PHYTOCHEMICAL AND MOSQUITO LARVICIDAL STUDIES OF *STACHYTARPHETA URTICIFOLIA* (SIMS)

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A THESIS SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE AWARD OF THE DEGREE OF MASTER OF SCIENCE IN CHEMISTRY IN THE SCHOOL OF PURE AND APPLIED SCIENCES, KENYATTA UNIVERSITY

OCTOBER 2022

DECLARATION

I declare that this thesis is my own work and it has not been submitted for any degree or examination in any other university.

Signature……………………………………….Date………………………………

Caroline F. Agola I56/22624/2011

SUPERVISORS

We confirm that the work reported in this thesis was carried out by the student under our supervision.

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Kenyatta University

DEDICATION

This work is dedicated to my children; Dave, George and Michelle for their love and support.

AKNOWLEDGEMENTS

I thank the Almighty God for His divine providence, good health and prayers answered. May all honour and glory be His forever!

I also sincerely thank my Kenyatta University supervisors; Dr. Alphonce Wanyonyi for his guidance, encouragement and constantly keeping me on my toes for the completion of this work and Dr. Ram Manohar for his encouragement. I wish also to acknowledge Dr. Philip Wafula of Embu University for his assistance in getting NMR data from Germany. Much thanks to Mr. John Ofula of zoology department of Kenyatta University for his assistance in rearing of mosquito larvae for bioassay studies.

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ABBREVIATIONS AND ACRONYMS

^{13}C NMR	Carbon- 13 nuclear magnetic resonance
^{1}H NMR	Proton nuclear magnetic resonance
CAM	Complementary or alternative medicine
CC	Column chromatography
CD_3OD	Deuterated methanol
$CDCl_3$	Deuterated chloroform
COSY	Correlation spectroscopy
DCM	Dichloromethane
DEPT	Distortionless enhancement by polarization transfer
DMSO	Dimethyl sulphoxide
EtOAc	Ethyl acetate
HMBC	Heteronuclear multiple bond correlation
HMQC	Heteronuclear multiple quantum correlation
IGRs	Insect Growth Regulators
IPT	Intermittent preventive therapy
IR	Infrared Spectroscopy
ITNs	Insecticide treated nets
LC_{50}	Lethal Concentration at 50%
LC_{90}	Lethal Concentration at 90%
LD_{50}	Lethal dose at 50%
LD_{90}	Lethal dose at 90%
NMR	Nuclear Magnetic Resonance
PTLC	Preparative thin layer chromatography
Rf	Retention factor
TLC	Thin layer chromatography
BSLT	Brine Shrimp lethality test
ICIPE	International Centre for Insect Physiology and Ecology
TMS	Trimethyl silane
UV	Ultraviolet Spectroscopy
VLC	Vacuum liquid chromatography
WHO	World Health Organization

ABSTRACT

Malaria is one of the most common and severe tropical diseases on the planet, transmitted by the *Anopheles gambiae* mosquito. Malaria affects more than 300 million people each year, according to the World Health Organization. Each year, these diseases are estimated to kill between 1.5 and 3 million people worldwide, with nearly 90 percent of all illnesses affecting pregnant women and children under the age of five. Chemotherapy, vaccine development, and vector control are currently used to prevent and control malaria. Vector management has been hailed as a critical tool in the fight against malaria transmission in light of the *plasmodium* species' increasing resistance to currently available antimalarial medications. Controlling mosquitoes during their larval stage is an additional effort made by humanity in an attempt to combat malaria. The use of natural compounds derived from plants to control the insect pests is a non-toxic and environmentally friendly strategy. Research has shown that compounds from the Vebenaceae family have been identified and shown to have larvicidal activity. The phytochemical and mosquito larvicidal properties of crude extracts and fractions derived from *Stacytarpheta urticifolia*, a member of the Vebenaceae family, were investigated in this work. The dry powdered stems of *S. urticifolia* were extracted using hexane, DCM, EtOAc and methanol for a total of 48 hours, with each extraction utilizing a different solvent. Phytochemical screening showed presence of terpenoids, alkaloids, tannins, steroids and flavonoids in the plant. For bioassay investigations, different concentrations of extracts were used. The larvicidal activity of the polar crude extracts was much greater than that of the non-polar extracts; MeOH LC_{50}=0.08, Hexane LC_{50}=10.59. The cytotoxicity tests also revealed that the EtOAc and methanol extracts were the more toxic to brine shrimp eggs; MeOH LC_{50}= $6.48x10^{-8}$, EtOAc LC_{50}=0.0015. Column chromatography and repeated PTLC on the bioactive crude extracts from Hexane, DCM, EtOAc and MeOH gave a white amorphous solid (EtOAc:MeOH, 7:3). Upon use of ^{1}H NMR, ^{13}C NMR, DEPT, HMBC and HSQC led to **SUS 1** an oleanane tritepene, 1,2,3,4,4a,5,6a,7,8a,9,10,11,12,12a,13,13a,14,14a,14b-icosahydro-2,3,11,12,13a-pentahydroxy-4,4,6a,12,14b-pentamethyl-10-oxobenzo[α]tetracene-12a-carboxilic acid. A white amorphous solid (EtOAc:MeOH, 7:3). Another oleanane tritepenoid with a sugar moiety, 8a-acetyloctadecahydro-6b,8,12-tihydroxy-4,4,6a,11,12b,14b-hexamethyl-2-(tetrahydroxo-3, 4, 5trihydroxy-6- (hydroxymethyl) - 2H- pyran - 2-yl oxy) picen - 6 (6aH, 6bH ,14Bh)-one, **SUS 3** was also obtained as white crystalline solid (EtOAc:MeOH, 7:3). In addition, two common plant sterols were also isolated from Chloroform: EtOAc (1:1); 3β-stigmasterol, **SUS 2** as white powder and 3β-sitosterol, **SUS 4** as colourless needlike crystals. The results from this study provides a basis for further research in malaria control.

CHAPTER ONE

INTRODUCTION

1.1 Background information

Malaria is a vector-borne disease that can be fatal and spreads primarily through anopheles mosquito bites. It is more common in humid settings where pools of water provide ideal hatching grounds for anopheles mosquitoes. Malaria parasites are spread from sick to healthy persons by the bite of the mosquito (WHO, 2008). When adult parasites enter the circulation, they begin to proliferate in the liver. The fast expansion of the parasite results in the death of red blood cells throughout the body. Annually, it is estimated that US$ 2 billion is spent on malaria prevention and treatment initiatives in Africa. It is the leading cause of illness and death in Kenya, claiming approximately 34,000 children under the age of five each year (WHO, 2010). There are many methods of controlling malaria which include chemotherapy, bite prevention like use of ITNs and IRS and mosquito larva control.

Mosquito population reduction is critical in public health domains, as mosquitoes are the primary vectors of a variety of tropical diseases, including malaria, dengue fever, filariasis and yellow fever. To control mosquito larvae, measures such as the use of organophosphorus (OP) pesticides, insect growth regulators (IGR) and bacterial larvicides have been implemented. As a result of pesticide resistance, additional treatments and dosages have been required, posing significant risks to the physical environment and human health. Due to their reputation as a source of biologically active chemical compounds that are considered to be safer and have fewer adverse effects on the environment and human health than synthetic chemical compounds, phytochemical extracts from plants have attracted considerable attention as potential sources of disease vector control products (Ghosh *et al.,* 2012).

1.2 The Malaria burden and epidemiology

Malaria is one of the most prevalent and severe tropical diseases on the planet, according to the World Health Organization. The disease is caused by parasites of the genus *Plasmodium*, with *Plasmodium falciparum* being the most virulent and difficult to cure of the bunch (Breman *et al*., 2006). Throughout its life cycle, *Anopheles gambiae* undergoes a complete metamorphosis, passing through four distinct stages: egg, larva, pupa, and adult, which takes approximately one month. (Plate 1.1).

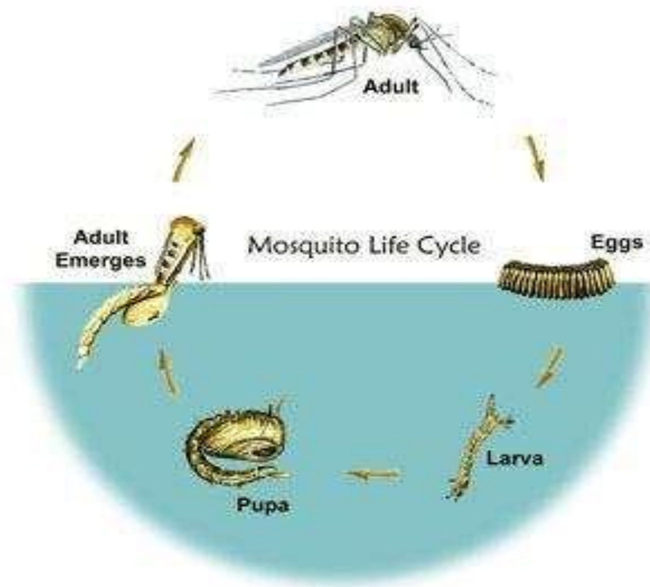


Plate 1. 1: Mosquito lifecycle
Source: WHO, (2010).

According to the World Health Organization, over 300 million people contract malaria each year (Snow and Omumbo, 2006). Each year, malaria infections claim between 1.5 and 3 million lives worldwide, with pregnant women and children under the age of five bearing a disproportionate share of the burden (Steketee *et al*., 2001) (Plate 1.2).

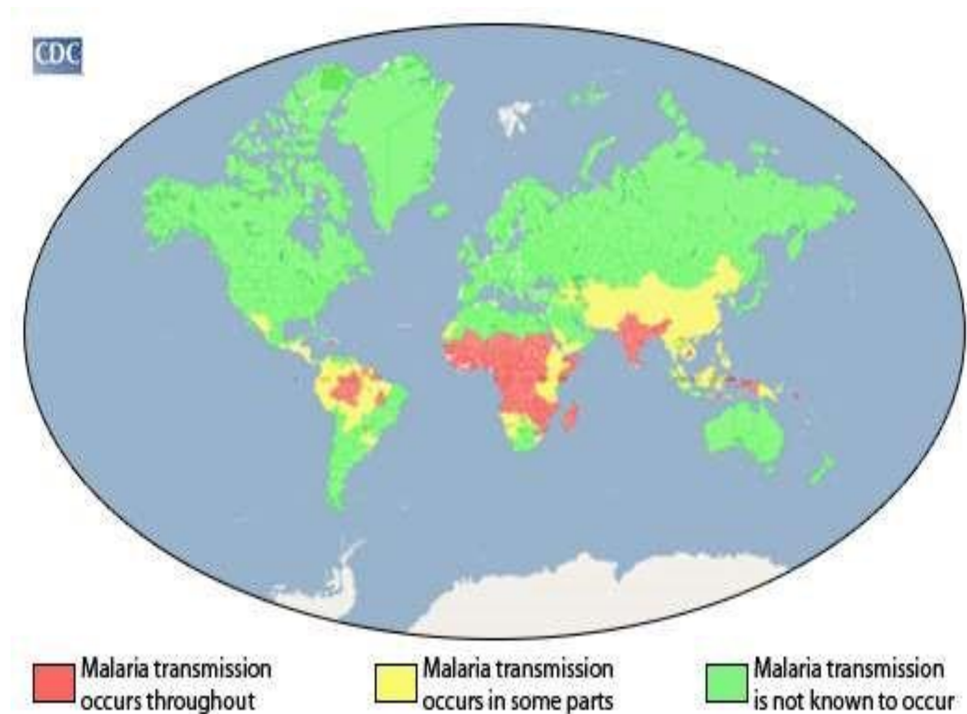


Plate 1. 2: World Malaria Distribution

Source: WHO, (2018).

1.3 Economic implications of malaria

Africa loses USD 12 billion each year to malaria. Malaria is responsible for 40% of all public health spending in Africa. It is estimated to kill approximately 584,000 people, the majority of whom are African children. More than 70% of Kenyan population lives in the disease endemic regions (Plate 1.3) including Western, North Eastern and coastal parts of the country.

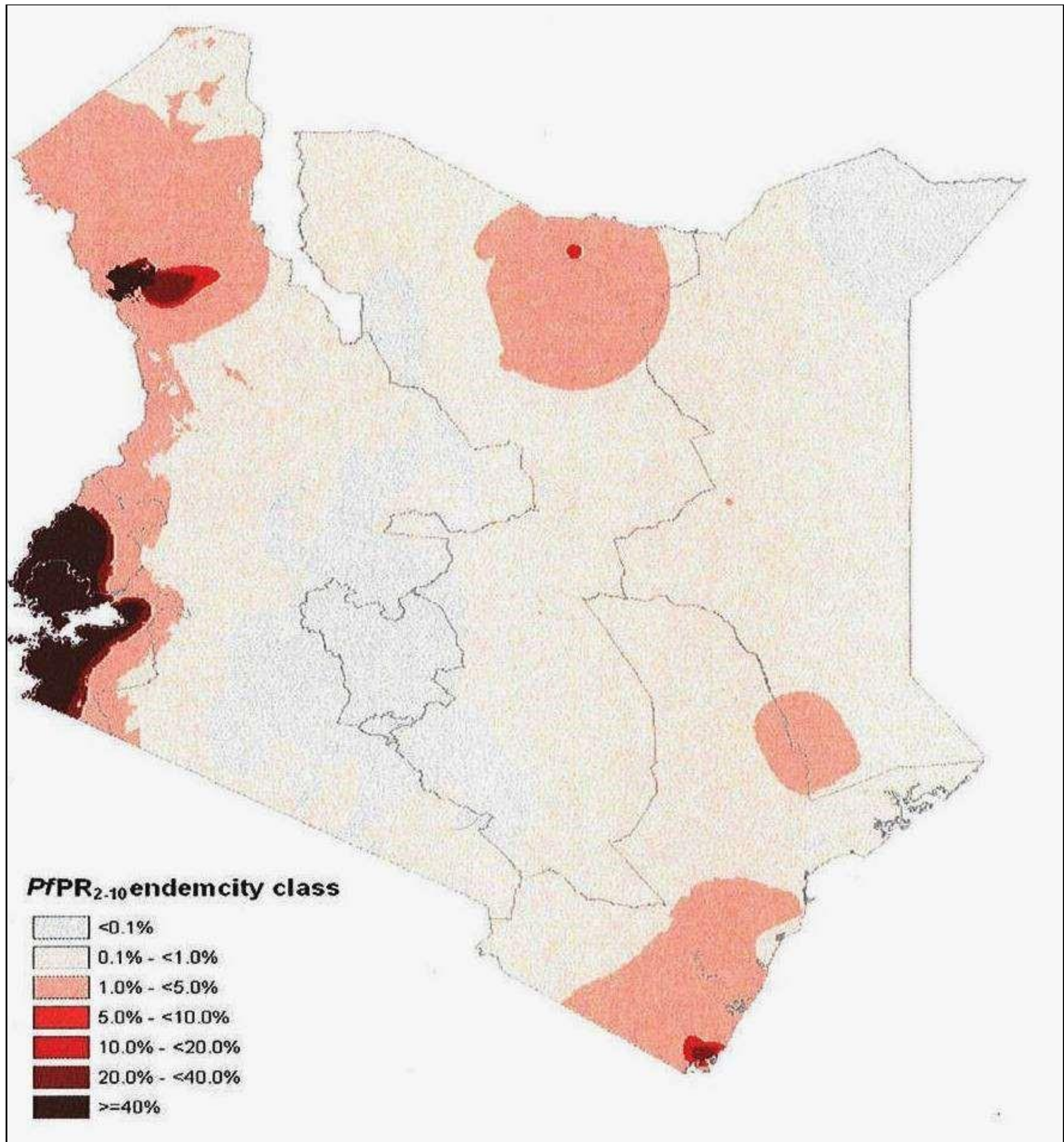


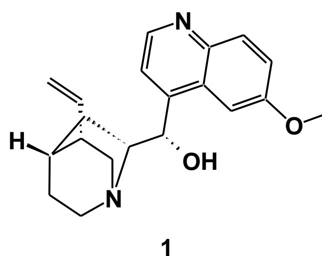
Plate 1.3: Malaria Distribution in Kenya
Source: WHO, (2018).

1.4 Malaria prevention/control strategies

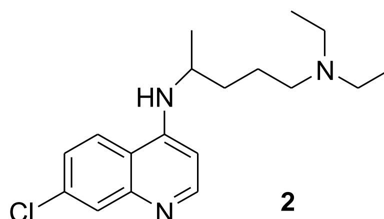
Chemotherapy, vaccine research, and pest management are some of the current malaria control strategies being used.

1.4.1 Chemotherapy

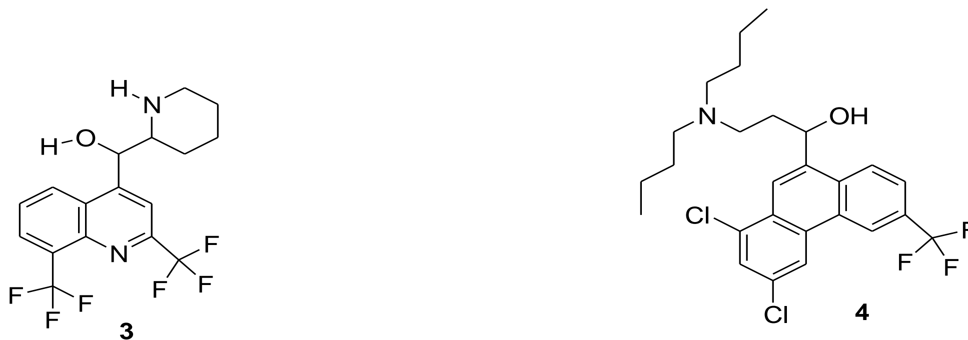
Current first line drugs for malaria treatment can be classified into three broad classes including: quinine derivatives, antifolates, artemisinin derivatives and combination of two or more drugs belonging to above groups (Breman *et al*., 2006). Quinine (**1**), an antimalarial drug derived from the cinchona tree's bark, is used to treat malaria.



Chloroquine (**2**) is a synthetic antimalarial which has become the cornerstone of antimalarial chemotherapy for many years.



Mefloquine (**3**) and Halofantrine (**4**) have also been used to treat malaria infections (Breman *et al*., 2006).



1.4.2 Vaccine development

There is presently no malaria vaccine available due to the parasite's growing resistance to antimalarial drugs. However, vaccination studies have resulted in the development of a partially successful subunit vaccine, RTS, S/AS01, which is currently undergoing trials in sub-Saharan Africa. It is possible that such vaccinations will fail to provide the necessary protection in the malaria-endemic neighborhood. There is a pressing need for a multi-stage vaccination that contains antigens from all three phases of the parasite lifecycle: pre-erythrocytic, erythrocytic, and sexual. This will prevent the spread of the parasite at all stages. Furthermore, a thorough knowledge of the prospective vaccine targets as well as the mechanisms by which immunity is induced is essential for the creation of an efficient malaria vaccination (Ishizuka, 2016).

1.4.3 Vector control

With increasing drug-resistance of the plasmodium species against existing drugs, many interventions have been put in place to fight the malarial menace, including breaking the mosquito lifecycle. This has been achieved through eradication of mosquito larvae and pupae (Matasyoh *et al*., 2010). In order to prevent the spread of mosquito-borne illnesses such as yellow fever, fillariaris, and Japanese encephalitis, vector management is an essential component of disease prevention (Votandoost and Vaziri, 2001; Radhika *et al*., 2011). The female mosquitoes usually lay their eggs on water, after which the eggs undergo complete meta-morphosis (Plate 1.1) for a period between 10 to 14 days (Yasin, 2018). The WHO in 2002 classified vector control methods into three categories according to the effect to be obtained;

1.4.3.1 Methods of reducing human-vector contact

This category includes all methods that creates a barrier between the vector and humans, such as use of mosquito nets, house protection and use of repellents.

1.4.3.2 Methods aimed at increasing adult vector mortality

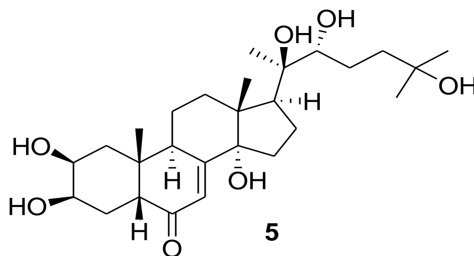
These methods are aimed at reducing life expectation and the chances of the parasite completing all its stages of development. They also ensure a reduction in the vector density. They include indoor residual spraying and use of ITNs (WHO, 2002).

1.4.3.3 Methods aimed mainly at reducing vector density

The impact of vector control on the transmission potential has a direct relationship to the reduction of the vector density. In order to effectively reduce vector densities, there is need for the treatment of vector breeding places, with the aim of total elimination or a reduction of breeding in the treated sites. This category includes all forms of larval control such as source reduction by environmental management, space spraying of insecticides which is applicable for controlling epidemics of mosquito-borne diseases, biological control where predators are used such as larvivorous fish (WHO, 2002). Finally, Larviciding; use both of biological and chemical insecticides and IGRs. As opposed to sanitation methods, use of larvicides has been reported to have little residual effect (WHO, 2002).

1.4.3.4 Hormonal vector control

This method focusses on the disruption of mosquito hormonal reproduction system and hence a reduction on population size. The ecdysteroid 20-hydroxyexdysone (**5)** is one such hormone whose key role is to control moulting and metamorphosis in insects.

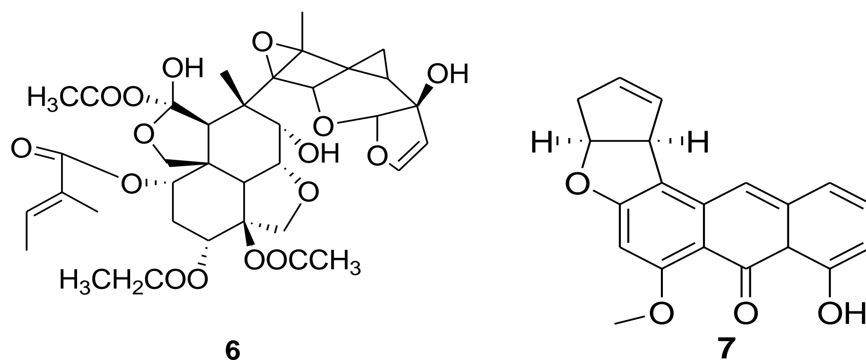


The hormone is also known to reduce reproductive fitness, and the lifespan of virgin females, even at the lowest dose (Michel, 2017).

Recently, the emphasis on mosquito vector control has shifted significantly away from convectional chemicals and toward target-specific, biodegradable, and ecologically benign insecticides that are typically derived from plants (Mudalungu *et al*., 2013).

1.5 Larvicides from plant sources

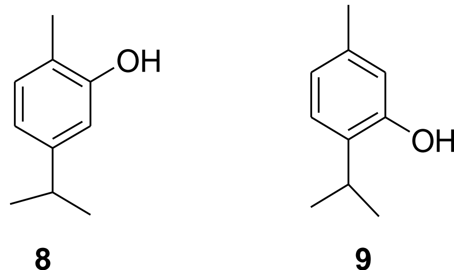
Most natural larvicidal and insecticidal compounds have been reported from plants. Plants are known to generate pesticidal chemicals as a chemical defense mechanism against predators or illness, according to scientific evidence. *Azadirachta indica* (Meliaceae) known as neem in India, is one of the most widely researched plants for mosquito control (Ouma, 2012). Azadirachtin **(6)** is an insecticidal compound, isolated from it. Though neem products show a high larvicidal activity, which is attributed to the epoxide ring function, they do not show adulticidal action (Ouma, 2012). Sterigmatocystin **(7)** isolated from the plant *Laggera alata* (Asteraceae), was aslo reported to possess larvicidal properties against 3rd intar mosquito larvae of *Anopheles gambiae* (Matasyo *et al.,* 2010).



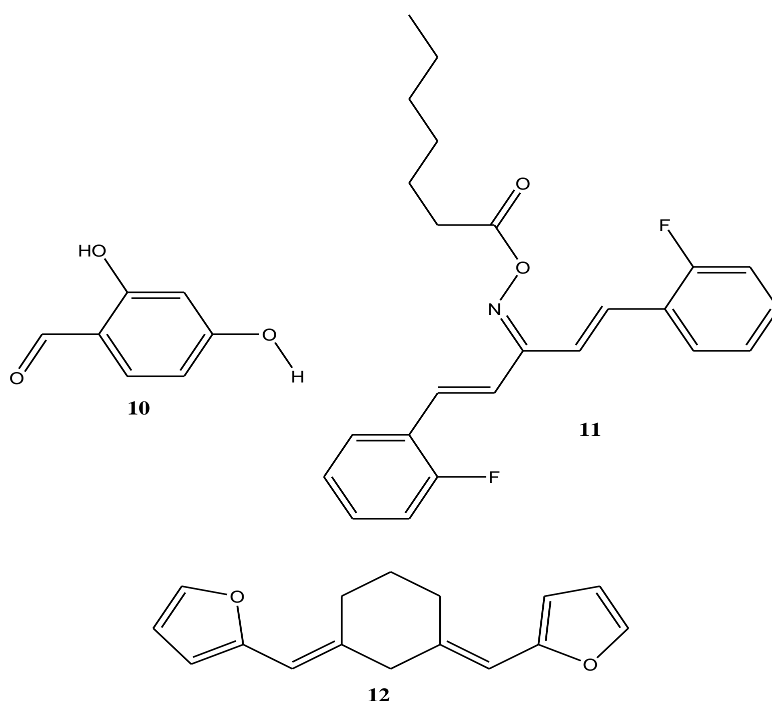
Saponins were discovered in the bark of the shrub *Quillaja saponaria*, which grows in China, Peru and Chile's arid zone. They are either steroids or triterpene glycosides with a variety of biological effects, including anti-inflammatory, antiallergy, antiviral, and molluscidal properties, all of which have been linked to cancer. It was found that young larvae of both *Aedes. aegypti* and *Culex. piperins* were susceptible to saponin application (Cruz *et al.,* 2022).

Essential oils have have been reported as potential bioactive compounds against insects. A study was carried on essential oils obtained from the shoot of *Lippia sidoides* in which co-distilled water called hydrolate was produced. Pure hydrolate caused larval mortality of *Ae.*

Aegypti and it was revealed that its main constituents were carvacrol **(8)** and thymol **(9).** It was also found that thymol **(9)** was the active essential oil in hydrolate (Carvalho *et al.,* 2003).



Other compounds from plants which have been found to possess larvicidal activities include O-heptanoyl oxime **(11)** and (2E,6E)-2,6-bis(furan-2-ylmethylene)cyclohexane **(12)**.



1.6 Statement of the problem

Malaria is one of the most prevalent and life-threatening vector-borne infections in the world, affecting about 100 million people each year (Breman *et al*., 2006). There is increased resurgence of malaria in many parts of the tropics, and the danger of importation by nonmalarious countries. This has been attributed to increasing resistance of *P. falciparum*

to drugs and the Anopheles vector to insecticides. Studies have shown that the ITNs and intermittent preventive therapy (IPT) have proved to be unaffordable and inaccessible especially in many rural areas of Africa (Breman *et al.*, 2006).

Despite the fact that malaria has made a considerable contribution to the global burden of illness, it remains a serious hindrance to socio-economic development in poor nations. Malaria is the primary cause of illness and mortality in most countries in Sub-Saharan Africa and has been for several decades. Globally, one kid dies of malaria every 40 seconds, according to Geissbhler *et al*. (2007) and Baraza (2011), respectively. In 2017, it was predicted that total spending for malaria prevention will reach US$ 3.1 billion (WHO, 2018)

Controlling mosquitoes during the larval stage is an extra measure that has been put in place in an attempt to prevent malaria. Given the shortcomings of current malaria interventions and the absence of a viable vaccine, it is critical to investigate and develop alternative strategies that are environmentally friendly, environmentally safe, and based on biodegradable plant products that are non-toxic to both non-target and target organisms (Kumar, *et al*., 2012).

The purpose of this study therefore was to carry out phytochemical studies to determine the presence of bioactive compounds that have been reported in literature and to test them for larvicidal activity against *A. gambiae* larvae. The bioactive fractions were then exposed to isolation and structure elucidation to find out the structures of the isolated compounds. This is in an attempt to expand the larvicidal drug arsenal which is in accordance with the current trends towards the use of natural products to find solutions to human problems.

1.7 Justification of the study

In recent history, the most effective methods of controlling mosquito larvae have been organophosphorus insecticides, insect development regulators, and bacterial larvicides (Rozendaal, 1997). By contrast, widespread use of these larvicides has resulted in a significant disruption of natural biological control systems, resulting in an increase in wild mosquito populations (Croft and Brown, 1995). A frequent consequence of these practices is the evolution of more resistant bacteria (WHO, 2010). As a result of this growing resistance,

numerous treatments and excessive dosages have been developed, posing significant risks to the environment and human health.

Given the rate at which these trends are progressing, it is critical to develop targeted mosquito control options that can assist in the establishment of a successful resistance management strategy. Plant extracts have long been recognized as a source of bioactive chemical compounds that could be used to develop disease vector control products. They frequently attack a variety of distinct target locations, significantly reducing the likelihood of developing mosquito resistance. [Centers for Disease Control] (Kostyukovsky *et al*., 2002).

The purpose of this study was to determine the phytochemical and larvicidal activity of *S. urticifolia* (Sims) extracts against the malaria vector, *Anopheles gambiae*. The findings from the study will help expand research in the area of malaria control and structures used as templates in larvicidal drugs.

1.8 Hypothesis

Stachytarpheta urticifolia (Sims) contains secondary metabolites that can be extracted, isolated, and identified.

1.9 Objectives of the study

1.9.1 General objective

The broad objective of the study was phytochemical and mosquito larvicidal studies of *Stachytarpheta urticifolia* (Sims).

1.9.2 Specific objectives

(i) To screen for phytochemicals present in hexane, DCM, Ethyl acetate and methanol crude stem bark extracts from of *S. urticifolia* (Sims).

