# ISOLATION, CHARACTERIZATION AND ANTIMICROBIAL ACTIVITY OF EXTRACTIVES FROM *Cola nitida* (Vent) Schott et Endl. SEED

**By**

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## ABSTRACT

This study obtained the methanolic extract of *Cola nitida* seed, fractionated the extract and isolated the bioactive compounds and characterized the isolated compounds using relevant spectroscopic techniques and evaluated their antimicrobial activity. This was with a view to establishing the secondary metabolites in *Cola nitida* seed extract and their antimicrobial potentials.

The crude methanolic extract of *C. nitida* was partitioned with *n*-hexane, ethylacetate and *n*-butanol. Fractionation of the ethylacetate fraction was carried out using Accelerated Gradient Chromatography (AGC) and column chromatography using silica gel kieselgel 60G 0.040-0.063 mm and 60 – 120 mesh size respectively as separating phase for fractionation. Structure elucidation of the isolated compounds was done using Nuclear Magnetic Resonance (NMR),Infrared Spectroscopy and Gas Chromatography-Mass Spectrometry. The antimicrobial activity of crude extract, solvent fractions and isolated compound(s)was determined using the agar well diffusion method.

The isolated compounds were 1,3,7-Trimethyl-1*H*-purine-2,6(3*H*,7*H*)dione (caffeine) **(1)** and *n*-Hexadecanoic acid **(2)**was identified as the major constituents of solid 2. The result of the antimicrobial screening revealed that the crude extract had the most antibacterial activity followed by the ethyl acetate fraction. The antibacterial activity of compound 1 was dependent on the test organism. Among the Gram- positive organisms, *Bacillus cereus* was the most susceptible (zones of inhibition, 7 to 17 mm) while the most susceptible among the Gram-negative organisms was *Escherichia coli* (zones of inhibition, 7 to 19 mm). Compound 1, the ethyl acetate and butanol fractions

demonstrated antifungal activities (zones of inhibition, 7 to 11 mm) which were lower than the crude extract (zones of inhibition, 11 to 15 mm) against the tested fungi.

The study concluded that the demonstrated antimicrobial activity of *Cola nitida*justified the ethnomedicinal use of the plant in the management of infectious diseases and to combat emerging drug-resistant strain of pathogenic microorganisms.

**Keywords :** the methanolic extract, *Cola nitida* seed, methanolic extract.

**Supervisor:** Dr. J. K. Adesanwo

xiv, 79p

# CHAPTER ONE

# INTRODUCTION

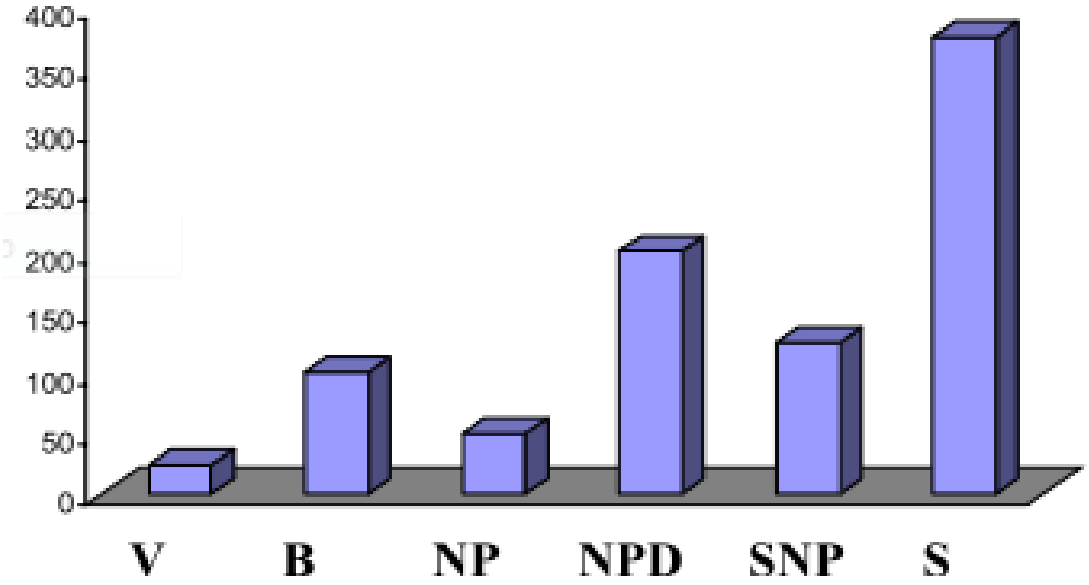
## 1.1 Medicinal Plants

Medicinal plants are the richest bio-resource of drugs of traditional medicine, modern medicines, nutraceuticals, food supplements and pharmaceutical intermediates (Hammer *et al*., 1999). Nature has been a source of medicinal agents for thousands of years and an impressive number of modern drugs have been isolated from natural sources, many based on their use by traditional healers. Renewed interest in the therapeutic potential of medicinal plants therefore is not only to validate the ethno-pharmacological uses of plants, but to also isolate and characterize the active components (Dias *et al*., 2012; Rates, 2001). Moreover, some compounds obtained from natural sources have been used as leads or precursors in the chemical synthesis of drugs (Newman and Cragg, 2007). Despite the efforts made in the treatment and management of diseases with the use of synthetic drugs, the search for safe, affordable and improved natural agents is ongoing. Modern pharmaceutical industries in the world largely rely on secondary metabolites (phytochemicals) from plants for new drug discovery (Cosa *et al*., 2006). One of the most common practices involves the use of crude plants extract which contains a broad spectrum of secondary metabolites with unknown biological activities. The recent interest in the use of herbal medicines has been attributed to the belief that they are comparatively less toxic than the synthetic drugs (Yusuf *et al*., 2007).

## 1.2 Natural Products

Natural products have played an important role in the health care and prevention of diseases for thousands of years. The earliest civilizations of the Chinese, Indians and North Africans provide written evidence for the use of natural products for curing diseases (Phillipson, 2001). The earliest known written document is a 4000 years old Sumerian clay tablet that records remedies for various illnesses (Kong *et al*., 2003). For instance, mandrake (*Mandragora officinarum*) was prescribed for pain relief, turmeric (*Curcuma longa*) possesses blood clotting activity, as well as antimalarial and antitumour properties, roots of endive plants (*Cichorium endivia*) were used for treatment of gall bladder infections, and raw garlic (*Allium sativum*) was prescribed for digestive and circulatory problems. These are still being used in several countries as alternative medicines.

According to a study conducted by the World Health Organization, about 80% of the world's population relies on traditional medicine. About 121 drugs prescribed in the USA come from natural sources, 90 of which come directly or indirectly from plant sources. 47% of the anticancer drugs in the market come from natural products or natural products mimics (Newman and Cragg, 2007). Between the years 1981-2002, about a hundred anticancer agents have been developed, of which 25 are natural products derivatives, eighteen are natural products mimics, eleven are derived from natural products pharmacophore and nine are pure natural products (Newman *et al*., 2003). A graphical representation of the contribution of natural products to drug discovery is given in Figure 1.1.



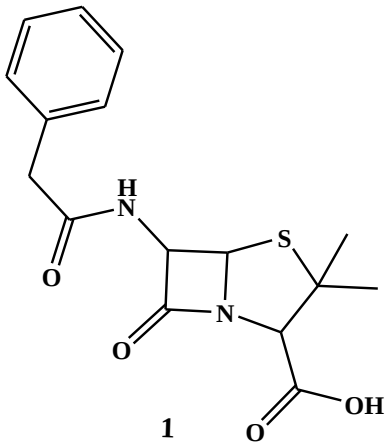

V: Vaccine; B: Biological; NP: Natural products; NPD: Natural products derivatives; SNP: Synthesis derived from Natural products; S: Synthesis.

**Figure 1.1: Distribution of Natural Products as Drugs (Newman *et al.*, 2003)**

The study of natural products involves isolation in a pure form of the secondary metabolites and investigation of their structures, formation, use and purpose in organisms (Croteau *et al*., 2000). Natural products appear to function primarily in defense against predators and pathogens and in providing reproductive advantage as intraspecific and interspecific attractants. They may also create competition advantage as poisons of rival species (Newman *et al*., 2000). Natural products have a very significant contribution to the health care system of both developed and developing countries.

### 1.2.1 Natural Products from Microorganisms

Microorganisms as a source of potential drug candidates were not explored until the discovery of penicillin, $C_{16}H_{18}N_2O_4S$, **(1)** in 1929 from a filamentous fungus *Penicillum notatum* (Wainwright, 1993; Bennett and Chung, 2001). Since then a number of terrestrial and marine microorganisms have been screened for drug discovery. Microorganisms have a wide variety of potentially active substances which have led to the discovery of antibacterial agents like the cephalosporins, $C_{19}H_{21}N_3O_5S$, **(2)** isolated from *Cephalosporium acrimonium*, antidiabetic agents like acarbose, $C_{28}H_{49}NO_{14}$, **(3)** and anticancer agents like epirubicin, $C_{27}H_{29}NO_{11}$, **(4)** (Chin *et al*., 2006).

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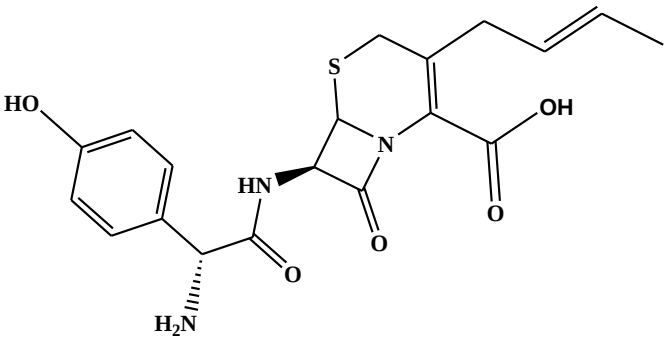


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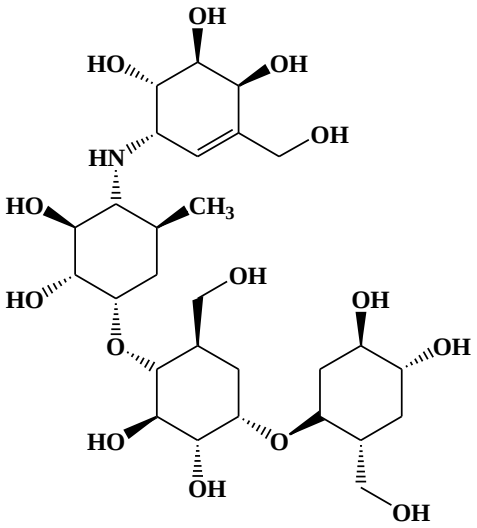


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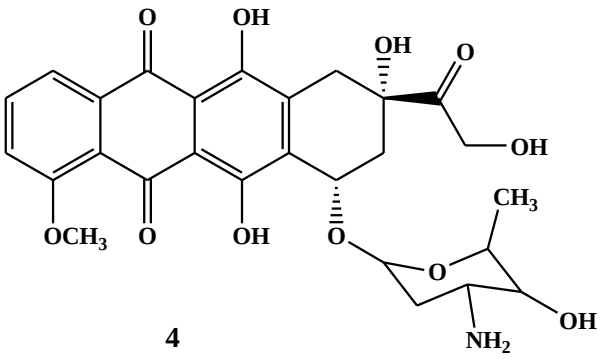


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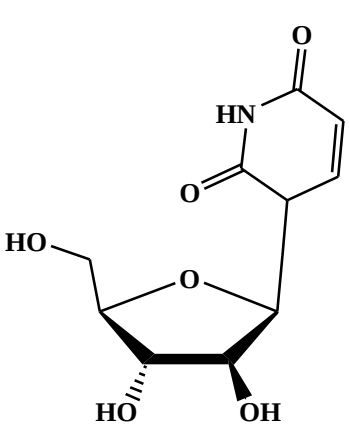
### 1.2.2 Natural Products from Marine Organisms

Sponges are sessile organisms which lack a nervous, digestive and circulatory system and maintain a constant flow of water through their bodies to obtain food, oxygen and to remove wastes. All sponges are filter or current feeders (McConnell *et al*., 1994). The first biologically active compounds to be isolated from marine organisms are spongouridine, $C_{11}H_{15}NO_6$, **(5)** and spongothymidine, $C_{12}H_{17}NO_6$, **(6)** from the Carribean sponge *Cryptotheca crypta*. These compounds are nucleosides and show great potential as anticancer and antiviral agents. About 70% of the Earth surface is covered by the oceans thus providing significant biodiversity for research into new drug discovery. Many marine sponges live a sedentary life and as such synthesize very potent and complex chemical agents as their means of defense against predators (Haefner, 2003). These chemical agents can serve as possible remedies to various ailments.

