PHYTOCHEMICAL AND ANTIBACTERIAL STUDIES OF *Combretum glutinosum* STEM BARK

BY

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ABSTRACT

This study was conducted to investigate the methanol extract of *Combretum glutinosum* for its phytochemical contents and antitussive potential. Powdered *Combretum glutinosum* was cold macerated with 80% methanol to obtain crude methanol extract of *C. glutinosum* stem bark, coded (M). Qualitative screening of the crude methanol extract (M) revealed the presence of saponins, flavonoids, steroidal compounds, tannins, alkaloids and reducing sugars and absence of anthaquinones. Quantitative screening of the crude methanol extract (M) also confirm that the plant is rich in polar constituents, such as saponins (606.072 ± 1.471)>>>>flavoniods (561.794 ± 1.419) >>>tannins (102.746 ± 0.561)>>alkaloids (45.762 ± 0.118) mg/100g. The total (crude) saponins content of the crude methanol extract (M) was extracted using diethylether. Acid hydrolysis of total crude saponins was carried out and the sapogenin fraction was chromatographed on silica gel column using hexane: chloroform (100:0 to 0:100) to obtain major subfractions (coded Mss_1 - Mss_4). The total crude alkaloid was extracted from crude methanol extract (M) by defating with hexane to yield an acidic and a lipophillic portion. The acidic portion was further basified and re-extracted with chloroform to yield chloroform soluble portion (crude alkaloids), coded M_A, which was purified through preparative thin layer chromatography. GC-MS analysis of Mss_1-Mss_4 revealed triterpenoidal and steroidal compounds, GC - MS of M_{A1} revealed Pyrolidine alkaliods, Alkaloidial amine, and Piperidines alkaloids, GC - MS of M_H revealed alkane, alcohols, esters, organometallic compound and dichlorobenzene. The antitussive activity of Methanol extract (M), ethyl acetate fraction (M_E), butanol fraction (M_B), and residual fraction (M_R) of *C. glutinosum* stem bark on selected microorganism, *K. pneumonia, P. aeruginosa, E. Coli* showed activity against the isolate with zones of inhibition ranging from 15.00±0.83- 29.00±1.40.The antibacterial potential of *Combretum glutinosum* justifies its usage in the treatment of cough

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LIST OF ABBREVIATIONS

Mg/cm^3	Milligram per centimeter cube
h	Hour
GC-MS	Gas chromatography Masss Spectrometry
TLC	Thin layer chromatography
PTLC	Preparative thin layer chromatography
MIC	Minimum inhibitory concentration
MBC	Minimum bactericidal concentration
IUPAC	International Union of Pure and Applied Chemistry
UV	Ultraviolet
WHO	World Health Organization

CHAPTER ONE

1.0 INTRODUCTION

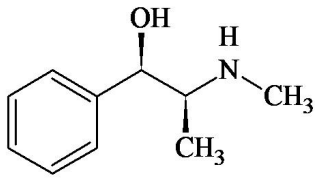
1.1 Background to the Study

Nature has been a source of medicinal agents for thousands of years since the beginning of mankind. In Nigeria, almost all plants are medicinal and are associated with some medicinal value, so also in Africa. According to the World Health Organization (WHO), about 80% of the population around the world depends on traditional medicine, mostly herbal remedies, for their primary health care needs (Hu *et al*., 2017). African continent have a long history with the use of plants and in some African countries up to 90 % of population rely on medicinal plants as source of drugs (Jamshidi *et al*., 2018). A medicinal plant is any plant, which in one or more of its organs contains active ingredients that can be used for therapeutic purposes or contains foundation compounds that can be used for the synthesis of useful drugs (Saadat *et al*., 2018). The application of medicinal plants, especially in traditional medicine is currently well acknowledged and established as a viable profession. The variety of plants used in a community reflects the duration of a people's presence in a certain location, their medicinal knowledge, the diversity of plants present and the availability of plants with a possible medicinal use (Ayuba *et al.,* 2015). Organs of medicinal plants, contain substances that can be used for therapeutic purpose. It may be in the form of vegetable drugs which may either be organized (material which possess a cellular structure for example, leaf, bark, flower, stem, root.) or unorganized drugs (a cellular structural medicinal agent, such as gums, balsams and latex). Such plant materials may be utilized in the form of decoctions in cold water or warm water, concoctions, preparation of soups, and drinks. They can also be used as infusions often made by pouring water on a specified plant material and allowing the mixture to stand for about 15 minutes (Yudharaj *et al*., 2016).

Due to poverty, ignorance and unavailability of modern health facilities, most people, especially the rural ones are still forced to practice traditional medicine for their ailments. A vast knowledge of how to use the plants against different illnesses may be expected to have accumulated in areas where the use of plants is stwn medicinal plants in Nigeria; although the applications vary from culture to culture, believe, weather and other factors. Many of these plants are not widely distributed, some grow well in the rain forest, while, others in savannah regions (Ugboko *et al*. 2020).

There have been records of advances made in the modern (synthetic) medicine, there are still a large number of ailments or infection (diseases) for which suitable drugs are yet to be found. This has brought an urgent need to develop safer drugs (both for man and his environment) for the treatment of several ailments, such as; inflammatory disorders, diabetes, liver diseases, and gastrointestinal disorder. Through recent researches on herbal plants or medicine, there have been great developments in the pharmacological evaluation of various plants used in traditional systems of medicine (Amudha *et al*., 2018). Consequently, plants can be described as a major source of medicines, not only as isolated active principles to be dispensed in standardized dosage form, but also as crude drugs for the population. Modern medicines and herbal medicines are complimentarily being used in areas for health care program in several developing countries, such as, countries in Africa, Asia and some parts of Europe (Simbo, 2010). Due to different outcomes on herbal plants, plant products surfaces all over the world due to the belief that many herbal medicines are known to be free from health and environmental effects. The fear of the masses in the utility of synthetic drug or modern drugs is always accompanied with its single or multiple adverse or health effects (Oladeji, 2016).

In this modern setting, some drugs are sometimes marketed for uses that were never contemplated in the traditional healing systems from which they emerged. In some countries, herbal medicines are subject to rigorous manufacturing standards, this is not so everywhere. In Nigeria, for example, where herbal products are sold, they are subjected to the same criteria for efficacy, safety and quality as are other drug products (Calixto, 2000). Researchers have shown that different plants contain different bioactive components at different concentrations. The higher the amount of the important phytochemical in medicinal plants, the greater the therapeutic potency or medicinal importance of the plants. For the past two decades, there has been an increasing interest in the investigation of different extracts obtained from traditional medicinal plants as potential sources of new antimicrobial agents. The therapeutic potency of a medicinal plant is due to the presence of some bioactive components, which can be ascertained using phytochemical screening, such as; phytochemical tests and thin layer chromatography. Phytochemical constituents (secondary metabolites) are produced as by-products and are not directly useful to the plant, but rather give plants their medicinal value. Example of some secondary metabolites are alkaloids I, tannins II, saponins III, flavonoids IV, triterpenoid V phenols VI .