(ii) To determine *In-vitro* larvicidal activities of hexane, ethyl acetate, DCM and methanol extracts of the stem bark of *S. urticifolia* (Sims) against *Anopheles gambiae.*

(iii) To determine *In-vitro* cytotoxicity of hexane, ethyl acetate, DCM and methanol stem bark extracts of *S. urticifolia* (Sims) using the Brine Shrimp cytotoxicity assay.

(iv) To determine the larvicidal activities through bio-assay guided fractionation of the plant stem bark extracts by chromatographic techniques (CC, TLC, and HPLC).

(v) To find out the active compounds using conventional spectroscopic techniques (UV, IR, NMR).

1.10 Significance of the study

Mosquito control is critical in public health because mosquitoes are the primary vector for a variety of diseases, including malaria, dengue fever, filariasis, and yellow fever, among others (James, 2001). This study was carried out on *S. urticifolia*, a plant in the family Verbenaceae and genus *Stachytarpheta* to help control the malaria menace through sustainable and less harmful natural way by eradicating the malaria vector at the larval stage. *S. urticifolia* yielded potent larvicidal agents hence form a basis of further research.

CHAPTER TWO

LITERATURE REVIEW

2.1 Family Verbenaceae

The Verbenaceae family is a member of the Lamiales order and may be found in every terrestrial habitat (Judd *et al*., 2002). There are several different types of development habits in this tropical family. There are around 98 genera and 3,000 species in the family. They can be either low shrubs, herbs, or trees in their natural habitat. Flowers are arranged in spikes (Clement *et al*., 2015). Several genera from this family are being investigated due to their medicinal and larvicidal properties (Salimena, 2000).

2.1.1 Ethnobotanical uses of the family Verbenaceae

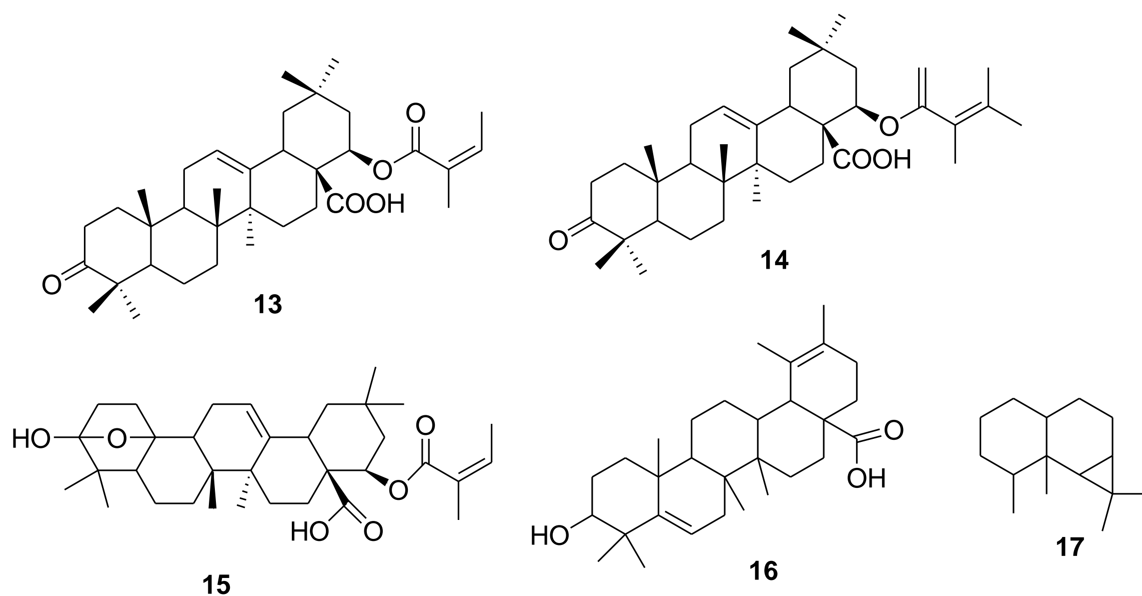
Tradition has it that members of this family have been utilized to heal a variety of diseases over the years. Many different Lantana species have been used to cure a broad variety of illnesses over the years. Various components of the plant have been used to treat a variety of conditions in traditional Chinese medicine, including bronchitis, wounds, rheumatisms, and ulcers; malaria; cancer; and hypertension. Additionally, decoctions have been used topically to cure leprosy and scabies (Eliana, 2014). Coughs, colds, dysentery, diarrhea, malaria, and diabetes are among the maladies that members of the *Lippia* species have been used to cure by both traditional healers and ordinary people. *Lippia multiflora* has been used to treat coughs, colds, dysentery, diarrhea, malaria, and diabetes. The leaves and stems of *Lippia. nodiflora* have been used in the form of tea infusions to treat malaria, menstrual disorders and gonorrhea. Adulticidal activity against various mosquito species has been reported for essential oils extracted from the leaves of *L. camara* (Dua *et al*., 2010).

2.1.2 Phytochemistry and pharmacology of the family Verbenaceae

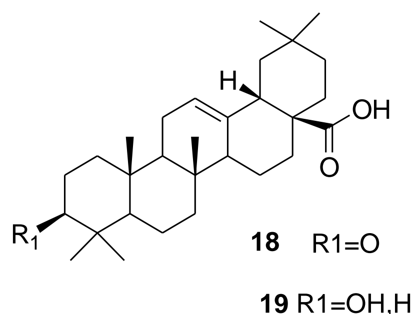
There have been a plethora of bioactive chemicals identified from members of this family (Ghisalberti, 2000). Chemical analysis of *Lantana* species revealed the presence of pentacyclic triterpenoids of the oleanane series, flavonoids and glycosides, in addition to monoterpenes, sesquiterpenes, steroids, and quinones. Crude extracts, essential oils, and

isolated chemicals have all been demonstrated to possess a variety of biological effects, including antiprotozoal, antiviral, antioxidant, antiproliferative, and cytotoxic activity (Sousa, 2012; Grace-Lynn *et al*., 2012). The methanolic extract of *L. camara* was found to be toxic to a variety of mosquito species at extremely low doses. It was discovered to be effective against *Cx. quinquefasciatus* and *An. stephensi*, with LC_{50} and LC_{90} concentrations of 35.36 and 107.42 ppm, respectively, for both species (Periaswamy *et al*., 2015).

Lantadene A **(13)** and lantadene B **(14)** extracted from *Lippia* were found to exhibit insecticidal activity (Wachter *et al*., 2001), while Camaric acid **(15)** isolated from *Lantanaviburnoides var kisii* showed larvicidal activity (Innocent *et al*., 2020). 3-hydroxy-10, 19-en-urs-28-oic acid **(16)** and Calarane **(17)** isolated from *Lantana camara* using n-hexane showed the antimicrobial activity.



Lippia species essential oils have been shown to have antimicrobial, antifungal, larvicidal, and antiseptic properties (Pascual *et al*., 2001). A hexane fraction of an ethanol extract of *L. lupulina* roots produced the triterpenes oleanonic (**18**) and oleanolic (**19**) acids, both of which exhibited insecticidal activity against Sitophilus oryzae (L.) (Mahato and Kundo, 1994).



2.2 The genus Stachytarpheta

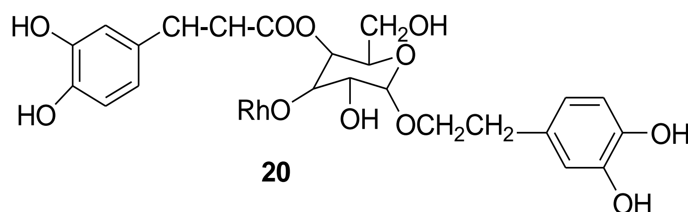
Around 113 species comprise the genus *Stachyterpheta* Vahl (Verbenaceae), which is found in North and South America, Asia, and Africa (Barbola *et al*., 2006). They come in a variety of shapes and sizes, including herbs, shrubs, vines, and even trees. Their leaves are usually opposite or whorled, simple or palmately complex, or exstipulated, and are typically opposite or whorled. *S. indica* (Linn.) Vahl, *S. angustifolia* (Mill.) Vahl, and *S. cayannensis* (Rich.) Vahl are all known species of *S. cayannensis* (Rich.) Vahl in Kenya (Zhou *et al*., 2015).

2.2.1 Ethnobotanical uses of the genus *Starchytarpheta*

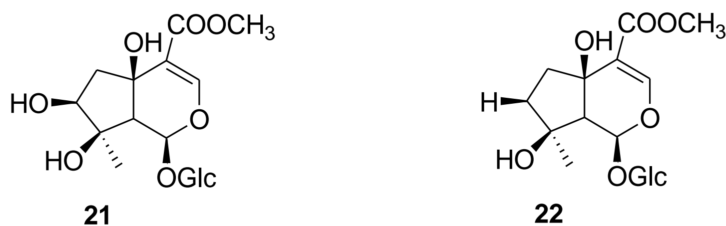
Different *Stachytarpheta* species have all been used ethnopharmacologically to treat a variety of illnesses including dysentery, vermifuge, gonorrhea, cataract, ulcers in children's ears, and heart disease. Other species have been used as purgatives, vermifuges, expectorants, diuretics, emmenagogues, sorethroat gargles, and general tonics, among other applications (Sanders, 2001). Women in Nigeria have been reported to employ *S. Jamaicans* to treat menstruation abnormalities and other feminine diseases, according to local reports. Quite often, its leaves have been used to treat dysentery and intestinal worms (Almeida *et al*., 1998). The boiled leaves have also been used in Malaysia to treat ulcers and anti-periodic medicine for malaria (Rao *et al*., 2006; Sasidharan *et al*., 2011). *S. angustifolia* has been used to treat sexually transmitted diseases (Enwuru *et al*., 2008) and diabetes (Ogbonnia *et al*., 2009).

2.2.2 Phytochemistry and pharmacology of the genus *Starchytarpheta*

This plant genus has been linked to the production of a number of phytochemicals. *S. jamaicensis* contains an iridoid glycoside known as verbascoside or acetoside (**20**), which has been isolated and studied (Liu *et al*., 2003). In addition to its antioxidant capabilities, this potent plant component has been shown to have neuroprotective, antiviral and antibacterial qualities, as well as liver protecting, cardioactive and antitumorous effects (Sanz *et al*., 1994; Daels–Rakotoarison *et al*., 2000; Sheng *et al*., 2002; Liu *et al*., 2003)



Additional species, including *S. cayennensis*, *S. indica, S. australis, S. cayennensis mutabilis,* and *S. glabra,* have been found to contain the iridoids lamiide (**21**) and ipolamiide (**22**) (Roengsumran *et al*., 2002). Some of these phytochemicals have been demonstrated to contain a variety of biological activities including antibiotic, anticancer, anti-inflammatory and antinociceptive properties as well as hepatoprotective and laxative properties, among others (Roengsumran *et al*., 2002).



Different extracts of the leaves and stems of *L. camara* have also been shown to exhibit larvicidal and antifeedant properties, according to the research (Mohammad *et al*., 2017). Flavonoids, terpenes, phenols, and steroids have all been found in *S. jamaicensis*, according to Nazar *et al*., 2011. *Cx. quinquefasciatus* and *aegypti* mosquitoes were used in the screening to see whether it has larvicidal and insecticidal properties (Idu *et al*., 2007).

2.3 Stachytarpheta urticifolia

The annual herbaceous weed *S. urticifolia* grows to a height of 60–120 cm. On tall bracts, it blooms in clusters of small reddish-purple to deep blue flowers (See Fig. 2.1). Leslie (2003) did a study on this topic and opined that it is a half-woody plant with an erect and branching morphology. The stems are terete with a slight slant on the younger stems. The leaves range in shape from elliptic to oblong-ovate, measuring 2.5–10 centimeters in length and featuring pointed ends and serrated edges. The petiole base is decurrent (Figure 2.2). The spikes are terminal, relatively thin, between 10 and 30 centimeters in length, 3-4 millimeters in thickness, green and continuous. The calyx is minuscule in size, oblique in shape, and equipped with four teeth. The corolla is a dark blue color and measures 1 centimeter in length and width. The calyx encases the fruit, which is appressed to and partially buried in the rachis. The fruit is smooth and oblong in shape, and measures approximately 4 millimeters in length (Sivaranjani *et al*., 2013).

Plate 2.1: A photograph of *Stachytarpheta urticifolia* (Sims)

The blooms have five wide petal lobes and are tubular in form (Bostock *et al*., 2010). (See Plate 2.2.)

Plate 2.2: A photograph of the aerial parts of *S. urticifolia* (Sims)

2.3.1 Ethnobotanical uses of *Starchytarpheta urticifolia* (Sims)

This plant has a variety of folkloric applications across the globe among them to expel worms in young children, to treat dysentery, in Haiti an infusion made from the plant leaves is used as an anthelmintic. Extracts from the leaves and stems of the plant are used to treat dyspepsia, allergies, asthma, fevers, and liver problems (Modi and Shah, 2022). It is applied topically to treat ulcers, lesions, cuts, and wounds on the skin. According to Clement (2015), ethnomedical usages, macerated leaves and roots should be applied topically to treat painful skin wounds, inflammation, and other maladies.

2.3.2 Phytochemistry and pharmacology of the *S. urticifolia*

Saponins, flavanoids, terpenoids, steroids, glycosides, quinines, alkaloids, tannins, and phenols were discovered in various extracts of this family's root, stem, leaf and inflorescence (Modi and Shah, 2022). Additionally, it has been suggested that members of this family are capable of a wide variety of biological functions. According to scientific evidence, these phytochemicals are responsible for the biological activity observed in plant extracts (Mohammad *et al*., 2017).

Although this plant is used by the traditional healers, no phytochemical constituents and biological activity have been reported. More so, no pure compounds have been reported. Hence, the current study was designed to evaluate the phytochemical constituents, the larvicidal property of *S. urticifolia* stem extracts and isolation and characterization of the isolated compounds.

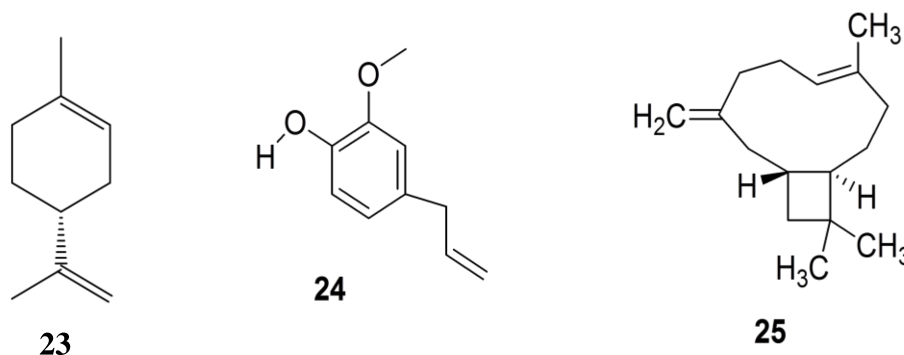
2.4 Bioactive phytochemicals

Phytochemicals are natural bioactive compounds which are present in plants (Murali *et al*., 2021). Phytochemicals have a variety of biological properties such as antioxidant, antimicrobial, larvicidal and cytotoxicity (Craig, 1997). The common phytochemicals are saponins, flavanoids, terpenoids, steroids, glycosides, quinines, alkaloids, tannins and phenols (Modi and Shah, 2022).

Terpenenoids are a huge and diverse family of natural chemicals that are generated principally by a diverse range of plant and animal species, as well as insects, bacteria, and other microbes. Essential oils extracted from a wide variety of plants and flowers are mostly composed of terpenes and terpenoids, which are the major ingredients. These compounds are typically denoted by the formula (C_3H_8)n. The carbon atoms to hydrogen atoms ratio is generally 5:8, and the isoprene unit is generally connected from head to tail. They can be made up of two, three, or more isoprene units and can take the form of open chains or cyclic structures, depending on the application (Eberhard, 2006). Terpenes have been documented for their role as insect growth regulators and as agents of communication and defense between plants and animals (Ammar *et al*., 2017).

2.4.1 Larvicidal terpenes

The hunt for effective natural larvicides or pesticides that are low in environmental toxicity has intensified in recent years (Chantraine *et al.,* 1998). There have been just a few researches that have looked specifically at the larvicidal efficacy of isolated essential oil components. In the hunt for novel ways to restrict the spread of *Aegypti,* sixteen aromatic and aliphatic terpenes were examined, and all of them were shown to have a larvicidal impact. *R*-limonene **(23)** exhibited the highest activity (LC_{50}, 25 ppm), followed by carvacrol **(8)** (LC_{50}, 69 ppm), thymol **(9)** (LC_{50}, 81 ppm), and eugenol **(24)** (LC_{50}, 88 ppm). Other terpenes exhibited LC_{50}>100 ppm. Bicyclic monoterpenes and β-Caryophyllene **(25)** exhibited the highest LC_{50} values (Santos *et al*., 2008).



2.4.2 Biosynthesis of terpenoids

Terpenoids are biosynthesized from isopentenyl pyrophosphate, $CH_2=C(CH_3)CH_2CH_2OPP$, which is formed via mevalonic acid, $CH_2OH–CH_2C(OH,CH_3)CH_2CO_2H$ (Ammar *et al*., 2017). Two isopentenyl pyrophosphates are linked together in the biosynthesis process to form geranyl pyrophosphate (GPP) (C_{10}), a critical intermediate in the formation of monoterpenes; GPP and isopentyl pyrophosphate are then linked together to form farnesyl pyrophosphate (C_{15}), a critical intermediate in the formation of sesquiterpenes. Following that, various combinations of these C_5, C_{10}, and C_{15} units are used to synthesize the higher terpenoids, the most common being triterpenoids formed by the condensation of two farnesyl units and carotenoids formed by the condensation of two geranylgeranyl units (Figure 2.1). Because the majority of natural terpenoids have cyclic structures with one or more functional groups, the final stages of synthesis require cyclization and oxidation, or other structural modifications, in addition to other synthesis processes (Ammar *et al*., 2017).

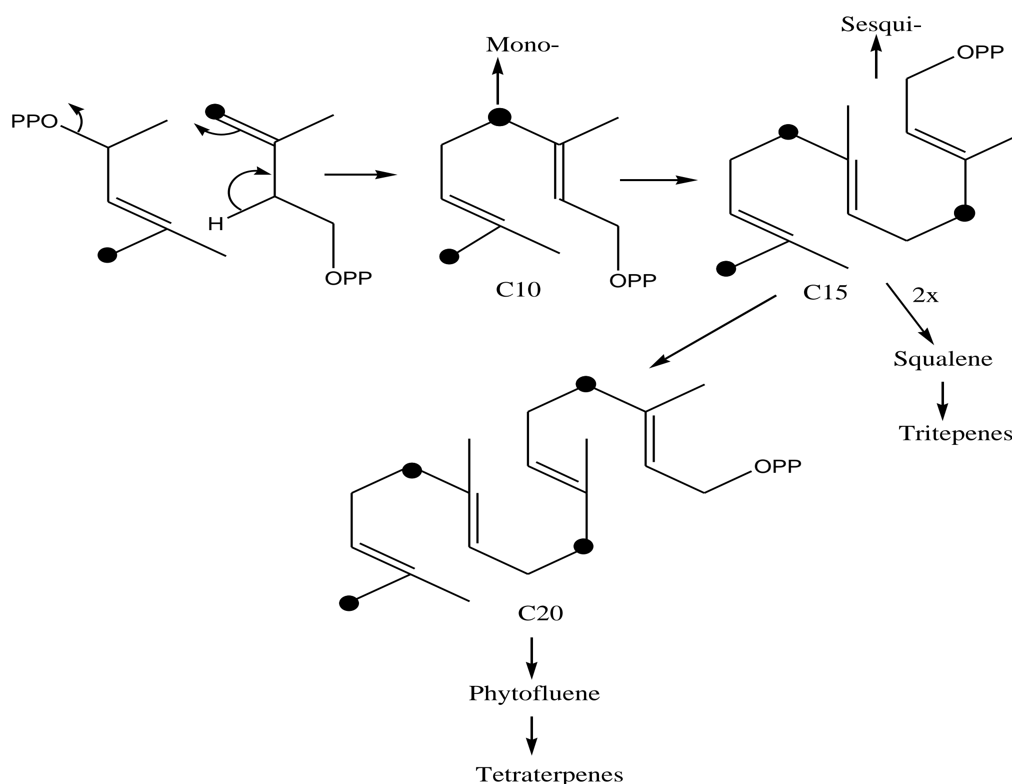


Figure 2.1: Biosynthesis of terpenoids

2.4.3 Bioassay

A bioassay can be used to determine the concentration or potency of physical, chemical, or biological agents by measuring and comparing the magnitude of the test's response to that of the standard over time using an appropriate biological system. An important use of bioassay is the estimate and finding of biologically active chemicals. It also has a significant role in the sensitivity and specificity of pharmaceutical applications. Chemical approach is a very complicated procedure that necessitates a large chemical dose and a chemical composition that demonstrates the drug's pharmacological activities against it (Sunil, 2015).

2.4.3.1 Mosquito larvicidal bioassay

To determine the activity range of the substances under investigation, mosquito larvae are exposed to a wide range of test concentrations as well as a control. After determining the mortality of larvae over a wide range of concentrations (four to five concentrations yielding between 10% and 95% mortality in 24 or 48 hours), the lethal concentration (LC_{50}) and lethal concentration (LC_{90}) values are determined over a narrower range of concentrations (four to five concentrations yielding between 10 and 95% mortality in 24 or 48 hours). Using strainers, screen loops, or droppers, transfer batches of 25 third or fourth instar larvae to tiny disposable test cups or containers holding 100–200 mL of water each. Substitute larger, healthier larvae for small, sick, or injured larvae (WHO, 2005).

Water should be kept between 5 and 10 cm deep in cups or containers; any deeper levels may result in an increase in the number of deaths. To the cups of 100 mL or 200 mL water, add the required volume of dilution, starting with the lowest concentration. Using tap water containing 1 ml alcohol, four or more replicates of each concentration are established concurrently with an equal number of controls (or the organic solvent of choice). To ensure accuracy, each test should be repeated three times on three different days. If the control group has a high mortality rate, each test cup should be supplied with larval food to allow for prolonged exposure (WHO, 2005).

The test containers are maintained at a temperature of 25–28 degrees Celsius, with a preferred photoperiod of 12 hours of light followed by 12 hours of darkness (12L:12D). After a 24-hour exposure, the mortality of larvae is determined. A 48-hour reading may be required for slowacting pesticides. To calculate the percent mortality, the number of moribund larvae is added to the number of dead larvae. By inserting a needle into the siphon or cervical area of the larva's body, it is possible to elicit movement in dead larvae. Moribund larvae are those that are unable to rise to the surface or do not exhibit the typical diving behavior observed in other larvae when the water is disturbed. If the control group has a mortality rate of between 5 and 20%, the treatment groups' mortality rates should be adjusted using Abbott's formula.

$$Mortality\,(\%) = \frac{X-Y}{X}100 \text{ ....(Equation 1)}$$

Where X = percentage survival in the untreated control and Y = percentage survival in the treated sample (WHO, 2005).

2.4.4 Spectroscopic techniques for structural elucidation of terpenoids

Different spectrophotometric methods are used for the identification, estimation and structural elucidation of terpenoids. Among them are ultraviolet spectroscopy, infrared spectroscopy, nuclear magnetic resonance spectroscopy, and mass spectroscopy (Hanson, 2001).

2.4.4.1 Ultraviolet (UV) and Infra –red (IR) spectroscopy

UV spectroscopy, as the name implies, focuses on the electron structure of molecules and provides critical information about the presence or absence of functional groups in a compound (Silverstein *et al*., 1991). The use of infrared spectroscopy for the identification and structural elucidation of novel terpenoids is common practice. It provides information on a large number of functional groups. Similarly, (Nita and Rajesh, 2014).

2.4.4.2 Nuclear magnetic resonance spectroscopy (NMR)

NMR spectroscopy, which includes both ^{1}H-magnetic resonance and ^{13}C-magnetic resonance, is one of the most essential instruments for providing a significant amount of information necessary for the elucidation of the structure of a compound. The combination of 1D selective and 2D NMR techniques such as DEPT, COSY, TOCSY, ROESY, 2D-IN ADEQUATE, HMQC, HMBC, HETCOR and selective INEPT are of great value for the structure elucidation of various terpenoids including the saponins and glycosides consisting of a sugar moieties (Manasa, 2014).

2.4.4.3 Mass spectrometry (MS)

This procedure can produce a precise molecular weight of the chemical, as well as a complicated fragmentation pattern that is frequently characteristic to that particular compound, and it is very inexpensive (Silverstein *et al.,* 1991). Fast atom bombardment spectroscopy (FAB-MS) affords the exact molecular ion peak along with diagnostic fragmentation pattern of the terpenoid molecule. It is an important tool for the structure determination. FAB-MS in both positive and negative modes, combination of GC-MS or HPLC-MS spectrometry coupled with computerized analysis and Tandem MS spectral analysis have been advantageously employed in recent years for the structure determination of various terpenoids (Manasa, 2014).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Research design

The plant material was collected from Uriri Sub-county of Migori County at 9.00 a.m. It was then dried and ground into powder before use in sequential extraction. Phytochemical analysis was done according to standard methods. Bioassays were then done on the crude fractions followed by isolation of compounds from the bioactive fractions. The isolated compounds were then identified using spectroscopic techniques.

3.1.1 General procedures.

After being cleaned with water and soap, all of the glass apparatus was chemically cleaned by leaving them to soak overnight in new chromic acid to remove any remaining residue. Before usage, they were washed in distilled water for 12 hours, followed by a final rinse in acetone, and then dried at 110^0C in an electric oven for an hour before being used.

3.1.2 Laboratory equipment and instruments

The plant materials were pulverized in a laboratory mill (Christy and Norris Ltd., Chelmsford, England) and weighed on a top-loading analytical balance during the research at the Jomo Kenyatta University of Agriculture and Technology (JKUAT). Gallen Kamp melting point equipment was used to determine the melting points of the isolated compounds (Sanyo, West Sussex and UK). The spots on the TLC plates were observed using an ultraviolet light with both long and short wavelengths, (ENF-240 C/F, Supertonics Corporation, Westbury, United Kingdom) (365 nm and 254 nm respectively).

3.1.3 Chromatographic materials and solvents

The study used analytical TLC pre-coated plastic sheets (polygram Sil G/UV 254) and aluminum sheets (Alugram Sil G/UV 254) measuring 20 by 20cm to determine the optimal solvent systems, separations, extract complexity, and purity of isolated compounds. Matcher-Nagel Gmbh and Co. in Frankfurt, Germany supplied the sheets. Vacuum liquid

chromatography (VLC) was used as the stationary phase, with Kiesegel silica gel 60G slurry packing (0.04-0.6 mm Merck, Germany). Column chromatography experiments were conducted using glass columns with internal diameters ranging from 1.5 to 4.0 cm and lengths ranging from 15 to 80 cm. They were densely packed using a slurry process with Kiesegel silica (Merck, 70-230 mesh/ 0.63-0.2 mm). Purification was then accomplished with Sephadex LH 20, a sieve material packed via the slurry process.

They were acquired from Kobian Kenya Ltd in Nairobi and included hexane, dichloromethane (DCM), dimethyl sulphoxide (DMSO) (analar grade), ethyl acetate (EtOAc), acetone (MeCOMe), and methanol (MeOH), all of which were employed as organic solvents.

3.1.4 Spray reagents

To detect and visualize the isolated compounds on the TLC plates, the following reagents were used:

i. A solution containing 0.5 mL p-anisaldehyde, 10 mL acetic acid, 85 mL methoxyethanol, and 5 mL concentrated sulfuric acid produces anisaldehydesulphuric acid (Krishnaswamy, 2003)

ii. A combination of concentrated sulfuric acid and distilled MeOH, made by combining 5ml of concentrated sulfuric acid with 95ml of distilled MeOH (Krishnaswamy, 2003).

iii. Drangerdoff reagent, prepared by mixing 0.5 g of bismuth nitrate and 10 ml of concentrated hydrochloric acid in one beaker and 4 g of potassium iodide is dissolved in a little water in another beaker. The two solutions are then mixed to give a dark orange solution. (Krishnaswamy, 2003).

3.1.5 Detection of compounds

Spots on the chromatograms were detected with p-anisaldehyde spraying or the Drangerdoff reagent after they had been visualized under UV light at 254 nm and 365 nm and then sprayed with p-anisaldehyde.

3.1.6 Nuclear magnetic resonance (NMR)

TMS was used as an internal standard in both ^{1}H and ^{13}C NMR. ^{1}H was measured using a frequency of 200 MHz while ^{13}C NMR was done at 75 MHz on the same machine. The investigations were carried out in CD_3OD on a Bruker-300 machine. A Bruker-300 machine was used to record HMBC, COSY, NOESY, HSQC, and DEPT NMR studies. Chemical modifications induced by TMS were quantified in (ppm). The maximum multiplicities are denoted by the symbols s (singlet), d (doublet), t (triplet), q (quartet), dd (dual of doublet), bd (broad doublet), and m, (multiplet). Constants of coupling were determined in hertz (Hz).

3.1.7 Bioassays

Larvicidal bioassays were done on 4th instar larvae of *Anopheles gambiae* while cytotoxicity tests were done on brine shrimp eggs hatched in artificially prepared sea water, prepared by dissolving 38 g of sea salt in 1 litre of distilled water for hatching the shrimp eggs. The 4th instar larvae were chosen due to their size which is about 3.5 mm after 3 to 4 days and that they can be influenced by food and temperature.

3.1.8 Larvicidal data analysis

For analysis, all of the replicates' data were combined. LD_{50} and LD_{90} values were computed using SPSS 11.5 software using the Log dosage-Probit mortality regression line.

3.2 Plant collection, identification and pretreatment

S. urticifolia was collected from Kenya's Migori County. Dr. Paul Kirika, a botanist at the National Museums of Kenya, identified and authenticated the plant, which was deposited in their herbarium under voucher specimen number CFA001 2018. The stems were cleaned under running tap water and dried for 7-14 days in the open air in the shade.

3.2.1 Solvent extraction for *S. urticifolia*

3.2.1.1 Extraction procedure for *S. urticifolia* stem bark

For 48 hours, hexane, dichloromethane, ethyl acetate, and methanol were used in sequence of increasing polarity to extract the ground plant material. For 48 hours, 2kg of powdered material was immersed in 2000 mL of hexane, stirring occasionally. Filtration was then carried out in a vacuum chamber. Figure 3.1 illustrates the sequential extraction.