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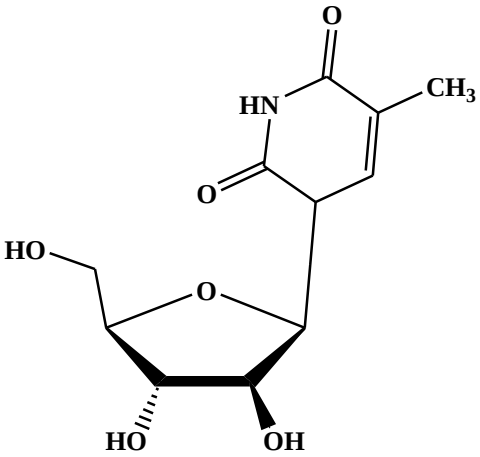


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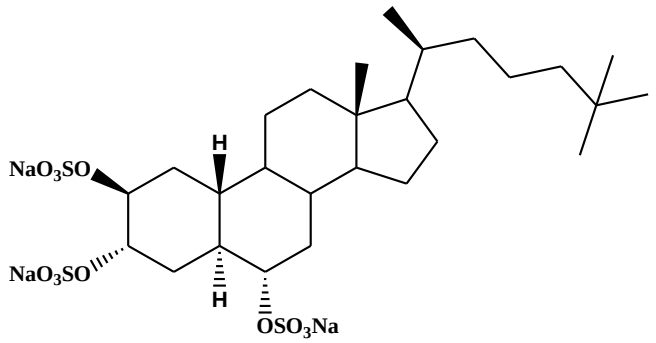
Marine sponges are also good source of unusual sterols. Some of these sterols have phylogenetic significance. These sterols are of interest to understanding the function of biological membranes. The sulphated and alkaloidal sterols exhibit antimicrobial activity.

Halistanol, $C_{27}H_{45}Na_3O_{12}S_3$, **(7)** isolated from *Halichondria mooriei* and a sterol $C_{27}H_{43}NaO_4S$, **(8)** from *Toxadocia zumi* inhibited the growth of *Staphyloccocus aureus* and *Bacillus subtilis* at 100µg/disc and 50µg/disc (Fusetani *et al*., 1981; Mukku *et al*., 2003; Mayer *et al*., 2007).

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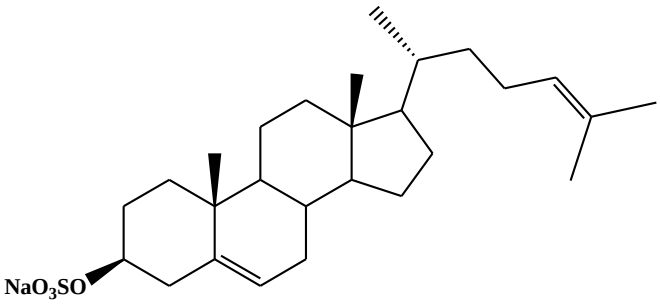


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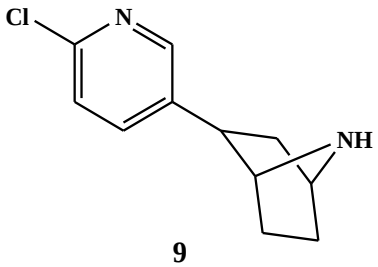
### 1.2.3 Natural Products from Animals

Animals have been a source of interesting compounds that has been used as drugs for years. Epibatidine, $C_{11}H_{13}N_2Cl$, **(9)** isolated from the skin of an Ecuadorian poison frog is tenfold more potent than morphine (Spande *et al*., 1992). Venoms and toxins from animals have also been used as intermediates in the synthesis of antibiotics. Teprotide (pro-trp-pro-arg-glu-ile-pro-pro), a peptide extracted from a Brazilian viper *Bothrops*

*jararaca* (Ferreiara *et al*., 1970) led to the development of captopril, $C_9H_{15}NO_3S$, **(10)** which is an effective antihypertensive drug.

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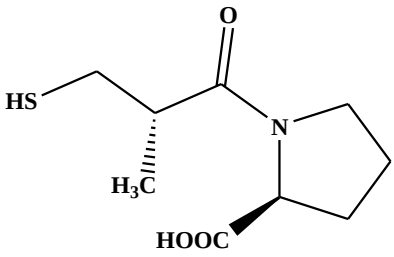


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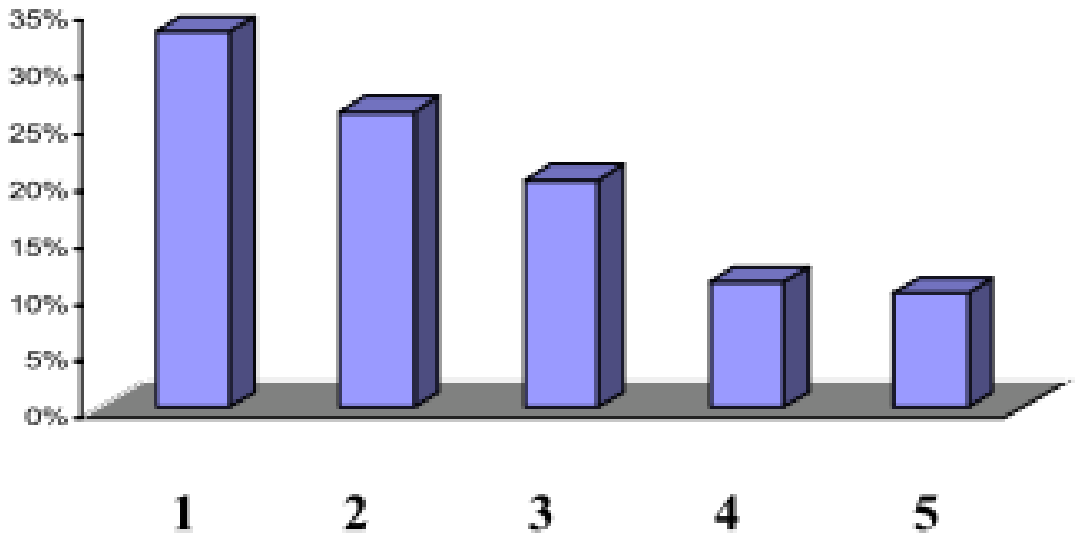
### 1.2.4 Natural Products from Plants

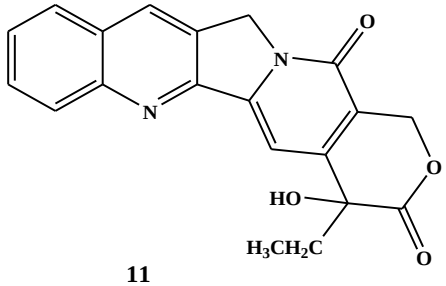
The use of plants as medicines has a long history in the treatment of various diseases. The earliest known records for the use of plants as drugs are from Mesopotamia in 2600 B.C. and are still a significant part of herbal remedies (Koehn and Carter, 2005). The demand for natural products of plant origin is on the increase especially in the western countries as shown in Figure 1.2.




1: Europe (33%) 2: Asia (26%) 3: North America (20%) 4: Japan (11%) 5: Others (10%)

**Figure 1.2: World Market for Drugs of Plant Sources (Dev, 1999)**

Camptothecin, $C_{20}H_{16}N_2O_4$ **(11)** isolated from *Camptotheca acuminata*, is too insoluble for drug use but its synthetic analogues topothecan, $C_{23}H_{23}N_3O_5$ **(12)** and irinothecan, $C_{33}H_{38}N_4O_6$ **(13)** are used in the treatment of gastric, rectal, colon and bladder cancers (Wani *et al*., 1971).

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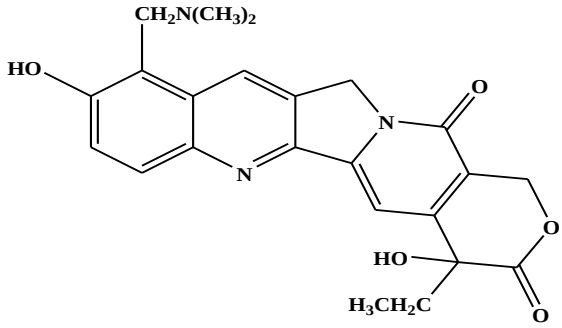


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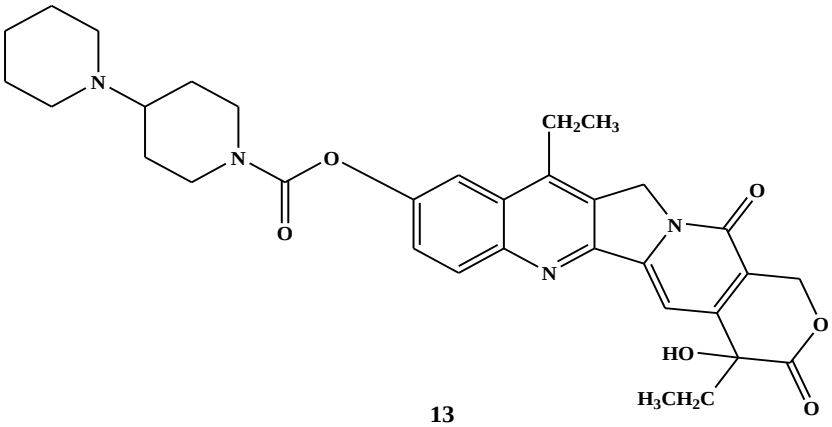


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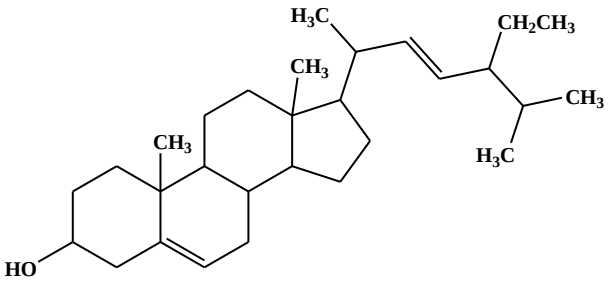
The development of oral contraceptives and other synthetic steroidal hormones stems from the isolation and characterization of plant steroids. Stigmasterol, $C_{29}H_{48}O$ **(14)**

isolated from *Glycine max* serves as an intermediate for the synthesis of progesterone, a hormone secreted by the corpus luteum that controls pregnancy. Ergosterol, $C_{28}H_{44}O$ **(15)** a plant sterol found abundantly in fungi is the precursor of vitamin D. Antheridiol, $C_{29}H_{42}O_5$ **(16)** is also a fungal sterol and a sex hormone of the aquatic fungus *Achlya bisexualis*. It is secreted by the female strain of the fungus and induces formation of antheridial hyphae (Salisbury and Cleon, 1978; Thorton and Boyd, 1992). These have also helped in the development of adrenocortical hormones; an example is cortisone, $C_{21}H_{28}O_5$ **(17)**.