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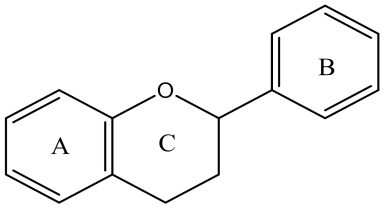

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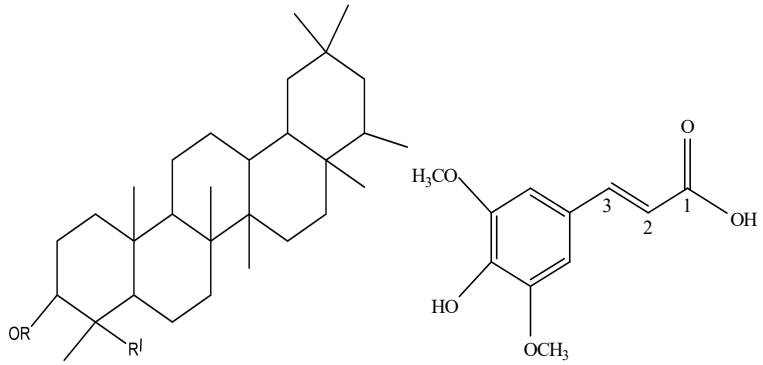

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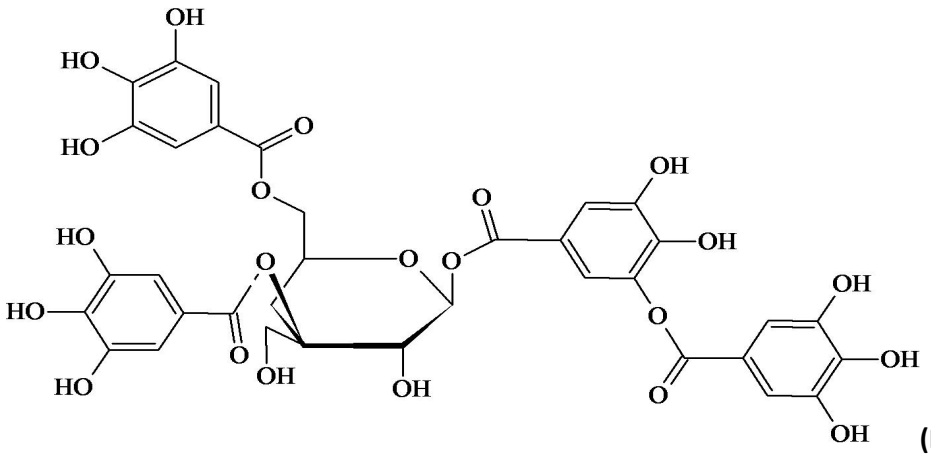

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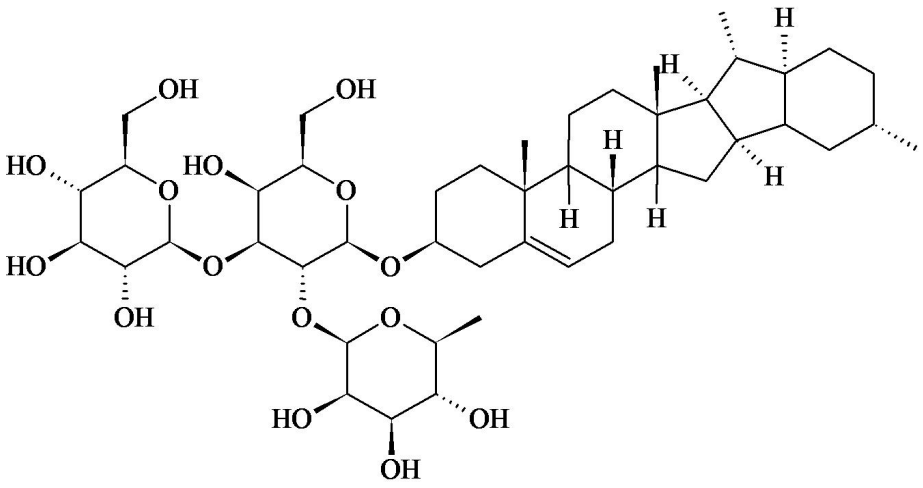

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Medicinal plants contain a wide variety of secondary metabolites or compounds that dictate the therapeutic potency of the plants. For example, antimicrobial activities of some phytochemical constituents, such as, flavonoids and tannins were revealed to be active against *Bacillus cereus, Staphylococcus aureus* and some other pathogenic bacteria (Mohan and Savithramma, 2019). Flavonoids are strong antioxidant and effective antibacterial substances active against large number of microorganisms. They occur naturally in fruits, vegetables and beverages; they provide flower color pigment (Alsemini *et al*., 2017)

Tannins are bitter plant polyphenolic compounds, which have property of binding to protein to form reversible and irreversible complexes due to presence of a number of phenolic hydroxyl groups in their structures. The healing properties of medicinal plants could be due to the presence of tannins. They are known to possess astringent, anti-inflammatory, anti-diarrheal, antioxidant and antimicrobial properties (Jaiswal *et al.,* 2018). Saponins exert various biological benefits, such as, anti-inflamatory, anti-diabetic, and anti-micriobial. Saponins were reported as a major component acting as antifungal secondary metabolite (Ewansiha *et al*., 2012). They have been traditionally used in pesticides and molluscides in addition to their industrial applications, such as, surface-active agents. They help in controlling cardiovascular diseases and in controlling cholesterol in humans (Shang *et al*., 2010)

1.1.2 Problems facing the use of medicinal plants in Nigeria

Despite the recent advancement in herbal medicine, one of the most difficult issues to contend with in translating traditional herbal practices into conventional 'Western' medicine is the individualization of prescriptions containing multiple herbal and other ingredients (Calixto, 2000). In Nigeria, lack of funds, lack of adequate standardization and safety regulations, inadequate quality control, are common problems faced in the

use of medicinal plants. Another challenge is extinction of some plant species. Clinicians working with herbal products are still unfamiliar with the herbal medicine, and they sometimes do not realize the necessity of adequate dosage (Orji *et al*., 2013).

Lack of information on the social, biochemical and economic benefits that could be derived from the industrial utilization of medicinal plants is also another concern. In addition, there are little incentives for standardization of products, little information on the market potential and trading possibilities of these medicinal plants. This result in under use or less exploitation in the real potential of these plants (Ekwensili *et al*., 2011) For many, however, including virtually all of the most common products in the market place, such information is incomplete or unavailable. This in part is due to the complexity of herbal and botanical preparations as they are not pure compounds. It is also a function of the traditionally-held belief that the synergistic combination of several active principles in some herbal preparations is responsible for their beneficial effects (Ekeanyanwu, 2011)

Drugs are still obtained commercially for the most part by extraction from only few species of plants, with more species of higher plants, more useful drugs remain to be discovered. There is a great demand and potential for medicinal plant research as shown by the growing market in medicinal herbs. These problems, when fully addressed, will help the future development of phytomedicines.

1.1.3 Cough

Coughing is a symptom of respiratory illness that prevents talking and causes chest and thorax pain. Current available therapies to treat cough are expectorants, antitussives and glucocorticoids. Antitussives are medicines that suppress coughing also known as cough suppressants (Saraswathy *et al*., 2014). They are thought to work by inhibiting a

coordinating region for coughing located in the brain stem, disrupting the cough reflex arc. Examples include codein®, Diphenhydramine® and dextromethorphan®. Antitussive agents which are used to suppress dry, painful, and patient-disturbing coughs, suppress only one symptom without influencing the underlying condition (Shahnaz *et al*., 2016). Expectorants are natural ingredient or medications that help clear mucus from the air ways. Most available antitussive drugs, especially the synthetic ones, have a number of serious side effects which include; drowsiness, dizziness, nausea, and gastrointestinal upset (Cammaerts *et al*., 2021). Hence, it is very important to find new drugs with effective and minimal side effects in clinical experience. Recently, attention is being paid to natural products and traditional herbs, which have side effects when compared to the synthetic drugs that are used to prevent and treat this symptom (Saha *et al.*, 2011).

Adverse effects, such as; depression of the respiratory center, decreased secretion in the bronchioles, inhibition of ciliary activity, increased sputum viscosity, decreased expectoration, hypotension, and constipation are the limitations to the therapy. Therefore, researchers need to search for an effective antitussive that can effectively alleviate chronic cough without side effects.

