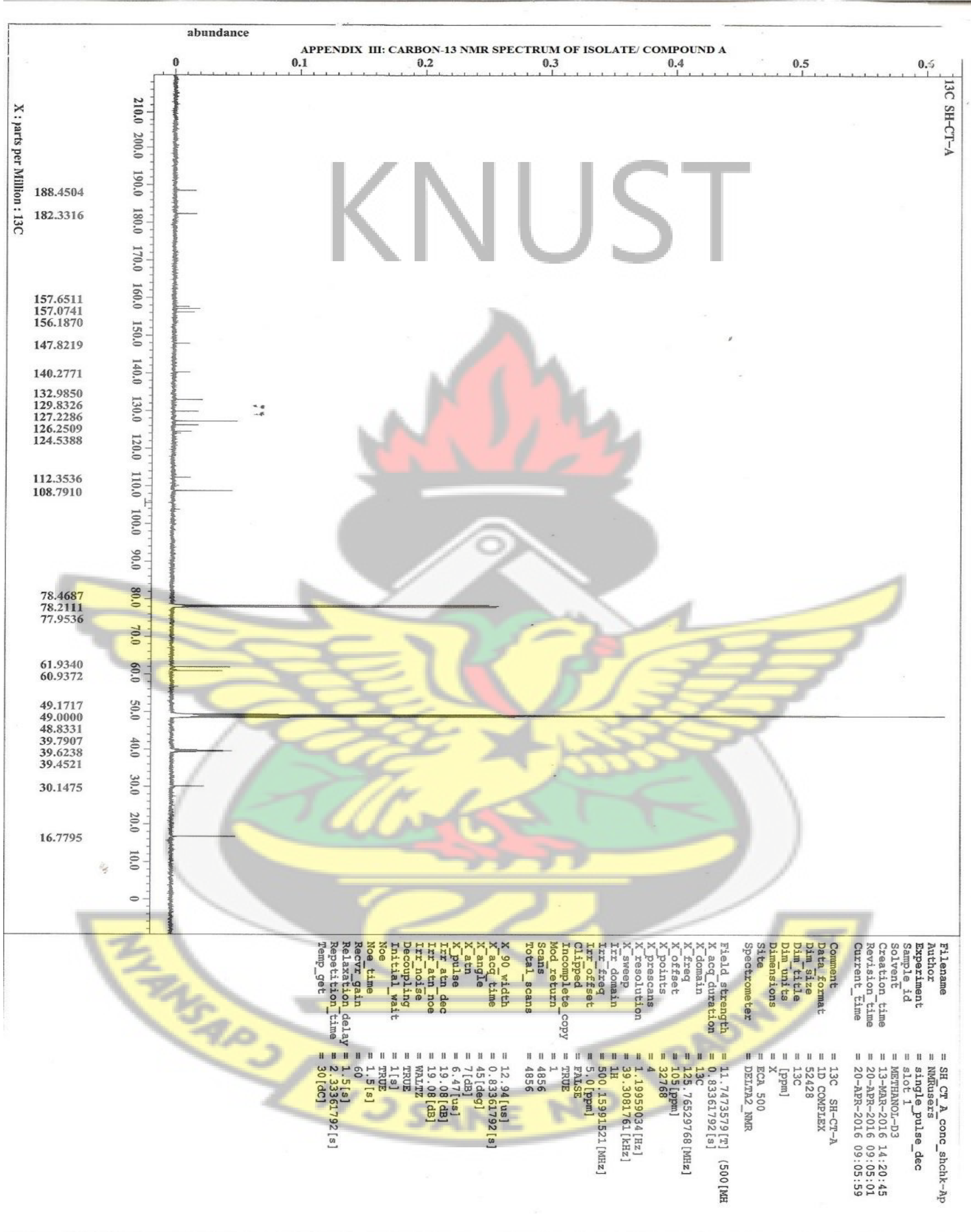




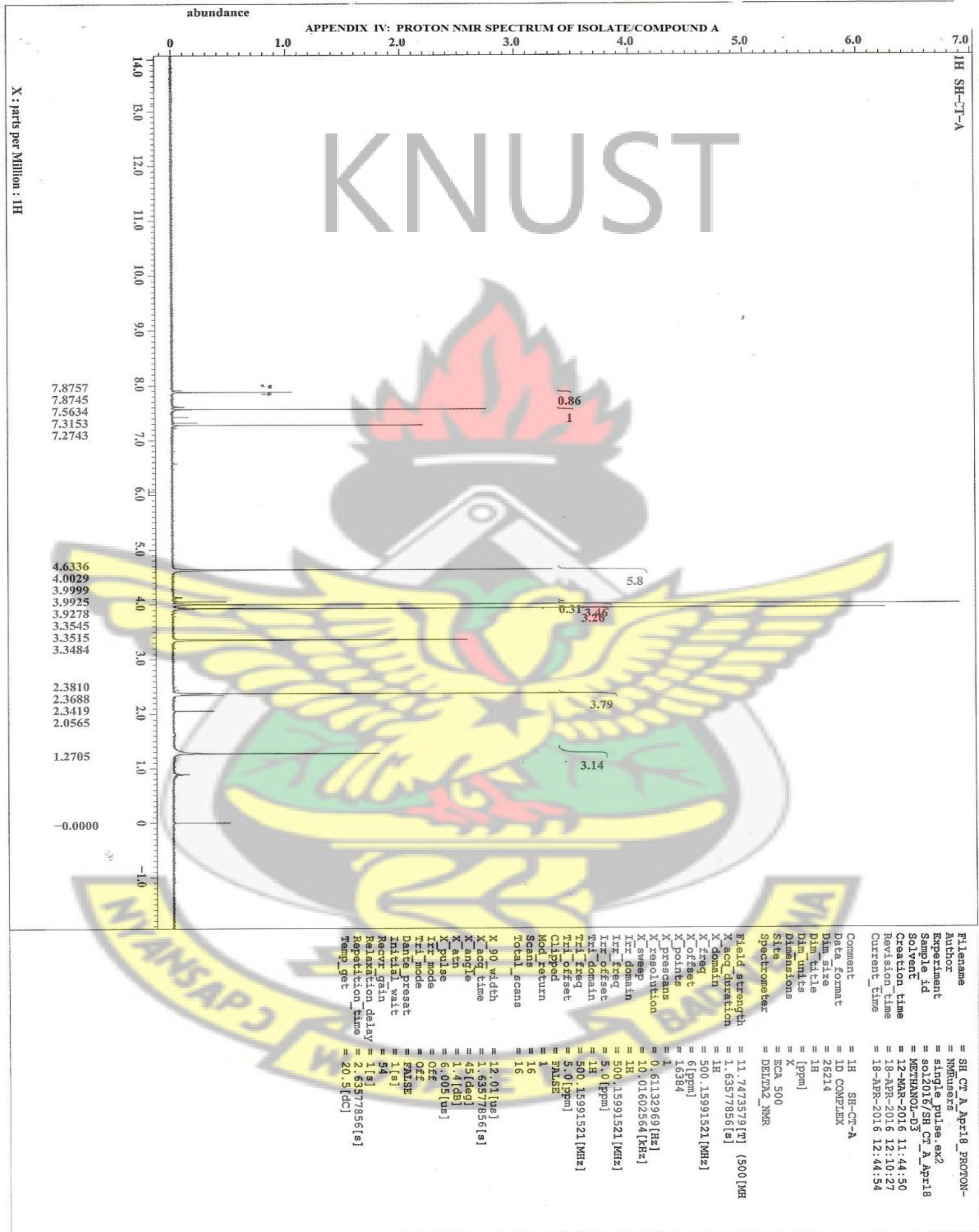




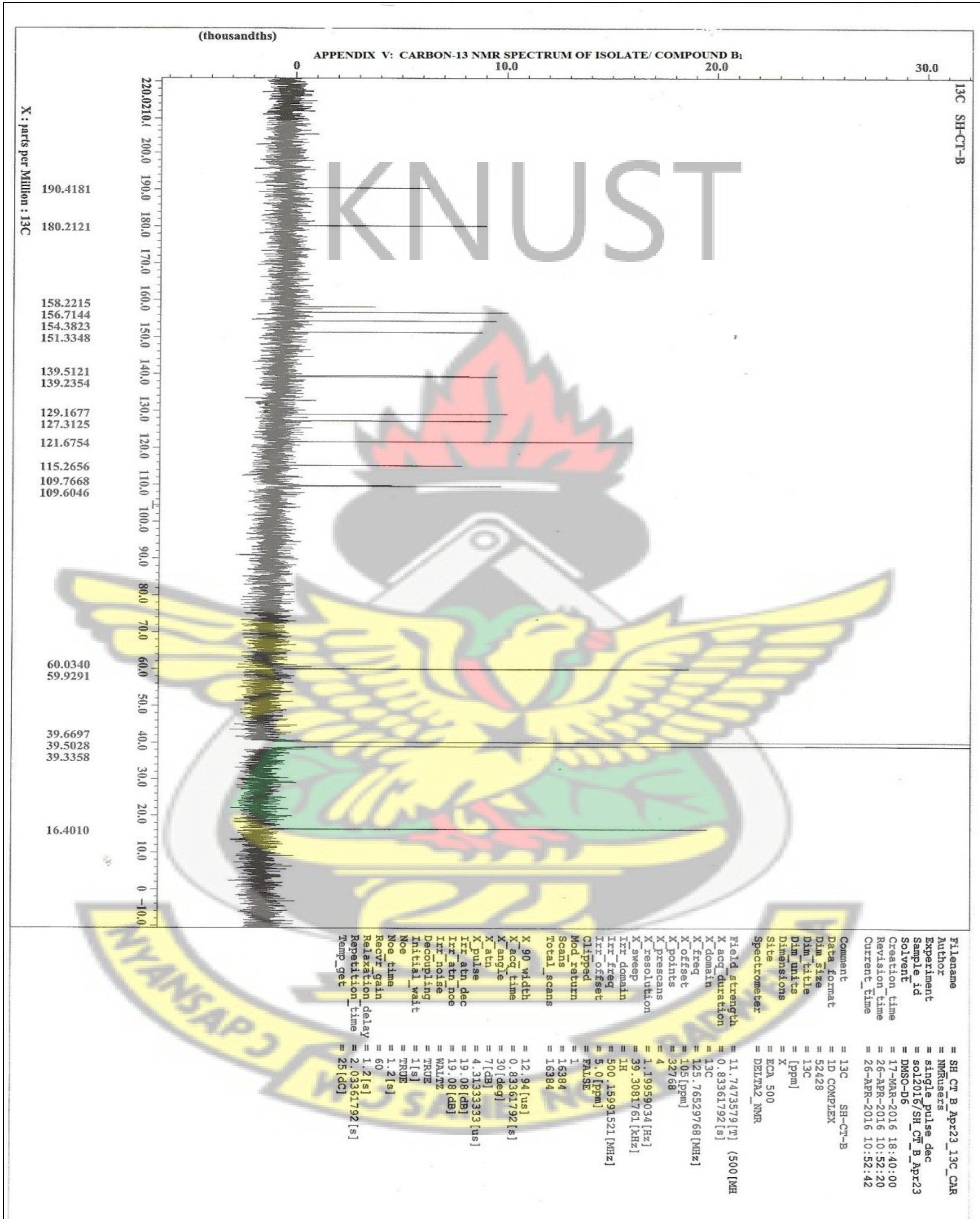
APPENDIX III: CARBON-13 NMR SPECTRUM OF ISOLATE/COMPOUND A



APPENDIX IV: PROTON NMR SPECTRUM OF ISOLATE/COMPOUND A



APPENDIX V: CARBON-13 NMR SPECTRUM OF ISOLATE/COMPOUND B1



APPENDIX VI: PROTON NMR SPECTRUM OF ISOLATE/COMPOUND B_1