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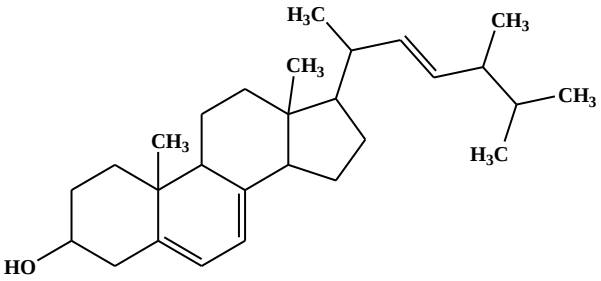


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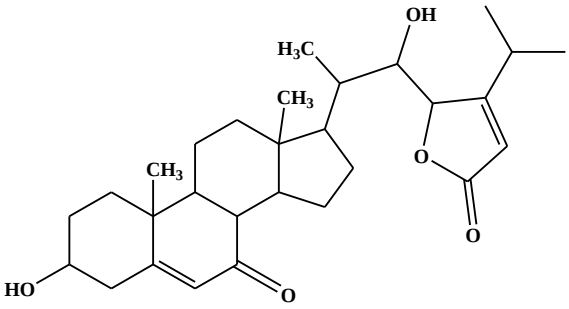


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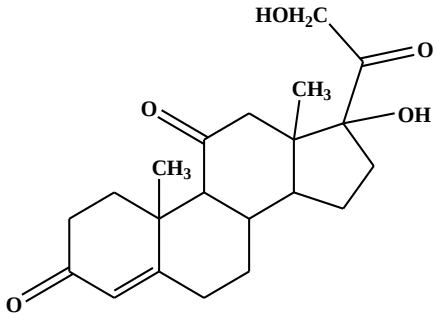


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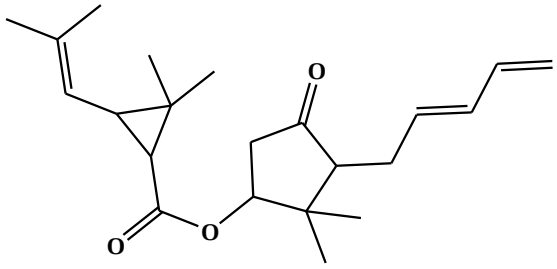
Natural products from plants in addition to the above, also serve as sources of agrochemicals and pesticides. Pyrethrine, $C_{22}H_{32}O_3$ **(18)** isolated from *Chrysanthemum cinerarifolium* have been used in the semisynthesis of other pyrethrine-based insecticides, rotenone, $C_{23}H_{22}O_6$ **(19)** which is also used as an insecticide is isolated from *Derris elliptica* and other species from the *Fabaceae* family. Nicotine, $C_{11}H_{15}N$ **(20)** an alkaloid found abundantly in the *Solanaceaea* family (the nightshade) is a natural insecticide.

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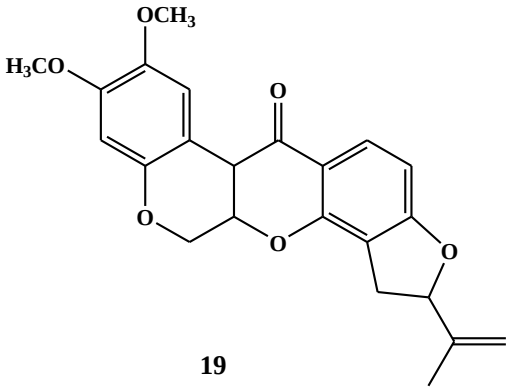


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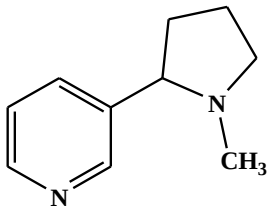


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### 1.3 Rationale for the Study

Extracts of *C. nitida* is used in the Nigerian traditional medicine in the management of diseases caused by pathogenic microorganisms. Based on ethnomedicinal uses, the activities demonstrated by the seed extract could be linked to the presence of phytochemicals, antimicrobial compounds in the extract. This study therefore aims to isolate and identify the secondary metabolites in the methanolic seed extract of *C. nitida* and evaluate them for antimicrobial activities.

### 1.4 Statement of Research Problem

It has been observed that chemically synthesized drugs have adverse side effects associated with them. Microbial resistance to available drugs is also on the increase. From the ethnopharmacological uses of *C. nitida* seed extract, few reports have indicated

possible uses of the seed extract in the management of infections. This study therefore intends to investigate the antimicrobial compounds in *C. nitida*.

### 1.5 Specific Objectives of Research

The specific objectives of the research work are to:

(i) extract the powdered seeds of *C. nitida* with 100% methanol to obtain the crude extract of the seeds;

(ii) carry out liquid-liquid fractionation of the crude extract with organic solvents of varying polarities to obtain the respective solvent fractions;

(iii) isolate compounds from the most active fraction and characterize isolated compounds using spectroscopic techniques such as nuclear magnetic resonance (NMR), and mass spectrometry (MS); and

(iv) determine the antimicrobial potential of the crude extract, solvent fractions and isolated compound(s).

### 1.6 Expected Contribution to Knowledge

This study will provide information on the secondary metabolites (phytochemicals) in *Cola nitida* seed extract and establish their antimicrobial potential.

# CHAPTER TWO

# LITERATURE REVIEW

## 2.1 The Family *Stercullaceae*

Cola is an evergreen tree in the family of the flowering plants *stercullaceae*. The most common, widely distributed and consumed being the *Cola nitida* (Vent.) Schott et Endl, and *Cola acuminata* (Pal. De Beauv.) Schott et Endl. Others members of the family are *Cola anomala*, *Cola verticillata* (Thonn.) Stapf, *Cola pseudoacuminata* and *Cola minelli* (Lovejoy 1980; Opeke 1992; Blumenthal, 2000; Adebayo and Oladele, 2012). It grows to a height of about 20 feet (Grieve, 2001). The cola nut is a capsule-shaped fruit composed of fleshy, irregularly shaped polycotyledonous seeds. The colour of seed of cola varies from pink, red to white when fresh and which becomes brown and hard when dry. The bitter and astringent taste of the seeds is why they are called nuts (Sharma *et al*., 2011).

*Cola* occupies a unique place in West African culture, where it is widely consumed and this habit cuts across most cultures in Nigeria. It is often still said that "he who brings

cola brings life." It is of particular importance in the social life and religious customs of the people of West Africa and in all types of traditional gatherings, colanuts are highly esteemed channels of blessings. About 90% of the colanuts produced in Nigeria is consumed within the country while 10% is exported (Quarco, 1973).

The family contains about 125 species native to the tropical rain forest region of Africa. Of these species, the most common in Nigeria are *Cola nitida* called Obi Gbanja in yoruba language and Gooro in hausa, *Cola acuminata* called Obi Abata or Obi Alawe in yoruba language, *Garcinia cola*, called Orogbo in yoruba language and *Buchholzia coriacea* popularly known as wonderful cola. *Cola acuminata* has special religious significance in the South-western part of Nigeria as it is used by most "Ifa priest" as medium of divination and spiritual enquiry.

Traditionally, the nuts are chewed as a masticatory substance to stimulate the flow of saliva, suppress hunger, fatigue, thirst and sleep. It is also claimed by the consumers that cola seeds strengthen the dental gums, suppress gout and related diseases. The cola nut husk meal is also reported to have nutritional similarity to cocoa pod meals, and is therefore used in the manufacture of poultry feeds (Olubamiwa *et al*., 2002; Hamzat and Adeola, 2011). The pod is also used in the manufacture of liquid detergents and organic fertilizers (Blades, 2000). Extracts of the seeds is used in the manufacture of dyes and cola group of beverages and drinks (Ajiboye and Afolayan, 2009).

In folk medicine, powdered cola seeds mixed with honey is used in the treatment of cough. Traditional treatment of circumcision wounds and other skin infections have also been performed using various cola concoctions (Mboto, 2000). The caffeine in the nut is

a known bronchiodilator, expanding the bronchial air passages, hence the use of the seed extract in the treatment of whooping cough and asthma (Blades, 2000). Extract from roots, leaves, stem bark and seeds of *C. nitida* are used extensively in folk medicine (Olorunisola *et al*., 2014) *C. nitida* seeds produce a state of euphoria, well-being and enhance alertness. It has also been found to increase physical energy and suppress hunger; it is therefore used as a stimulant.

The seeds are therapeutically and industrially important because of their xanthine content. Traditional and other social functions are not complete without cola in Nigeria and other West African countries (Atawodi *et al*., 1995). Aqueous extract of *Cola nitida* seed have also been found to induce gastric acid secretion in the body (Ibu *et al*., 1986; Tende *et al*., 2011). In Africa, cola seeds are used as masticatory agents for their stimulating effect. The ethanolic extract of the leaves and seed has also been found to exhibit antiemetic activity and used in the management of dysentery. Powdered seeds are applied to cuts to stop bleeding. These properties have been attributed to the richness of the seeds in purine alkaloids, polyphenols and sugar (Niemenak *et al*., 2008).

Cola nuts have also been found to have a degenerative effect on the internal organs of rats as reported by Ikegwounu *et al*. (1981). Extracts of the seeds and leaves have been used in the treatment of sexual impotence and erectile dysfunctions in Western Uganda. In Benin, extract of *Cola acuminata* is used with other herbs to treat primary and secondary sterility. The fruits are also said to be laxative and diuretic when administered orally (Kamatenesi-Mugisha, 2005). In Nigeria, the methanolic extract of the seed is commonly used against emesis gravidarum and migraine (Suherman *et al*., 2013).

Aqueous extract of the seeds have been used as flavourants in carbonated soft drinks (Buraimoh *et al*., 2014). It was in 1886 that the druggist John Pemberton invented the popular soft drink, Coca- Cola, by combining coca and cola extracts for use as headache and hang-over remedy. Cola seed are also used in liquers, ice creams, confectionary and baked products. In Europe, cola seeds are used as condiments in mineral water, wine and various pharmaceutical formulations. It has also been listed as a food additive in the United States (Burdock *et al*., 2009;). Ethanolic extracts of the seed have been found to have antioxidant activity in the protection of the red cells from oxidation and degradation. The effectiveness of certain antioxidant substances among them cola extracts, in the protection of red cells with respect to their window times of survival has been analysed. In the presence of an oxidizing agent (potassium ferricyanide), lysis of red cell membrane, oxidation of exposed haemoglobin and methemoglobin formation were observed for 12 hours. 70% methanol extract of *C. acuminata* and *C. nitida* were implicated red cell viability and survival. (Atolaye, 2009; Adebayo *et al*., 2012). The ethanol extracts of the seed also show high trypanocidal activity (Kubata *et al*., 2005).

In Indonesia, aqueous extract of *C. nitida* seeds have been found to be an effective chemopreventive agent against hepatocellular carcinoma (Suherman *et al*., 2013). Ethanolic extract of *C. nitida* have neurodegenerative effects (Buraimoh *et al*., 2014). In Nigeria, aqueous extract of *Cola nitida* have been shown to improve the release of reproductive hormones in rat (Adisa *et al*., 2010).

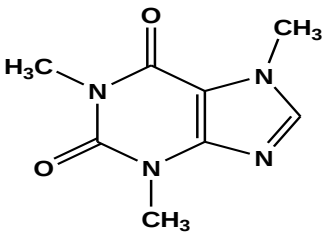
In Trinidad and Tobago, *Cola acuminata* is used for induce childbirth, improve fertility and in treatment of female genital diseases. It has also been used in the management of diabetes, diarrhea, dysentry and hypertension (Lans, 2006; Lans, 2007). An activity

attributed to the high purine alkaloids content. Aqueous extracts of *C. acuminata* and *C. nitida* seeds have also been found to demonstrate an improved cardiovascular effect on the rhythmic activity of the heart (Chukwu, 2006; Salahdeen *et al*., 2015). High caffeine intake has also been implicated to have effects on the oestrous cycle of rat when compared with rats that were fed with decaffeinated extract of kola nuts (Benie *et al*., 2003; Boylan *et al*., 2008). Also high caffeine consumption by preganant women in Africa may possibly trigger reproductive hazards and future occurrence of miscarriage (Liston, 1998; Leviton and Cowan, 2002; Signorello and McLaughlin, 2004; Weng *et al*., 2008; Koczkowski, 2009; Peck *et al*., 2010).