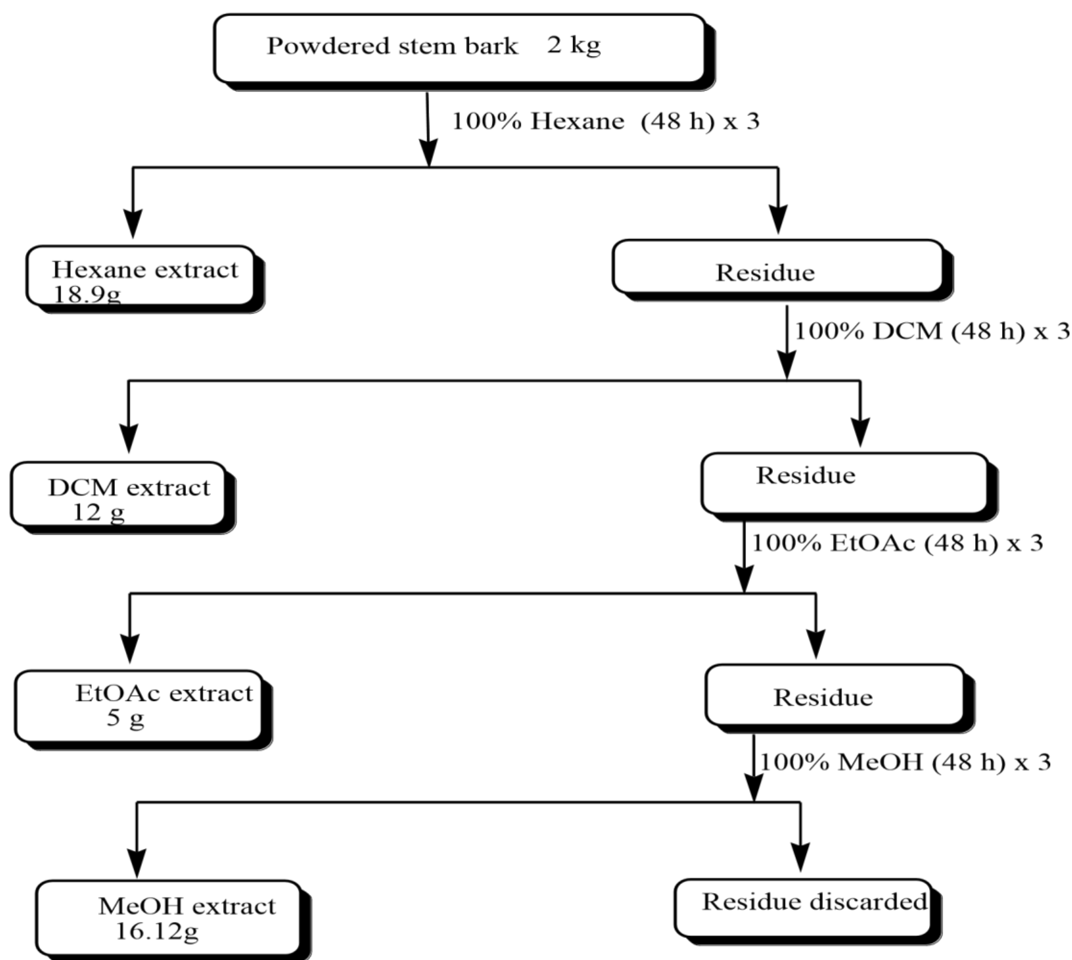


Figure 3.1: Sequential extraction of *S. urticifolia* (Sims) stem bark

The filtrate was then concentrated to yield 10.1 g using a rotary evaporator (vacuum evaporator) set to 45 ^{0}C. The remaining residue was soaked in another 2000 mL of hexane and left for another 48 h. On filtration and concentration, this second extraction yielded 5.2

g of crude extract. This procedure was repeated on the remaining residue which yielded 3.6 g of crude extract. The three portions were combined to yield an 18.9 g hexane extract. The remaining residue was added 2000 mL of dichloromethane and left for 48 hours with occasional swirling. It was then filtered under vacuum using a Buchner funnel and the filtrate concentrated at 45 ^{0}C using a rotary evaporator (vacuum evaporator). For an additional 48 hours, 2000 mL of dichloromethane was added to the remaining residue. The procedure was repeated to yield 4.20 g of crude extract. A third dichloromethane extraction was done using the same procedure to yield 3.30 g of crude extract. The three samples were combined to give 12 g of dichloromethane extract.

To the remaining residue 2000 mL portions of EtOAc were added in accordance with the above procedure to yield 2.5 g, 1.8 g and 0.7 g for the first, second and third extractions, respectively. 5.0 g of EtOAc extract was obtained by combining these. The same procedure was repeated with 2000 mL portions of methanol to obtain 9.50 g, 4.30 g, and 2.32 g for the first, second, and third extractions. 16.12 g of crude methanol extract was obtained by combining these three components. All crude extracts were then carefully sealed, labeled, and stored at -20 ^{0}C in pre-weighed sample bottles in a deep freezer for subsequent use.

3.3 Phytochemical analysis

Standard procedures outlined by Harborne (1984), were used to analyze the crude extracts for the presence of plant phytochemicals such as flavonoids, tannins, saponins, terpenoids, steroids (Obadoni and Ochuko, 2001).

3.3.1 Test for flavonoids

This procedure is referred to as the Shinoda test. 0.5 g of each crude extract was dissolved in ethanol, warmed and then filtered. Three magnesium chips and two drops of concentrated hydrochloric acid were then added to the filtrate. The presence of flavonoids was indicated by a pink coloration. (Trease and Evan, 2002).

3.3.2 Test for tannins

This test is also referred to as the ferric chloride test. 0.5 g of each crude extract was dissolved in 5 mL distilled water and added to a tube containing 20 mL boiling distilled water. The tube was then heated for one hour. We added a few drops of ferric chloride and allowed it to develop properly. Tannins were distinguished by their blue-black coloration (Ndam *et al*., 2016).

3.3.3 Test for saponins

The frothing test is used to determine this. This is accomplished by dissolving half a gram (0.5 g) of the crude extract in three milliliters of hot distilled water and shaking vigorously for one minute. There was persistent foaming, indicating the presence of saponins (Ndam *et al.,* 2016).

3.3.4 Test for terpenoids

This is sometimes referred to as the Salkowsky test. 1 g of the extract dissolved in ethanol followed by 1 mL acetic anhydride and a few drops of concentrated sulphuric acid. The transition from pink to violet in the color spectrum indicates the presence of terpenoids. (Sofowora, 1993).

3.3.5 Test for alkaloids

This is the Drangendorf test, in which 0.5 g of each crude extract is dissolved in dichloromethane and then spotted on a thin layer chromatographic plate developed in a 20% hexane/ethyl acetate solution. The chromatogram was then sprayed with newly manufactured Dragendorf's reagent in a fume room. A positive reaction was denoted on the chromatogram by an orange or darker-colored patch against a yellow background (Ndam *et al*., 2016).

3.3.6 Test for steroids

The Liebermann-Buchard test was used to establish this. 0.5 mL dichloromethane, 0.5 mL acetic anhydride, and three drops concentrated sulphuric acid were added to a 0.5 g crude extract. To confirm the presence of steroids, a blue-green tint was used (Ndam *et al*., 2016).

3.4 Bioassays of the crude extracts of *S. urticifoila*

3.4.1 *In-vitro* bioassays

Samples from the crude extracts from hexane, DCM, ethyl acetate and methanol were prepared into different concentrations and used for both larvicidal and cytotoxicity tests.

3.4.2 Preparation of extracts for bioassays

Extracts of hexane, dichloromethane, ethyl acetate, and methanol were prepared at various concentrations for anti-larvicidal experiments. Each extract was prepared as a 1.0 percent stock solution by dissolving 200 mg in 20 ml analytical grade acetone. The mixture was vigorously shaken in a screw-cap vial with the mouth covered with aluminum foil. Five test concentrations (0.0001 mg/ ml, 0.001 mg/ ml, 0.01 mg/ ml, 0.1 mg/ ml, and 1 mg/ ml) were obtained by serially diluting the stock solution tenfold in acetone (WHO, 2006).

3.4.3 Larvicidal assays

A. gambiae larvae were obtained and raised in the laboratory from the ICIPE insectarium in Nairobi. Larvae were fed a 1:3 ratio of powdered dog biscuits and Brewer's yeast and kept in an insectarium maintained at a temperature of 25-29 ^{0}C and a relative humidity of 75-85 percent (Arivoli *et al*., 2015). Larvicidal bioassays were performed on larvae of the fourth instar obtained from the stock culture (Shivakumar *et al*., 2013). The WHO laboratory and field testing protocols for mosquito larvicides (2005), as well as the Rahuman *et al.* technique, were used to determine the larvicidal activity of crude extracts (2008).

Batches of 25 fourth instar larvae were placed in a 250 mL beaker containing 200 mL water containing test extract using screen loops. We prepared a control solution of 2 mL acetone in 200 mL water. Each concentration was increased by a factor of four. The test flasks were maintained at a constant temperature of 25–28 ^{0}C and subjected to a 12-hour photoperiod followed by a 12hour period of darkness. After 24 hours, larval mortality was determined and corrected it using Abbot's formula (Arivoli, 2012; Shivakumar *et al*., 2013). The number of dead larvae was added to the number of moribund larvae, and the percent mortality was calculated using Abbot's method, as shown in equation 1(Section 2.4.3.1).

3.4.4 *In vitro* cytotoxicity assay of crude extracts

3.4.4.1 Preparation of test extracts

In 2 mL of hexane, 20 mg of hexane extract was dissolved. 1000, 100, 10, 1, and 0.1 parts per million (g/mL) were the final concentrations. Each concentration was replicated three times. Additionally, a control test was conducted using the same volume of sea water but without the plant extract. This procedure was repeated with dichloromethane, ethyl acetate, and ethanol crude extracts.

3.4.4.2 Brine shrimp lethality test

This experiment was conducted according to the protocol previously reported (Olowa and Nuneza, 2013). ICIPE, Nairobi, provided the brine shrimp eggs. To create artificial seawater for hatching the shrimp eggs, 38 g of sea salt was dissolved in 1 liter of distilled water. Seawater was placed in a small plastic container with a dark and a light zone. Shrimp eggs were introduced into the chamber's dark side, while the light above attracted the hatched shrimp. For two days, the shrimp were allowed to hatch and develop into larvae. Seawater was placed in a small plastic container divided into two zones: dark and light. The dark side of the chamber was introduced with shrimp eggs, while the light above attracted the hatched shrimp. The shrimp were allowed to hatch and develop into larvae for two days. When the shrimp larvae were ready, each test tube was filled with 4 mL of artificial seawater and ten brine shrimps were added. Thus, each dilution included a total of 100 shrimp. The volume was then increased to 5 mL each test tube using fake seawater. Under the illumination, the

test tubes were left exposed. After 24 hours, the number of surviving shrimps was counted and recorded. Additionally, the percentage mortality was estimated by dividing the number of dead larvae by the total number of larvae and multiplying by 100%.

3.5 Preliminary analysis of crude extracts

To ascertain the complexity of the secondary metabolites present in the tested extracts, each crude extract was subjected to thin layer chromatography (TLC) on silica gel (60F254) (Merck) coated plates using the standard 1D ascending method. Through color development, the generated plates were air dried and sprayed with Dragendorff and anisaldehyde spray reagents to detect alkaloids, flavonoids, glycosides, saponins, and terpenoids, respectively. The hue of the spots/bands was determined, as well as their retention durations (R_f) values.

3.5.1 Monitoring of isolated compounds

Using thin capillary tubes, the samples were spotted on TLC plates. Pre-coated TLC plates

(analytical grade) were used to determine the optimal solvent systems for separations, the complexity of the extracts, and the purity of the isolated compounds. At room temperature, the loaded plates were developed using the ascending solvent technique in glass jars (development tanks).

3.6 Fractionation of the crude extracts

The ethyl acetate (5 g) and dichloromethane (12 g) crude extracts (Figure 3.1) were selected for isolation since they showed high larvicidal activity. The EtOAc crude extract was subjected to several chromatographic steps (Figure 3.2). The crude extracts were separated and purified using both CC and VLC on silica gel. Dry packing was performed on the VLC column using silica gel (Kiesegel 60G, Merck, Germany), which was then consolidated with hexane and sucked by vacuum to dry and effect proper packing.

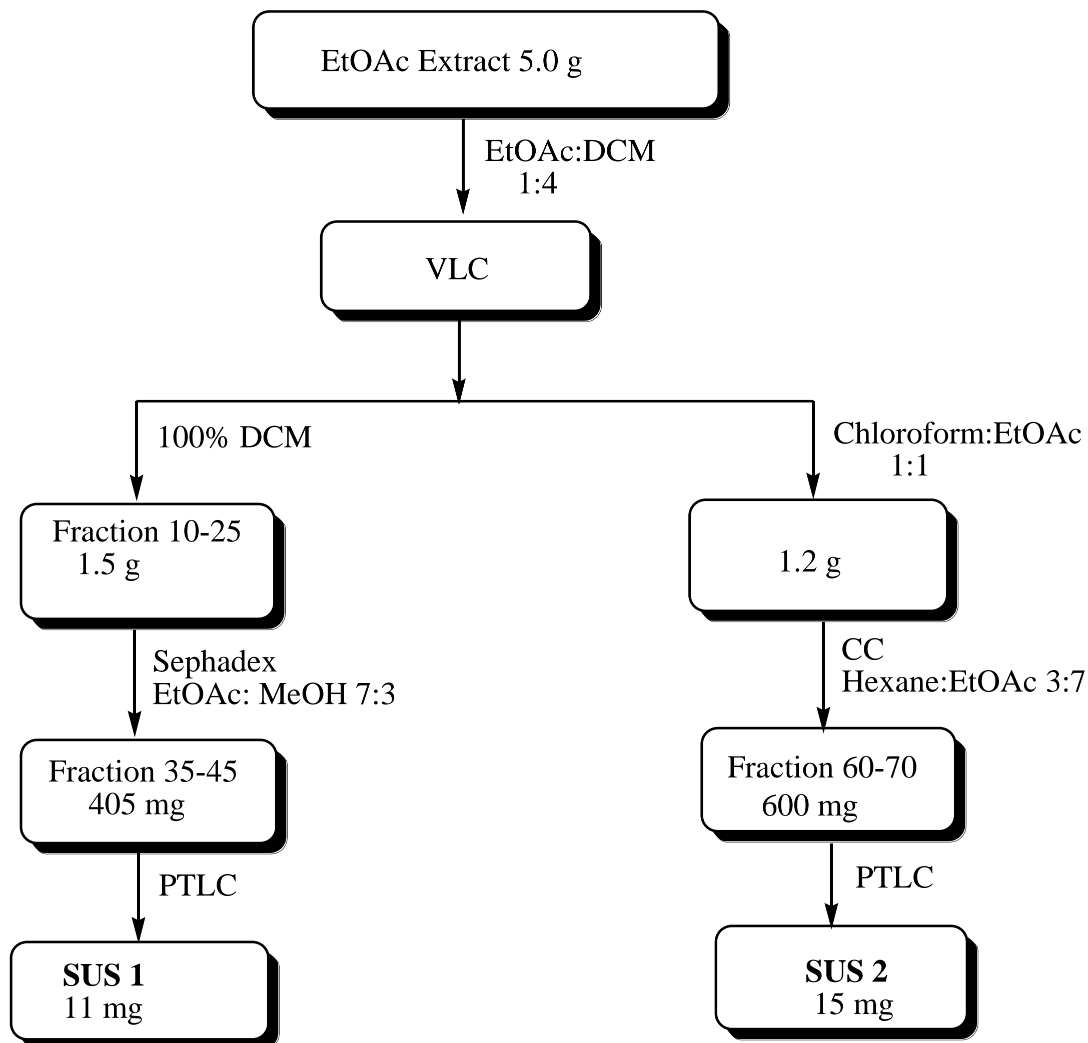


Figure 3.2: Chromatographic separation of crude EtOAc stem bark extract of *S urticifolia* (Sims)

Separation and elution were carried out with increasing polarity bi-solvent solutions, beginning with 100 percent DCM. This gave 30 fractions from which fraction 10-25 were combined based on the similarity of TLC profiles. Upon concentration they yielded 1.5 g fraction. The polarity was then increased to Chloroform/EtOAc 1:1 which gave 1.2 g fraction.

3.7 Isolation and purification of the compounds

The first fraction (1.5 g) of 100 percent DCM (Figure 3.2) was packed in 30cm x 2cm Sephadex LH 20 columns (a sieve gel technique) and eluted with EtOAc/MeOH (7:3).

Following that, a series of PTLCs were performed (70 percent EtOAc in MeOH). After that, the chromatogram was viewed under UV illumination set to 254 nm for inactive compounds and 366 nm for active compounds. Following this, the plate was developed in ammonia vapor (to determine the presence of flavonoids) or sprayed with a detecting agent such as a 5% solution of sulphuric acid in methanol or p-anisaldehyde and baked for ten minutes at 110^0C. Spots that showed homogeneity (R_f = 0.37, 70% EtOAc in MeOH) were combined and concentrated together to yield 11 mg of a pure compound coded as **SUS 1.** The same procedure was repeated for the second fraction (1.2 g) (Chloroform/EtOAc 1:1) which yielded 15 mg of **SUS 2**.

The DCM (12 g Figure 3.1) crude extract was also subjected to several chromatographic techniques similar to the EtOAc fraction above using the same equipment. A solvent system of 20% MeOH on DCM gave 2.0 g fraction which was then packed in a smaller column and eluted using 70% EtOAc in MeOH (Figure 3.3). This gave a total of 80 fractions where fraction 55-65 were combined based on similarity on TLC profile to give 300 mg sample. On exposure to several PTLC and concentration using 70% EtOAc in MeOH, a white amorphous solid was obtained coded as **SUS 3.**

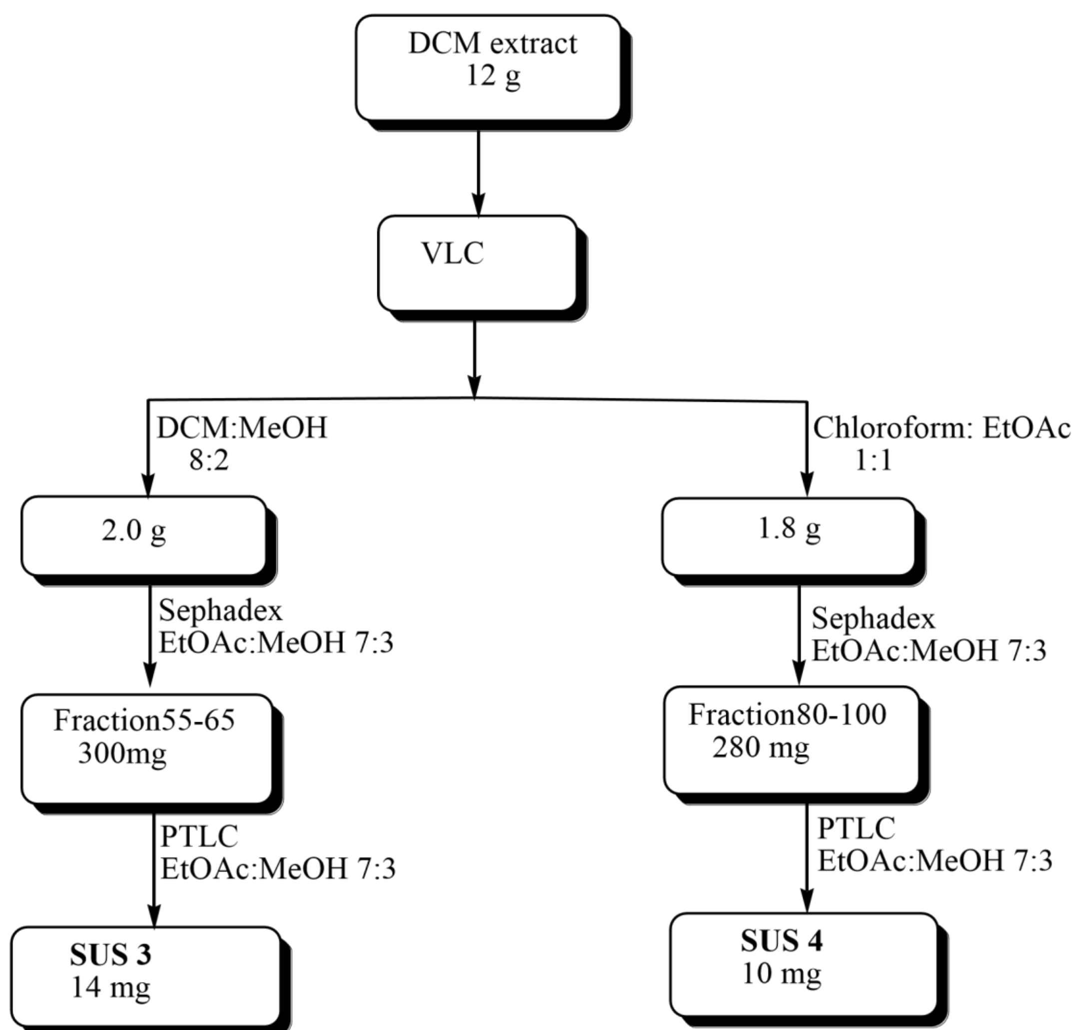


Figure 3.3: Chromatographic separation of crude DCM stem bark extract of *S urticifolia* (Sims)

The final fraction, 1.8 g (Chloroform:EtOAc, 1:1), was absorbed on silica gel and subjected to column chromatography with increasing polarity solvents from 100 percent DCM to 100 percent EtOAc to get 120 fractions. On exposure to PTLC, fractions 80-100 were combined to give colourless needlelike crystals named compound **SUS 4**.

3.8 Physical and spectroscopic data of isolated compounds

The physical data obtained for the isolated compounds **SUS 1**, **SUS 2, SUS 3 and SUS 4** included the melting point, the appearance and the retention factor (R_f). The spectral data determined were ^{13}C NMR, ^{1}H NMR, COSY, DEPT, HMBC, HSQC, IR and MS spectra as

indicated in the appendices. These were obtained according to the procedures on section 3.1.6 and 3.1.7.

3.8.1 SUS 1

A white crystalline solid (70% EtOAc/MeOH) 11 mg; M.p 206-209^{0}C; R_f of 0.37 (50% Benzene: Ethyl acetate); ^{1}H NMR (CD_3OD, δ (ppm), 200 MHz) δ 5.8 (1H, d, J=2.4Hz), 3.94 (1H, s), 3.84 (1H, m), 3.4 (1H, m), 3.14 (1H, m), 2.39 (1H, m), 2.36 (1H, m), 2.12 (2H, m), 1.95 (1H, m), 1.93 (1H, m), 1.87 (2H, m), 1.78 (3H, m), 1.73 (1H, m), 1.58 (1H, m), 1.43 (2H, m), 1.28 (1H, m), 1.18 (2H, m), 0.96 (2H, s), 0.88 (1H, s); ^{13}C NMR (CD_3OD, δ, 75MHz) δ 18 (C-26), 21.0 (C-25), 21.0 (C-28),21.4 (C-22), 24.4 (C-23, C-24), 27.3 (C-18), 28.9 (C-10), 31.8 (C-11), 32.5 (C-14), 32.8 (C-7), 35.1 (C-16), 37.3 (C-1), 39.3 (C-4), 42.4 (C-6), 49.5 (C-8), 50.5 (C-9), 51.7 (C-5), 68.5 (C-3), 68.7 (C-2), 71.3 (C-19),77.9 (C-17), 78.4 (C-20), 85.2 (C-12), 122.1 (C-15), 138 (C-13), 167 (C-27), 206 (C-21).

3.8.2 SUS 2

White powder in (Chloroform: EtOAc (1:1)) 30 mg; M.p. 174-176^{0}C; R_f of 0.7 in hexane-DCM (1:1). ^{1}H-NMR ($CDCl_3$, MHz) δ 0.64 (3H, s), 0.92 (3H,s), 1,00 (3H, s), 1.02 (3H, s), 1.07 (3H, s), 1.25 (3H, m), 1.55 (m), 3.51 (1H, m), 5.05 (1H,dd), 5.15 (1H, dd), 5.35 (1H, m); ^{13}C-NMR ($CDCl_3$, 100 MHz) δ 11.9 (C-18), 12.2 (C-29), 19.4 (C-19), 21.1 (C-11,C-21) 24,3 (C-15), 19.1 (C-27), 25.4 (C-28) 28.3 (C-16), 31.7 (C-7), 31.8 (C-8), 31.9 (C-2, C-25, C-26) 36.1 (C-20), 36.5 (C-10), 37.3 (C-1), 39.8 (C-12), 42.3 (C-4, C-13), 50.2 (C-9), 51.3 (C-24), 56.0 (C-14), 56.9 (C-17), 71.8 (C-3), 121.7 (C-6), 129.3 (C-23), 138.3 (C-22), 140.8 (C-5).

3.8.3 SUS 3

A white amorphous solid (70% EtOAc/MeOH) 14 mg; M.p 197-199 ^{0}C; R_f of 0.87 (50% Benzene: Ethyl acetate); ^{1}H NMR (CD_3OD, δ (ppm), 200MHz) δ 4.21(1H, m), 4.1(1H, m). 3.61 (1H, m), 3.56 (1H, m), 3.55 (1H, m) 3.4 (1H, m) 3.07 (1H, m) 3.03 (1H, m), 2.89 (1H, m), 2.6 (1H, m), 2.4 (2H, m) 1.9 (2H, m), 1.85 (1H, m), 1.82 (1H, m), 1.78 (1H, m), 1.67 (2H, m), 1.64 (2H, m), 1.45 (3H, m), 1.27, m, 1.20 (3H, m), 0.98, m 0.95 (2H, m), 0.85 (4H, m) 0.6 (1H, m), 0.45 (1H, m); ^{13}C NMR (CD_3OD, δ, 100MHz) δ 17.2 (C-27), 17.4 (C-25), 18.2 (C-23,C-24), 21.0 (C-29), 28.9 (C-10), 29.4 (C-21, C-26), 32.8 (C-15), 34.2 (C-11, C-22), 35.2 (C-12), 35.7 (C-3), 37.5 (C-20), 38.5 (C-8), 38.9 (C-13, C-16), 39.3 (C-1), 45.6 (C-6), 48.7 (C-4, C-17), 49.0 (C-9), 54.4 (C-5), 59.3 (C-18), 61.1 (C-6'),70.0 (C-4'), 70.1 (C-5'), 73.5 (C-2'), 75.1 (C-2), 76.7 (C-19), 76.8 (C-3'), 83.6 (C-14), 100.7 (C-1'), 211 (C-7), 214 (C-28).

3.8.4 SUS 4

Colourless needlelike crystals (Chloroform: EtOAc 1:1) () 20 mg; M.p. 131-133 ^{0} C; R_f of 0.6 in pure DCM; 1H-NMR ($CDCl_3$, 200 MHz) δ 0.90 (9H, s), 1.0 (3H, s), 1.06 (3H, s), 1.25 (6H, m), 1.50 (2H, m), 3.22 (1H, m), 5.20 (1H, t); ^{13}C-NMR ($CDCl_3$, 100 MHz) δ 14.1 (C-18), 14.1 (C-29), 17.5 (C-27), 18.0 (C-26), 21.4 (C-11), 23.3 (C-15, C-19, C-21, C-23), 23.4 (C-28), 26.2 (C-23), 28.1 (C-16), 29,4 (C-25), 31.1 (C-7), 31.3 (C-2), 31,7 (C-7), 31.9 (C-8), 33.8 (C-22), 34.8 (C-20) 37.0 (C-10), 37.1 (C-1), 39.7 (C-12) 41.5 (C-13), 42.1 (C-4) 46.9 (C-24), 47.7 (C-9), 55.2 (C-14), 59.1 (C-17) 79.1 (C-3), 124.4 (C-6), 139.6 (C-5).

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1 Crude extract yields

The dry ground (2.0 kg) stem of *S. urticifolia* (Sims) was extracted sequentially for 48 hours using solvents of increasing polarity (2000 mL). Hexane was used as the first solvent, followed by DCM, ethyl acetate, and finally methanol. For subsequent work, these crude extracts were kept at a temperature of 4^{0}C. Weighing the crude extracts yielded the following percentage yields (Table 4.1).

Table 4. 1: Masses and percentage yields of sequential extraction of *S. urticifolia* (Sims) stem

Solvent extract	Mass (g)	% Yield
Hexane	18.9	0.945
DCM	12 .0	0.6
EtOAc	5.0	0.25
MeOH	16.12	0.806

The highest percentage yield was obtained from hexane extract at 0.945 percent, while the lowest percentage yield was obtained from ethyl acetate extract at 0.25 percent. The results indicated that the yield decreased as the solvent's polarity increased up to ethyl acetate. Methanol yield increased, which could be due to the fact that methanol is the most polar solvent in comparison to the other solvents, and thus dissolved all remaining compounds (Adetutu *et al*., 2011).

4.2 Cytotoxic effects of crude extracts of *S. urticifolia* on brine shrimp eggs

The results of *in-vitro* cytotoxic activities of solvent extracts of *S. urticifolia* against brine shrimp eggs are summarized in table 4.2.

Table 4.2:Raw data on cytotoxicity of crude solvent extracts of *S. urticifolia* against brine shrimp eggs

Plant Extract	Concentration (ppm)	Number of subjects	Number surviving after 24h	Percentage mortality
Hexane	0.1	100	40	60
	1	100	32	68
	10	100	29	71
	100	100	23	73
	1000	100	20	80
DCM	0.1	100	25	75
	1	100	11	89
	10	100	6	94
	100	100	6	94
	1000	100	3	97
Ethyl Acetate	0.1	100	20	80
	1	100	17	83
	10	100	11	89
	100	100	8	92
	1000	100	6	94
Methanol	0.1	100	10	90
	1	100	8	92
	10	100	6	94
	100	100	4	96
	1000	100	2	98

The crude extracts showed high mortality against brine shrimp eggs even at low concentrations, for instance at 0.1 ppm, hexane = 60%, DCM =75%, Ethyl acetate = 80% and MeOH = 90%. The mortality was highest in methanol with all the concentrations recording 90% and above. A control set with 5 mL of artificial seawater in a test tube gave 0.0% mortality (Lim *et al.*, 2017). There was a general increase in mortality from hexane to methanol. This information was analyzed on probit to get LC_{50} values for the different solvent extracts (Table 4.3).