The most common bacterial agents responsible for respiratory disorder that causes cough are *Streptococcus pneumoniae, Haemophilus infuenzae, Klebsiella pneumoniae, Pseudomonas aeruginosa, Haemophilus influenza, Escherichia coli,* and *Mycobacterium tuberculosis* (Serges *et al*., 2019).

Klebsiella pneumoniae and *Pseudomonas aeruginosa* often cause infection of the lungs, this happens when the bacteria enter the respiratory tract. Symptoms of K. *pneumonia* include, fever, cough, chest pain, trouble breathing or shortness of breath (Uzoamaka *et*

al., 2017). *E.coli* is a bacterium found in the gut, most strains of *E.coli* are not harmful, but some types can cause illnesses in humans including cough, sneeze, vomiting, fever, respiratory illnesses, pneumonia, and meningitis (Serges *et al*., 2019). Chronic cough cause damage to the bronchi, leaving people with disease susceptible to serious infections with bacterial pathogens. People with cough are at risk of an accelerated decline in lung function.

Many medicinal plants having strong antibacterial, antibiotic and antifungal properties have been identified as capable of treating respiratory disorders. The herbal remedies are now known as cough suppressants, expectorants, mucolytics and nasal decongestants, some act to smoothen the lungs and others to stimulate the heat and mind and for some, a combination of many actions (Onyedum *et al*., 2013).

1.2 Statement of the Research Problems

Cough is the commonest symptom for which most patients seek medical attention, affecting a large proportion of the general population. It may be the beginning and also a warning sign of an impending disease (Holzinger *et al*., 2014). Respiratory disease represents an enormous and increasing health care and economic burden. Survey at some part of Nigeria revealed that, patient with cough reported a reduction in quality of life, disturbed sleep, impaired daily activities including sports, job career, physical activity, social activities, and house hold chores (Onyedum *et al*., 2013).

Symptoms of cough include the runny or stuffy nose, sore throat, hoarseness and shortness of breath (Powers *et al.,* 2015). These signs make it difficult for a person to remain physically and mentally active, contributing to an increased in medical problems like the postnasal trip, gastroesophageal reflux and chronic obstructive pulmonary diseases and vulnerability to headache, sleep disrupt, dizziness, vomiting.

Challenge of cough treatment is that the new medication is of limited benefits and often more expensive making it impossible for patients with poor resources and people in underdeveloped countries living in abject poverty to be faced with problems of supply, purchasing and using orthodox medicine, because only a few persons can afford the commodity, thereby limiting the availability of such drugs to rural dwellers and the less privilege to benefit from them.

Treatment challenges could also lead to under treatment, unnecessary treatment and poor control, increase adverse drug reaction, increased mortality and poor quality of life. Furthermore, cough has shown some resistance to antibiotics despite progress using non-steroidal inflammatory drugs and therapeutic agent (Simeon *et al*., 2017). However, the unpleasant adverse effects associated with these drugs and their inability to alleviate or provide long-term reduction could be challenging. The undesirable side effects of these orthodox medicines such as constipation, depression of the respiratory centre, over dependence, drowsiness and death from their use in humans makes them highly unsatisfactory. The scientific justification of some medicinal plant used traditionally as antitussives are yet to be established. Saponins and alkaloidal contents of some plant in the Combretaceae family are yet to be investigated.

1.3 Justification of the Study

Medicinal plants are an important source for the discovery of novel bioactive compounds, which have served and continue to serve as lead molecules for the development of new drugs.

Combretum glutinosum stem bark has been traditionally useful in the treatment of several bacteria caused ailments such as cough, tuberculosis, diarrhea and syphilis (Dodehe *et al*, 2012). The species of this family have also been reported to contain an

appreciable amount of alkaloids, triterpenes (Gairola *et al*., 2018) and steroidal compounds (Mazid *et al*., 2016), which have also been detected in various organ of *Combretum glutinosum* (Silje *et al.,* 2011). These classes' of compounds amongst others are known to exhibit various biological activities including antibacterial, anti-inflammatory, anti-plasmodial *activities* (Yahaya *et al.,* 2014). In view of severe undesirable side effects of synthetic agents, there is growing focus to follow systematic research methodology and to evaluate scientific basis for *Combretum glutinosum,* the traditional herbal medicine claimed to possess antitussive activity.

Therefore, the stem bark of *Combretum glutinosum* was investigated for its phytochemical content as well as its antitussive efficacy. This will help validate the traditional use of the plant and will probably increase the arsenal of effective anti-bacterial.

1.4 Scope and Limitation of the Study

The study is focused on extraction, fractionation, and identification of one or more compounds present in the methanol stem bark of *Combretum glutinosum.* Furthermore, the crude methanol extract and its fractions will be evaluated for their antitussive potentials in comparison with a standard drug.

1.5 Aim and Objectives of the Study

The aim of this work is to carry out phytochemical and antibacterial studies of *Combretum glutinosum* stem bark.

The specific objectives are as follows:

i. Qualitative and quantitative screening of 80 % crude methanol extract (M) of *C. glutinosum* using standard methods.

ii. Investigations of the total (crude) saponins content of crude methanol extract, (M).

iii. Determination of the total (crude) alkaloidal content of crude methanol extract, (M).

iv. Antitussive screening of the crude extract, (M) and its partitioned fractions

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 *Combretum glutinosum*

2.1.1 Taxonomy

Kingdom: Plantae

Order: Myrtales

Family: Combretaceae

Genus: *Combretum*

Specie: *C. glutinosum*

Common and Local names; Wuyandamo (Hausa) damoruli (fulani) and aragba (Yoruba) (Adelanwa *et al*., 2015).

2.1.2 Plant description

Combretum glutinosum are bushy shrubs, having a height of 12 meters. Leaves are spear shaped which ranges from 4-10 cm. They are also membranous, having a blunt base, the thick leathery green leaves are glutinous above when young, strongly reticulate and densely hairy beneath. The leaves differ in shape and size, even on the same tree. The blades are elliptic, 9-18 cm long and 4-8 cm across.

The stem bark is grey-black and may be smooth or rough with fissures on the upper surface and red to orange slash. Young stems are velvety, and greyish in colour.

The petals and filaments are greenish-yellow in colour, 2.5-3 mm diameter. The flowers have 4 petals and are densely hairy (Silje *et al*., 2011).

The fruit typically measures 2.5–4 centimetres (0.98–1.57 in) long and 1.5–3 centimeters (0.59–1.18 in) across. The seeds are oil bearing and one kilogram contains about 20,000 seeds.

The trunk is usually twisted and low branched, with a rounded, open crown. The lower branches characteristically point downwards (Alowanou *et al*., 2015).

Plate I: *Combretum glutinosum* Plant

2.1.3 Ethno-medicinal use

Combretum glutinosum has been found useful in various parts. The water extract of *Combretum glutinosum* and infusion of the leaves are used for jaundice, severe cough and bronchitis. The macerations of the roots are used to enhance wounds healing and the water extract of its roots is used as a purgative. The wood smoke is used for the rheumatic pains (Coulibaly *et al*., 2019)

In Nigeria, it has been known for the treatment of bronchitis, tuberculosis, pneumonia, hepatic disease, diuretic and as antihypertensive remedies. Traditional healers in Africa and Asia use species of the *Combretaceae* for many medicinal purposes such as infertility in women, venereal diseases including syphilis, and ear ache (N'diaye *et al*., 2017).

2.1.4 Non medicinal use

In Nigeria, the fresh young leaves of *Combretum glutinosum* tree, though bitter-tasting are occasionally eaten as a vegetable. The leaves, stem and root bark, collected from the wild are important source of yellow to brownish dyes for cotton textiles. The leaves are widely gathered as fodder for livestock and are fed to animals as food (Heustis *et al*., 2018). The flowers are faintly fragrant and are used as ornamentals. The gum extract from the stem bark of *C. glutinosum* is used to fill the teeth of human.