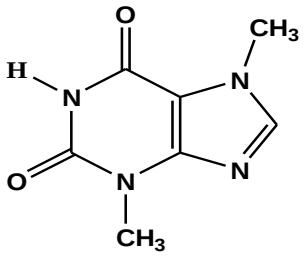
A study conducted on athletes in London also reveals that caffeine and theobromine have been found to increase physical and cognitive performance of athletes. This assertion is in agreement with the fact that caffeine is a stimulant of the cardiovascular system (Magkos and Kavouras, 2005; Hogervorst *et al*., 2008; Glaister *et al*., 2008).

**2.2 Compounds identified in *C. nitida* (Vent.) Schott et Endl.**

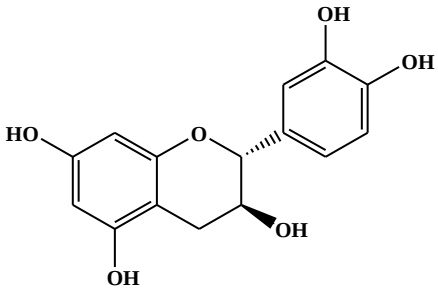
Phytochemical investigation of the seed crude extract of *C. nitida* using reversed-phase high performance liquid chromatography afforded flavonoids: catechin, epicatechin and alkaloid caffeine (Niemenak *et al*., 2008). Analysis of the 80% aqueous methanolic extract of the seed using Waters 600 E high performance liquid chromatography equipped with 990 photodiode array detector indicated the presence of phenolic compounds such as epicatechin, d-catechin, and alkaloids caffeine and theobromine (Odebode, 1996; Boudjeko *et al*., 2009). The structures are given below:



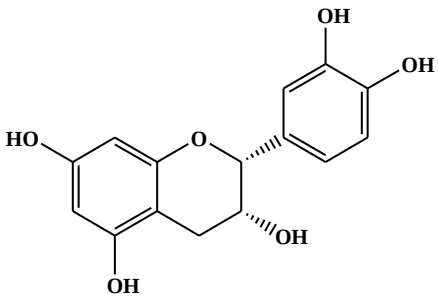
Caffeine



Theobromine



Cathechin



Epicathechin

## 2.3 Description of Analytical Techniques

### 2.3.1 Chromatographic Methods

Chromatography is a method of choice in handling the problem of isolation of pure compounds of interest from a complex natural mixture. Therefore, the chromatographic methods used in this research are briefly described.

#### 2.3.1.1 Thin Layer Chromatography (TLC)

TLC involves the use of a particulate sorbant spread on an inert sheet of glass, plastic, or metal as a stationary phase. The solvent is allowed to travel up the plate carrying the sample that was initially spotted on the sorbant just above the solvent level. Depending on the nature of the stationary phase, the separation can either be partition or adsorption chromatography. The advantage of TLC is in its ability to detect a wide range of compounds using reactive spray reagents, visualization under UV light or reaction with iodine in iodine tank. TLC analysis of a mixture is important to further separation and fractionation of an extract. TLC also makes it possible for purified samples to be scrapped off the plate and analyzed (Snyder and Kirkland, 1979; Guiochen, 2001)

#### 2.3.1.2 Accelerated Gradient Chromatography (AGC)

AGC is a chromatographic technique that is simple, faster and more efficient than the traditional classical column chromatography which is gravity-fed, with sample or mobile phase which is generally used only once. Therefore, packing a column has to be repeated for each separation and individual fractions are collected manually. In AGC, it is pressure (between 10 to 12 millibars) using a pump that is used to drive the mobile phase through the column, hence AGC is also called Medium Pressure Liquid Chromatography (Baekstrom, 1993; Ajibesin *et al*., 2011; Sodipo *et al*., 2012). A rapid controlled flow of

solvent is obtained with convenience. The essential features of the AGC used in this research include the gradient mixer, reservoir, column, magnetic stirrer, and pump.

#### 2.3.1.3 Column Chromatography

Column chromatography consists of a column of particulate material such as silica or alumina that has a solvent passed through it at atmospheric pressure. The column is usually made of glass. Most column chromatography rely on gravity to push the solvent the stationary phase. The sample is dissolved in suitable solvent or adsorbed in silica and loaded onto the column. The solvent elutes the sample through the column allowing sample components to separate. The solvent is usually changed stepwise and fractions are collected. The fractions are monitored by TLC. The advantage is that no expensive equipment is required and the technique can be scaled up to hold larger sample size. (Snyder and Kirkland, 1979; Guiochen, 2001). The size of the column depends on the sample weight and method of sample loading.

### 2.4 Extraction Methods

Extraction is the separation of medicinally active portion of plants and animals tissues using selective solvents through standard procedures. (Tiwari *et al*., 2011) During extraction solvent diffuse into the sample material and solubilize the phytochemicals based on their affinity for the solvent used for the extraction. Extraction is an important step in obtaining a medicinally active extract and it is imperative that proper action must be taken to ensure that the potential bioactive compounds are not lost, distorted or destroyed during preparation of the extract (Ncube *et al*., 2008; Sasidharan *et al*., 2011).

If the plant was selected based on the ethnomedicinal usage, it is therefore proper to prepare the extract as described by the traditional healers in order to mimic as closely as possible traditional herbal drug. The type of solvent used for extraction ranges from non-polar to polar solvents depending on the targeted components and as the target compounds may be non-polar, polar or thermally labile, the suitability of extraction methods must therefore be considered. It is desired to extract the phytochemicals in the form in which they occur in the plant matrix; therefore in this study solid-liquid extraction method at room temperature was used.

### 2.4.1 Plant Tissue Homogenization

This method of extraction has been widely used by researchers. Dried or wet, fresh plant parts are grinded in a blender to fine particles, put in a certain quantity of solvent and shaken vigorously for 5 to 10 minutes or left for 24 hours after which the extract is filtered. The filtrate is then dried under a reduced pressure. Some researchers have also centrifuge the filtrate for clarification of the extract (Das *et al*., 2010). This method is suitable for the extraction of thermolabile components.

### 2.4.2 Serial Exhaustive Extraction

This is another common method of extraction that involves successive extraction with solvent of increasing polarity from a non-polar (hexane) to a more polar solvent (methanol). This ensures that a wide polarity range of compounds are extracted. This method gives a way of defatting plant material and therefore makes subsequent chromatographic fractionation and separation easy and finer (Das *et al*., 2010; Tiwari *et al*., 2011).

### 2.4.3 Maceration

In this method, finely grinded or coarsely grinded plant material is kept in contact with the extracting solvent in a stoppered container for a defined period of time with frequent agitation to solubilize the components. This method is particularly useful in the extraction of thermolabile compounds as no heat is applied (Ncube *et al*., 2008). The extracting solvent diffuses into the plant materials and solubilizes the phytochemicals.

### 2.4.4 Soxhlet Extraction

This method cannot be used for extraction of thermolabile compounds as prolonged heating may lead to degradation of the desired compounds. The advantage of this method lies in the fact that the same solvent is recycled for extraction thus an economical method. Soxhlet extraction is required if the desired compounds have limited solubility in the solvent (Nikhal *et al*., 2010).

### 2.4.5 Percolation

This is the method most frequent used in the extraction of active metabolites in the preparation of tinctures. A percolator (a narrow, cone –shaped vessel open at both ends) is generally used. The plant material is moistened with an appropriate amount of specified menstrum and allowed to stand for four hours in a closed container. The mass is then packed and the top of the percolator is closed. Additional menstrum is added to form a layer above the mass. The mixture is then allowed to macerate for 24 hours in the closed percolator. The outlet of the percolator is then opened and the liquid is slowly drained. The marc is then pressed and the expressed liquid is added to the percolate. The

percolate then is filtered or centrifuged for clarification (Handa *et al*., 2008; Sasidharan *et al*., 2011).

### 2.4.6 Sonication

Sonication involves the use of ultrasound with frequencies ranging from 20 kHz to 2000 kHz; this increases the permeability of the cell walls of the plant materials and produces cavitation. Although the method is very useful but its wide scale application is limited due to high cost. One disadvantage of sonication is the occasional but deleterious effects of ultrasound energy on the plant active constituents due the possible formation of free radicals leading to undesirable modification in the active compounds (Handa *et al*., 2008). Ultrasound has the main advantages of shorter reaction and preparation time, use of small plant materials, efficient and minimum expenditure of solvent (Ishtiaq *et al*., 2009; Gupta *et al*., 2012).

### 2.4.7 Decoction

This method is useful in the extraction of water soluble and heat stable compounds from plant materials by boiling the plant materials in water for 15 minutes, cooling and straining and passing sufficient cold water through the plant marc. Decoction is one of the oldest traditional extraction methods employed by traditional healers (Gupta *et al*., 2012)

## 2.5 Extraction Solvents

Successful extraction of bioactive compounds from plant materials is largely dependent on the type solvent used in the extraction procedures. The properties of a good solvent to be in the extraction of phytochemicals from plant sources include:

- the solvent must have rapid physiologic absorption by the plant materials
- the solvent must have rapid rate of evaporation at low heat
- the solvent must not cause the extract to complex, dissociate or react with the extract. This is important because the crude extract may contain the last traces of solvent that is not removed.
- the solvent must have a relatively low toxicity to test animals most especially if the extract is to be used for bioassays.

The choice of solvent is influenced by what is intended with the extract and the choice will also depend on the targeted compounds to be extracted. The various solvent that are used in the extraction of phytochemicals are:

### 2.5.1 Water

Water is a universal solvent used most commonly by the traditional healers in the extraction of secondary metabolites with antimicrobials activity (Tiwari *et al*., 2011). Though traditional healers use primarily water, plant extracts from organic solvents have been found to give better activity than water extract (Eloff, 1998). Care must be taken when using water for extraction. This is due to the fact that water is a good medium for the growth of microorganisms which may attack the extract and cause degradation (Das *et al*., 2010).

### 2.5.2 Alcohols

The higher activity of alcoholic extracts compared to the aqueous extract can be attributed to the presence of higher amount of polyphenols in alcoholic extract. This shows that alcohols are more efficient in cell walls and seed degradation which have unipolar character thereby causing polyphenols to be released from cells. Alcohols also have a preservative effect on their extract in that the enzyme polyphenols oxidase which active in water extract are inactivated in the presence of alcohols (Bimakr, 2010). Alcohol – water mixtures have also been used for extraction. Extracts obtained from 70 % methanol has been found to contain higher concentration of flavonoids and has been used by many researchers (Lin *et al*., 1999). Additionally, ethanol was found easier to penetrate the cellular membrane to extract the intracellular compounds (Wang, 2010). Since nearly all of the identified compounds from plants that are active against microorganisms are aromatic or saturated organic compounds, they are most often obtained through initial ethanol or methanol extraction. Aqueous methanol has also been found to extract a wide range of phytochemicals, this due to the expanded range in polarity of the solvent with water (Cowan, 1999).

### 2.5.3 Acetone

Acetone dissolves both lipophilic and hydrophilic compounds from plants materials. It is miscible with water, volatile and has low toxicity to bioassays. It is a very useful extracting solvent especially for antimicrobial studies where more phenolic compounds are to be extracted. A study reported that extraction with aqueous acetone was better than

aqueous methanol (Lapornik *et al*., 2005). Acetone and water were found to extract saponins which have antimicrobial activity (Ncube *et al*., 2008).