Table 4.3: Slope and intercept of probit regression line and LC_{50} for the extracts of *S. urticifolia* against brine shrimp eggs

Extract	Slope	intercept	LC_{50} (µg/mL)
Hexane	0.1331	5.4094	0.0008
Dichloromethane	0.2987	6.0659	0.0003
Etyl acetate	0.2899	5.8227	0.0015
Methanol	0.1942	6.3962	6.48×10^{-8}

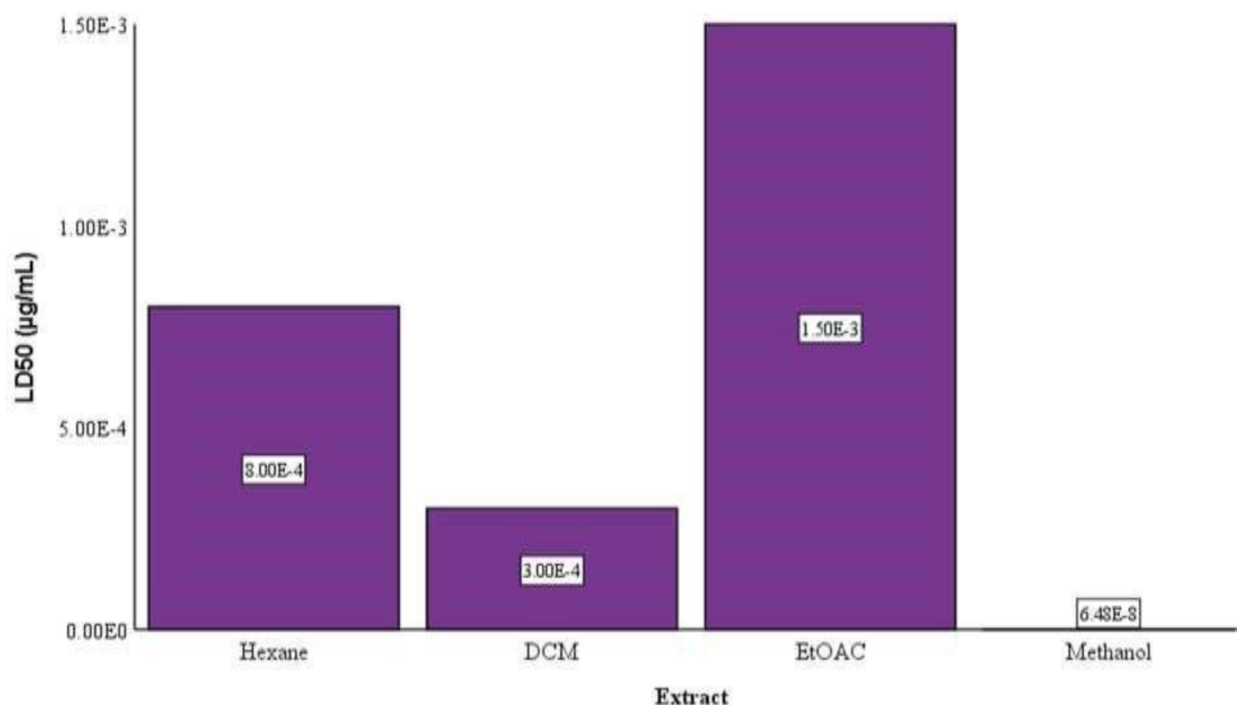


Figure 4.1: LC_{50} of crude solvent extracts of *S. urticifolia* against brine shrimp eggs

(E in the bar graph scale denotes x 10 e.g. 5.00E-4 is the same as 5.00×10^{-4})

From the results, it was observed that the LC_{50} of the extracts against the brine shrimp was lowest in methanol ($LC_{50} = 6.48 \times 10^{-8}$) followed by DCM ($LC_{50} = 0.0003$). Ethyl acetate showed the highest LC_{50} (0.0015). All the plant extracts showed high toxic activity against the brine shrimp eggs. This supports the fact that most of reported chemical insecticides used as mosquito larvicides show negative impact on the predators or natural enemies of mosquito larvae (Lawler *et al.*, 2007). The bioassay data also reveals that the cytotoxicity levels of different extracts was dependent on their concentration. This led to the cytotoxicity analysis of different solvent systems starting with hexane (Table 4.4). Linear graphs were

also generated to represent these relationships. Generally, the level of cytotoxicity increases with increasing concentration (Hassan *et al*., 2017).

Table 4.4: Probit analysis data of hexane extract of *S. urticifolia* against brine shrimp eggs

Conc (µg/mL)	Observed Responses (%)	Expected Responses (%)	Residual	Probability	Log C	Probit
0.1	60	60.9	-1.025	0.609	-1	5.28
1	68	65.9	1.897	0.659	0	5.41
10	71	70.6	0.234	0.706	1	5.54
100	73	75.0	-2.04	0.75	2	5.67
1000	80	79.1	0.935	0.791	3	5.81

The hexane extract showed high toxicity against the brine shrimp eggs. At 0.1, 1, 10, 100 and 1000 µg/mL, it showed percentage mortality of 60, 68, 71, 73 and 80%, respectively against 0.0% on a negative control of pure DMSO and artificial sea water (Baravalia *et al.*, 2012). The cytotoxicity of hexane was increasing with increasing concentration (Hassan *et al*., 2017).

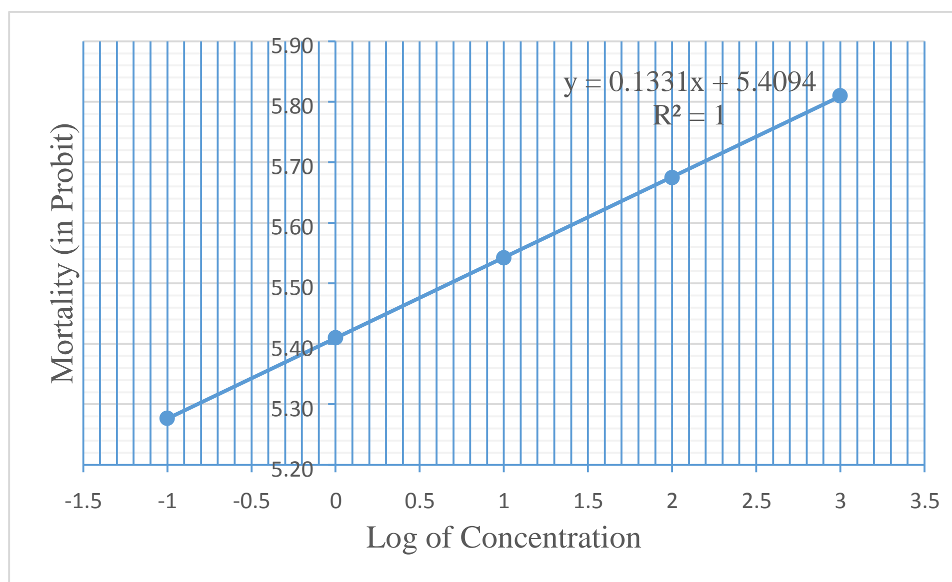


Figure 4.2: LC_{50} of Hexane crude solvent extracts of *S. urticifolia* against brine shrimp eggs

The probit graph gave a line with a positive gradient. At 0.1, 1, 10, 100 and 1000 µg/mL, it showed probit percentage mortality of 5.28, 5.41, 5.54, 5.67 and 5.81%, respectively. The high toxicity showed by hexane extract against brine shrimp pointed to the fact that it could contain bioactive substances (Asaduzzaman *et al.*, 2015).

Table 4.5: Probit analysis data of DCM extract of *S. urticifolia* against brine shrimp eggs

Conc (µg/mL)	Observed Responses (%)	Expected Responses (%)	Residual	Log C	Probability	Probit
0.1	75	77.754	-3.174	-1	0.778	5.77
1	89	85.656	2.874	0	0.857	6.07
10	94	95.226	-1.596	1	0.952	6.37
100	95	91.405	2.375	2	0.914	6.66
1000	97	97.548	-0.448	3	0.975	6.96

The DCM extract also showed high toxicity against brine shrimp eggs. At 0.1, 1, 10, 100 and 1000 µg/mL, it showed percentage mortality of 75, 89, 94, 94 and 97%, respectively.

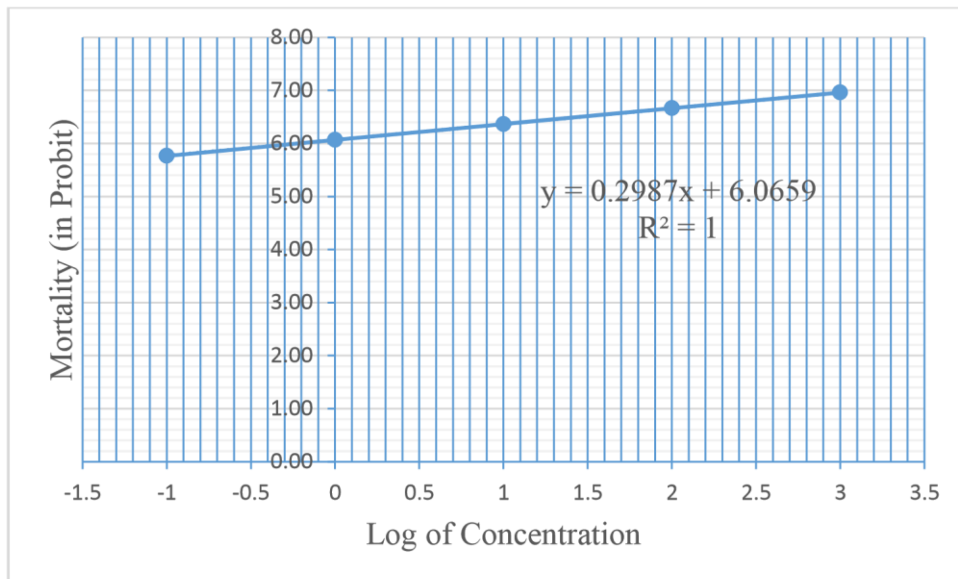


Figure 4.3: Mortality of brine shrimp eggs as a function of concentration of DCM *S. urticifolia* extract

The DCM probit mortality at log of concentration -1, 0, 1, 2 and 3 were 5.77, 6.07, 6.37, 6.66 and 6.96, respectively. These high values also indicated the possibility of bioactive compunds (Shaheen and Mominur, 2019).

Table 4.6: Probit analysis data of Ethyl acetate extract of *S. urticifolia* against brine shrimp eggs

Conc (µg/mL)	Observed Responses (%)	Expected Responses (%)	Residual	Probability	Log C	Probit
0.1	66	70.323	-4.553	0.703	-1	5.53
1	83	79.47	3.93	0.795	0	5.82
10	89	86.693	2.707	0.867	1	6.11
100	92	91.941	-0.221	0.919	2	6.40
1000	94	95.451	-1.771	0.955	3	6.70

The EtOAc extract also showed high mortality of brine shrimp eggs. At 0.1, 1, 10, 100 and 1000 µg/mL, it showed percentage mortality of 66, 83, 89, 92 and 94%, respectively. The mortality was increasing with increasing concentration of the extract (Hassan *et al*., 2017).

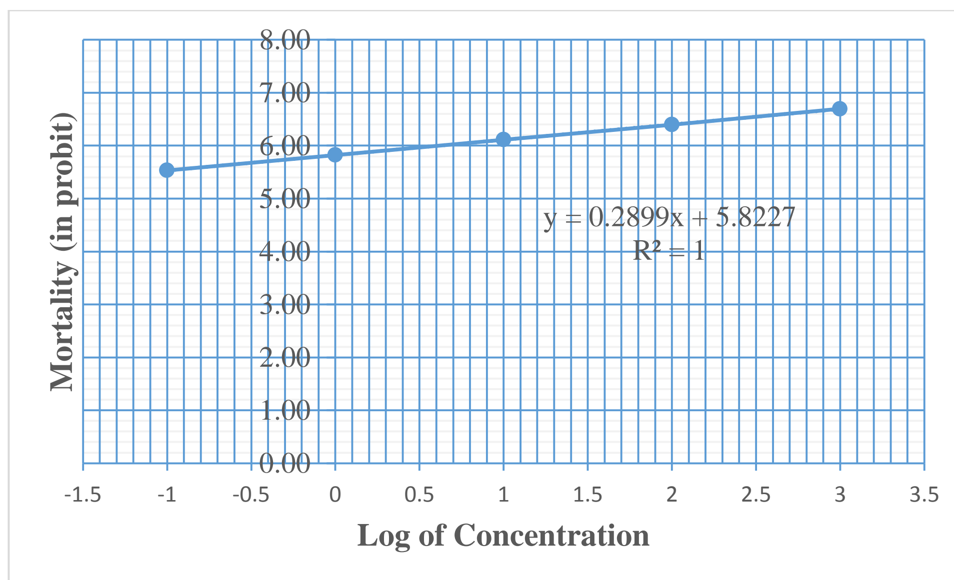


Figure 4.4: Mortality of brine shrimp eggs as a function of concentration of ethyl acetate *S. urticifolia* extract

The probit mortality values were high; 5.53, 5.82, 6.11, 6.40 and 6.70 at concentrations of 0.1, 1, 10, 100 and 1000 µg/mL, respectively. Lawi *et al*., (2018) reported that EtOAc extracts showed high mortality against brine shrimp eggs.

Table 4.7: Probit analysis data of methanol extract of *S. urticifolia* against brine shrimp eggs

Conc (µg/mL)	Number of Subjects	Observed Responses	Expected Responses	Residual	Probability	Log C	Probit
0.1	100	90	88.49	1.15	0.885	-1	6.20
1	100	92	91.864	-2.144	0.919	0	6.40
10	100	94	94.432	1.238	0.944	1	6.59
100	100	96	96.313	-0.483	0.963	2	6.79
1000	100	98	97.64	0.24	0.976	3	6.98

The methanol extract being most polar of the solvents used showed the highest mortality; 0.1 µg/mL= 90%, 1 µgmL= 92, 10 µgmL= 94, 100 µgmL= 96 and 1000 µg/mL= 98%.

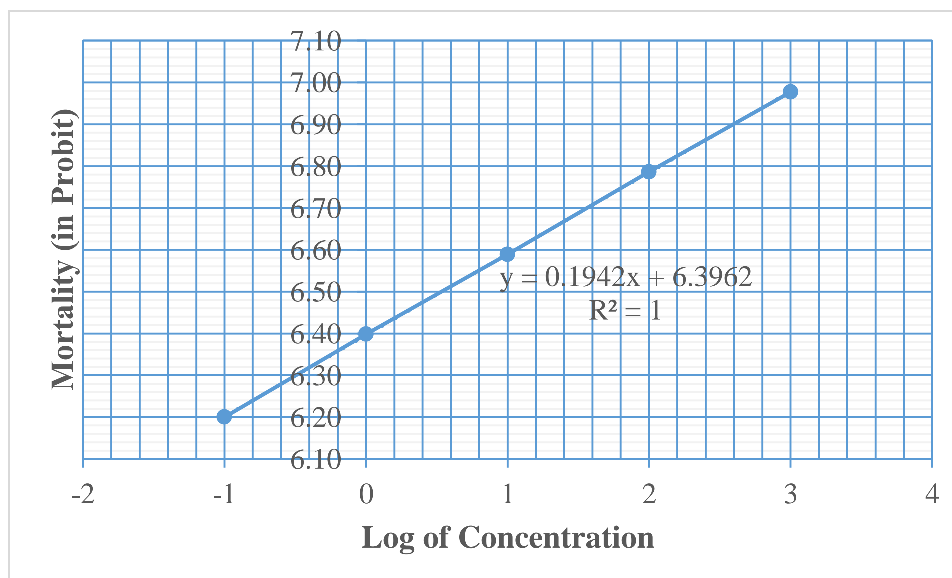


Figure 4.5: Mortality of brine shrimp eggs as a function of concentration of *S. urticifolia* MeOH extract

The MeOH linear graph was the steepest of all the extracts. Methanolic extracts have been reported to poses high cytotoxicity against brine shrimp eggs (Gosh *et al*., 2015). This increment in percent mortality due to increase in concentration of brine shrimp eggs shown

by *S. urticifoli*a indicates the presence of cytotoxic principles in the extracts. Phytochemical screening done on the crude extracts revealed the presence of alkaloids and steroids. The cytotoxic effect observed could be a result of the presence of such compounds. Additionally, alkaloids and steroids have been implicated in the cytotoxic activity of plant extracts (Badami *et al*., 2003; Vijayan *et al*., 2004). The high toxicity of *S. urticifolia* extracts in a bioassay for brine shrimp lethality also indicates the presence of bioactive compounds in the plant (Asaduzzaman *et al.*, 2015).

4.3 Larvicidal activities of crude solvent extracts of *S. urticifolia*

The results of *In-vitro* larvicidal activity assays of the various solvents extracts of *S. urticifolia* against 4th instar larvae of *A. gambiae* are shown on table 4.8. It was observed that the extracts from polar solvents showed higher mortality levels than those from non-polar solvents at lower concentrations (Table 4.8)

Table 4.8: Raw data on In-vitro larvicidal activity of solvent extracts of *S. urticifolia* against 4th instar larvae of *A. gambiae*.

Plant Extract	Concentration (ppm)	Number of larvae surviving after 24 h			Total number surviving	% mortality
		Test 1	Test 2	Test 3		
Hexane	1	8	7	7	22	26.66
	10	6	5	5	16	46.7
	100	4	3	3	10	66.7
	1000	0	0	0	0	100
DCM	1	6	5	5	16	46.7
	10	3	4	4	11	63.3
	100	2	2	2	6	80
	1000	0	0	0	0	100
EtOAc	1	5	3	3	11	63.3
	10	4	3	3	10	66.7
	100	2	1	2	5	83.3
	1000	0	0	0	0	100
MeOH	1	3	2	1	6	80
	10	1	2	1	4	86.7
	100	0	0	0	0	100
	1000	0	0	0	0	100

For instance at 1 ppm the percentage mortality range was from 26.66 to 80 for hexane to MeOH. At high concentration (1000 ppm) all the crude extracts recorded 100 percent mortality. When the above raw data was analyzed on probit software according to section 3.1.9, the LC_{50}, LC_{75}, LC_{95} and LC_{99} values obtained are given in table 4.9 below.

Table 4. 9: *In-vitro* larvicidal activity of solvent extracts of *S. urticifolia* against 4th instar larvae of *A. gambiae*

Extract	Slope	intercept	LC_{50} (ppm)	LC_{75} (ppm)	LC_{95} (ppm)	LC_{99} (ppm)
Hexane	0.7668	4.214	10.59	80.29	1479.54	11451.92
Dichloromethane	0.644	4.7942	2.09	23.28	747.63	8548.83
Etylacetate	0.5113	5.1492	0.51	10.65	841.59	18111.20
Methanol	0.6777	5.7356	0.08	0.81	21.96	222.38

The hexane extract showed the lowest in-vitro larvicidal activities at all tested concentrations (LC_{50} = 10.59, LC_{95} = 1479.54). A previous study on mosquito larvicidal action of the *Lantana camara* extract against larvae of *Aedes aegypti* showed a LC = 30.71 mg/L (Irrusappan and Nisha, 2018). This supports the studies conducted by other researchers to the effect that plants in this family contain larvicidal metabolites (Kumar *et al*., 2011). A control set up containing 2 mL of acetone in 200 mL of water showed a zero activity. From this observation, it was noted that highly polar extracts possess higher larvicidal activities, methanol LC_{50} = 0.08, ethyl acetate LC_{50} = 0.51 and dichloromethane LC_{50} = 2.09, compared to less polar hexane extracts (LC_{50} = 10.59). This further pointed to the fact that the active phytochemical principles responsible for the larvicidal properties may be polar compounds (Irrusappan and Nisha, 2018). These results corroborate with those of cyclic terpenes, isolated from Hexane, DCM, EtOAc and MeOH extracts of *S. urticifolia* (Santos *et al*., 2008).

When a bar graph for mean probit was drawn for LC_{50} to compare the different effects of the various solvent extracts under the study, it gave the shape below.

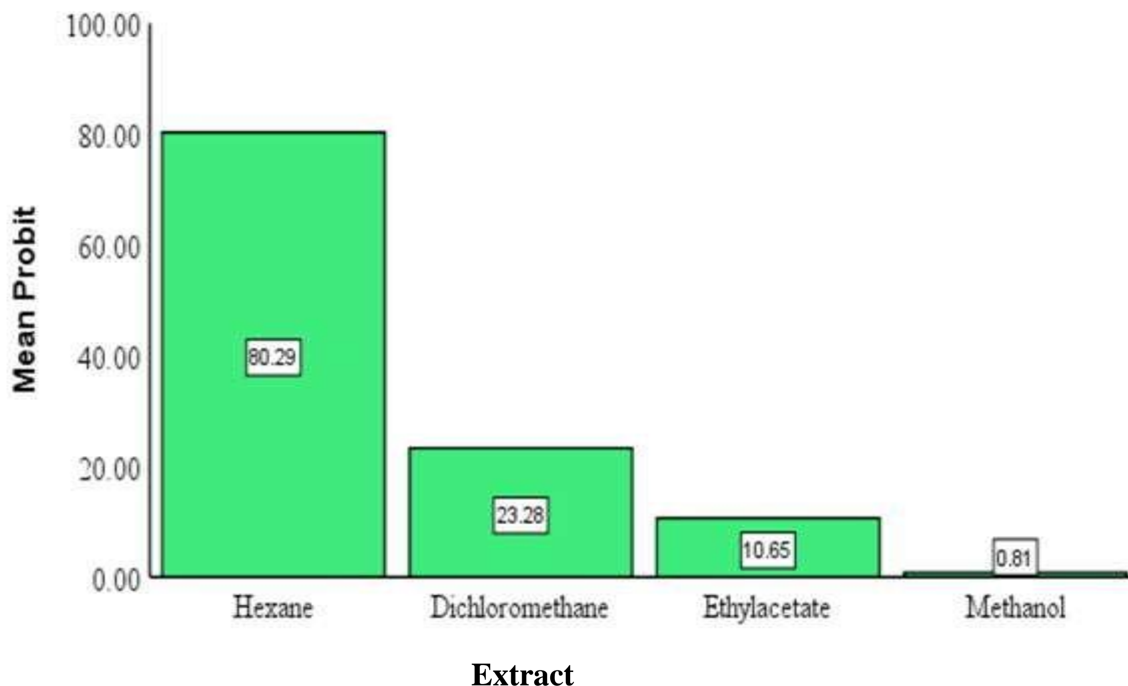


Figure 4.6: LC_{50} for various extracts on larvae of *A. gambiae*

The Hexane extract showed the lowest activity against *A. gambiae* 4th instar larvae, mean probit LC_{50} = 80.29, DCM = 23.28, EtOAc = 10.65 while the methanolic extract being most polar was quite effective against *A. gambiae* 4th instar larvae (mean probit LC_{50} = 0.81).

The mortality of the larvae of *A. gambiae* was found to be dependent on the concentration of the crude extracts (Omondi, *et al.,* 2017). Individual solvent analysis and linear graphs were then drawn showing the relationship between the mortality of the *A. gambiae* 4th instar larvae against the concentration of the various solvent extracts starting with hexane, followed by DCM, ethyl acetate and MeOH.

4.4: Probit analysis data of Hexane extracts on larvae of *A. gambiae*

Concentration (ppm)	Number of Subjects	Observed Responses	Expected Responses	Residual	Probability	Log C	Probit
1	30	8	6.472	1.528	0.216	0	4.21
10	30	14	14.77	-0.77	0.492	1	4.98
100	30	20	23.186	-3.186	0.773	2	5.75
1000	30	30	28.056	1.944	0.935	3	6.51

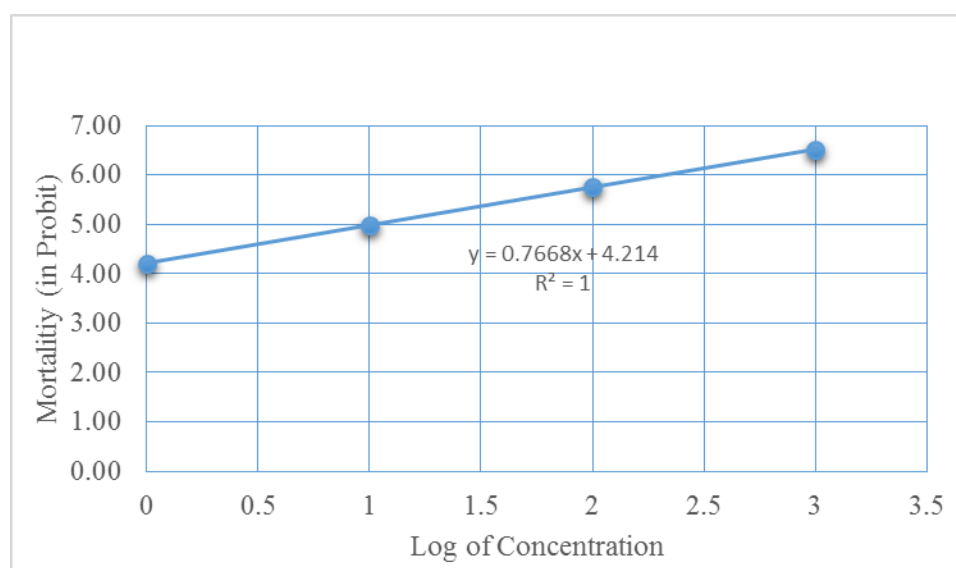


Figure 4.7: Mortality of larvae of *A. gambiae* as a function of concentration of hexane

Even though there was reduced activity of the hexane extract (Rahuman and Venkatesan, 2008; Raghavendra *et al*., 2009) it showed increase in activity at higher concentrations. Mostafa *et al*. (2017) reported that larvicidal activity of extracts increases with increase in concentration. At concentrations of 1, 10, 100 and 1000 ppm, it showed percentage mortality of 26, 46, 66 and 100% respectively.

4.5: Probit analysis data of DCM extracts of larvae of *A. gambiae*

Concentration (ppm)	Log Conc	Number of Subjects	Observed Responses	Expected Responses	Probability	probit
1	0	30	14	12.567	0.419	4.80
10	1	30	19	20.085	0.669	5.44
100	2	30	24	25.809	0.86	6.08
1000	3	30	30	28.732	0.958	6.73

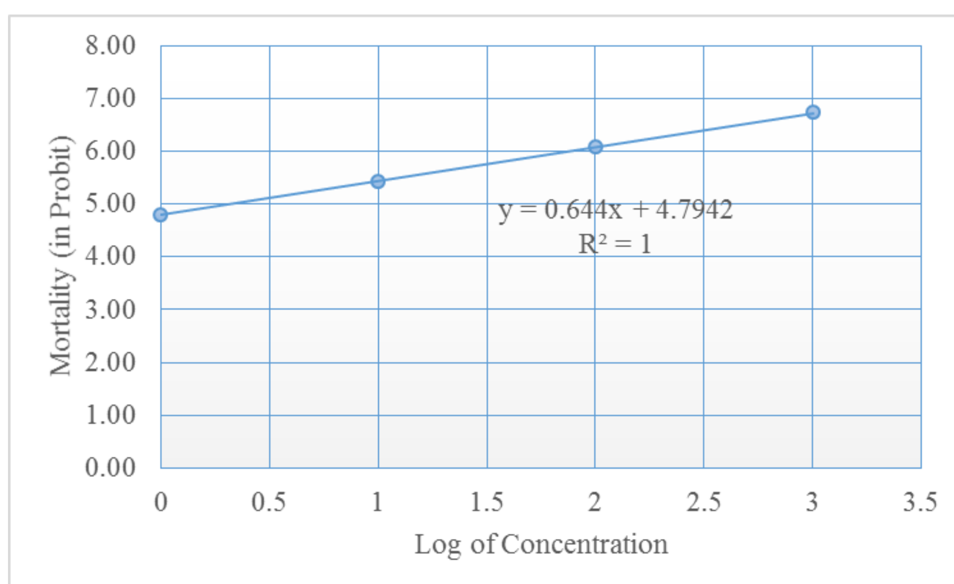


Figure 4.8: Mortality of larvae of *A. gambiae* as a function of concentration of DCM

The DCM extract also showed increased activity at high concentrations. It showed mortalities of 46, 63, 80 and 100% for concentrations of 1, 10, 100 and 1000 ppm respectively against 0.0% mortality for the control. Tennyson *et al*. (2012) reported 100% mortality after 48 h of exposure DCM extract against 4th instar mosquito larvae.

Table 4.12: Probit analysis data of ethyl acetate extracts of larvae of *A. gambiae*

Concentration (ppm)	Number of Subjects	Observed Responses	Expected Responses	Residual	Probability	Log of C	Probit
1	30	19	16.795	2.205	0.56	0	5.15
10	30	20	22.364	-2.364	0.745	1	5.66
100	30	25	26.371	-1.371	0.879	2	6.17
1000	30	30	28.605	1.395	0.954	3	6.68

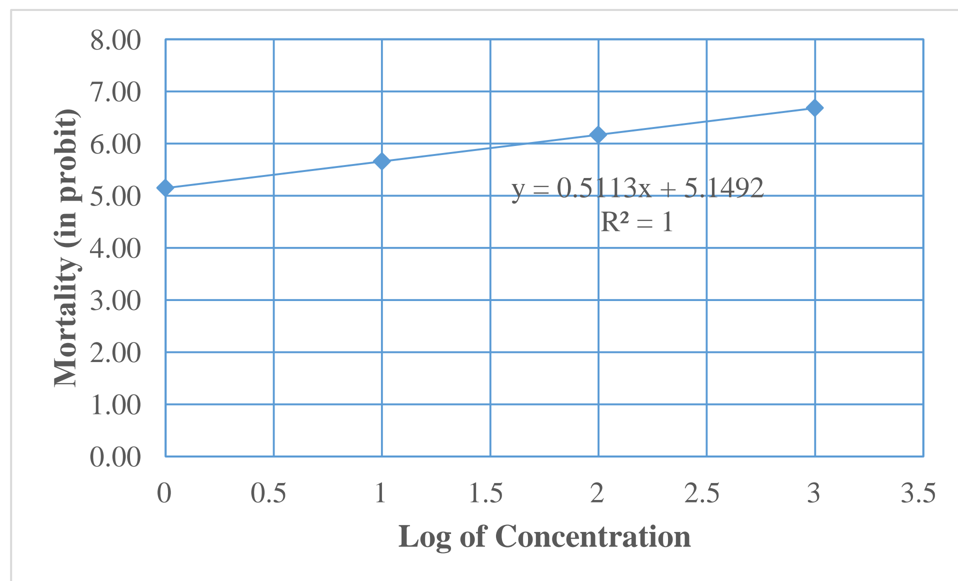


Figure 4.9: Mortality of larvae of *A. gambiae* as a function of concentration of ethyl acetate

The ethyl acetate extract showed high percentage mortality even at low concentrations (Tennysson *et al*., 2012). At 1, 10, 100 and 1000 ppm, it showed percentage mortalities of 63, 66, 83 and 100%, respectively.