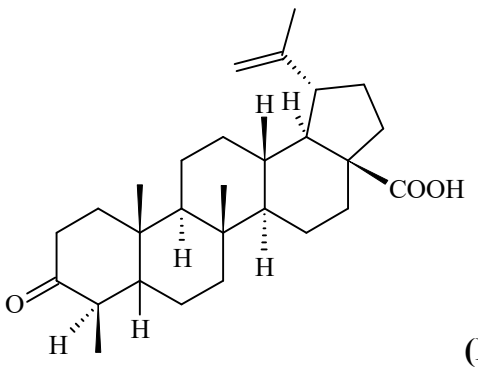
The wood gotten from *C. glutinosum*, is hard and extremely durable, it is used in construction, also used as framework for huts, tools handle, and general carpentry. It makes a good source of fuel wood and charcoal. The smoke of the woods is used for fumigation

The alkaline ash of *C. glutinosum* is used in Senegal in indigo-dyeing and the root and bark yield yellow and brownish yellow dyes for cotton textiles. These dyes are used in

Nigeria, Burkina Faso and Benin to dye leather and mats made of vegetable fibre. These dyes are also used in the preparation of the internationally renowned bogolan textiles. (Coulibaly *et al.,* 2019).

2.2 Phytochemistry

Triterpenoid molecules, such as betulonic acid **(I),** cabraleone **(II),** betulonal **(III)** and betulinol **(IV)** were isolated from hexane and dichloromethane extracts of leaves of *Combretum glutinosum* (N'diaye *et al*., 2017). 2,4,-di-tertbutylphenol **(V)** and Bamyrin**(VI)** was isolated from pet ether stem bark extract of *Combretum glutinosum* **(**Amako *et al*., 2015). Combreglutinin **(VII)**, a hydrolysable tannin have also been isolated from the leaves of *C. glutinosum*. Gallic acid **(VIII)**, ellagic acid **(IX)**, were isolated from leaves of *Combretum glutinosum* (Alowanou *et al*., 2015). Three tannins which are 2, 3 (S)- hexahydroxydiphenoyl- D-glucose **(X)**, punicalin **(XI),** Punicalagin **(XII)** have also been isolated from the stem bark of *Combretum glutinosum*s (Marquet and Jansen, 2008). A pentacyclic triterpenoid, friedelan-3-one **(XIII)** was identified in the stem bark of *Combretum glutinosum* (Amako and Nnaji, 2016).



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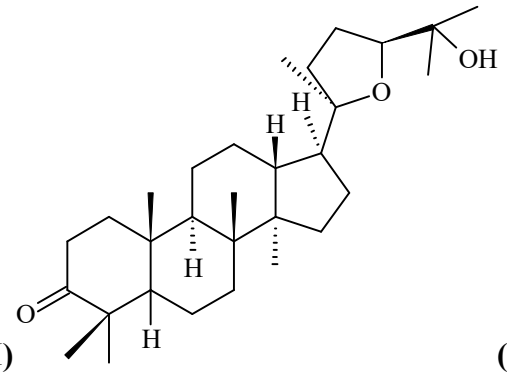

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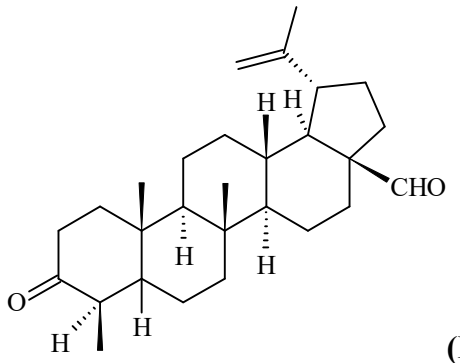

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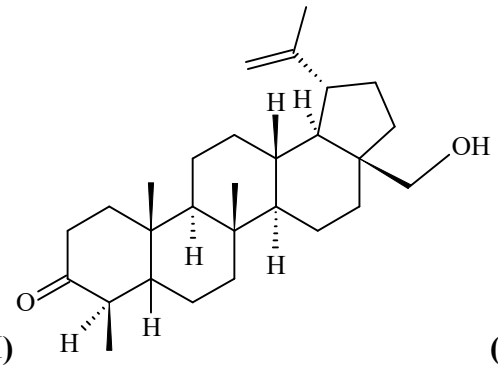

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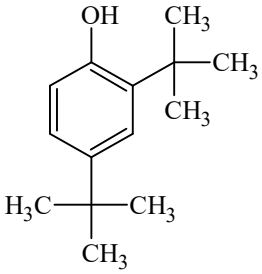

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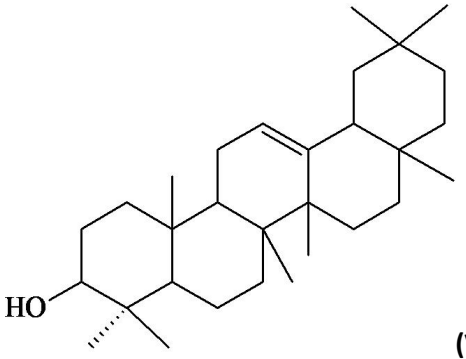

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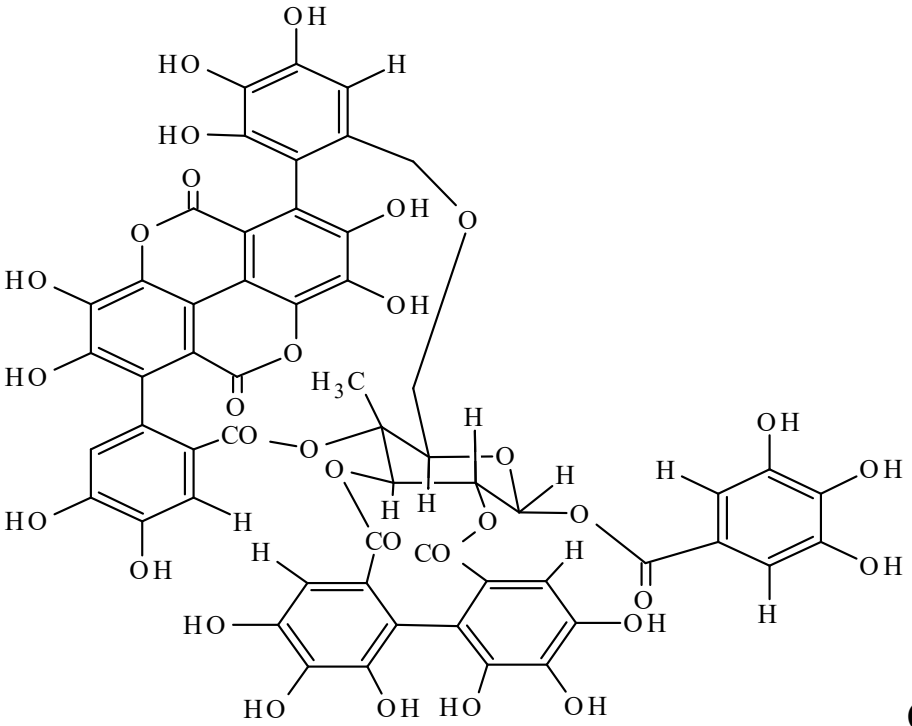

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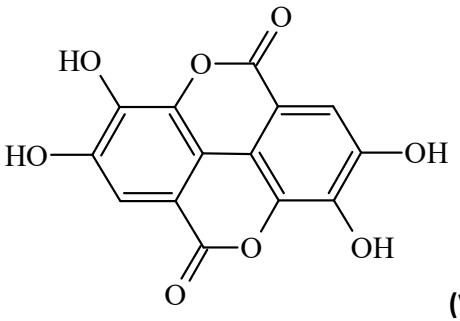