### 2.5.4 Chloroform

Terpenoids lactones have been obtained by successive extraction dried barks with n-hexane, chloroform and methanol with activity concentrated in the chloroform extract. Occasionally, tannins and terpenoids are found in the aqueous extract but they are more often obtained with less polar solvents (Cowan, 1999).

## 2.6 Antimicrobial Sensitivity Testing

Antimicrobial sensitivity or antibiotic sensitivity testing is the determination of the susceptibility of micro-organisms (bacteria, viruses and fungi) to antibiotic or potential antimicrobial agents. The performance of antimicrobial sensitivity testing is important to confirm susceptibility of tested microorganisms to chosen antimicrobial agents and to detect resistance in individual microorganisms. Susceptibility testing is also important with species that may possess acquired resistance mechanisms. For example, members of the *Enterobacteriaceae*, *Pseudomonas* species, *Staphylococcus* species, *Enterococcus* species, and *Streptococcus pneumoniae (Jorgensen, 1993; Doern et al., 1994; Barenfanger et al., 1999). Antimicrobial susceptibility testing is an in-vitro procedure for determining the susceptibility of a microorganism to an antimicrobial agent. This helps in the selection of appropriate antimicrobial agent for the treatment of an infection (Humphrey and Lightbrown, 1952).*

### *2.6.1* Antimicrobial Sensitivity Testing Methods

A number of Antimicrobial sensitivity testing methods are available worldwide and their usage varies from one country to another. The aim of the method is to predict how the tested microorganisms will respond to the antimicrobial agents. The methods involves culturing a sample of the microorganisms to obtain a pure isolate and testing to determine which antimicrobial agent inhibits the growth of (Bacteriostatic) or kill (Bactericidal) the tested microorganisms ( CLSI, 2013).

**2.6.1.1 Broth Dilution Test**

One of the earliest antimicrobial sensitivity testing method was the macrobroth tube-dilution method (Bauer *et al*., 1966; Ericsson and Sheriss, 1971). This method involves preparing two-fold dilutions of antimicrobial agents for example 1, 2, 4, 8, 16 µg/mL in a liquid growth medium dispensed in testubes. The antimicrobial agents in the testtubes are then inoculated with a standardized microorganisms' suspension of $1.0 \times 10^5$ CFU/mL to $5.0 \times 10^5$ CFU/mL. Following overnight incubation, the tubes are then examined for visible microbial growth as evidenced by turbidity (Jorgensen and Turnidge, 2007). The lowest concentration of the antimicrobial agent that prevents growth is the minimum inhibitory concentration (MIC). The precision of this method was considered to be ± 1 two-fold concentrations due the manual preparation of serial dilution of the antimicrobial agent. The advantage of this method is the generation of a quantitative result (MIC). This method has principal disadvantages in that it is tedious and involves manual preparation of antimicrobial agent for each test, the possibility of errors and relatively large amount of reagents and space for each test. The miniaturization and mechanization of the test by the use of small, disposable plastic microdilution trays has made broth dilution practical and popular. Standard trays contain 96 wells, each containing a volume of 0.1 mL that

allows approximately 12 antimicrobial agents to be tested in a range of 8 two-fold dilutions in a single tray. Microdilution procedure is reproducible and convenient (Ballows, 1972).

### 2.6.1.2 Antimicrobial Gradient Method

The antimicrobial gradient diffusion method uses the principle of establishment of an antimicrobial concentration gradient in an agar medium as a means of determining susceptibility (Citron *et al*., 1991). The E-Test is a commercial version available in the United States. It employs a thin plastic test strips that are impregnated on the underside with a dried antimicrobial concentration gradient. As many as 5 or 6 test strips may be placed in a radial fashion in the agar plate inoculated with the microorganism's suspension. The whole set-up is then allowed to stand overnight. The test strips are then viewed from the top of the plate. The gradient diffusion method has intrinsic flexibility and is most suitable for antimicrobial assays in which the MIC of one or two antimicrobial agents are to be determined. Generally, E-Test results correlated well with MIC obtained from by broth or agar dilution methods (Brown and Brown, 1991; Baker *et al*., 1991).

### 2.6.1.3 Disk Diffusion Method

This method is simple, practical and has been well standardized. It is carried out by applying a microbial suspension of approximately $1.0 \times 10^8$ CFU/mL to $2.0 \times 10^8$ CFU/mL to the surface of a Mueller-Hinton agar plate (Bauer *et al*., 1966). Commercially prepared, fixed concentration, paper antibiotic disks are then placed on the inoculated agar surface. Plates are then incubated for 16 to 24 hours at 35 °C. The zones of growth

inhibition around each of the antibiotic disk are measured to the nearest millimeter. The diameter of the zone is related to the susceptibility of the isolates and to the diffusion rate of the antimicrobial agent through the agar medium (Korgenski and Daly, 1998). The results obtained from disk diffusion method are qualitative in nature that is the result is categorized as susceptible, intermediate or resistant rather than an MIC. The advantages of the disk diffusion method are the test simplicity that does not require any special equipment. The provision of categorical results that are easy to interpret and also flexibility in the selection of disks. It is the least costly of all susceptibility testing methods (Nijs *et al*., 2003).

# CHAPTER THREE

# MATERIALS AND METHODS

## 3.1 Materials Used in this Study

The following materials were used in this research:

### 3.1.1 Chemicals

Methanol, Ethylacetate, Chloroform, *n*-Hexane, *n*-Butanol, Silica Gel (kieselgel 60G 0.040-0.063 mm and 60-200 mesh), Pre-coated TLC Plates (MERCK, silica gel 60 $F_{254}$

0.2mm), Accelerated Gradient Chromatography (AGC Baeckstrom Separo AB), and Gallenkamp Melting Point Determination Apparatus.

All the solvents were redistilled before use.

### 3.1.2 Micro-organisms

The following standard bacteria and fungi of National Collection for Industrial Bacteria (NCIB) and Locally Isolated Organisms (LIO) used in this research work were obtained from the Department of Microbiology, Obafemi Awolowo University, Ile – Ife:

*Micrococcus luteus* (NCIB 196), *Pseudomonas fluorescence* (NCIB 3756), *Bacillus cereus* (NCIB 6349), *Clostridium sporogenes* (NCIB 532), *Pseudomonas aeruginosa* (NCIB 950), *Shigella dysenteriae* (LIO), *Bacillus stearothermophillus* (NCIB 8222), *Escherichia coli* (NCIB 86), *Klebsiella pneumoniae* (NCIB 418), *Bacillus subtilis* (NCIB 3610), *Shigella flexneri* (LIO), *Staphylococcus aureus* (NCIB 8588), *Bacillus polymyxa* (LIO), *Corynebacterium pyogenes* (LIO), *Enterococcus faecalis* (NCIB 775), *Proteus vulgaris* (LIO), *Aspergillus niger* (LIO), *Aspergillus fumigatus* (LIO), and *Candida albicans* (LIO).

### 3.1.3 Spectroscopic Instruments

Nuclear Magnetic Resonance ($^{1}$HNMR) spectroscopic data was recorded on a 400 MHz Agilent Technologies NMR Machine and 100 MHz for $^{13}$CNMR. Chemical shifts of signals were reported in parts per million (ppm). Gas Chromatogram was recorded on Agilent Technologies 7890A and Mass Spectroscopic data was recorded on Agilent Technologies model 5975 C. The stationary phase used was HP 5 MS column with length

30 m and internal diameter 0.320, thickness 0.25 μL. The mobile gas used is helium gas. Detector temperature was set at 250 °C.

### 3.2 Plant Collection

Seeds and twigs of *C. nitida* were collected at Alaro Farm Settlement, Ile-Ife. It was identified and authenticated at Herbarium Section, Department of Pharmacognosy, Obafemi Awolowo University Ile-Ife by a Botanist, Mr Ogunlowo, I. I. with voucher number: FPI 2052.

### 3.3 Preparation of Plant Material

The seeds were washed with distilled water, drained, cut into pieces, and air-dried at room temperature and ground to powder.

### 3.4 Extraction Procedure

Powdered seeds (2.0 kg) were extracted with distilled methanol in a 10 L stoppered jar with frequent agitation for 24 hours. The extract was filtered through cotton plug and filtrate was concentrated in vacuo using rotary evaporator at 40 °C to obtain the crude extract. This was further dried over activated silica in desiccator to constant weight of 80 g. Extraction afforded a 4 % yield of extract.

### 3.5 Liquid-liquid Fractionation of Crude Extract

The crude extract obtained was suspended in distilled water in a separatory funnel (2 L) and successively partitioned with *n*-hexane, ethylacetate and *n*-butanol to obtain the respective solvent fractions.

### 3.6 Antimicrobial Screening

The crude extract and the solvent fractions were spotted on pre-coated TLC plates and developed with solvent system ethylacetate - methanol (8:2). The chromatogram was then placed in a petri-dish and overlayed with a medium seeded with test microorganisms. This was then incubated at 37 °C for 24 hours, after which a spray reagent thezolyl blue tetrazolium was added. The antimicrobial activity of the various extracts showed that ethylacetate fraction demonstrated the highest activity against most of the microorganisms used and was selected for further fractionation. All Thin Layer Chromatography (TLC) analyses were performed at room temperature using pre-coated plates (MERCK, silica gel 60 $F_{254}$ 0.2 mm). Detection of spots was by ultraviolet light (254 and 366 nm) and staining with iodine crystals.

### 3.7 Fractionation of Ethyl Acetate Fraction

Accelerated Gradient Chromatography (AGC) using silica gel (kieselgel 60 G 0.043-0.063 mm) as stationary phase was used to fractionate ethylacetate extract (Plate 3.1).

**Plate 3.1: Accelerated Gradient Chromatography (AGC)**

The ethylacetate extract was dissolved in methanol and adsorbed in silica gel. The mixture was allowed to dry under a stream of air until it flows. The AGC column (50 × 2.5 cm) was packed dry with silica gel as the separating phase using a long stemmed

funnel and it was tapped to allow captured air pockets to escape. The adsorbed extract was then loaded onto the packed column. A filter disc was inserted and the column was connected to the pump. The column was saturated with 100% *n*-hexane and was eluted with solvent mixtures in Table 3.1. A total of 106 fractions in test tubes were collected.

### 3.7.1 Bulking of Fractions

Tubes 1-7 were developed on TLC plates using *n*-hexane:ethylacetate (4:1) solvent system, tubes 8-23 were developed on TLC plates using *n*-hexane:ethylacetate (3:2; 2:3), tubes 24-76 were developed on TLC plates using *n*-hexane:ethylacetate (1:4;3:7;0:4), tubes 77-106 were developed on TLC plates using ethylaceate:methanol (4:1;9:1) solvent systems. The tubes with similar TLC profile were pooled to give four combined fractions coded as XA, XB, XC and XD (Table 3.2). The chromatogram obtained from the development of fractions 24-76 contain major spots previously observed in the ethylacetate extract developed with the same solvent system and it was selected for further fractionation.

#### 3.7.1.1 Fractionation of Combined Fraction XA (CFXA)

Open column chromatography using silica gel (60 – 120 mesh) as stationary phase was used to fractionate CFXA. The sample was dissolved in chloroform and adsorbed in silica gel. The mixture was allowed to dry under a stream of air. A glass column was packed with silica gel slurry as the separating phase and the extract (adsorbed in silica) was loaded onto the column. The column was eluted with solvent mixtures in Table 3.3.