Table 4.13: Probit analysis data of methanol extracts of larvae of *A. gambiae*

Concentration (ppm)	Number of Subjects	Observed Responses	Expected Responses	Residual	Probability	Log of C	Probit
1	30	24	22.953	1.047	0.765	0	5.72
10	30	26	27.649	-1.649	0.922	1	6.42
100	30	30	29.476	0.524	0.983	2	7.12
1000	30	30	29.924	0.076	0.997	3	7.75

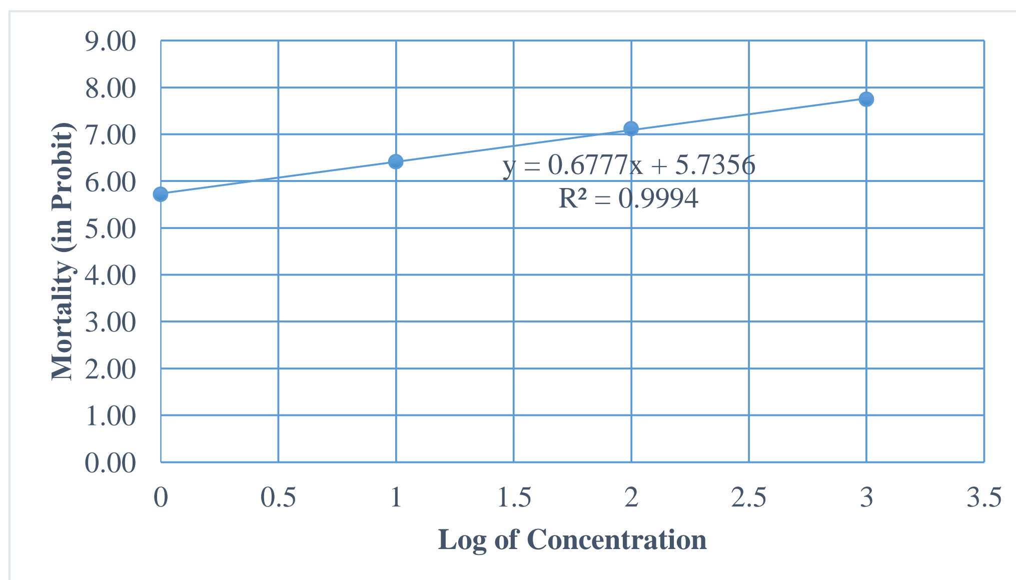


Figure 4.10: Mortality of larvae of *A. gambiae* as a function of concentration of methanol

The methanolic results above shows a probit mortality of 5.72, 6.42, 7.12 and 7.75 at concentrations 1, 10, 100 and 1000 ppm, respectively as opposed to 0.0% for the control system. (Devan *et al*., 2015) reported that methanolic extracts were very effective against the 4th instar larvae of all the three mosquito species.

4.6 Phytochemical analysis

This was performed on crude hexane, DCM, EtOAc, and MeOH extracts. *S. urticifolia* phytochemical screening revealed the presence of flavonoids, steroids, tannins, terpenoids and alkaloids (Table 4.14). The presence of the phytochemical was indicated by (++) for a lot of it (+) for just detected while non- detection was shown by (-).

Table 4.14: Phytochemical analysis of crude extracts of *S. urticifolia*

Fraction	Hexane	Dichloromethane	Ethyl acetate	Methanol
Flavonoids	++	++	-	-
Saponins	-	-	-	-
Alkaloids	-	+	+	+
Steroids	+	+	-	-
Terpenoids	++	++	++	++
Tannins	-	-	-	+

Key: ++ High concentration + Just detected - not detected

Terpenoids were found in all solvent extracts, whereas tannins were found only in the methanol extract. In hexane and dichloromethane extracts, flavonoids and steroids were detected. Saponins were absent from all extracts. This is supported by the fact that this family of plants contains plenty of terpenes (Salimena, 2000). Terpenoids and alkaloids have also been reported to contain larvicidal properties (Irrusappan and Nisha, 2018). This corroborates with the findings which showed the structures proposed from the DCM and EtOAc extracts to be terpenoids. The larvicidal activities were also found to be higher in the same solvent systems (Mostafa *et al*., 2017).

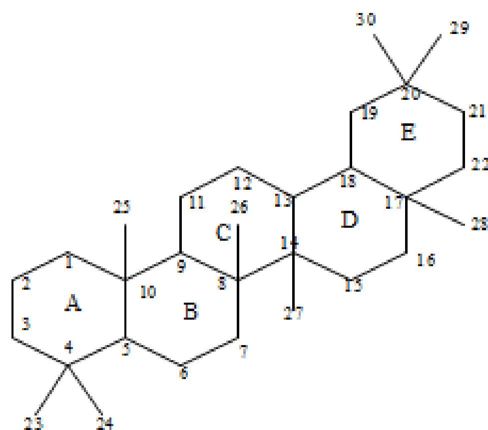
4.7 Structural elucidation of compounds isolated from *S. urticifolia*

4.7.1 Structural elucidation of compound SUS 1

SUS 1 (11mg) was isolated as a white amorphous solid (EtOAc/MeOH, 7:3) with a refractive index of 0.37 and a melting point of 206-209 ^{0}C. When developed spots were sprayed with anisaldehyde, they turned purple, indicating that the compound is a terpenoid.

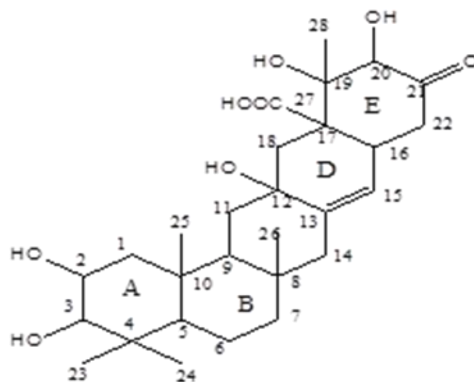
Five methyl groups were detected in the ^{1}H NMR (200 MHz) spectrum of SUS 1 (Appendix 1a) (δ_H 0.88, 0.96 and 1.18), seven methylene groups (δ_H 1.78, 1.43, 2.12, 1.87, 1.97, 1.73, 1.28, 1.58 and 1.93), six methine groups (δ_H 3.94, 2.36, 3.14, 3.4, 2.39 and 3.84). The compound also showed olefinic proton at δ_H 5.8 attributed to a double bond attributed to cyclic terpenoids (Agrawal and Dharam, 1992).

The ^{13}C NMR (100 MHz) broadband spectrum of the compound (Appendix 1b) revealed 28 signals which according to the DEPT spectrum (Appendix 1c) were classified as five methyl groups(δ_C, 18.1, 21.0 and 24.4), assigned positions 23, 24, 25, 26 and 28; nine quaternary carbon atoms (δ_C 206.5, 167.9, 85.2, 71.3, 39.3, 49.5, 138, 77.9 and 28.9) assigned to positions 21, 27, 12, 19, 4, 8, 13, 17 and 10 respectively; seven methylene groups; δ_C 42.4 (6), 37.3 (1), 27.3 (8), 32.5 (14), 32.8 (7), 31.8 (11), 27.3 (18) and 21.4 (22) and seven methine groups (δ_C 122.1 (15), 35.1 (16), 51.7 (5), 50.5 (9), 68.5 (3), 68.7 (2) and 78.4 (20). The carbon skeleton was found to be a derivative of oleanane type of tritepenoids whose structure is shown below.



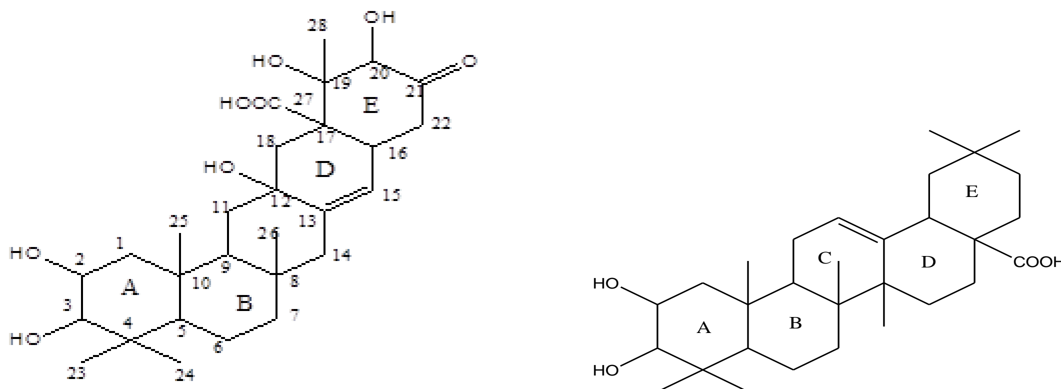
Structure of an oleanane

The oleananes structures have been reported to contain aldehydic or keto groups in either ring-A or E (Zhou *et al*., 2015). Aldehyde keto group in a compound can be detected by the appearance of a ^{13}C signal with methine characteristics resonating between δc 204.0 to 209.2 in the ^{13}C spectrum (Agrawal and Dharam, 1992). Based on this δc 206 was assigned to position 21. The compound was proposed to be a modified oleanane triterpene with the carbon skeleton below.



Proposed Carbon skeleton for SUS 1

The presence of olefinic hydrogen at δ_H 5.7 was confirmed by the CH signal at δ_C 122.1 (position 15) on the ^{13}C spectrum. The methine and quaternary carbon atoms that are olefinic in nature are detected by the presence of chemical shifts between δ 121.0-125.5 (Agrawal and Dharam, 1992). The assignment of the –OH groups at positions (2) δc 68.7, (3) δc 68.5, (12) δc 85.2, (19) δc 71.3 and (20) δc 78.4 was based on the fact that the signals of hydroxylated carbon atoms usually resonate between 60-80 ppm (Agrawal and Dharam, 1992). The presence of a keto group is shown by the occurrence of a resonance between 198.4-219.0 ppm (Agrawal and Dharam, 1992), hence the assignment of a keto group at position (21). The ^{13}C NMR (100 MHz) and DEPT spectra for ring A and B (C1 –C10) for **SUS 1** were found to have similarities with those of Maslinic acid (Nguyen *et al*., 2017).



Structure of SUS 1 and Maslinic acid

Table 4.15: The ^{13}C-NMR and ^{1}H-NMR data for ring A and B for SUS 1 and Maslinic acid

C	**SUS 1** (CD_3OD)		**Maslinic Acid** (CD_3OD)	
	δc 75MHz	δH (200MHz), m, *J* in Hz	δC (125 MHz)	δH (500 MHz), m, *J* in Hz
1	48.5	1.78(m), 1.43 (m)	48.2	0.92 (m),1.95(m)
2	68.7	3.84 (m)	69.5	3.64 (dt,4.5,10.0)
3	85.2	3.94 (d)	84.4	2.93 (d, 10.0)
4	38.8	-	40.6	-
5	55.6	2.36 (m)	56.7	0.86 (m)
6	21.5	1.78 m, 1.43 m	19.6	1.15 (m), 1.58 (m)
7	42.5	2.12 m, 1.87 m	33.8	1.52 (m), 1.57 (m)
8	39.9	-	40.5	-
9	49.7	2.39 m	49.0	1.65 (m)
10	39.9	-	39.3	-

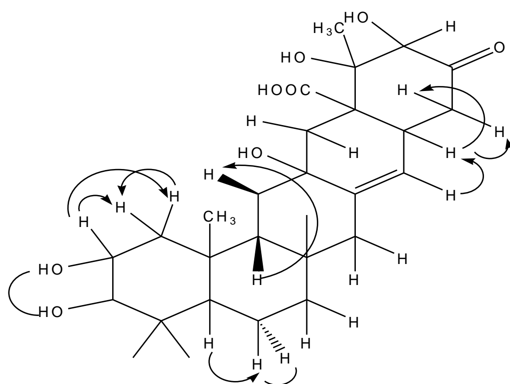
Both structures show quaternary carbon atoms at positions 4, 8 and 10, hydroxylated centres at carbon 2 and carbon 3 and methylene groups at position 1, 6 and 7. The δ_C for the quaternary carbon atoms are 38.8, 39.9 and 39.9 for **SUS 1** and 40.6, 40.5 and 39.3 for maslinic acid, respectively. The hydroxylated centres have δ_C of 68.7 and 85.2 in **SUS 1** and 69.5 and 84.4, respectively in maslinic acid. The methylene carbon atoms also show related δ_C of 48.5, 21.5 and 42.5 for SUS 1 and 48.2, 19.6 and 33.8, respectively for maslinic acid. The DEPT information for both **SUS 1** and maslinic acid were also found to be

corroborating; carbons at the same position being either a quaternary, a CH or a CH_2 as can be seen in table 4.17 below.

Table 4.16: The DEPT data for ring A and B for SUS 1 and maslinic acid

Carbon	SUS 1		Maslinic Acid	
	δC	DEPT	δC	DEPT
1	48.5	CH_2	48.2	CH_2
2	68.7	CH	69.5	CH
3	85.2	CH	84.4	CH
4	38.8	-	40.6	-
5	55.6	CH	56.7	CH
6	21.05	CH_2	19.6	CH_2
7	42.5	CH_2	33.8	CH_2
8	39.9	-	40.0	-
9	49.7	CH	49.0	CH
10	39.9	-	39.3	-

The COSY spectrum of the compound (Appendix 1d) showed important cross-peaks between the following protons: δ_H5.8/ δ_H 3.14; δ_H 3.14/ δ_H 1.78, 1.95; δ_H 2.39/ δ_H 1.93; δ_H 2.36/δ_H 1.78; δ_H 1.78/δ_H 1.43; 3.84/δ_H 1.78, 1.43; δ_H 1.78/1.43



Important COSY relations for SUS 1

This information is summarized in the table below.

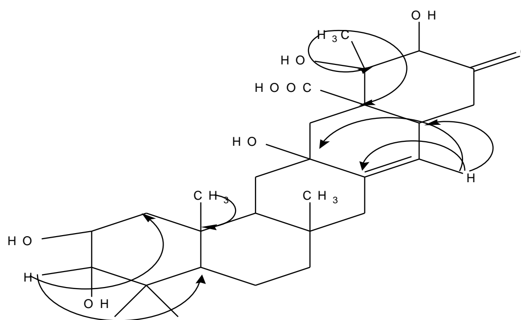
Table 4.17: Splitting patterns, COSY and major HMBC correlations of compound SUS 1

S/no	δC	δH, m, *J*	COSY (major)	HMBC (major)
1	48.5	1.78, m, 1.43, m,	H-2, H-20, H-5	C-27
2	68.7	3.84, m	H-1a, 1b	
3	85.2	3.94, s		C1 , C-5
4	38.8	Q		
5	55.6	2.36, m	H-6a	
6	21.05	1.78, m 1.43, m	H-6a, 6b	C-21, C27
7	42.5	2.12,m, 1.87, m		C-21, C- 12
8	39.9	Q		
9	49.7	2.39, m	H-11a	C-26
10	39.9	Q		
11	31.8	1.93, m, 1.58,m	H-9	
12	85.2	Q		
13	138	Q		
14	32.5	2.12. m, 1.87,m		
15	122.1	5.8,d, *J*=2.4	H-16	C-13, C-16, C-12
16	35.1	3.14,m	H-15	C-27, C-21, C-15
17	77.9	Q		
18	27.3	1.73, m, 1.28,m		C-27
19	71.3	Q		
20	78.4	3.4, m	H-22a, 22b	
21	206	Q		
22	21.4	1.78, m, 1.95,m	H-20	
23	24.4	0.96, s		
24	24.4	0.96, s		C-3
25	21.0	1.18, m		C-10, C-19, C-17
26	18	0.88, s		C-14
27	167	Q		
28	21.0	1.18, m		

From the HSQC spectrum of the compound (Appendix 1f) the following Carbon – Hydrogen correlations were established for all the protonated carbons δ_C 122.1/ δ_H 5.8; δ_C 78.4 / δ_H 3.4; δ_C 68.7/ δ_H 3.84; δ_C 51.7/ δ_H 2.36; δ_C 50.5/ δ_H 2.39, δ_C 42.4/ δ_H 1.78, 1.43; δ_C 37.3/ δ_H 1.73, 1.43; δ_C 35.1/ δ_H 3.14; δ_C 32.5/ δ_H 1.87, 2.12; δ_C 31.8/ δ_H 1.93, 1.58; δ_C 27.3/ δ_H 1.73, 1.28; δ_C 24.4/ δ_H 0.96; δ_C 21.4/ δ_H 1.78, 1.95; δ_C 18.1/ δ_H 0.88; δ_C 21.0/δ_H 1.18; δ_C

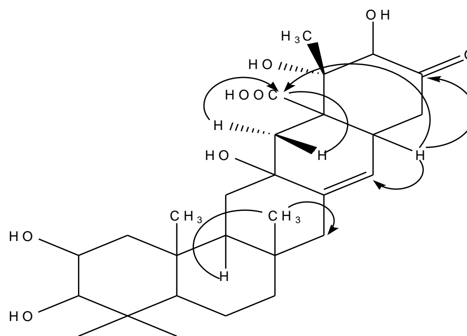
32.8/δ_H 2.12,1.87; δ_C 68.5/δ_H 3.94. These correlations justified the allocation of the carbons and the hydrogens in their respective positions within the structure.

The HMBC spectrum of the compound (Appendix 1e) revealed important three bond C/H coupling signals. Thus, three bond multiple correlations was observed between the proton (δ_H 5.8) and three carbon atoms (δ_C 85.2, 35.1 and 138). The proton (δ_H 3.94) showed three bond multiple correlation with two carbon atoms (δ_C 37.3 and 51.7). Key HMBC correlations were also observed for methyl protons hence enabling their placement. The methyl protons (δ_H 0.96) showed strong correlation with δ_C 68.5. The methyl protons (δ_H 1.18) showed correlation with three carbon atoms (δ_C 28.9, 71.3 and 77.9)



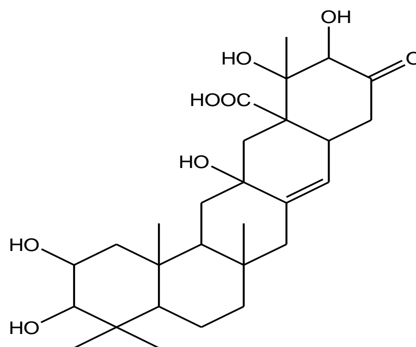
Major three bond Carbon - Hydrogen coupling for SUS 1

The methyl protons ((δ_H 0.88) showed correlation with one carbon (δ_C 32.5). The methylene protons (δ_H 1.73 and 1.28 showed a three bond correlation with one quaternary carbon (δ_C 167). The methine proton (δ_H 3.14) showed a three bond correlation with three carbons (δ_C 167, 206 and 122.1). Another methine proton (δ_H 2.39) showed an HMBC correlation with one methyl carbon (δ_C 18.0).



Major three bond carbon - hydrogen coupling for SUS 1

From the above spectral information and the melting point, the structure of the compound was found to be an oleanane triterpene suggested to be 1, 2, 3, 4, 4a, 5,6a, 7,8a, 9,10, 11, 12, 12a, 13,13a,14,14a,14b-icosahydro-2,3,11,12,13a-pentahydroxy-4,4,6a,12,14b-pentamethyl-10-oxobenzo[α]tetracene-12a-carboxilic acid. (**SUS 1**). The compound was found to be missing in the list of isolated compounds from this plant species.

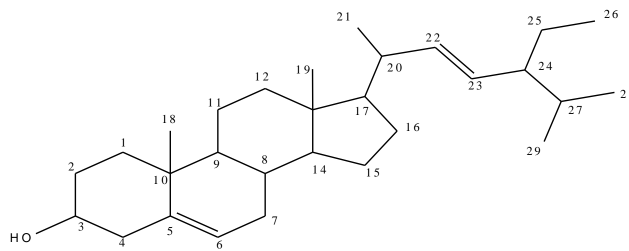


SUS 1

4.7.2 Structural elucidation of compound SUS 2

SUS 2 (15 mg) was isolated as a white powder with a melting point of 174-176 ^{0}C from the chloroform: ethyl acetate (1:1) fraction. TLC analysis revealed that the compound had an R_f of 0.7 in chloroform: ethyl acetate (1:1). When p-anisaldehyde was sprayed onto the chromatogram, it developed a purple color, indicating that the compound is a triterpenoid (Brenan and Sarada, 2011). The ^{1}H-NMR spectrum (Appendix 2a) revealed several triterpenoid-specific signals between 1.25 and 0.64 MHz. The diagnostic chemical shift values for C-18 and C-19 were 1.07 and 1.25 for the angular methyl protons, respectively. This is a property that cyclic terpenoids exhibit (Manasa, 2014). Four methyl groups were discovered at 0.92, 0.93, 1.00, and 1.02. (Chaturvedula and Prakash, 2012). At 5.51, the multiplet indicated the presence of a proton associated with a hydroxylated carbon atom. At 5.35, the presence of a doublet indicated the presence of a quaternary carbon atom with a double bond. At positions 5.15 and 5.05, two doublets of a doublet signal indicated the presence of a double bond at position 22, which is characteristic of a triterpenoid side chain (Kemp, 1993).

Compound **SUS 2** revealed twenty-nine carbon atoms in its ^{13}C-NMR (100 MHz) spectrum (appendix 2c), indicating that it is a modified triterpenoid. It detected signals at 140.8 and 121.7, confirming the presence of olefinic carbons and attributing the more deshielding signal to the bridge's quaternary carbon (C-5). The signal at 138.3 and 129.3 was assigned to the olefinic carbons C-22 and C-23 in the side chain, respectively (Rajput and Rajput, 2012). The peak at 71.8 was assigned to C-3 due to the presence of the hydroxyl group found in this class of compounds (Maniafu *et al.*, 2009). The three signals at 36.1, 36.5, and 42.3 were identified as quaternary carbon atoms C-20, C-10, and C-5, respectively. Through the use of HMQC and HMBC experiments, correlations between the major signals were established (appendix 2e and 2f). Due to the fact that the signal at 121.7 was correlated with 5.35 in the HMQC spectrum, the signals were assigned to C-6 and H-6, respectively. The correlation between the peak at 71.8 and 3.51 indicates the presence of a hydroxyl group at C-3. The spectral data (table 4.18) were strikingly similar to those of 3-stigmasterol, whose structure is depicted below (Rajput and Rajput, 2012). This is the first time this compound from *S. urticifolia* has been reported, as it was not previously discovered in the collection of isolated natural products from this plant.



Stigmasterol

Table 4.18: ^{1}H- and ^{13}C-NMR data for SUS 2 and reported data of 3β-stigmasterol

	SUS 2		3β-stigmasterol	
No	δ ($CDCl_3$, J, Hz)	δc ($CDCl_3$)	δ (MeOH, J)	δc (MeOH)
1		37.3		37.2
2	1.55 (m)	31.9	1.43 (m)	31.9
3	3.51 (m)	71.8	3.25 (m)	71.8
4		42.3		42.3
5		140.8		140.8
6	5.35 (1H, d)	121.7	5.14 (1H,m)	121.7
7		31.7		31.7
8		31.8		31.9
9		50.2		50.1
10		36.5		36.5
11		21.1		21.1
12		39.8		39.9
13		42.3		42.3
14		56.0		56.8
15		24.3		24.3
16		28.3		28.3
17		56.9		56.1
18	1.07 (3H,s)	11.9	1.07 (3H,s)	11.9
19	1.25 (3H,s)	19.4	1.26 (3H,m)	19.4
20		36.1		36.2
21	0.64 (3H,s)	21.1	0.91(3H,s)	18.8
22	5.15 (1H, m)	138.3	4.62 (1H, m)	129.3
23	5.05 (1H,m)	129.3	4.61 (1H, m)	121.7
24		51.3		45.8
25		31.9		29.2
26	1.02 (3H, s)	21.2	1.01(3H, s)	19.8
27	1.00 (3H,s)	19.1	1.00 (3H, s)	19.0
28		25.4		23.1
29	0.92 (3H,s)	12.2	0.97 (3H,s)	12.0

Based on the closeness in melting points, ^{I}H NMR, ^{13C} NMR and splitting patterns, it was therefore concluded that **SUS 2** is 3β-stigmasterol.

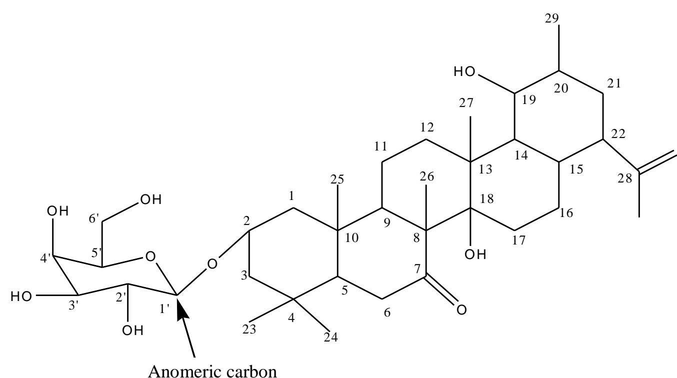
4.7.3 Structural elucidation of compound SUS 3

Compound **SUS 3** (14 mg) was isolated as a white crystalline solid (EtOAc/MeOH, 7:3), R_f = 0.87 and melting point of 197-199 ^{0}C. The TLC also gave a positive test for a terpenoid

when sprayed with anisadehide. The ^{1}H NMR (200MHz, CD_3OD) spectrum (Appendix 3a) showed signals due to seven methyl groups (δ_H 0.85, 0.85, 0.85, 0.6, 1.75, 1.45 and 2.1); Twenty methylene protons (δ_H 3.4, 3.61, 1.27, 1.67, 1.90, 1.20, 1.64, 0.95, 1.20, 1.20, 1.45, 1.290, 1.90, 0.45, 1.85, 1.87, 1,40, 2.4 and 1.82) and 11 methine protons (δ_H 3.07, 2.89, 4.21, 0.98, 2.60, 3.55, 0.85, 2.40, 3.56, 4.10 and 3.03).

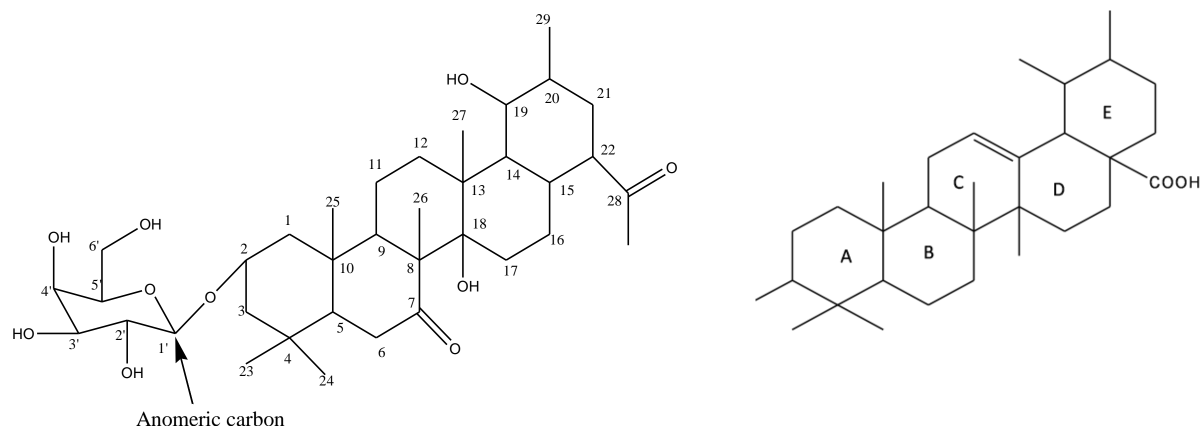
The ^{13}C NMR (100MHz, CD_3OD) broadband spectrum of the compound (Appendix 3b) showed the presence of a main structure or a glycone with 29 carbon atoms (a triterpene) and carbon range indicating a sugar moiety of 6 carbon atoms. The DEPT spectrum (Appendix 3c) showed the presence of eight quaternary carbon atoms (δ_C 48.7, 211 , 38.5, 83.6, 214, 38.9 and 28.9) assigned positions 4 and 17, 7, 8, 14, 28, ; seven methyl groups (δ_C 18.2 (24), 29.4 (26), 26.7, 21.0 (29), 17.2 (27) and 17.4

(25); ten methylenes (δ_C 61.1 (6'), 35.7 (3), 45.6 (6), 32.8 (15), 38.9 (16), 34.2 (22), 29.4 (26), 35.2 (12), 34.2 (11) and 33.9 (1), and eleven methine groups (δ_C 37.5 (20), 76.8 (3'), 73.5 (2'), 100.7 (1'), 54.4 (5), 76.7 (19), 49.0 (9), 75.1 (2), 70.1 (5), 70.0 (4') and 59.3 (18). The high chemical shift of δ_C 75.1 at position 2 showed the presence of an oxygenated centre thus influenced the placement of the sugar at *ortho* position in the first ring. The anomeric carbon was assigned a chemical shift of δ_C 100.7 since it is surrounded by several oxygenated centers (Agrawal *and* Dharam 1992). The carbon skeleton was proposed to be as show below.