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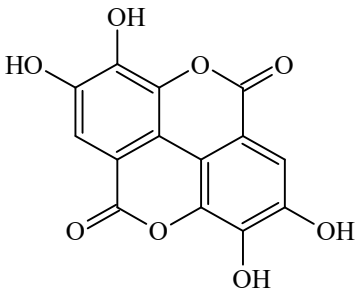

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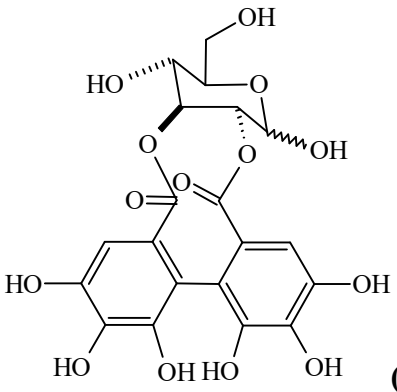

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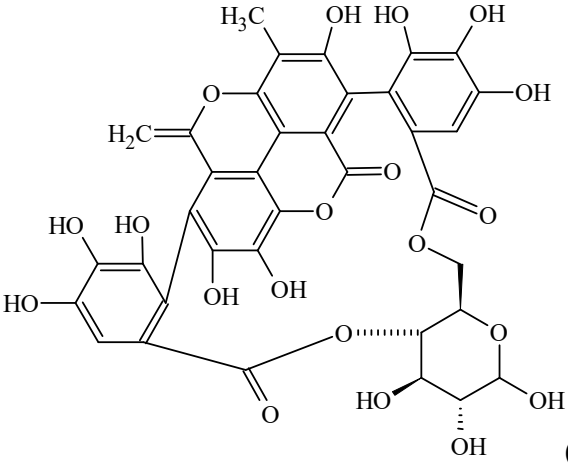

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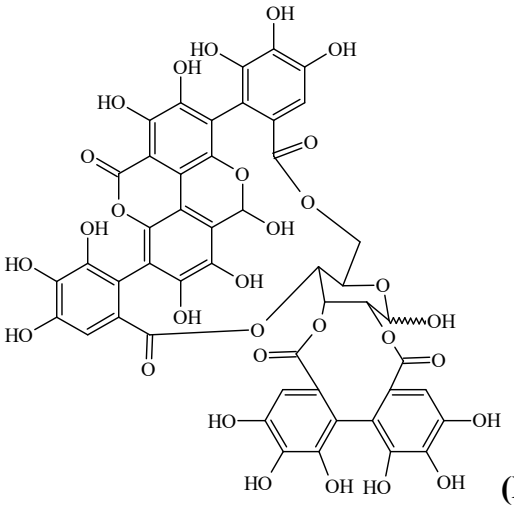

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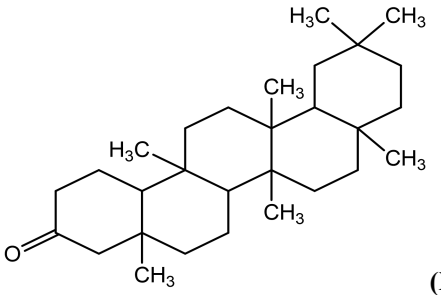

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2.3 Biological Activities

The methanolic and ethyl acetate extracts of *Combretum glutinosum* leaves showed significant anti-sickling activity against SS sickle type (Cheikhsall, *et al,* 2017). The anthelmintic properties of leave powder extracts of *C. glutinosum* was effective on the three life - cycle stages (eggs, larvae and adult worms) of the parasitic nematode, *Haemonchus contortus*, for the control of gastro-intestinal nematodes. The stem bark and leaves of *C. glutinosum* displayed significant antipyretic effect (Ghildyal *et al*., 2010).

Methanol and hydromethanol extracts of leaves of *C. glutinosum* showed antimalarial activity against a chloroquine resistant *Plasmodium falciparum* strain *invitro* (Quattara *et al*., 2010). The ethanolic extract of leaves, stem and roots revealed significant anticercarial activity against cercariae of *Schistosoma mansoni (*Ahmed *et al.,* 2017). The leave extract of *Combretum glutinosum* Perr ex DC showed good hypoglycemic activity on the glycaemia level of Han Wister rat (Balde *et al.,* 2019). *Combretum glutinosum* leaves showed significant activity in Cytotoxicity studies on cancerous cells. Antitumor activities of methanolic extracts showed activity in leukemic and Lung carcinoma cells (Uthman and ChabulaMota, 2015).

CHAPTERTHREE

3.0 MATERIALS AND METHOD

3.1 Materials and Instruments

Rotary evaporator (Griffin, Gallenkamp), pre-coated TLC plates, silica gel (mesh 60-120), water bath (Griffin, Gallenkamp), weighing balance, fume cupboard, freeze dryer, separatory funnel (1L), capillary tubes, microscope, slides, freezer, developing tank, *GC-MS (GCMS-QP2010SE).*

3.1.2 Chemical/Reagent/Solvents

All inorganic reagents and organic solvents were of analytical grade.

3.2 Extraction Procedures

3.2.1 Collection of plant material

Combretum glutinosum stembark was collected in December, 2018 at Gidan Kwano Campus, Minna, Niger State, Nigeria. The stem bark of *C. glutinosum* was air-dried for (4) four weeks till a constant weight was obtained. The dried sample was pulverized using wooden mortar and pestle and stored in a polythene bag for further use.

3.2.2 Extraction of plant material

Powdered *Combretum glutinosum* (400 g) was cold macerated with 80% methanol in an extracting jar for 72 hours with regular manual shakes. The mixture was filtered using whatman filter paper no 1 and the filtrate concentrated using a rotary evaporator to obtain an extract that was finally dried over a water bath at 40°C and labelled as crude methanol extract of *C. glutinosum* stem bark, M.

3.3 Phytochemical Screening of the Methanol Extract of *C.glutinosum*

The methanol (M) extract was subjected to preliminary qualitative and quantitative screening to detect the presence/absence of some secondary metabolites. Tests were carried out in accordance with the methods described by Amako *et al*., (2015).

3.3.1 Qualitative screening

3.3.1.1 *Test for saponins (frothing test)*

The crude extract (0.2 g) was added to 10 cm^3 of distilled water in separate test tubes and both were shaken for 2 minutes. Formation of 1 cm layer of foam indicates the presence of saponins.

3.3.1.2 *Test for alkaloids*

The crude extract was dissolved in dilute hydrochloric acid and filtered.

Mayer's test: About 6 drops of Mayer's reagent (Potassium mercuric iodide) was added to 2 cm^3 of the filtrate. Formation of a yellow coloured precipitate indicates the presence of alkaloids.

Wagner's test: 2 cm^3 of filterate was treated with 6 drops of Wagner's reagent. Formation of dark reddish precipitate indicates the presence of alkaloids.

Dragendroff's test: 2 cm^3 of the filtrate was treated with Dragendroff's reagent (solution of potassium bismuth iodide). Formation of red precipitate indicates the presence of alkaloids.

3.3.1.3 *Test for flavonoids (alkaline reagent test)*

The crude extract (0.2 g) was mixed with 5 cm^3 of dilute NaOH. Formation of an intense yellow colour which becomes colorless on addition of dilute HCl indicates the presence of flavonoids.

3.3.1.4 *Test for tannins*

The crude extract (0.2 g) was dissolved in distilled water (10 cm^3) and few drops of 10% $FeCl_3$ solution added. A formation of a green precipitate is taken as positive.