Table 3.1: Fractionation of Ethylacetate Fraction

| *n*-hexane (mL) | Ethylacetate (mL) | Methanol (mL) | Volume (mL) | Tubes |
|---|---|---|---|---|
| 100 | - | - | 200 | 1-7 |

| | | | | |
|---|---|---|---|---|
| 50 | 50 | - | 100 | 8-16 |
| 25 | 75 | - | 100 | 17-23 |
| 12.5 | 87.5 | - | 100 | 24-32 |
| 6.25 | 93.75 | - | 100 | 33-38 |
| 3.13 | 96.87 | - | 100 | 39-46 |
| 1.56 | 98.44 | - | 100 | 47-55 |
| 0.78 | 99.22 | - | 100 | 56-61 |
| - | 100 | - | 200 | 62-76 |
| - | 50 | 50 | 100 | 77-84 |
| - | 25 | 75 | 100 | 85-93 |
| - | 12.5 | 87.5 | 100 | 94-99 |
| - | - | 100 | 100 | 100-106 |

Table 3.2: AGC subfractions of Ethylacetate Fraction of *C. nitida*

| Test tubes | Combined Fractions |
|---|---|

| | |
|---|---|
| 24 – 76 | XA |
| 8 – 23 | XB |
| 1 – 7 | XD |
| 77 – 106 | XC |

Table 3.3: Fractionation of Combined Fraction XA (CFXA)

| Solvents | Proportions | Volume (mL) | Tubes |
|---|---|---|---|

| | | | |
|---|---|---|---|
| *n*-hexane | 100% | 200 | 1 – 5 |
| *n*-hexane:EtOAc | 60:40 | 100 | 6 – 14 |
| *n*-hexane:EtOAc | 50:50 | 100 | 15 – 40 |
| *n*-hexane:EtOAc | 20:80 | 100 | 41 – 70 |
| *n*-hexane:EtOAc | 100% | 200 | 71 – 77 |
| EtOAc:MeOH | 90:10 | 50 | 77 – 80 |

### 3.7.1.2 Bulking of Fractions and Isolation of Compound 1

Tubes 36 - 67 were developed on TLC plates using *n*-hexane:ethylacetate (1:4) solvent system, tubes 68 - 80 were developed on TLC plates using 100% ethylacetate. Tubes with similar TLC profile were bulked together to give two combined fractions XA1 (tubes 36 - 67) and XA2 (tubes 68 - 80). Combined fraction XA1 was analyzed by TLC and developed with using chloroform:methanol (4:1) and *n*-hexane:ethylacetate (1:4) as separate solvent systems. The chromatograms obtained were visualized under ultraviolet light (254 and 366 nm) and only one spot was observed. Iodine staining of the chromatograms also reveals one brown spot. Combined fraction XA1 was then labelled compound 1. The chromatogram of compound 1 is presented in Plate 3.2. The melting point of compound 1 was also determined.

**3.7.1.3 Fractionation of Combined Fraction XB (CFXB)**

Fraction XB was further concentrated in vacuo using a rotary evaporator and a yellow solid was obtained. This was then dissolved in chloroform and chromatographed using open column chromatography packed with silica gel slurry of mesh size 60 – 120 as the separating phase. It was adsorbed in silica gel and loaded on the column. The column was eluted with solvent mixtures in Table 3.4. Fractions collected from the column were concentrated by continuous air blow. Test tubes numbers 6 to 11 were found to contain a white sticky solid which was labeled as compound 2. Compound 2 was then analyzed by Gas Chromatography-Mass Spectrometry (GC-MS) and Nuclear Magnetic Resonance (NMR).

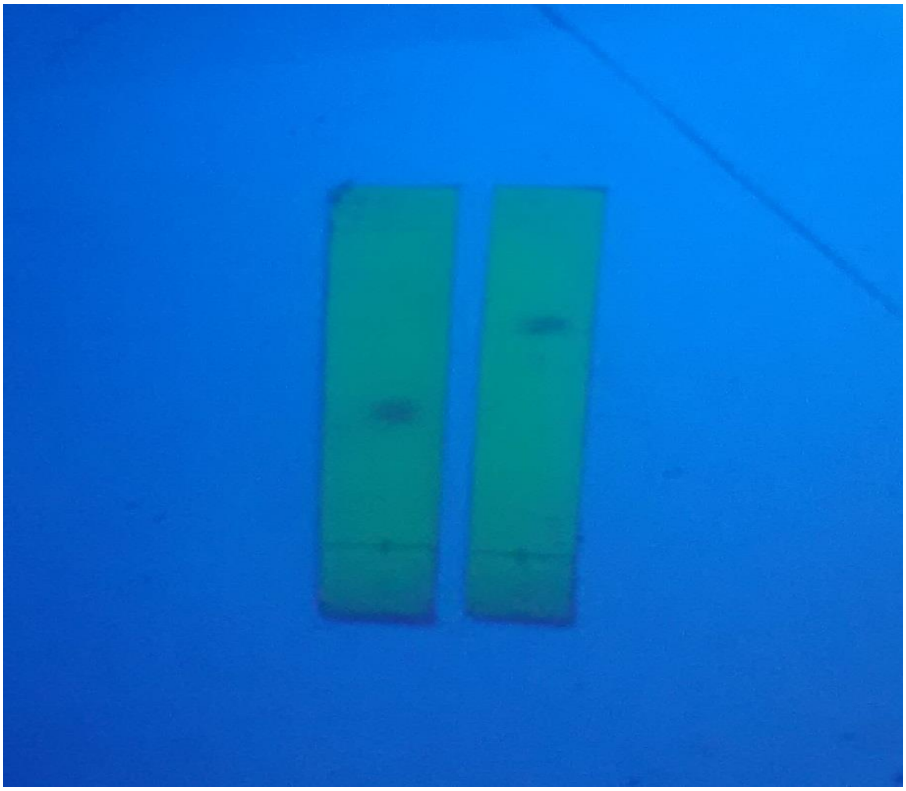


**Plate 3.2: Chromatogram of Compound 1 with two different Developing Solvents**

Table 3.4: Fractionation of Combined Fraction XB (CFXB)

| Solvents | Proportions | Volume (mL) | Tubes |
|---|---|---|---|
| *n*-hexane | 100% | 50 | 1 – 3 |
| *n*-hexane:EtOAc | 95:05 | 100 | 4 – 14 |
| *n*-hexane:EtOAc | 90:10 | 100 | 15 – 28 |
| *n*-hexane:EtOAc | 80:20 | 100 | 31 – 43 |

### 3.8 *In-vitro* Antimicrobial Activity of Plant Extracts and Isolated Compound(s)

The antimicrobial activity of the crude extract, solvent fractions and isolated compound was determined using the agar-well diffusion method described by Russell and Furr (1977) and Irobi *et al*. (1994). The bacterial isolates were first grown in nutrient broth for 18 hours before use, while the fungal isolates were allowed to grow on potato dextrose agar medium at 25 °C until they sporulate. The fungal spores were harvested after sporulation by pouring mixture of sterile glycerol and distilled water onto the surface of the plate and later scraping the spores with a sterile glass rod. The harvested fungal spores and the bacterial isolates were standardized to $OD_{600nm}$ 0.1 before use. 100 µL of the standardized bacterial suspension was evenly spread on Mueller-Hinton agar (Lab M) using a glass spreader while the same volume of the fungal spore suspension was spread on Potato dextrose agar (Agyare *et al*., 2013).

Wells were then bored into the agar media using a sterile 6 mm cork borer and the wells were filled with the solution of the plant extracts taking care not to allow spillage of the solution to the surface of the agar medium. The plates were allowed to stand on the laboratory bench for 1 hour to allow proper diffusion of the extracts into the media. The bacterial isolates were thereafter incubated at 37 °C for 24 hours after which they were observed for zones of inhibition. Plates containing fungal isolates were incubated at 25 °C for 96 h and later observed for zones of inhibition. The effects of the plant extracts at concentration of 10 mg/mL on the test organisms were compared with that of Ampicillin and Nystatin, standard clinical commercial antibiotic each at a concentration of 1 mg/mL.

The effect of the extracts on fungal isolates was compared with nystatin. Dimethylsulfoxide (DMSO) was used as a dissolving solvent and negative control.

The antimicrobial activity was carried out at the Department of Microbiology and the tested microorganisms of the National Collection for Industrial Bacteria (NCIB) and locally isolated organisms (LIO). They were obtained from the Department of Microbiology, Obafemi Awolowo University, Ile – Ife. The tested organisms were *Micrococcus luteus* (NCIB 196), *Pseudomonas fluorescence* (NCIB 3756), *Bacillus cereus* (NCIB 6349), *Clostridium sporogenes* (NCIB 532), *Pseudomonas aeruginosa* (NCIB 950), *Shigella dysenteriae* (LIO), *Bacillus stearothermophillus* (NCIB 8222), *Escherichia coli* (NCIB 86), *Klebsiella pneumoniae* (NCIB 418), *Bacillus subtilis* (NCIB 3610), *Shigella flexneri* (LIO), *Staphylococcus aureus* (NCIB 8588), *Bacillus polymyxa* (LIO), *Corynebacterium pyogenes* (LIO), *Enterococcus faecalis* (NCIB 775), *Proteus vulgaris* (LIO), *Aspergillus niger* (LIO), *Aspergillus fumigatus* (LIO), and *Candida albicans* (LIO).

# CHAPTER FOUR

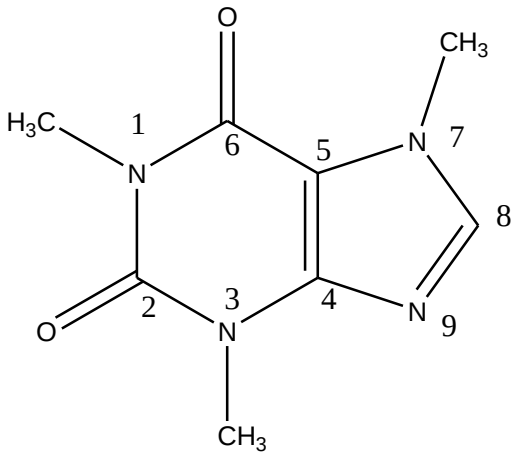
# RESULTS AND DISCUSSION

## 4.1 Structure Elucidation of Compound 1

### 4.1.1 $^{1}$H-NMR of Compound 1

The Proton Nuclear Magnetic Resonance ($^{1}$H-NMR) of **Compound 1** was recorded on a 400 MHz Agilent Technology Machine. Chemical shifts of signals were reported in parts per million (ppm). Compound **1** was obtained as a white crystalline solid with melting point of 230-233 °C. The $^{1}$HNMR of Compound **1** confirmed the presence of four proton signals which is consistent with the literature data for the proton nuclear magnetic resonance of caffeine. The spectrum (appendix i) showed a singlet peak at δ 7.46 ppm in the aromatic region. This signal is due to the olefinic proton signal at carbon 8 (C-8). This olefinic proton is deshielded by the two neighboring electronegative nitrogen atom thereby decreasing the electron density around the proton and therefore the proton appeared at higher δ values. The anisotropic effect of adjacent pi (π) electrons and lone pair of electron on the nitrogen atom also causes the signal to appear downfield. The three methyl signals were observed δ at 3.97, 3.54, and 3.36 ppm. The high δ value of the three methyl groups is due to the electron withdrawing effect of electronegative nitrogen atoms (Niemenak *et al*., 2008). The COSY experiment (appendix ii) showed that there

was no correlation among the proton signals. The structure of **Compound 1** is given in Figure 4.1.




1,3,7-trimethyl-1*H*-purine-2,6(3*H*,7*H*)-dione
Compound 1

**Figure 4.1: Structure of Compound 1**

### 4.1.2 $^{13}$C-NMR of Compound 1

The $^{13}$Carbon Nuclear Magnetic Resonance ($^{13}$CNMR) of **Compound 1** was recorded on a 100 MHz Agilent Technology Machine. Chemical shifts of signals were reported in parts per million (ppm). $^{13}$CNMR spectrum (appendix iii) of **Compound 1** confirmed the presence of eight $^{13}$C signals. Carbonyl carbon atoms C-6 and C-2 appeared at δ 155.3 and 151.6 ppm respectively. The δ values of carbonyl carbon atoms C-6 and C-2 are low compared to the δ value (205 ppm) of free unconjugated carbonyl carbon. This is due to the mesomeric effect of the lone pair electrons on nitrogen atom and double bond of carbon atoms C-5 and C-4 which releases electron to these carbonyl carbons thereby increasing the electron density (shielding effect) around carbonyl carbon atoms C-6 and C-2. This increase in the shielding effect therefore causes lower δ values.