The Carbon skeleton for SUS 3

The ^{13}C-NMR for **SUS 3** was found to have similarities in splitting patterns and DEPT information of rings D and E in the skeleton with that of Ursolic acid (Kumari, 2017).



Structure of SUS 3 and Ursolic Acid

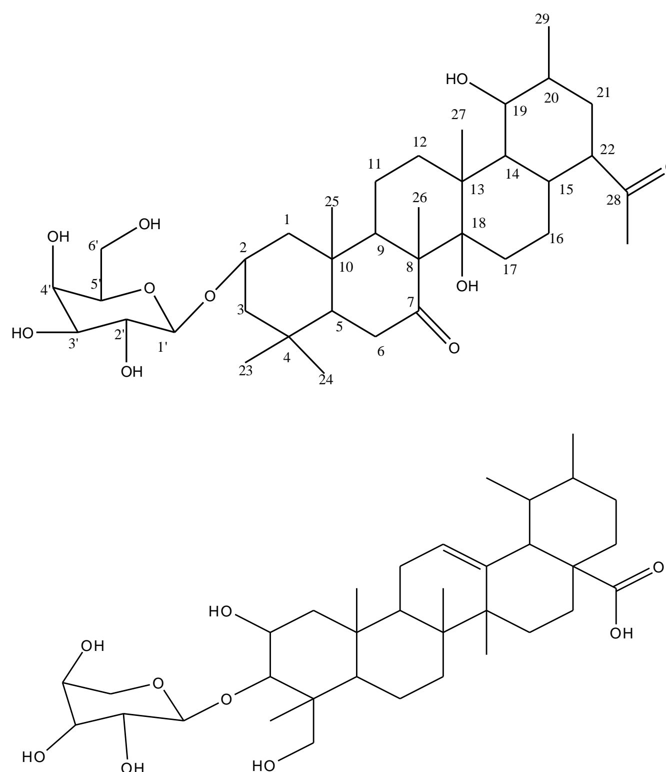
Table 4.19: ^{13}C-NMR data for SUS 3 and reported data for ursolic acid for rings D and E

	SUS 3		Ursolic acid	
C	δc (CD_3OD, 100 MHz)	DEPT	δc($CDCl_3$, 100 MHz)	DEPT
16	38.8	CH_2	34.2	CH_2
17	48.7	CH_2	46.2	CH_2
18	59.3	-	55.8	-
19	76.7	CH	73.4	CH
20	37.5	CH	35.5	CH
21	29.4	CH_2	30.8	CH_2
22	34.2	CH	36.8	CH_2
28	214	-	18.7	-
29	21.0	CH_3	21.0	CH_3

The δc values for positions 16 to 20 were slightly higher in **SUS 3** than in Ursolic acid. This can be attributed to the presence of hydroxylated centres at positions 18 and 19 in the **SUS 3** structure. At position 21, the δc is 30.8 in ursolic acid while it is 29.4 in **SUS 3**. This could be due to the presence of an OH group in carbon 28 of ursolic acid whereas there is keto group at the same position in **SUS 3**. The higher δc (36.8) at C-22 can also be attributed to the OH group at C-28 in ursolic acid as compared to δc 34.2 for the same carbon in SUS 3. There is a significance difference in δc at C-28; **SUS 3** is 214 while for ursolic acid 18.7. This is due to the presence of two adjacent methylene carbons on either side of C-28 in

ursolic acid and only one adjacent CH_2 in **SUS 3.** C-29 in both compounds gave similar values of δc 21.0.

The ^{13}C-NMR (100 MHz) chemical shifts for the sugar (C1' – C5') in **SUS 3** were also found to have similarities with that of 3-*O*-*α*-L-arabinopyranosyl asiatic acid (Bora *et al.*, 2018) below.



Structure of SUS 3 and 3-*O*-*α*-L-arabinopyranosyl asiatic acid

The anomeric carbon in **SUS 3** had a δc of 100.6 while that of the Asiatic acid showed δc of 106.3. Both values fall within the range of anomeric carbons in glycosidic compounds (Agrawal and Dharam, 1992).

Table 4.20: ^{13}C NMR and DEPT data for SUS 3 and *3-O-α-L-arabinopyranosyl* asiatic acid

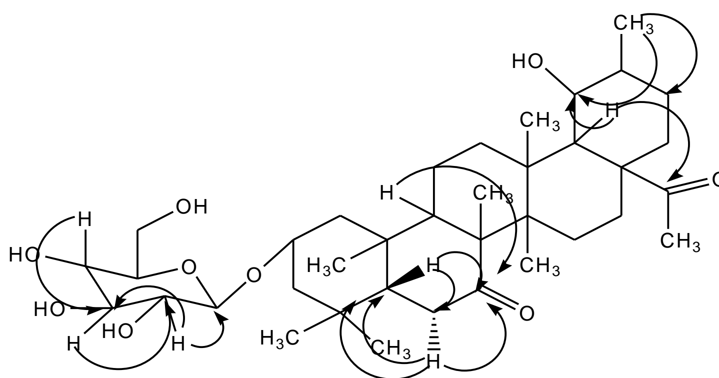
	SUS 3		***3-O-α*-L-arabinopyranosyl asiatic acid**	
C	δc ($CDCl_3$)	**DEPT**	δc (MeOH)	**DEPT**
1’	100.6	CH	106.3	CH
2’	73.5	CH	72.9	CH
3’	76.8	CH	74.6	CH
4’	70.0	CH	70.1	CH
5’	70.1	CH	67.8	CH_2

Carbons 2’, 3’ and 4’ all showed δc ranging from 70.0 to 76.8. This was attributed to the presence of OH groups attached to these positions. However, a slight difference was noted at carbon 5’ where in **SUS 3** it is bonded to carbon 6’ which also bears an –OH group hence more deshielded (δc = 70.1) as compared to δc = 67.8 in the Asiatic acid which is a methylene carbon.

The HSQC spectrum of the compound (Appendix 3e) showed the following carbon-hydrogen correlations for all the protonated carbons, δ_C 33.9/δ_H 1.27, 1.67; δ_C 75.1/δ_H 3.56; δ_C 35.7/ δ_H 0.95,1.64; δ_C 45.6/δ_H 1.82, 2.4; δ_C 49.0/δ_H 2.4; δ_C 34.2/δ_H1.20,1.90; δ_C 35.2/δ_H 0.95, 1.64; δ_C 32.8/δ_H 1.40, 1.67; δ_C 38.9/ δ_H 0.45, 1.85; δ_C 59.3/δ_H 0.85; δ_C 76.7/δ_H 3.55; δ_C 37.5/δ_H 1.45, 2.60; δ_C 29.4/δ_H 1.20, 1.45; δ_C 34.2/δ_H 1.20, 0.90; δ_C 18.2/δ_H 0.85; δ_C 17.4/ δ_H 0.60; δ_C 29.4/ δ_H 1.78; δ_C17.2/δ_H 0.85; δ_C 21.0/δ_H 1.45; δ_C 100.7/δ_H 4.21; δ_C 73.5/δ_H 2.89; δ_C 76.8/δ_H 3.07; δ_C 70.0/δ_H 3.03; 7δ_C 70.1/δ_H 4.1 and δ_C 61.1/δ_H 3.4, 3.61. An overlap was observed between δ_C 18.0/δ_H 0.85 which enabled placement in two different methyl groups in the same chemical environment.

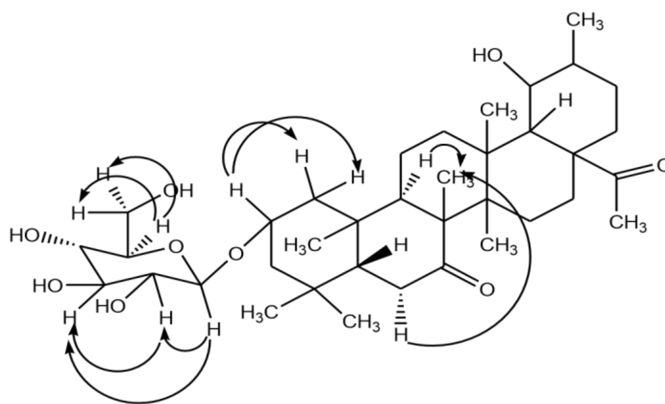
Important three bond C/H coupling signals were also revealed in the HMBC spectrum of the compound (Appendix 3f). The methine proton (δ_H 0.98) showed a correlation with two carbons (δ_C 45.6 and δ_C 211). The proton (δ_H 2.41) showed a three bond correlation with three carbons (δ_C 211, 54.4 and 48.7). The proton (δ_H 2.4) showed one correlation with carbon (δ_C 211). The proton (δ_H 1.20) also showed significant multiple bond correlation with the quaternary carbon (δ_C 38.5). The methine proton (δ_H 0.85) also showed a

correlation with the quaternary carbon (δ_C 214). The methylene proton (δ_H 1.45) showed a correlation with one methine carbon (δ_C 76.7). The methyl proton (δ_H 0.85) showed a multiple bond correlation with two carbons (δ_C 59.3 and (δ_C 76.7). The methyl proton (δ_H 1.45) showed a correlation with two carbons (δ_C 29.4 and δ_C 76.7). The carbon signal at (2) δc 75.1 indicated that a β-D-glucopyranosyl moiety was attached to the (2) position of the aglycone (Zhou *et al*., 2015).The following HMBC correlations were also observed in the sugar moiety; δ_H 2.89/δ_C 100.7, 76.8; δ_H 3.07/δ_C 73.5; δ_H 3.03/δ_C 76.8.



The major three bond Carbon - Hydrogen correlations.

The COSY spectrum (Appendix 3d) showed key cross peaks between the following protons δ_H 3.56/δ_H 1.27, 1.67; δ_H 1.82/δ_H 1.78; δ_H 2.4/δ_H 1.78; δ_H 4.21/δ_H 2.89; δ_H 2.89/δ_H 3.07; δ_H 4.21/δ_H 3.07 and δ_H 4.10/δ_H 3.40, 3.61.



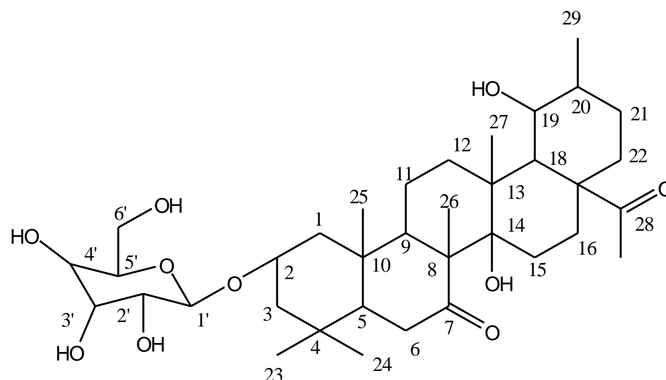
The major cross peak proton-proton correlations.

The major correlations are shown in table 4.21 below.

Table 4.21: Structure elucidation of compound SUS 3

S/no	δC	δH, m, *J*	COSY (major)	HMBC (major)
1	33.9	1.27, m, 1.67, m		
2	75.1	3.56, m	H-1a,1b	
3	35.7	0.95, m, 1.64, m		
4	48.7	q		
5	54.4	0.98, m		C-6, C-7
6	45.6	1.82, m, 2.41,m	H-26	C-5, C-4, C-7
7	211	q		
8	38.5	q		
9	49.0	2.4, m	H-26	C-7
10	28.9	q		
11	34.2	1.2, m, 1.9, m		C-13
12	35.2	0.95, m, 1.64, m		
13	38.9	q		
14	83.6	q		
15	32.8	1.4, m, 1.67,m		
16	38.9	0.45, m, 1.85, m		
17	48.7	q		
18	59.3	0.85, m		C-28
19	76.7	3.55m		
20	37.5	1.45, m, 2.6, m		
21	29.4	1.20, m, 1.45,m		C-19
22	34.2	1.2, m, 1.9,m		
23	18.2	0.85, m		
24	18.2	0.85,m		
25	17.4	0.6m		
26	29.4	1.78, m		
27	17.2	0.85, m		C-18,C-14
28	214	q		
29	21.0	1.45,m		C-21, C-19
1’	100.7	4.21,m	H-2’	
2’	73.5	2.89, m	H-3’, H-1’	C-3’, C-1’
3’	76.8	3.07, m	H-1’	C-2’
4’	70.0	3.03, m		C-3’
5’	70.1	4.1m	H-6’a,6’b	
6’	61.1	3.4, m 3.61, m	H-5’	

From the above comparisons and the spectral information the structure was proposed to be an ursane-type triterpene (Zhou *et al*., 2015) suggested as 8a-acetyl-octadecahydro-6b,8,12tihydroxy-4,4,6a,11,12b,14b-hexamethyl-2-(tetrahydroxo-3,4,5-trihydroxy-6-(hydroxymethyl)- 2H-pyran-2-yl oxy)picen-6(6aH,6bH ,14Bh)-one. SUS compound 3, the compound was isolated from the plant species for the first time, as it is not documented among isolated natural products.



SUS 3

4.7.4 Structural elucidation of compound SUS 4

Compound SUS 4 was isolated as colourless needlelike crystals from a chloroform:ethyl acetate (1:1) extract and had a melting point of 131-133^{0}C. In pure DCM, the compound had an R_f of 0.6 on TLC. When p-anisaldehyde was sprayed on the plates, the spot turned purple and then green, indicating that it was a triterpenoid (Khan *et al*., 2015).

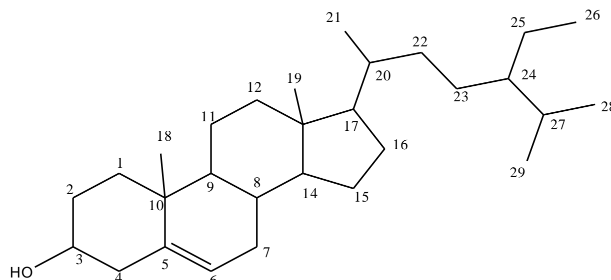
The proton ^{1}H-NMR spectrum (Appendix 4a) revealed three distinct regions: aliphatic, hydroxylated, and allylic, all of which strongly suggested the presence of a triterpenoid structure (Khan *et al*., 2015). At 5.20, the signal appeared as a triplet, indicating the presence of a quaternary carbon atom with a double bond. A multiple centered on 3.22 was observed when a proton germinal was attached to a hydroxyl group at C-3 in triterpenoids. Six signals corresponding to methyl groups were observed at 0.9 (3 CH_3), 1.00, 1.06 and 1.25, which are characteristic of a triterpenoid (Ahmad and Rahman, 1996). COSY correlations confirmed the presence of the characteristic peaks.

The ^{13}C-NMR spectrum (appendix 4c) revealed signals at the olefinic carbon atoms at 139.6 and 124.4, indicating the presence of a double bond at the end of a fused ring between C-5 and C-6 (Ahmad and Rahman, 1996). In the DEPT experiment, six methyl, eleven methylene, and nine methine carbon atoms were detected (Appendix 4d). The splitting patterns and ^{13}C-NMR peaks were consistent with previously published data for -sitosterol, as shown in table 4.9 (Rowshanul *et al*, 2007).

Table 4.22: ^{1}H-NMR and ^{13}C-NMR data for SUS 4 and reported data for β-sitosterol.

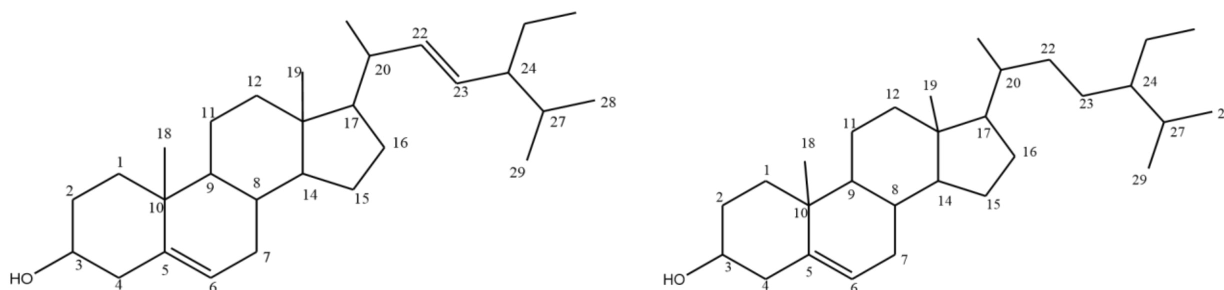
SUS 4			3 β-sitosterol	
No	δ ($CDCl_3$, J, Hz)	δc ($CDCl_3$)	δ (MeOH, J, Hz)	δc (MeOH)
1		37.2		37.2
2	1.55(m)	31.3	1.55(m)	31.9
3	3.22(m)	79.1	3.25 (m)	79.0
4	1,25 (d)	42.1	1.25(d)	42.3
5		139.6		140.8
6	5.20 (1H,t)	124.4	5.31 (1H,m)	121.7
7		32.1		31.7
8		31.9		31.9
9		47.7		50.1
10		37.0		36.5
11		21.4		21.1
12		39.7		39.9
13		41.5		42.3
14		55.2		56.8
15		23.3		24.3
16		28.1		28.3
17		59.1		56.1
18	1.06 (3H,s)	14.1	1.16 (3H,s)	11.9
19	1.25 (3H,s)	18.4	1.20 (3H, s)	19.4
20		34.8		36.2
21	0.90 (3H,s)	16.8	0.91 (3H, s)	18.8
22		33.8		34.0
23		26.2		26.1
24		46.9		45.8
25		29.4		29.2
26	1.00 (3H, s)	18.0	1.01 (3H, s)	19.8
27		17.5		19.0
28	0.90 ((3H,s)	23.4	0.98 (3H, s)	23.1
29	0.90 ((3H,s)	15.5	0.98 (3H, s)	12.0

SUS 4 was determined to be β-sitosterol based on the similarity of the ^{1}H-NMR and ^{13}C-NMR splitting patterns. Rahuman *et al*. (2008) demonstrated that β-sitosterol has larvicidal activity. This supports the fact that extracts containing this compound showed larvicidal activity in this study.



SUS 4

SUS 4 was found to have close ^{1}H NMR and ^{13}C NMR splitting patterns to those of **SUS 2.** However, there are differences at positions 22 and 23 brought about by the presence of a double bond in **SUS 2** and an absence of the same in **SUS 4.**



Structure of SUS 2 and SUS 4

These differences in the δc of **SUS 2** and **SUS 4** are illustrated in the table below.

Position	**SUS 2** δc ($CDCl_3$)	**SUS 4** δc ($CDCl_3$)
22	138.3	33.8
23	129.3	26.2

There are higher δc in **SUS 2** (138.3 and 129.3) due to the double bond as compared to δc 33.8 and 26.2, respectively for **SUS 4**

CHAPTER FIVE

CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

This study examined the larvicidal properties of *S. urticifolia* against mosquitoes (Sims). The study showed that:

i. The percentage yields of the extracts decreased with increasing polarity from 0.95 percent in hexane to 0.06 percent in DCM to 0.25 percent in EtOAc, but increased with increasing polarity to 0.806 percent in MeOH, indicating that the stem of Sims urticifolia (Sims) contains both non-polar and polar metabolites. The study shows that the stem of *S. urticifolia* (Sims) contains flavonoids, steroids, terpenoids, alkaloids and tannins.

ii. The cytotoxicity tests of the extracts against the brine shrimp eggs showed that the polar crude extracts of EtOAc and MeOH had high toxicity levels (LC_{50} =

0.005 and $6.48x10^{-8}$ ppm) respectively. Whereas the non-polar extracts showed (LC_{50}=0.0008 and 0.0003 ppm) for Hexane and DCM, respectively.

iii. The crude extracts from polar solvents; Ethyl acetate and methanol showed very high larvicidal activity (methanol LC_{50} = 0.08 and ethyl acetate LC_{50}=0.51) even though at high concentrations they were toxic to brine shrimp eggs.

iv. Two compounds, an oleanane triterpene;

1,2,3,4,4a,5,6a,7,8a,9,10,11,12,12a,13,13a,14,14a,14b-icosahydro-2, 3, 11, 12, 13 apentahydroxy-4, 4, 6a, 12, 14b-pentamethy l-10-oxobenzo [α] tetracene-12 acarboxilic acid (**SUS 1**) and an ursane-type triterpene 8a-acetyl-octadecahydro-6b, 8, 12-tihydroxy-4, 4, 6a, 11, 12b, 14b-hexamethy l-2-(tetrahydroxo-3,4,5trihydroxy-6-(hydroxymethyl)- 2H-pyran-2-yl oxy)picen-6(6aH,6bH,14Bh)-one (**SUS 3**) (EtOAc/MeOH, 7:3) were isolated for the first time from the plant species. In addition, two common plant sterols, 3β-stigmasterol (**SUS 2**) and

3βsitosterol (**SUS 4**), were also obtained from Chloroform: EtOAc (1:1). These were also reported for the first time from *S.urticifolia* (Sims).

5.2 Recommendations

i. The two new compounds were isolated in small quantities therefore they should be isolated in large quantities to enable further anti-larvicidal tests to determine their efficacy in pure form.

ii. To ascertain the in vitro interaction of larvicidal combinations, synergic effects tests should be conducted on crude extracts, isolated compounds, and conventional antilarvicidal compounds.

iii. Toxicity studies on the isolated compounds should also be conducted to ascertain any synergic or antagonistic effects on the crude extracts.

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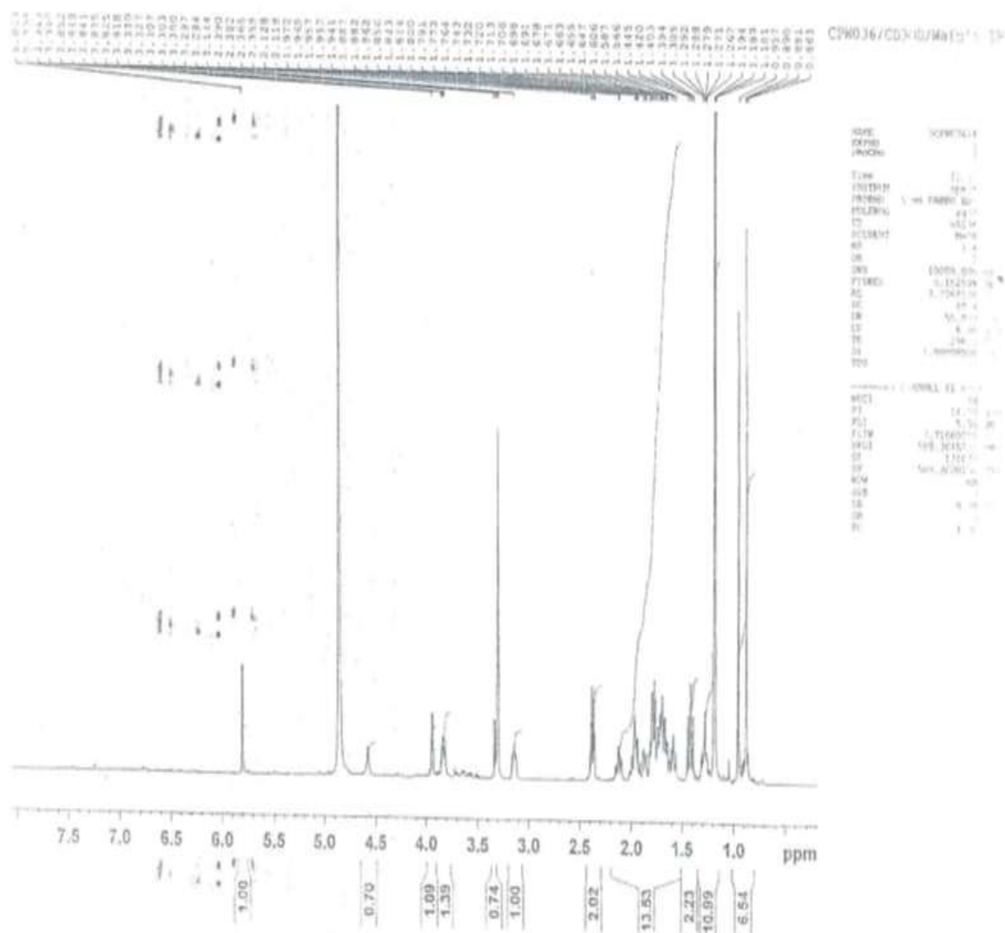
APPENDICES