3.3.1.5 *Test for steroidal compounds* (Salkowski's test)

The crude extract (0.2 g) was dissolved in chloroform (2 cm^3), and filtered and few drops of concentrated H_2SO_4 were added to the filtrate, formation of a golden, yellow reddish brown interphase indicates the presence steroidal nucleus

(Liebermann Burchard's test)

The extract (0.2 g), 5 drops of Conc H_2SO_4 were added. Formation of a reddish brown precipitate indicates the presence of steroidal nucleus

3.3.1.6 *Test for reducing sugars (Fehling's Test)*

The extract (0.2 g) was dissolved in 5 cm^3 distilled water and filtered. The filterate was then hydrolyzed with dilute HCl, neutralized with alkali and heated with Fehling's A and B solutions, formation of red precipitate indicates the presence of reducing sugars

3.3.1.7 *Test for phenols (ferric chloride test)*

The extract M (0.2g) was diluted with distilled water, 10 cm^3 was treated with 3-4 drops of $FeCl_3$ solution. The formation of deep bluish black colour indicates the presence of phenols.

3.3.2 Quantitative screening

3.3.2.1 *Determination of total flavonoids*

Total flavonoid content of the crude extract was determined by adding 0.5 g of each extract to a test tube containing 1.5 cm^3 of absolute methanol, 0.1 ml of 10% aluminum chloride, 0.1 ml of 1M sodium acetate and 2.8 ml of distilled water and incubated at

ambient temperature of 28^{O} C for 30 minutes. The absorbance was read at 415 nm and standard quercetin was used to prepare the calibration curve (Ezeonu *et al*., 2016).

3.3.2.2 *Determination of total alkaloids*

The crude extract was dissolved in 5 ml of mixture of 96% ethanol:20% H_2SO_4 (1:1) and then filtered. 1 ml of the filtrate was then added to a test tube containing 5 ml of 60% H_2SO_4 and allowed to stand for 5 minutes. Thereafter, 5 ml of 0.5% formaldehyde was added and allowed to stand at room temperature for 3 hours. The absorbance was read at a wavelength of 565 nm and vincristine extinction coefficient (E_{296}, ethanol{EtOH}= 15136M^1cm^1) was used as reference alkaloid.

3.3.2.3 *Determination of total tannins*

The crude extract (0.5 g) was weighed into a 50 ml beaker and 20 ml of 50% methanol was added, covered with para film, mixed thoroughly for homogeneity and heated on a water bath at 80^{0}C for 1 hour. Resulting mixture was then filtered into a 100 ml volumetric flask and 20 ml of distilled water, 2.5 ml of Folin-Denis' reagent, and 10 ml of sodium carbonate were added and mixed properly. The reaction mixture was then allowed to stand for 20 mins at room temperature for the development of a bluish-green coloration. The absorbance was taken at 760 nm, Standard tannic acid was used to prepare the calibration curve (Roghini *et al*., 2018).

3.3.2.4 *Determination of total saponins*

Saponin content of the crude extract was determined using the method described by Roghini *et al*. (2018). 0.5 g of crude extract was weighed and dissolved in 20 ml of 1N HCl and heated on a water bath at 80^{0}C for 4 hours. The reaction mixture was cooled, filtered and 50 ml of petroleum ether was added to the filterate and the organic layer was collected, evaporated to dryness and 5 ml of a mixture of acetone-ethanol (1:1), 6

ml of ferrous sulphate and 2 ml of concentrated sulphuric acid were added and allowed to stand for 10 mins. The absorbance was read at 490 nm and CO_2 was used to prepare the calibration curve.

3.4 Extraction and Hydrolysis of Total (crude) Saponins

3.4.1 Extraction of total (crude) saponins

The liquid-liquid extraction method was adopted as described by Ajuru *et al.* (2017). 20g of the crude methanol extract of *C. glutinosum* was solubilized in 150 cm^3 distilled water, filtered and the filterate partitioned in a seperatory funnel with 50 cm^3 x 4 portion of diethyl ether to afford the ethereal and the aqueous layer. The ethereal layer was collected and set aside, while the aqueous layer was further extracted with 100cm3 x 7 portion of normal butanol (n-BuOH). The combined BuOH fraction was washed 5x with 10cm^3 of 5% NaCl and the resulting butanol layer was further concentrated in-vacuo to afford the total saponins of MeOH extract of *C. glutinosum* coded Ms. Ms was weighed, and screened for the presence of saponins using a confirmatory test, froth and hemolysis test as well as TLC in ethyacetate: methanol: water (6:3:1.5) solvent system.

3.4.2 Acid hydrolysis of the total (crude) saponins

Acid hydrolysis of obtained total (crude) saponins, (Ms) was carried out to break the sapogenins from the sugars. The method of Leonia *et al* (2017) was adopted. 5 g of fraction (Ms) was refluxed with 2N H_2SO_4 in 10% methanol for 5 hours, the resulting mixture was filtered and the filterate treated with NH_4OH to regulate the mixture to pH 7.5. The resulting mixture was further treated with a methanol and the desired sapogenin was readily extracted from impurities and then concentrated *in-vacuo* to obtain a fraction, coded Mss while the residue was also concentrated *in-vacuo* to obtain

a fraction coded Ms_R. Both were screened for the presence of sapogenins and sugar moieties.

3.4.3 Fractionation of the Sapogenin Fraction

The sapogen in fraction (1 g) was fractionated using column chromatography. Column of length (30 cm) was packed with silica gel (30 g) by the wet method. Slurry was prepared using hexane, poured into the column and flushed severally to attain a good stability. 1 g of fraction Mss was mixed with a little quantity of silica gel, air-dried to form a solid mass and fed onto the packed column. Column was then eluted with increasing polarity of hexane: chloroform (100: 0 to 0: 100) in a desired ratio and flow rate. Similar sub-fractions collected were pooled based on their TLC profile to obtain sub fractions that were concentrated *in-vacuo* into 4 major sub fractions (coded Mss_1-Mss_4).

3.4.4 GC-MS Analysis of sub-fractions (Mss_1-Mss_4)

The analyses were performed on gas chromatograph-QP2010SE, Shimadzu, Japan. The oven was kept at 50 ^oC for 10 min, further increased to 240 ^oC at 21 ^oC/min. Helium gas was used as a carrier gas and the mass spectrometer functioned in scan mode from 40-450 m/z.

3.5 Extraction of Total (crude) Alkaloids

The methanol extract, M (10 g) was solubilized in water and hydrolyzed with 2M dil. HCl (2M). The mixture was then defatted with hexane to yield an acidic and a lipophilic portion. The acidic portion was basified with dil. NH_4OH and further re-extracted with chloroform to yield a chloroform-soluble portion (crude alkaloids) coded M_A and aqueous phase coded M_{AR}. The portions were washed with distilled water, dried with Na_2SO_4 and concentrated to dryness. M_A was then screened for the presence of crude

alkaloids and TLC in chloroform: methanol: ammonia (1: 1: 0.01) solvent systems which was sprayed with dragendroff reagent.

3.5.1 Preparation and activation of PTLC plate of total (crude) alkaloids (M_A)

TLC plates were prepared using silica gel as the stationary phase. Silica gel was mixed with water and made into slurry. The slurry was spread uniformly on the plate. The plates were coated after thorough prior cleaning and rinsing with distilled water. The plates were air dried for some time and then kept for activation. The plates were activated by heating the plate in an oven at 100 to110°C for 30 minutes.