Olefinic carbons atoms C-8; C-5 and C-4 appeared at δ values 141.5, 107.5 and 148.6 ppm respectively. This is because the C-6 carbonyl carbon atom is in conjugation with the pi (π) electrons of C-5 and C-4 carbon atoms, and by mesomeric effect, it increases the electron density around C-5 carbon atom and therefore makes C-5 carbon atom to be more shielded due to higher electron density and hence lower δ value of C-5 carbon atom compared to C-4 carbon atom. Signals due to the three methyl groups showed at δ 33.5, 29.7, 27.9 ppm. The $^{13}$CNMR spectroscopic data of **Compound 1** are in agreement with the literature data for Caffeine and shown in Table 4.1 (Niemenak *et al*., 2008; Boudjeko *et al*., 2009).

Table 4.1: $^{13}$C-NMR Spectroscopic Data of Compound 1

| Carbons | δ values (ppm) |
|---|---|
| C-8 | 141.5 |
| C-6 | 155.3 |
| C-5 | 107.5 |
| C-4 | 148.6 |
| C-2 | 151.6 |
| $N^1$-$CH_3$ | 29.7 |
| $N^3$-$CH_3$ | 33.5 |
| $N^7$-$CH_3$ | 27.9 |

### 4.1.3 DEPT-135 Experiment of Compound 1

The Distortionless Enhancement by Polarization Transfer (DEPT) Experiment (appendix iv) confirmed the presence of four $^{13}$carbon signals. This experiment is used to detect protonated carbons in organic compounds. Signal at δ value 141.5 ppm also observed in the $^{13}$C-NMR (appendix iii) is due to olefinic carbon C-8. The three signals at 33.5 ppm, 29.7 ppm and 27.9 ppm are in consistent with the signals for the three methyl carbons.

### 4.1.4 Infrared Data of Compound 1

The infrared spectroscopic data of **Compound 1** was recorded on a Thermo Scientific Nicolet iS5 Fourier Transforms Infrared Spectrometer at the Centre for Energy Research and Development, Obafemi Awolowo University Ile-Ife. The infrared spectrum was reported in wavenumber (appendix v). The infrared spectrum (Table 4.2) showed the presence of functional group C=N stretching frequency at 1615 $cm^{-1}$. This absorption frequency is low compared to the absorption frequency of unconjugated C=N functional group which absorbs at 1640 $cm^{-1}$ to 1660 $cm^{-1}$. This is as a result of the extended conjugation of the pi (π) electron system and the lone pair of electrons on nitrogen atom. Therefore mesomeric effect lengthens the bond (reduces the double bond character of C=N bond) and reduces the force constant and hence lower absorption frequency of C=N bond. Mesomeric effect also accounts for the lower absorption frequency of the olefinic double bond C=C stretching at 1609 $cm^{-1}$.

The spectrum also showed a medium intensity peak above 3000 $cm^{-1}$ which is typical of olefinic $sp^2$ C-H stretching frequency at 3112 $cm^{-1}$. $sp^3$ C-H stretching frequency at 2954 $cm^{-1}$ and $sp^3$ C-H bending vibration at 1485 $cm^{-1}$.

Table 4.2: Infrared Spectroscopic Data of Compound 1

| Functional groups | Frequency ($cm^{-1}$) |
|---|---|
| C=N stretching | 1615 |
| C=O stretching | 1665 and 1655 |
| C=C stretching | 1609 |
| C-N stretching | 1548 and 1285 |
| $sp^2$ C-H stretching | 3112 |
| $sp^3$ C-H stretching | 2954 |
| $sp^3$ C-H bending | 1485 |

The spectrum also showed two strong doublets in the region typical of conjugated amide functional group. The stretching vibrational frequency of the C=O absorbed at 1665 and 1655 $cm^{-1}$ and the polar C-N stretching vibrational frequency at 1548 $cm^{-1}$ and 1285 $cm^{-1}$.

### 4.2 Structure Elucidation of Compound 2

#### 4.2.1 $^{1}$H-NMR of Compound 2

The Proton Nuclear Magnetic Resonance ($^{1}$HNMR) of **Compound 2** was recorded on a 400 MHz Agilent Technology Machine. Chemical shifts of signals were reported in parts per million (ppm). Compound **2** was obtained from white sticky solid 2. The $^{1}$HNMR of **2** (appendix vi) showed the presence of methylene protons at upfield region δ values 2.24, 1.53, 1.21 and methyl protons at δ 0.78 ppm. No olefinic or aromatic proton was observed.

#### 4.2.2 $^{13}$C-NMR of Compound 2

The $^{13}$CNMR spectrum of **2** (appendix vii) showed the presence of a carbonyl carbon at δ value 179 ppm which is typical of the carbonyl carbon of carboxylic acids and esters. A weak signal was also observed at δ value 129.7 ppm which is due to signal as a result of olefinic carbon of the oleic acid, a constituent of solid 2. The signal at δ value 11.8 ppm is due to the methyl carbon. The other signals are due to the methylene carbons.

#### 4.2.3 Attached Proton Test Experiment (APT) of Compound 2

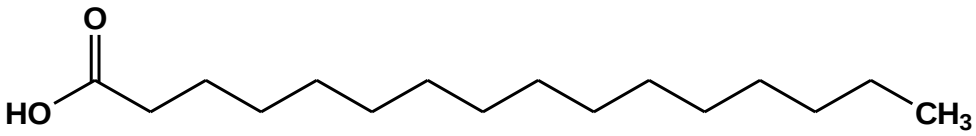
The APT experiment spectrum (appendix viii) showed that the methyl signal at δ value 11.8 ppm since it is the only signal on the negative axis, thus the terminal methyl. It is therefore concluded that compound **2** is long chain saturated fatty acid.

### 4.2.4 GC-MS of Compound 2

Gas Chromatogram was recorded on Agilent Technologies 7890A and Mass Spectroscopic data was recorded on Agilent Technologies model 5975 C. The stationary phase used was HP 5 MS column with length 30 m and internal diameter 0.320, thickness 0.25 μL. The mobile gas used is helium gas. Detector temperature was set at 250 °C. The total ion chromatogram obtained from the GC-MS of solid 2 was autointegrated by chemstation and the constituents were identified by comparison with database NIST 08 as shown in Table 4.3. From Table 4.3 *n*-Hexadecanoic acid has highest peak areas of 18.10 (100 %) and is the main constituents of solid 2**.** The mass spectrum showed m/z 60 (100) as the base peak, m/z 60 is due to the McLafferty rearrangement as a result of the presence of γ protons. The molecular ion peak at m/z 256 which corresponds to the molecular formular $C_{16}H_{32}O_2$. The m/z 74 (95) is also due to the McLafferty of the methyl ester of carboxylic acids. This could have originated from the derivatization of the carboxylic acid with hexamethyldisilazane. This is necessary so as to make the acid more volatile for GC-MS analysis. Other peaks observed in the mass spectrum of **Compound 2** are 83 (25), 129 (40). The structure of **Compound 2** is shown in Figure 4.2. The proposed fragmentation pattern of compound 2 is shown in Figure 4.3. Structures of other compounds detected in the GC-MS of solid 2 are shown in Figure 4.4.

## 4.3 *In-vitro* Antimicrobial Activity

The plant extracts showed varying inhibitory activities against the tested bacterial and fungal strains with the crude extract showing better activity than the other fractions and compound 1. The activity was against both the gram positive and gram negative




*n*-Hexadecanoic acid

**Figure 4.2: Structure of Compound 2**

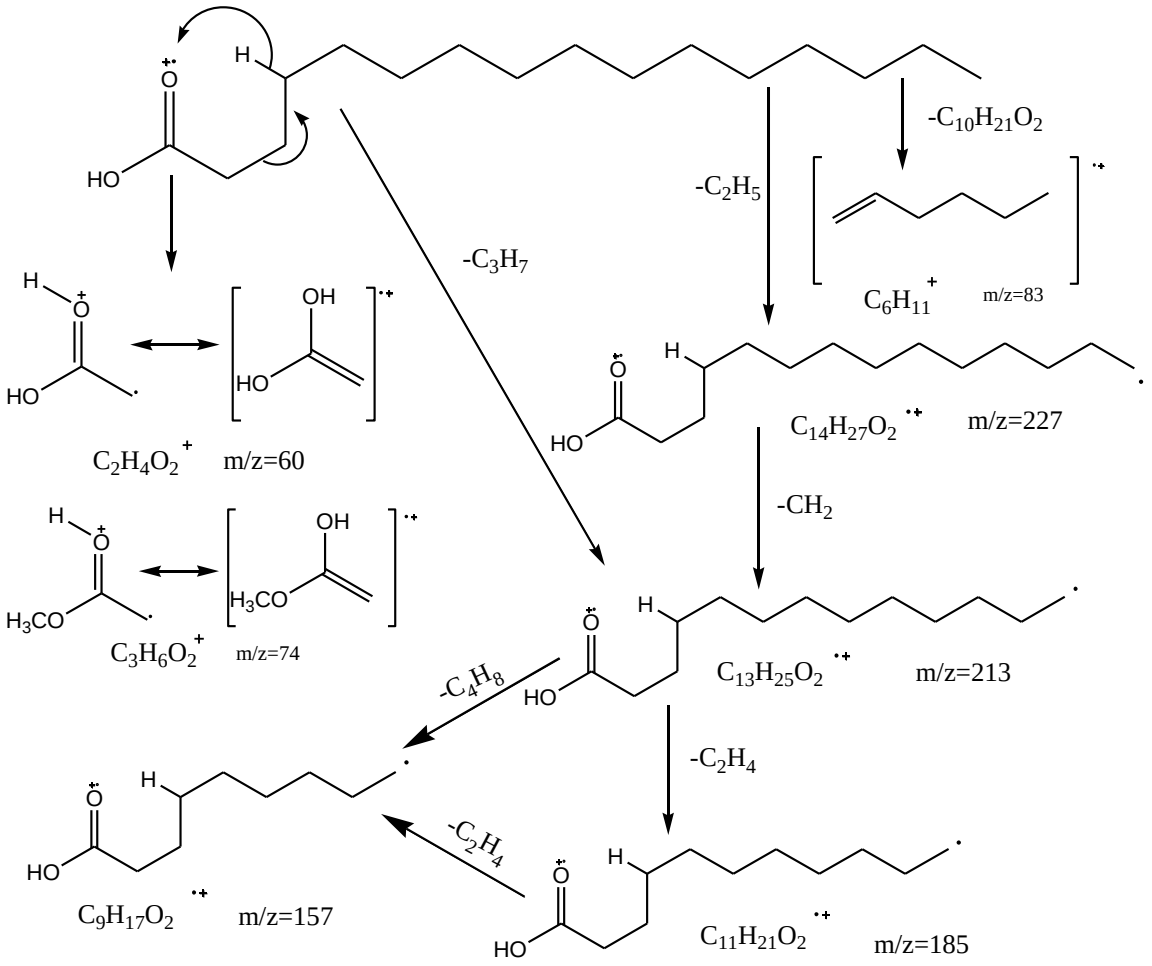
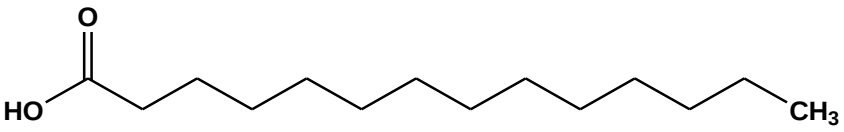