Appendix 1a: ^{1}H NMR for SUS 1



Appendix 1b: ^{13}C NMR for SUS 1

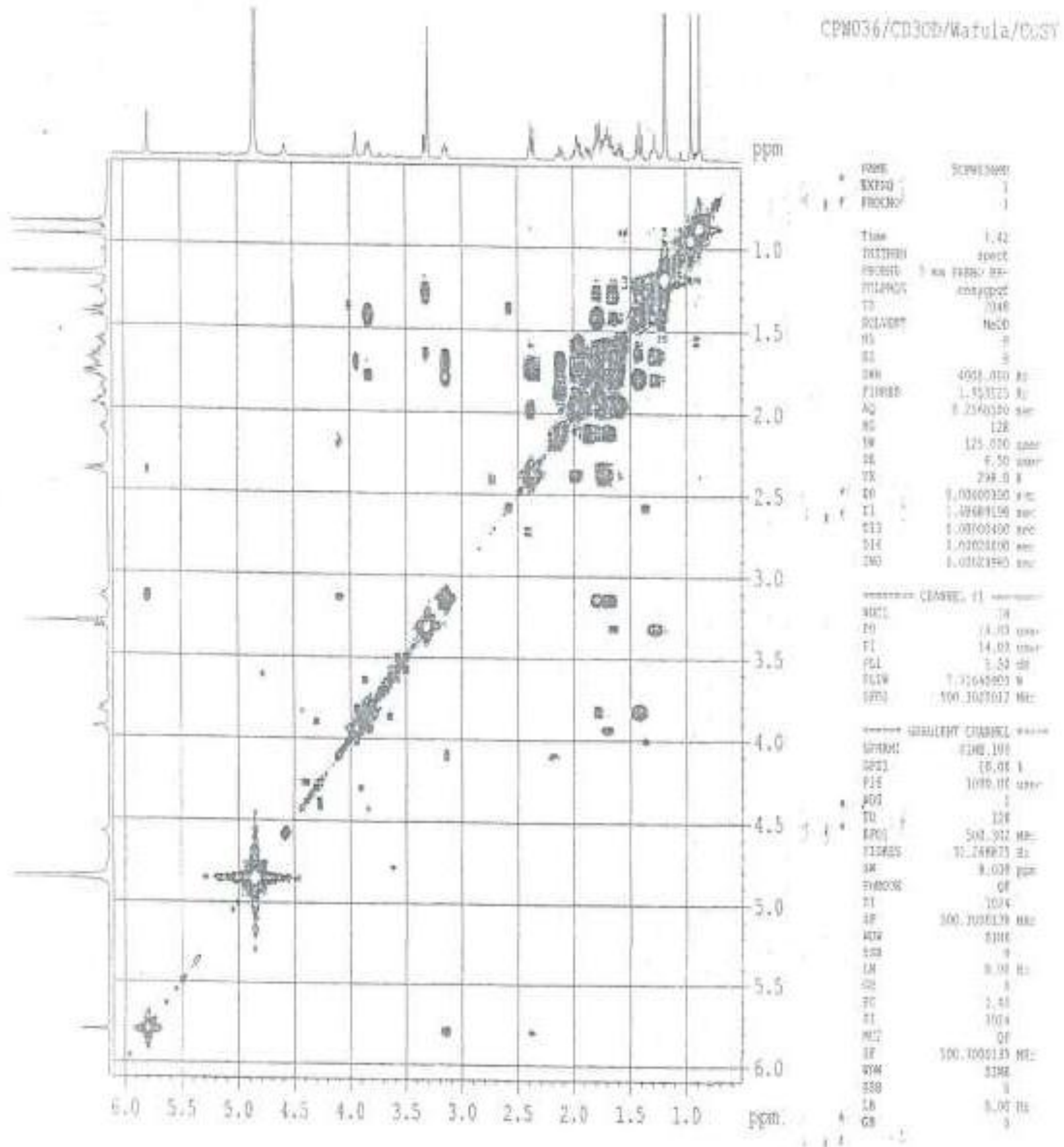
CPW036/CD3OD/Wafula/13C

206.453
167.983
122.124

200 180 160 140 120 100 80 60 40 20 ppm

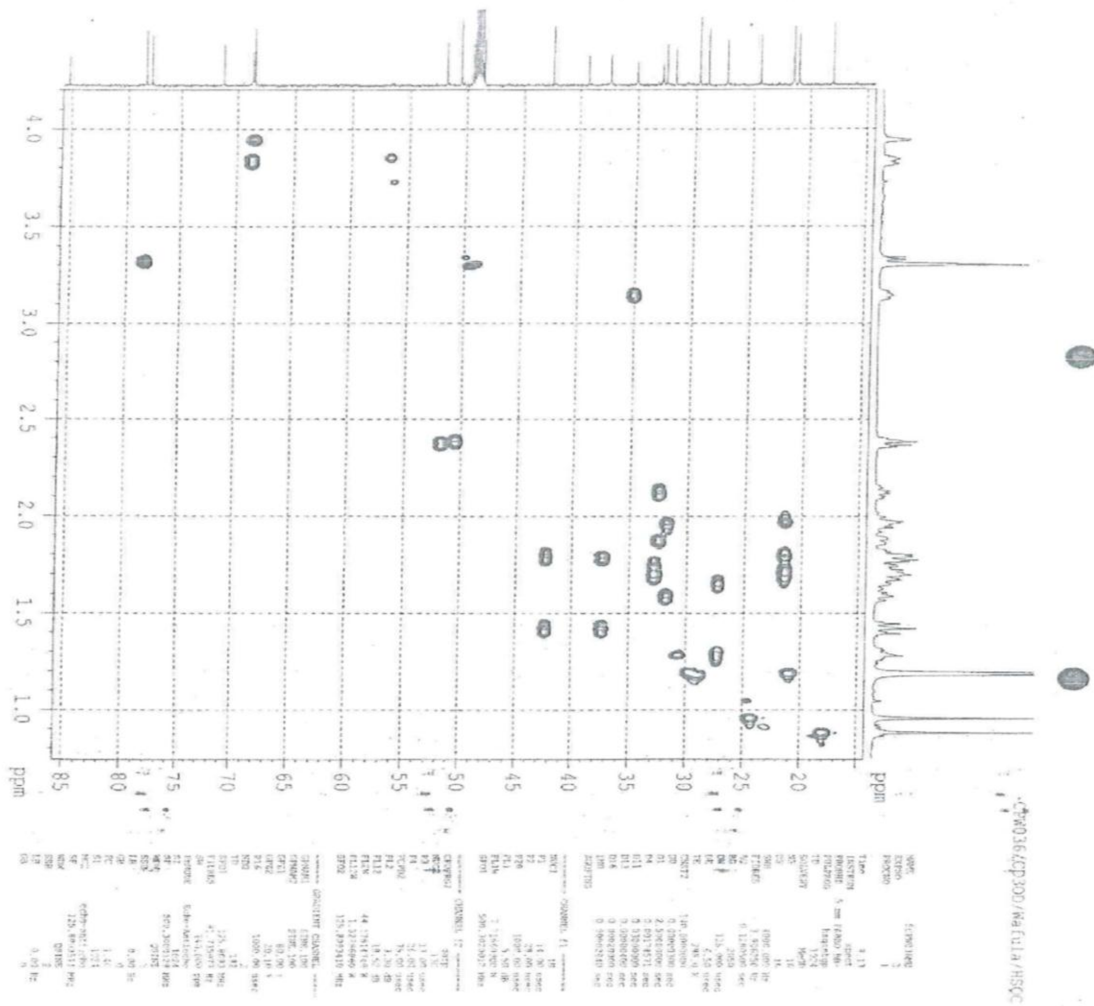
Appendix 1c: DEPT for SUS 1

Appendix 1d: COSY FOR SUS 1

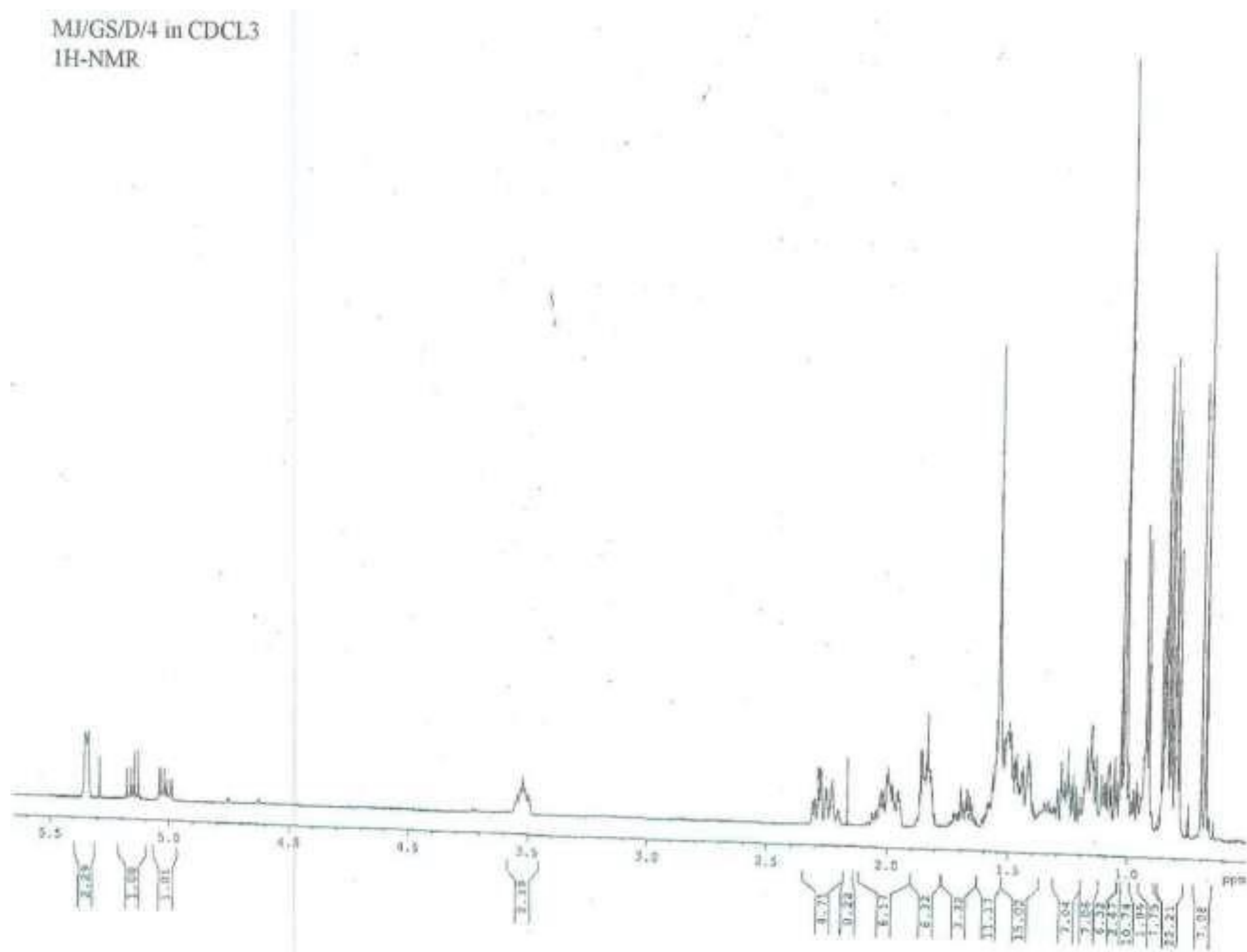


Appendix 1e: HMBC spectra for compound 1

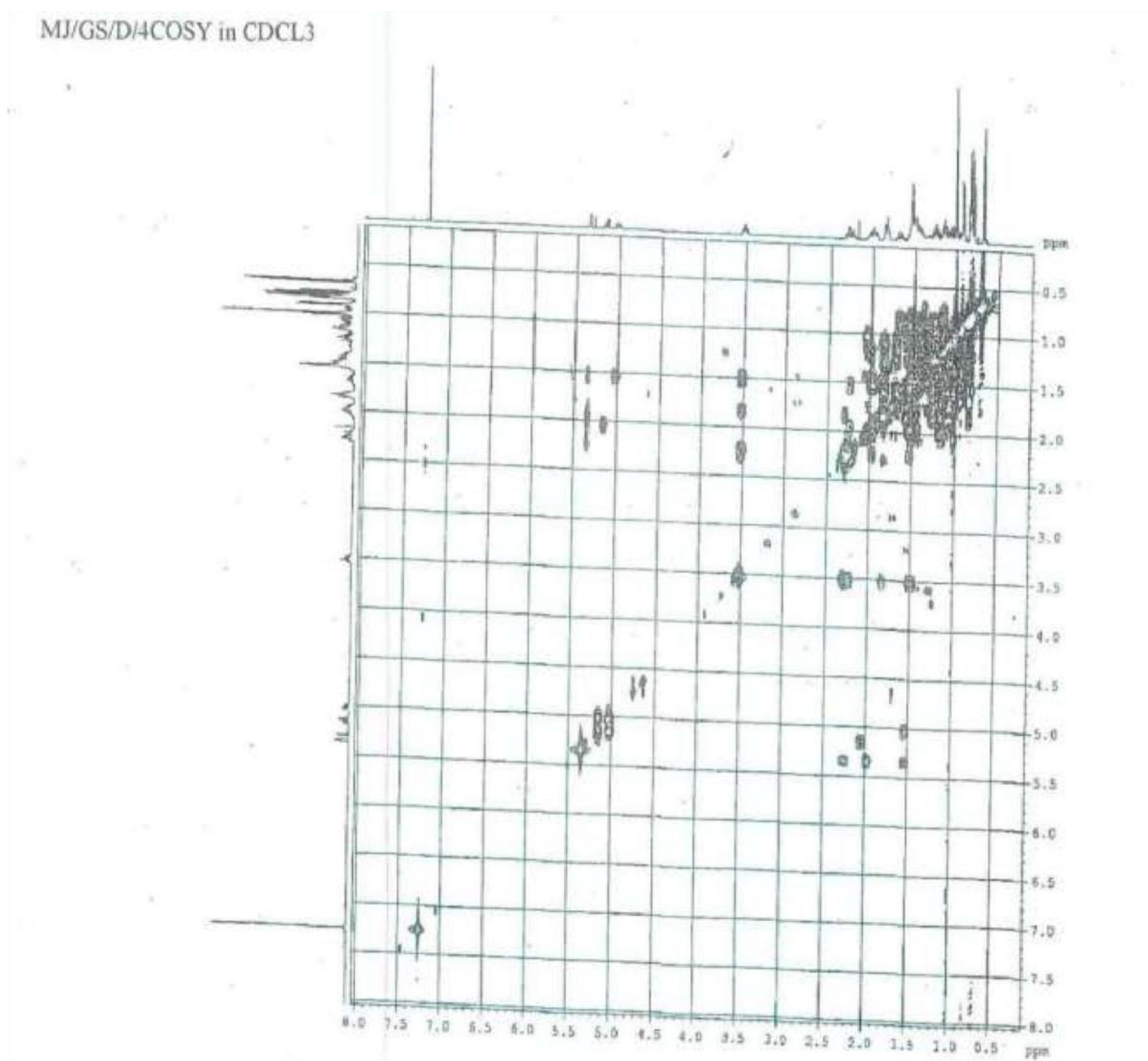
Appendix 1f: HSQC spectra for compound 1



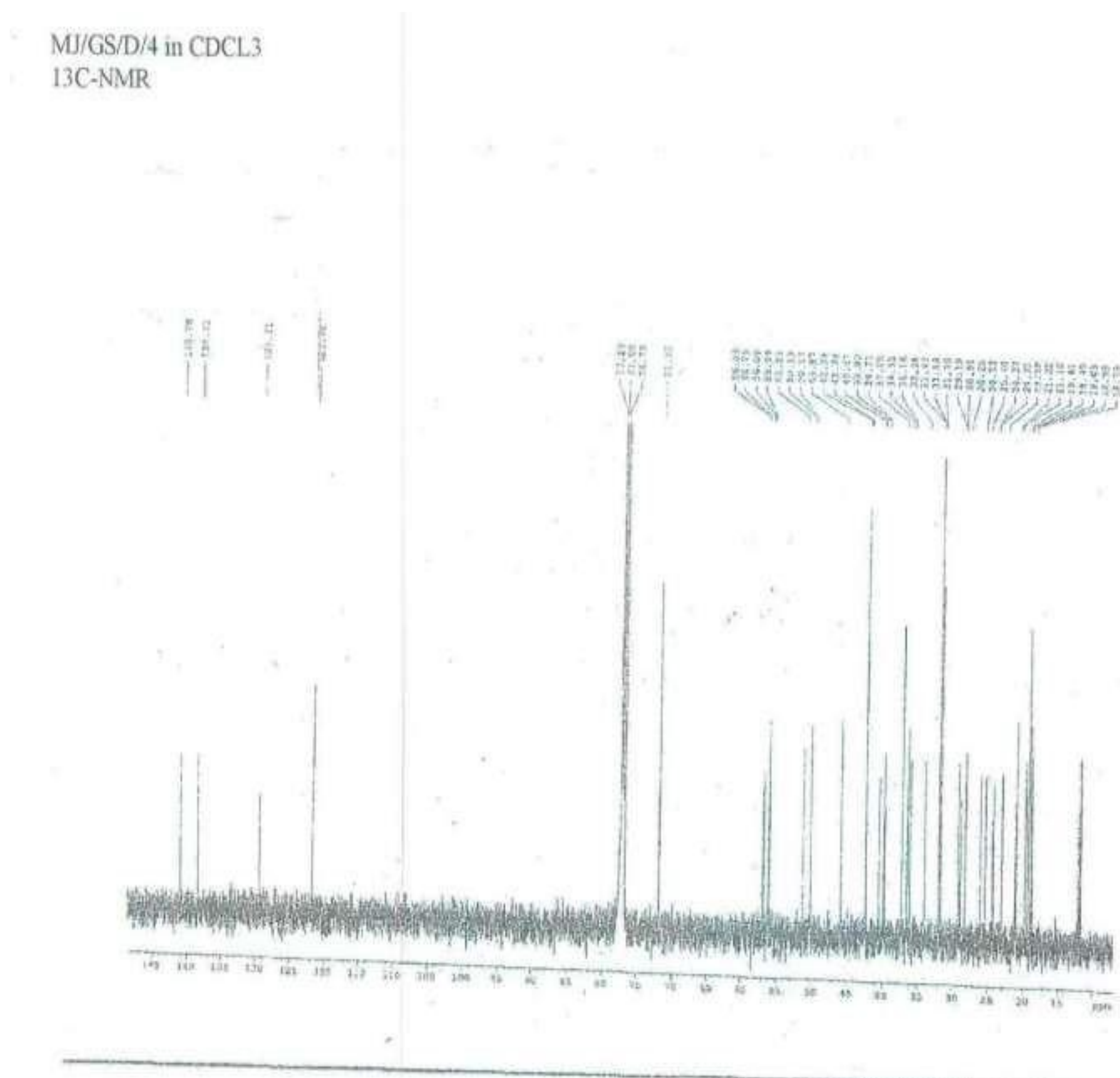
Appendix 2a: ^{1}H NMR spectrum of SUS 2



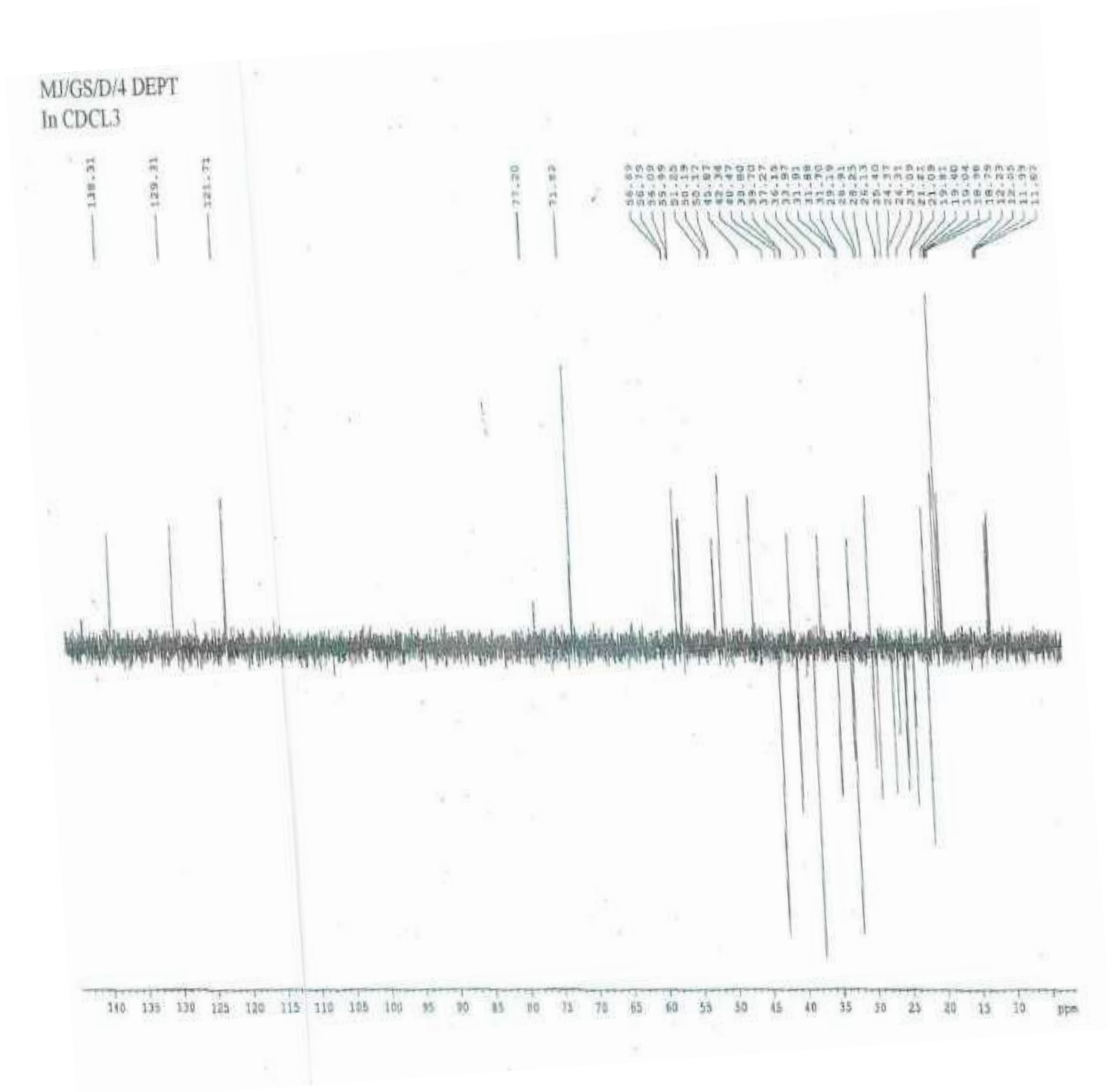
Appendix 2b: COSY spectrum of SUS 2



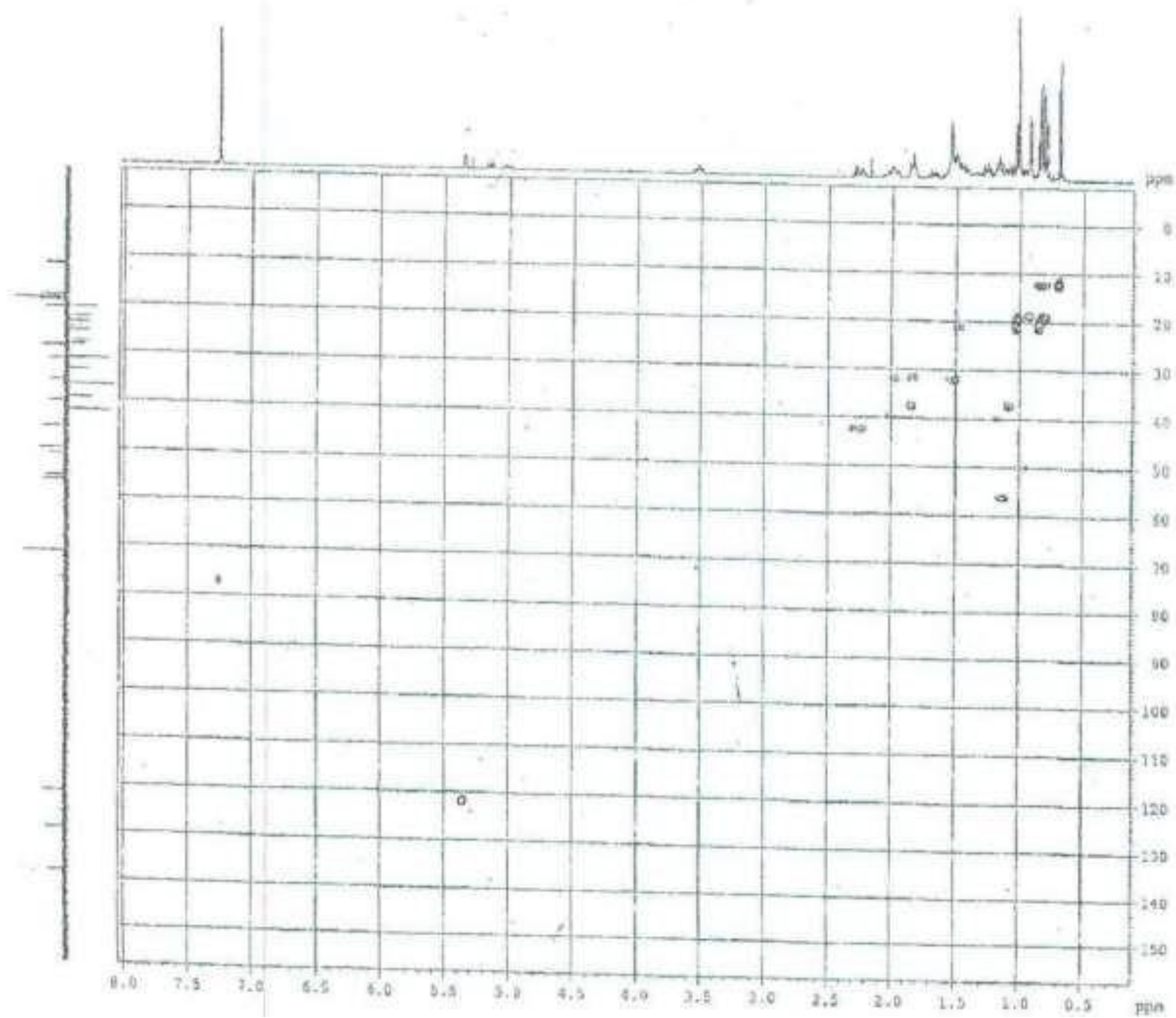
Appendix 2c: ^{13}C-NMR spectrum of SUS 2