The chloroform-soluble portion (crude alkaloids) was spotted on a preparative glass plate with the aid of a capillary tube and allowed to dry. Plate was transferred into a development tank using solvent system chloroform: methanol: ammonia (1: 1: 0.01), the developed bands were scrapped separately and then washed with Me_2CO and MeOH successively. The TLC of the Me_2CO portion reveal a single spot on and was coded $M_{A1.}$

3.5.2 GC-MS Analysis of total (crude) alkaloids (M_{A1})

The analyses were performed on gas chromatograph-QP2010SE, Shimadzu, Japan. The oven was kept at 50 ^oC for 10 min, further increased to 240 ^oC at 21 ^oC/min. Helium gas was used as a carrier gas and the mass spectrometer functioned in scan mode from 40-450 m/z.

3.6 Fractionation of Crude Methanol Extract of *Combretum glutinosum*

3.6.1 Partitioning of crude methanol extract (M)

Crude methanol extract of *Combretum glutinosum* stem bark, M, (30 g), was solubilized in 500 ml of distilled water in a 1L beaker until a homogenous mixture was obtained.

Mixture was allowed to stand for 24 hour and then filtered. The filtrate in a separatory funnel was partitioned exhaustively with n-hexane (5 x 100cm3). The resulting mixture was concentrated in-vacuo to obtain an hexane fraction, coded (M_H). The residual aqueous layer was further partitioned exhaustively and successively with ethyl acetate (13 x 100 cm^3) and butanol (20 x 100 cm^3) and concentrated over a water bath to afford the ethyl acetate, (M_E) and butanol (M_B) fractions, of *C. glutinosum* respectively. The residual aqueous portion was also concentrated over a water bath to yield the residual aqueous fraction coded (M_R).

3.6.2 GC-MS Analysis of hexane fraction (M_H)

The hexane fraction, (M_H) was subjected to GC-MS analysis only. Phytochemical screening and antitussive studies could not be conducted because of insufficient fraction yield

3.6.3 Qualitative and quantitative screening of the partitioned fractions

The partitioned fractions, (M_E, M_B, and M_R) obtained from partitioning of the crude methanol extract, of *Combretum glutinosum* stembark, (M) were subjected to preliminary qualitative and quantitative screening to detect the presence, as well as quantity of some secondary metabolites. Tests were carried out in accordance with the method described in sections 3. 3. 1 and 3. 3. 2.

3.7 Antimicrobial Assay

3.7.1 Test organisms

The bacterial strains used are *Staphylococcus aureus*, *Escherichia coli*, and *Psedomonas aeruginosa*. They were isolated from sputum of different individuals obtained from General hospital Minna, Niger state, Nigeria, in November, 2019.

3.7.2 Susceptibility tests

The agar plate-hole assay method was used to determine the growth inhibition of bacteria. Mueller hinton agar was prepared and 20 cm^3 portion each was poured into sterile petri dish and was allowed to solidify. 5mm sterile cock-borer was used to bore a holeper plate in the set agar. Crude methanol extract and its fractions were each introduced into the holes and allowed to stay for an hour to enable the extract diffuse into the agar. The plates were incubated at 37^0C for 24hrs. The diameter of zones of inhibition were observed, measured with a ruler and recorded (Amitan *et al*., 2014).

3.7.3 Minimum inhibitory concentration (MIC)

The Minimum Inhibitory Concentration (MIC) was determined using the broth dilution technique. Crude methanol extracts and its fractions was prepared by dissolving in Dimethyl-sulfoxid (DMSO), thereafter made up to different concentration in sterile distilled water and serially diluted (two-fold) to a working concentration ranging from 1.56mg/cm^3 to 100mg/cm^3 using nutrient broth and later inculcated with 0.2 ml suspension of the test organisms. After 24 hours of incubation at 37^0 C, the tubes were observed for the presence of turbidity. The lowest concentration where no turbidity was observed was determined and noted as the minimum inhibitory concentration (Amitan *et al.,* 2014).

3.7.4 Minimum bacterial concentration (MBC)

The Minimum Bacterial Concentration (MBC) was determined from the broth dilution resulting from the MIC tubes by sub culturing to antimicrobial free agar. The lowest concentration of the extract fractions which shows no growth were recorded as the minimum bacterial concentration (Amitan *et al*., 2014)

CHAPTER FOUR

4.0 RESULTS AND DISCUSSION

4.1 Description of Extract

The physical appearance and weight of crude methanol obtained from the extraction of stem bark of *C. glutinosum* using 80% MeOH are presented in Table 4.1

Table 4.1 Physical appearance of the methanol extract

Extract	Code	Colour/ Appearance	Weight(g)	% Yield
80% MeOH	M	Dark brown gummy mass	88.7	22.18
Residual	M_R	brown gummy mass	34.8	39.23

The extraction of *C. glutinosum* stem using cold maceration method with 80% MeOH, yielded the crude methanol extract, M (88.7g with 22.18% yield) are presented in Table 4.1.

Methanol is a good solvent for extraction of bioactive compounds, because it has high extractability and its polarity works on vast number of phytochemicals, including polar and nonpolar compounds (Edgar *et al*., 2016). Methanol is an amphiphillic compound, a compound that dissolve polar and nonpolar compounds (Tomislav *et al*., 2018). It easily evaporates and can be seperated from the plant material, without any damage to some classes of compounds (Kafaru *et al*., 2018).

4.2 Qualitative Screening of Crude Methanol Extract

The crude Methanol Extract M, was screened qualitatively for phytochemicals using standard procedures described by Amako *et al* (2015) the presence/absence of phytochemicals as presented in Table 4.2

Table 4.2: Qualitative phytochemical screening of crude methanol extract

Phytochemicals	Inference
Phenols	+
Flavonoids	+
Anthraquinones	-
Terpenes	+
Tannins	+
Saponins	+
Steroids	+

Key + = Present, - = abscent.

Qualitative screening of the crude methanol (M) of *C. glutinosum* using standard methods revealed the presence of saponins, flavonoids, steroidial compounds, tannins, alkaliods and reducing sugars and absence of anthraquinone as shown in Table 4.2. The methanol extract revealed the presence of steroidial compounds, flavonoids, saponins, tannins, alkaliods and reducing. This value was similar compared to the value reported by Yahaya *et al* (2012) on *C. glutinosum*. Tannins, saponins, and steroids have been found to be responsible for antibacterial, antifungal, and antiviral activities (Saraswathy *et al*., 2017). Saponins have a variety of biological effects, including antioxidant, antihepatotoxic, antibacterial, anticar-cinogenic, and antdiarrheal (Thalhammer *et al.,*2015). Flavonoids can be named as nature's biological response modifiers and has

been shown to posses anti-microbial, anti-allergic, anti-inflammatory & anti-cancer activities (Edeoga *et al*., 2015).

4.3 Quantitative Screening of Crude Methanol Extract

Quantitative screening of the crude methanol extract M, using various standard methods to reveal different quantity of phytochemicals as presented in Table 4.3

Table 4.3: Quantitative Phytochemical Screening of Crude Methanol Extract

Secondary metabolite	mg/100g
Alkaloids	45.762 ± 0.12^{d}
Saponins	450.908 ± 0.35^{a}
Tannins	89.053 ± 1.41^{c}
Flavonoids	398.879 ± 2.00^{b}

Qualitative screening carried out on the crude methanol extract (M) of *C. glutinosum*, revealed the presence of flavonoids, saponins, alkaloids and tannins as shown in Table 4.3. Quantitative screening of the extract also confirmed that the plant is rich in polar constituents, such as saponins (606.072 ± 1.471)>>>>flavoniods (561.794 ± 1.419) >>>tannins(102.746 ± 0.561)>>alkaloids (45.762 ± 0.118). This shows that saponins was significantly higher than all other tested phytochemicals.

4.4 Extraction and Hydrolysis of Total (crude) Saponins

4.4.1 Phytochemical screening of total (crude) saponins

4.4.1.1 *Qualitative screening of total (crude) saponins (Ms)*

Qualitative screening of total (crude) saponins revealed the presence/absence of some phytochemicals presented in Table 4.4.