**Figure 4.3: Proposed Fragmentation Pattern of Compound 2**

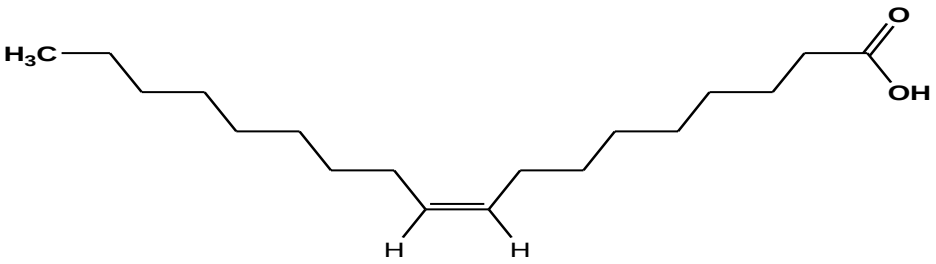
Table 4.3: Compounds detected in the GC-MS Analysis of Solid 2

| Compound Name | Formular | Retention time | Area | Corr. Area % |
|---|---|---|---|---|
| Tetradecanoic acid | $C_{14}H_{20}O_2$ | 11.31 | 0.74 | 4.11 |
| Nonanoic acid | $C_9H_{18}O_2$ | 12.17 | 0,48 | 2.66 |
| Pentadecanoic acid | $C_{15}H_{30}O_2$ | 12.56 | 0.15 | 0.84 |
| Dibuthylphthalate | $C_{16}H_{22}O_4$ | 12.88 | 0.77 | 4.27 |
| *n*-Hexadecanoic acid | $C_{16}H_{32}O_2$ | 13.21 | 18.10 | 100 |
| Methyl octyl ether | $C_9H_{20}O$ | 14.33 | 3.58 | 19.70 |
| Oleic acid | $C_{18}H_{34}O_2$ | 14.48 | 14.63 | 80.81 |
| Vaccenic acid | $C_{18}H_{34}O_2$ | 14.67 | 0.15 | 0.83 |

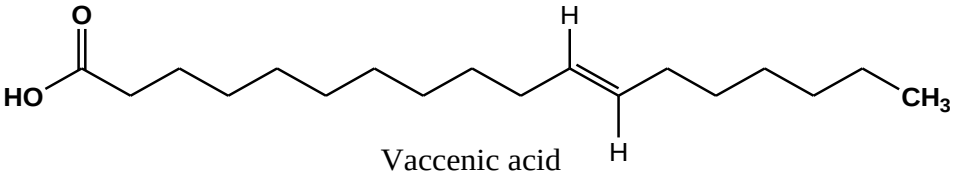
Corr. means Corrected Area in percentage



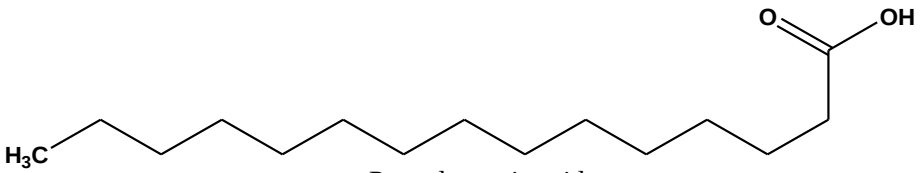

Tetradecanoic acid



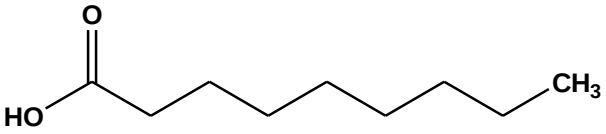

Oleic acid




Vaccenic acid




Pentadecanoic acid




Nonanoic acid

**Figure 4.4: Structure of Components detected in the GC-MS of Solid 2**

organisms. The most susceptible among the tested organisms was *Escherichia coli* and the activity appeared to be organisms dependent. The activity could be as a result of the morphological differences between these microorganisms (Nassar-Abbas and Halkman, 2004).

The Gram-positive bacteria have an outer peptidoglycan layer which is not an effective permeability barrier, which makes it more susceptible to most compounds while the Gram-negative bacteria have an outer phospholipidic membrane that contains lipopolysaccharides which makes the cell wall impermeable to most compounds (Mori *et al*., 1987; Zheng *et al*., 1996). The result of this study however showed that the Gram negative organism Escherichia coli had a greater inhibition than the other Gram positive organisms. Also some other Gram negative organisms which included *Shigella flexneri*, *Shigella dysenteriae* and *Pseudomonas spp* were not inhibited at all. This suggests that the activity may be organisms specific rather than being determined by cell wall characteristics.

The antimicrobial sensitivity testing (with zone of inhibition in mm) of the extracts at 10 mg/mL concentration against nine different Gram-positive and seven Gram-negative bacteria and three fungi were carried out. These microorganisms have been implicated in the pathogenesis of human infections (Swamy *et al*., 2014). Ampicillin (a standard clinical commercial antibiotic) was used at a concentration of 1 mg/mL and DMSO was used as a solvent and negative control. The result obtained as shown in Tables 4.4a to

4.4c indicate that ethylacetate fraction demonstrated better antimicrobial activity against most of the tested organisms compared to the other fractions. This activity is more pronounced against *Escherichia coli*, a Gram-negative bacterium. Compound 1 also demonstrated comparable activity against *Escherichia coli*. This is in agreement with literature data on antimicrobial activity of the methanolic activity of *Cola nitida* where Escherichia coli was also inhibited (Swamy *et al*., 2014).

However, the ethylacetate fraction was not active against the two *Pseudomonas* species, *Clostridium sporogenes, Corynebacterium pyogenes, Shigella* species, and *Candida albicans.* The activity of all the plant extracts did not compare significantly with that of standard drugs. The antimicrobial activity of the crude extract shows that methanol is a good solvent for the preparation of crude extract as evident from the Tables 4.4a to 4.4c, the crude extract demonstrated activity against most of the tested organisms. (Ahmed *et al*., 1998; Eloff 1998; Lin *et al*., 1999).

The antimicrobial activity of the crude extract can be attributed not only to a single bioactive compound but other bioactive compounds in concert (synergistic) action with other compounds in the extract and could be due to the strong presence of alkaloids in *C. nitida* seeds (Mori *et al*., 1987). The presence of tannins glycosides that binds to the cell wall of bacteria prevents the growth and protease activity of the bacteria and may accounts for the antimicrobial activity of butanol fractions which contains more water soluble polyphenols (flavonoids glycosides) and tannins (Jones *et al*., 1994).

The butanol fraction was also active against *Candida albicans* and *Aspergillus* species as evident from Table 4.4c. Compound 1 demonstrated antifungal activity that is

comparable to that of butanol fraction as it was active against all the fungal strains tested. Compound 1 also exhibited good antimicrobial activity against *Bacillus cereus* that compares favourably with ethylacetate activity as shown in Table 4.4a. Also from Table 4.4c, the hexane exhibited no antifungal activity against any of the tested organisms. This could be as a result of the fact that hexane fraction contains only fats, chlorophylls and waxes which are not secondary metabolites compounds with significant antimicrobial activity.

Table 4.4a: Antimicrobial Sensitivity Testing on Gram – Positive Bacteria with the Zone of Inhibition (mm)

| Organisms | Crude extract | Hexane fraction | Ethylacetate fraction | Butanol fraction | Compound1 | Ampicillin 1mg/mL | DMSO |
|---|---|---|---|---|---|---|---|
| *Bacillus subtilis*(NCIB 3610) | 13 | 0 | 11 | 7 | 8 | 23 | 0 |
| *Bacillus polymyxa*(LIO) | 11 | 0 | 9 | 0 | 0 | 21 | 0 |
| *Bacillus cereus*(NCIB 6349) | 17 | 7 | 13 | 9 | 11 | 25 | 0 |
| *Bacillus stearothermophillus*(NCIB 8222) | 13 | 0 | 10 | 0 | 7 | 23 | 0 |
| *Clostridium sporogenes*(NCIB 532) | 9 | 0 | 0 | 0 | 0 | 19 | 0 |
| *Micrococcus luteus*(NCIB 196) | 15 | 0 | 11 | 0 | 0 | 25 | 0 |
| *Enterococcus faecalis*(NCIB 775) | 12 | 0 | 9 | 7 | 0 | 17 | 0 |
| *Staphylococcus aureus*(NCIB 8588) | 14 | 8 | 11 | 8 | 0 | 15 | 0 |
| *Corynebacterium pyogenes*(LIO) | 10 | 0 | 7 | 0 | 0 | 20 | 0 |

Table 4.4b: Antimicrobial Sensitivity Testing on Gram – Negative Bacteria with the Zone of Inhibition (mm)

| Organisms | Crude extract | Hexane fraction | Ethylacetate fraction | Butanol fraction | Compound1 | Ampicillin 1mg/mL | DMSO |
|---|---|---|---|---|---|---|---|
| *Proteus vulgaris* (LIO) | 11 | 0 | 11 | 11 | 8 | 17 | 0 |
| *Klebsiella pneumonia* (NCIB 418) | 13 | 7 | 10 | 7 | 0 | 21 | 0 |
| *Shigella dysenteriae* (LIO) | 0 | 0 | 0 | 0 | 0 | 19 | 0 |
| *Escherichia coli* (NCIB 86) | 19 | 7 | 17 | 11 | 15 | 25 | 0 |
| *Pseudomonas aeruginosa*(NCIB 950) | 10 | 0 | 0 | 0 | 0 | 15 | 0 |
| *Shigella flexneri*(LIO) | 0 | 0 | 0 | 0 | 0 | 17 | 0 |
| *Pseudomonas fluorescence*(NCIB 3756) | 10 | 0 | 0 | 0 | 0 | 13 | 0 |

Table 4.4c: Antimicrobial Screening (Sensitivity Testing) on Fungi with the Zone of Inhibition (mm)

| Organisms | Crude extract | Hexane fraction | Ethylacetate fraction | Butanol fraction | Compound1 | Nystatin 1mg/mL | DMSO |
|---|---|---|---|---|---|---|---|
| *Aspergillus fumigatus* (LIO) | 14 | 0 | 11 | 11 | 8 | 17 | 0 |
| *Aspergillus niger* (LIO) | 15 | 0 | 10 | 9 | 11 | 19 | 0 |
| *Candida albicans* (LIO) | 11 | 0 | 0 | 9 | 7 | 17 | 0 |

# CHAPTER FIVE

# CONCLUSION AND RECOMENDATIONS

## 5.1 Conclusion

The use of *C. nitida* in the management of infections as a result of pathogenic microorganisms prompted us to investigate the antibacterial and antifungal activities of this plant extract.

Bioactivity guided fractionation of the ethylacetate fraction afforded 1,3,7-Trimethy-1*H*-purine-2,6(3*H*,7*H*)dione (caffeine) and *n*-Hexadecanoic acid. The structures of the isolated compounds were determined using Nuclear Magnetic Resonance, Infrared Spectroscopy and gas chromatograph-mass spectrometry.

The antimicrobial screening revealed that of all the solvent fractions, the ethylacetate fraction demonstrated the best inhibitory activity against most of the tested microorganisms including *Escherichia coli*, a Gram negative bacterium. However, no inhibitory activity was against *Shigella* species and *Pseudomonas* species. The butanol fraction demonstrated better antifungal activity than ethylacetate, being active against *Candida albicans* (yeast) and the two *Aspergillus* species (filamentous fungi).

This study therefore established that extracts of *C. nitida* could be used in the management of infections due to susceptible microorganisms and hence justifies the ethnomedicinal use of the plant in folk medicine.

## 5.2 Recommendations

Plant extracts have been found to contain many phytochemicals with unknown pharmacological activities which could serve as synthetic base for the development of affordable and potent antibiotics. It is therefore recommended that extract from other part of *C. nitida* plant like the stem barks and root barks should be investigated for biological activities like anticholinesterase activity, inhibition of denaturation of albumin activity, in-vitro anti-lipid peroxidation activity, anti-diabetic efficacy using enzyme inhibitory potential against hydrolytic enzymes such as α-glucosidase and α-amylase.



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