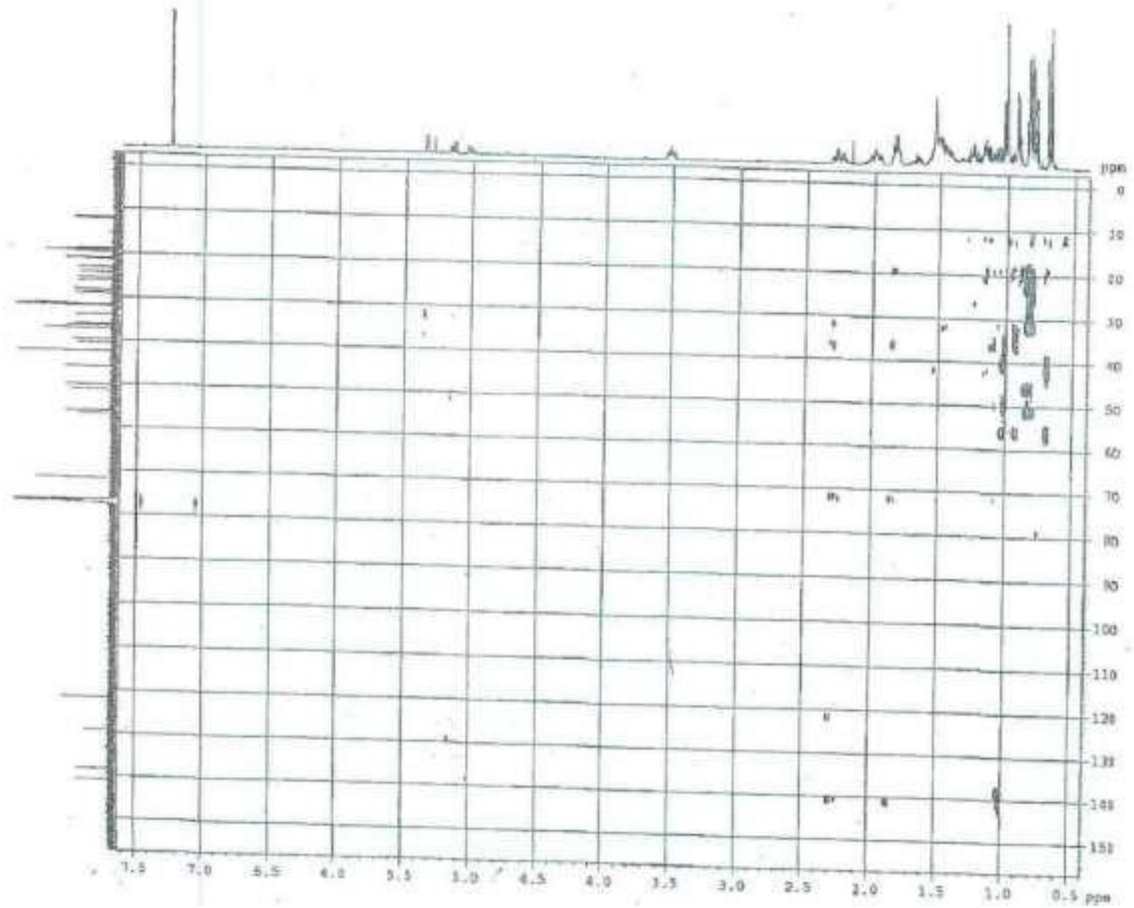
Appendix 2d: DEPT spectrum of SUS 2



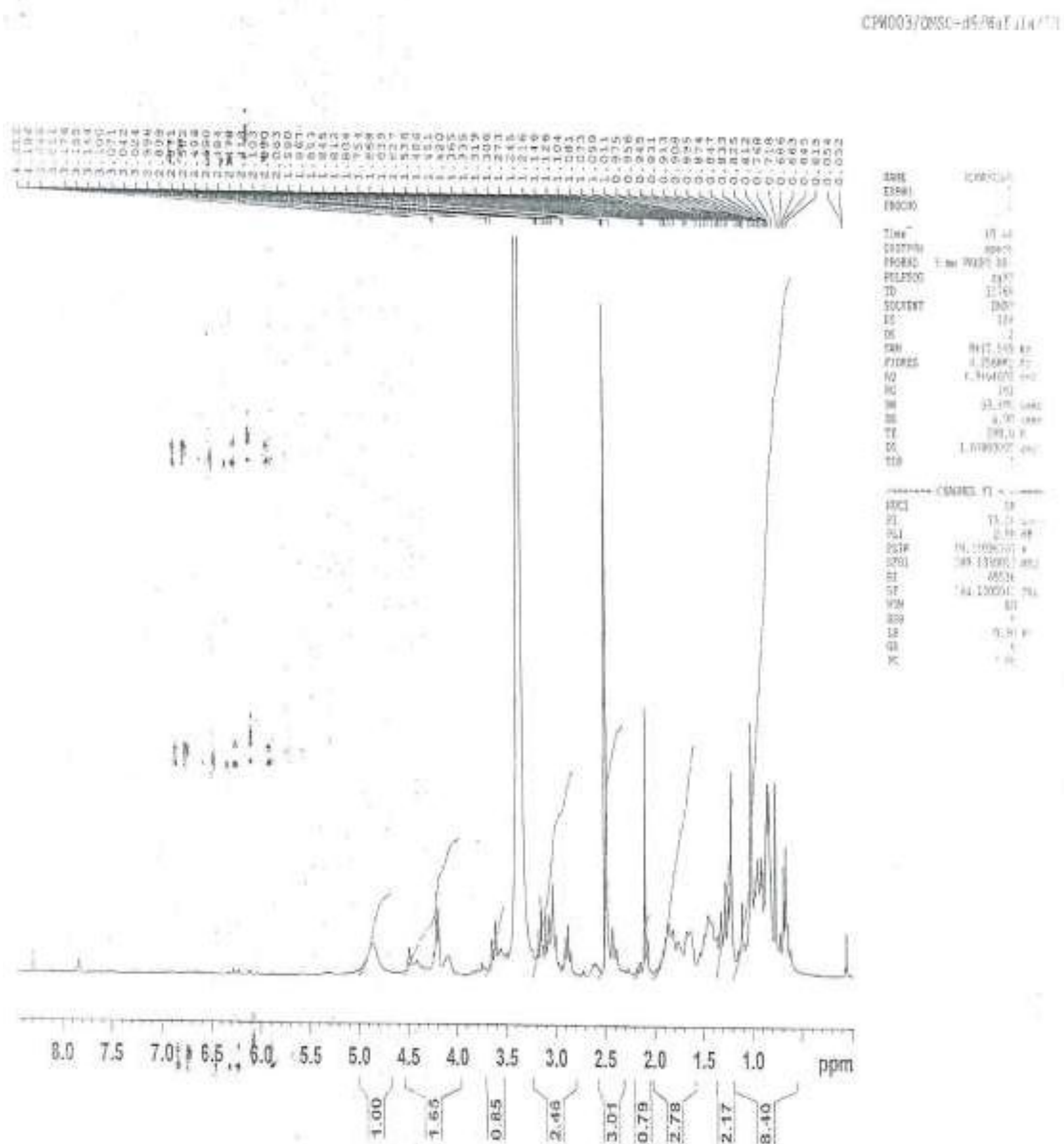
Appendix 2e: HMQC spectrum of SUS 2



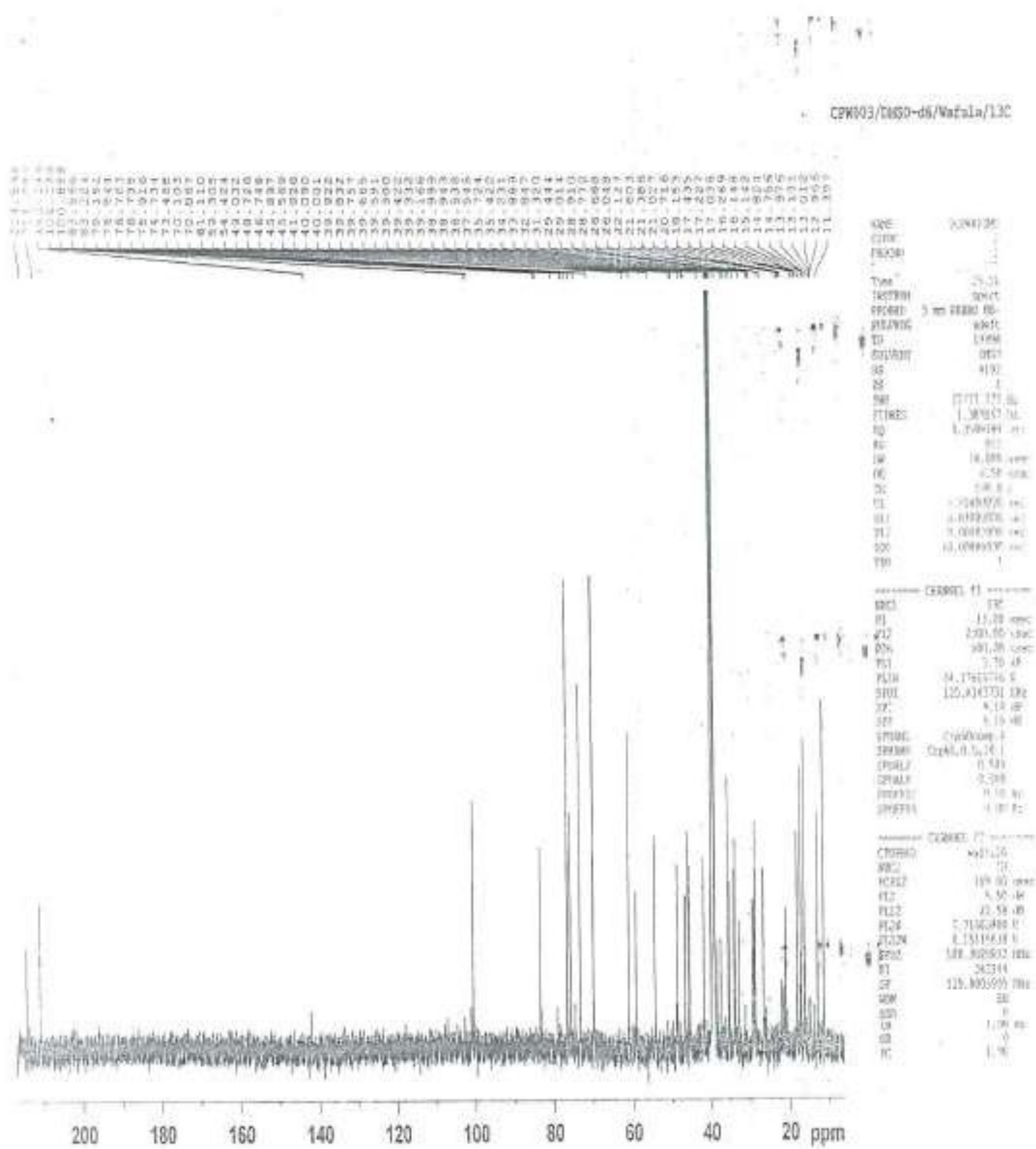
Appendix 2f: HMBC spectrum of SUS 2



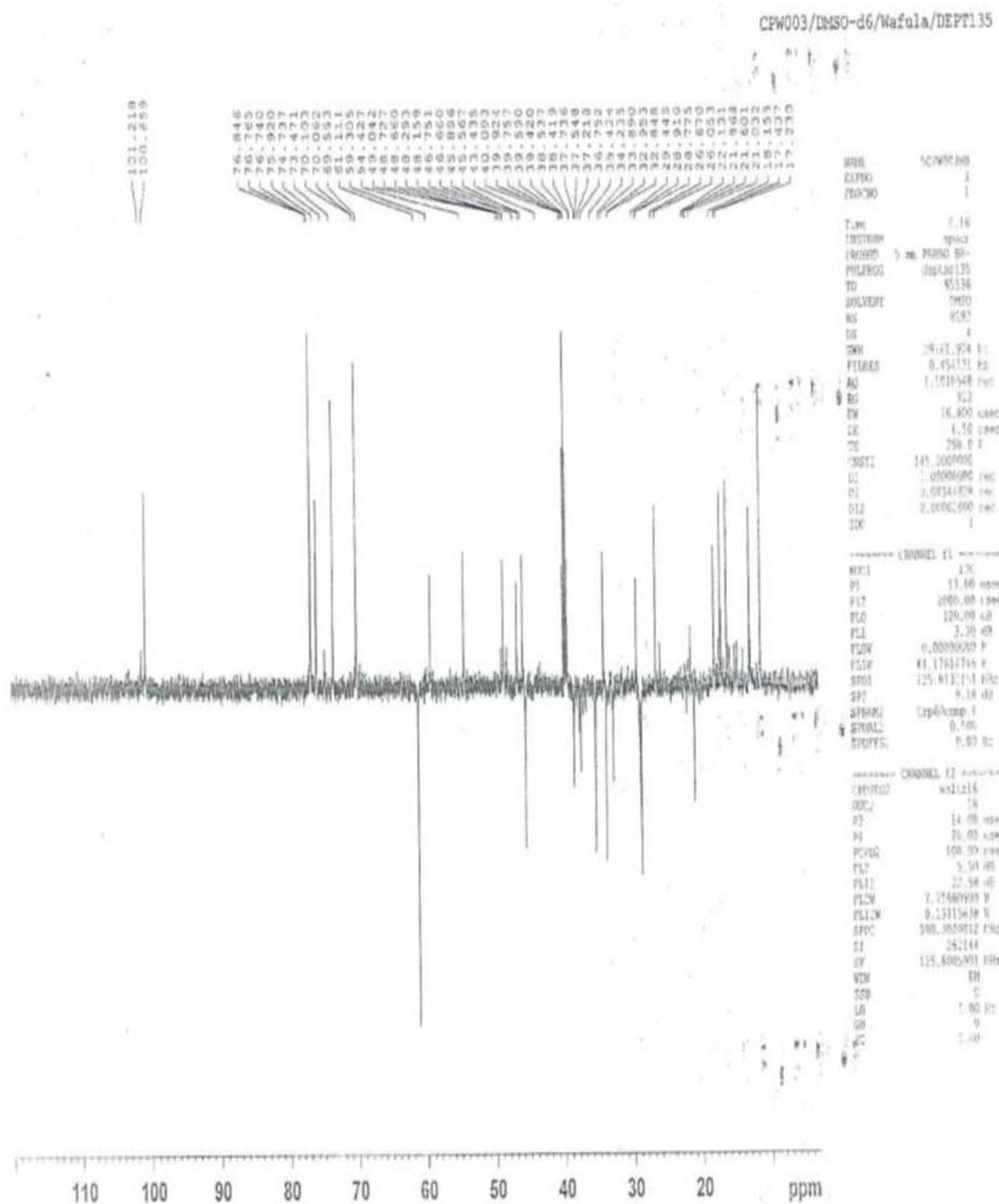
Appendix 3a: ^{1}H NMR for SUS 3



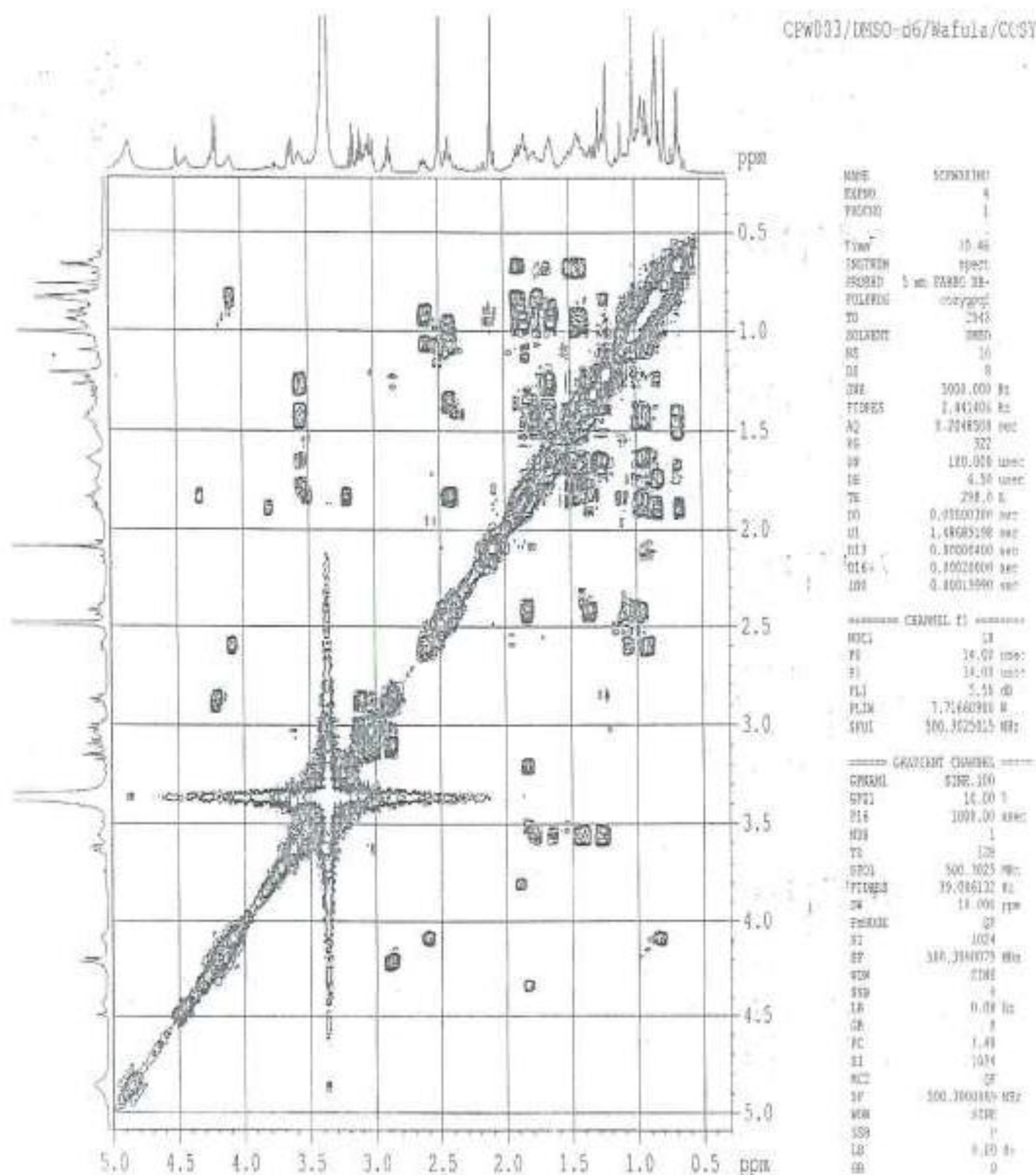
Appendix 3b: ^{13}C NMR for SUS 3



Appendix 3c: DEPT for SUS 3



Appendix 3d: COSY for SUS 3



Appendix 3e: HSQC for SUS 3

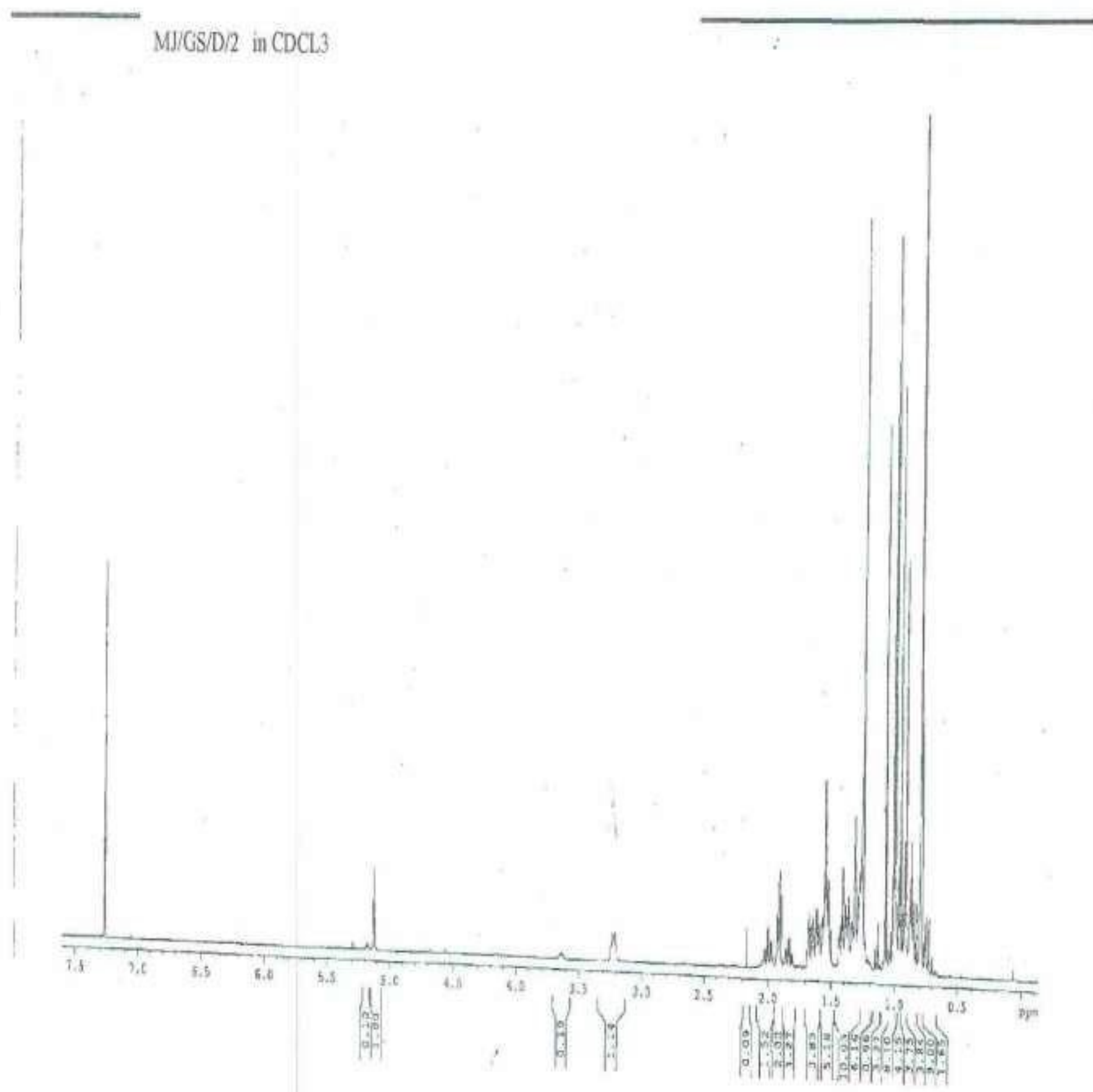
CTW003/DMSO-d6/Wafula/HSQC

ppm 10 20 30 40 50 60 70 80 90 100

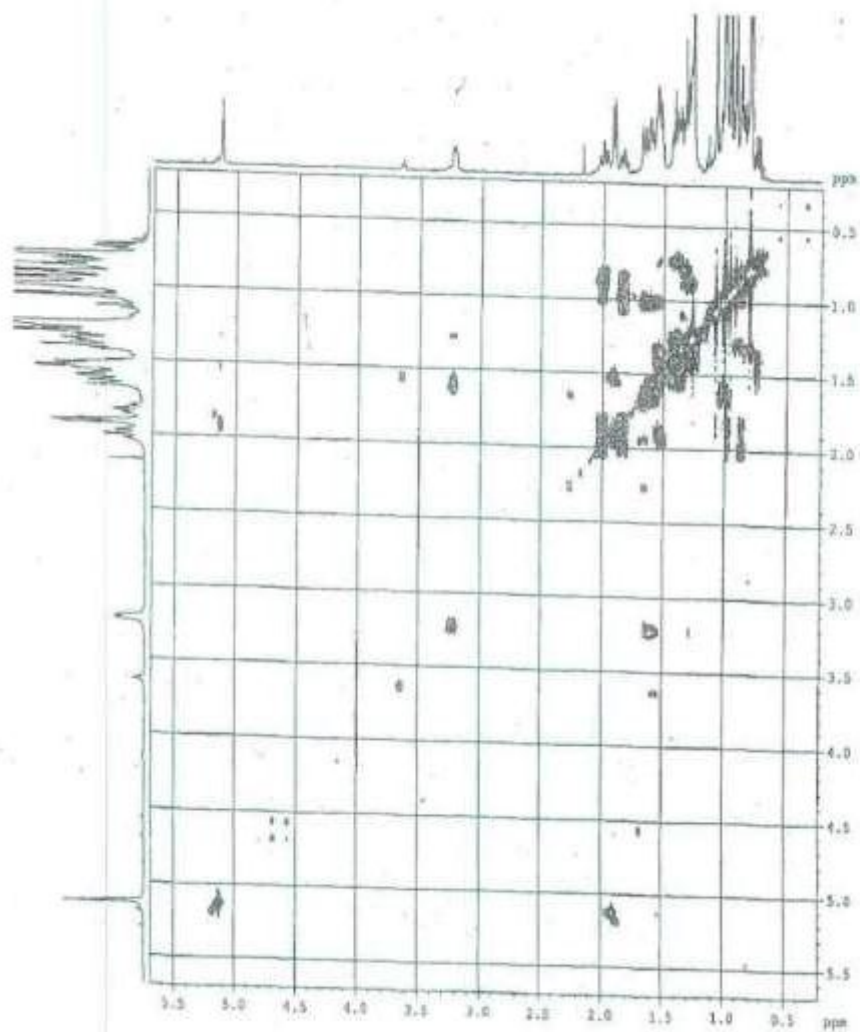
4.0 3.5 3.0 2.5 2.0 1.5 1.0 ppm

Appendix 3f: HMBC for SUS 3

Appendix 4a: ^{1}H-NMR spectrum of SUS 4



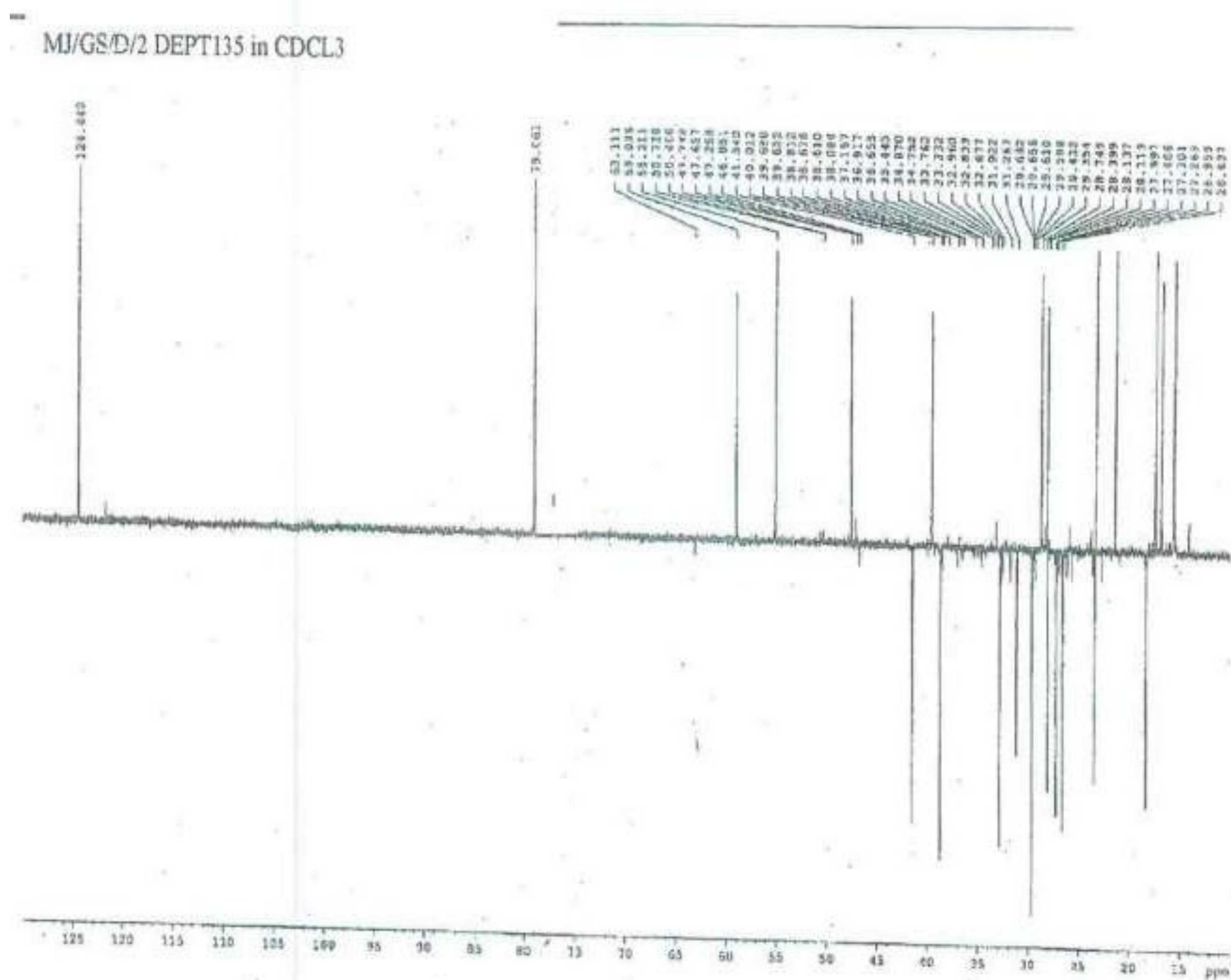
Appendix 4b: COSY spectrum of SUS 4



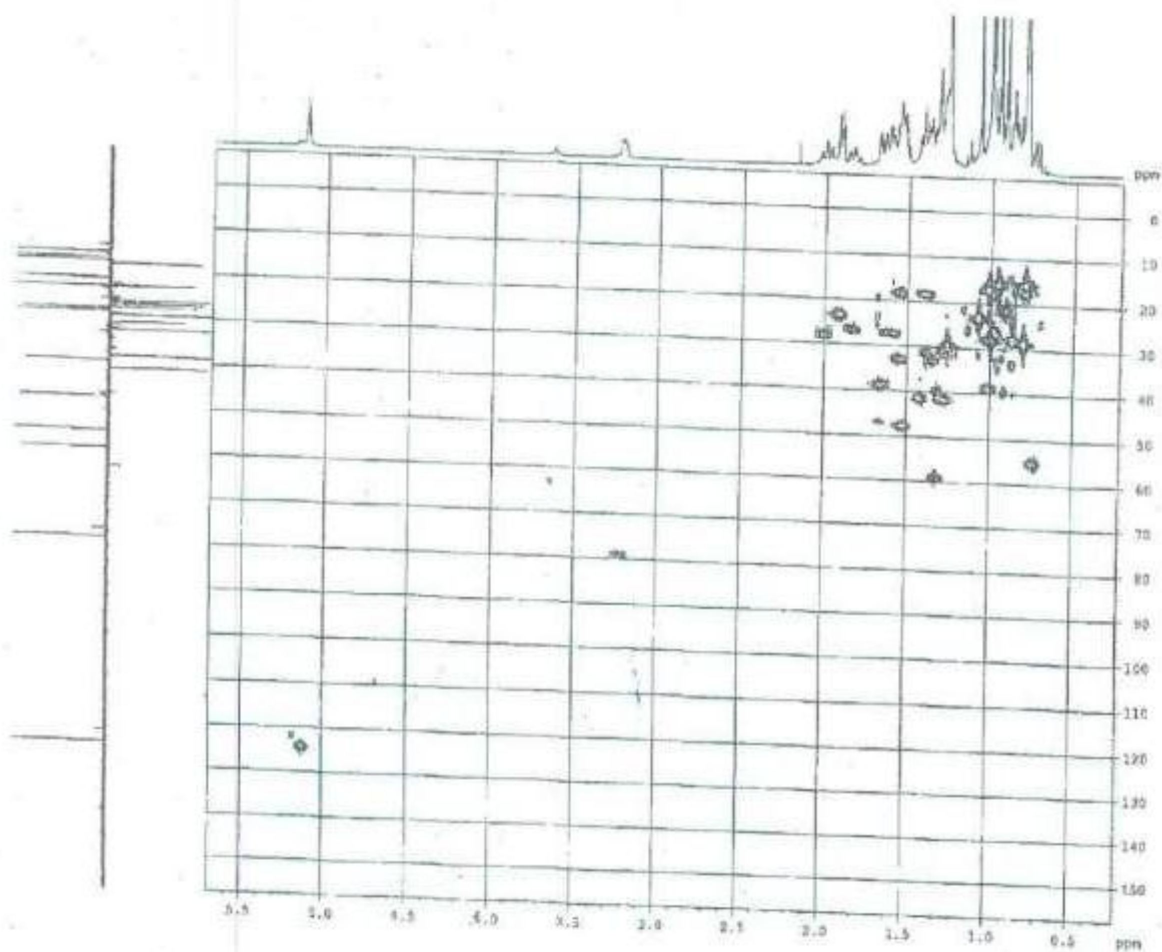
Appendix 4c: ^{13}C-NMR spectrum of SUS 4

MJ/GS/D/2 13C/CDCL3

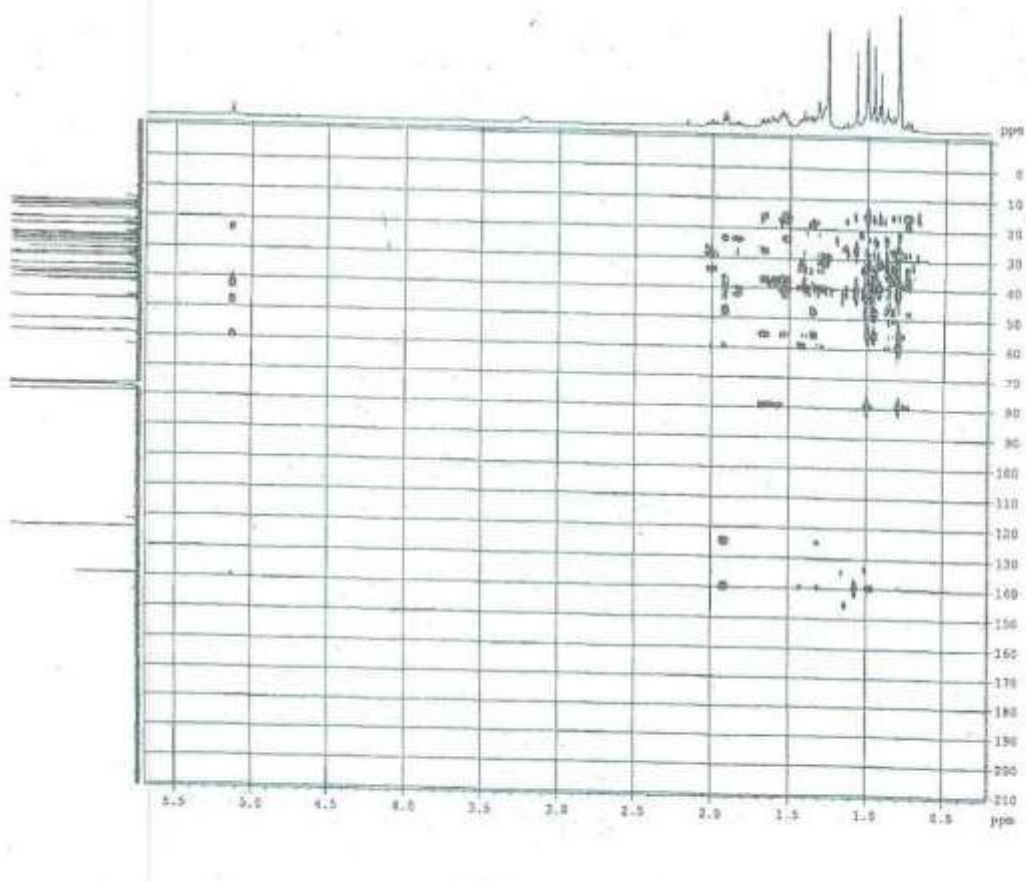
Appendix 4d: DEPT spectrum of SUS 4



Appendix 4e: HMQC spectrum of SUS 4



Appendix 4f: HMBC spectrum of SUS 4



KENYATTA UNIVERSITY
GRADUATE SCHOOL

E-mail: dean-graduate@ku.ac.ke

Website: www.ku.ac.ke

P.O. Box 43844, 00100
NAIROBI, KENYA
Tel. 8710901 Ext. 57530

Our Ref: I56/CE/22624/2011

DATE: 27th June, 2013

The Permanent Secretary,
Ministry of Higher Education, Science & Technology,
P.O. Box 30040,
NAIROBI

Dear Sir/Madam,

RE: RESEARCH AUTHORIZATION AGOLA CAROLINE FAITH– REG. NO. I56/CE/22624/2011

I write to introduce Ms. Agola Caroline Faith who is a Postgraduate Student of this University. She is registered for M.Sc degree programme in the **Department Chemistry.**

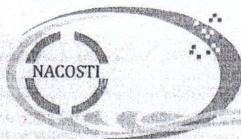
Ms. Agola intends to conduct research for a M.Sc proposal entitled, "Phytochemical, Antiplasmodial and Larvicidal Studies of Stachtarpheta Urticifolia (SIMS)."

Any assistance given will be highly appreciated.

Yours faithfully,

for **MRS. LUCY N. MBAABU**
FOR: DEAN, GRADUATE SCHOOL

DNN/rwm



NATIONAL COMMISSION FOR SCIENCE, TECHNOLOGY AND INNOVATION

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When replying please quote

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Uhuru Highway
P.O. Box 30623-00100
NAIROBI-KENYA

Ref: No. **NACOSTI/RCD/ST&I 5th CALL M.Sc/041**

Date: **5th June, 2014**

Carol Faith Agolla
Kenyatta University
P.O. Box 43844-00100
NAIROBI.

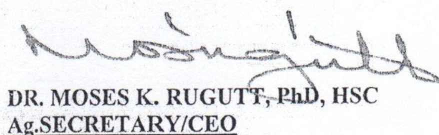
RE: SCIENCE, TECHNOLOGY AND INNOVATION RESEARCH GRANT (MSc/MA)

I'm pleased to inform you that, you have been awarded the Science, Technology and Innovation (ST&I) grant for your **Masters research proposal**.

National Commission for Science, Technology and Innovation (NACOSTI) has approved an amount of Kenya shillings one hundred and ninety thousand **(Ksh 190,000)** towards your MSc/MA research proposal titled ***'Phytochemical, Antiplasmodialand Larvicidal Studies of Stachytarpheta Urticifolia (SIMS)'.***

Find the enclosed ***Research Grant Contract Form (NCST/ST&I/CONTRACT/FORM 1C)*** that should be duly completed. You should attach a certified copy of your ***national identity card, detailed work plan, breakdown of the yearly budget and a letter accepting the grant offered. Your recent passport size photograph and an abstract of your proposal, not exceeding 500 words should be submitted in soft copy (Ms Word format) to the email:-*** postgraduates@nacosti.go.ke

Your duly signed contract form, acceptance letter and the abstract should be sent back to reach us not later than **18th June, 2014** for our further actions.



DR. MOSES K. RUGUTT, PhD, HSC
Ag.SECRETARY/CEO

cc: Vice Chancellor,
Kenyatta University