Table 4.4: Qualitative screening of total (crude) saponins

Phytochemicals	**Test**	**Observation**	**Inference Ms**
Saponins			
	Frothing	Persistent froth	+
	Hemolysis		+
Reducing sugars	Fehlings	Brick red ppt	+
Alkaloid	Dragedorff	Orange ppt	-
Phenol	$FeCl_3$	Bluish green colour	-
Triterpeniods	Liebermann	Pink colour	+
	Burchard's		
Steroidal compounds	Salkowski's	Red coulor	+

Keys: +=present, -= absent

Qualitative screening of total (crude) saponins (Ms) obtained from extraction (liquid liquid extraction) of methanol extract, M, revealed the presence of saponins using hemolysis and fronthing confirmatory test. Triterpeniod and steroidal compounds were also present.

4.4.1.2 *Thin Layer chromatographic profile of the total (crude) saponins*

The TLC of M_S total (crude) saponins, using ethylacetate; methanol : water (6:3:1) as mobile phase and spraying the developed chromatogram with Liebermann-Burchchards' reagent revealed, pink, violet and orange spots. This indicates the presence of steroid and triterpeniod compounds with R_f 0.22, 0.40 and 0.87 as shown in plate II and III

Plate II: TLC profile of the total (crude) saponins of *C. glutinosum* stem bark (Ms)

Solvent system :Ethylacetate : methanol : water (6:3:1)

Chromogenic reagent : Lieberman-Burchard's reagent

Plate III: TLC profile of the total (crude) saponins of *C. glutinosum* stem bark (Ms)
Solvent system: Ethylacetate: methanol: water (6:3:1)
Chromogenic reagent: Lieberman-Burchard's reagent.

Thin layer chromatography was carried out on the total (crude) saponins (Ms). A large number of solvent system was tried to achieve good resolutions. Solvent system Ethylacetate: methanol: water (6:3:1) was used with Liebermann-Burchards' spray reagent on the plate which gave a pink colour, indicating the presence of saponins and violet colour in plate III using ethylacetate : methanol : water (6:3:1) as solvent system, indicating the presence of triterpenoid, as described by Yahaya *et al*(2014)

4.4.3 Acid hydrolysis of the total (crude) saponins

Qualitative screening carried out on Mss, obtained from acid hydrolysis of total (crude) saponins Ms revealed the presence or absence of some phytochemicals presented in the Table 4.4

Table 4.4: Qualitative Screening of Mss

Phytochemicals	Test	Observation	Inference Mss
Triterpenoids	Liebermann Burchard's	Pink colour	+
Steroidal compounds	Salkowski's	Red colour	+
Reducing sugars	Fehling's	Brick red ppt	-
Saponins	Frothing	Persistent froth	-
	Hemolysis		-
Phenol	$FeCl_3$	Bluish green colour	-

Keys (−−) absence (+) present

Mss = sapogenin fraction

Acid hydrolysis of obtained total (crude) saponins, (Ms) carried out yielded two sub-fractions, glycone (sugar) and aglycone (sapogenin) moieties. The sapogenins were found to be steroidal and triterpenoidal as confirmed by the phytochemical screening carried out on Mss, in Table 4.4, and the thin layer chromatography in Plate II and III using Liebermann-Burchards' spray reagent on the plate which gave a pink and violet colour on the spot, indicating the presence of triterpeniods and steroidal compounds respectively as described by Arega et al (2018), Sub-fraction Ms_R were made up of majorly their sugar moieties, as confirmed by the test for reducing sugars.

4.4.4 Fractionation of Sapogenin Fraction

The sapogenin fraction, (Mss) from acid hydrolysis of the total (crude) saponins (Ms) was subjected to column chromatography packed with silica gel. Elution commenced with hexane with increasing polarity of chloroform. Collected eluent were subjected to TLC and pooled based on their TLC profile to obtain 4 major sub fractions (Mss_1-Mss_4). At hexane: chloroform (19: 1) revealed 2 spots, at hexane: chloroform (9: 1) revealed 4 spots,

hexane: chloroform (8: 2) revealed 5 spots, hexane: chloroform (1: 1) revealed 6 spots.

4.5 Extraction of Total (crude) Alkaloids

Qualitative screening carried out on M_A revealed the presence of some phytochemicals as presented in the Table 4.5

Table 4.5: Qualitative screening of M_A

Phytochemicals	Inference M_A
Alkaloids	+
Phenols	-
Tannins	-
Reducing sugars	-
Saponins	-

Keys (−) absence (+s) present

M_A = crude alkaliods

The total (crude) alkaloids, M_A, obtained from the methanol extract of *C. glutinosum,* M, was subjected to qualitative screening, revealed the presence of alkaloid using Dragendorff's reagent and the absence of phenolic compounds, and reducing sugars.

4.5.1 Thin layer chromatographic profile of (M_A)

The TLC of (M_A) using solvent system chloroform: methanol: ammonia (1 : 1: 0.01) as mobile phase and spraying the developed chromatogram with dragendroff spray revealed an orange spot which indicates the presence of alkaloids in plate III

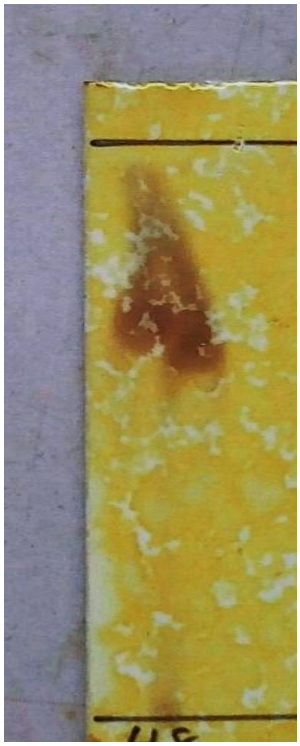


Plate IV: TLC profile of the total (crude) alkaloid of *C. glutinosum* stem bark (M_A)

solvent system : Chloroform : Methanol : Ammonia (1 : 1: 0.01)

Chromogenic reagent : Lieberman-Burchard's reagent. Tlc revealed an orange spot with dragendroff spray which indicates the presence of alkaloid in plate IV

4.6 Description of Partitioned Fractions

The physical appearance and weight of the Partitioned fractions obtained from the partitioning of stem bark of *C. glutinosum,* M are presented in Table 4.6

Table 4.6: Physical appearance and weight of the Partitioned fractions

Fraction	Code	Colour/ Appearance	Weight(g)	% Yield
Hexane	M_H	Yellow	Not significant	-
Ethyl acetate	M_E	brown gummy mass	44.8	50.51
Butanol	M_B	brown gummy mass	49.5	55.81
Residual	M_R	brown gummy mass	34.8	39.23

M_E =ethyl acetate fraction

M_B= butanol fraction

M_R=residual fraction

The partition using butanol had higher yield than ethyl acetate. This also confirms that the plant is very rich in polar compounds since the order of polarity is n-BuOH>>EtOAc> Hexane. The extraction yield showed differences which suggest that different solvent used for partitioning would extract different compounds depending on the polarity of the solvent.

4.7 Qualitative Screening of the Partitioned Fractions

The partitioned fractions were screened qualitatively for phytochemicals using standard procedures described by Amako *et al* (2015) for the presence/absence of phytochemicals as presented in Table 4.7

Table 4.7: Qualitative phytochemical screening of the partitioned fractions

Phytochemicals	**Test**	**Observation**	**Fraction**		
			M_E	**M_B**	**M_R**
Saponins	Frothing	persistent froth	+	++	++
Alkaloids			+	++	+
Flavonoids	NaOH, Shinoda	Colourless, pink	+	++	+
Steriods	Salkowski's	Red colour	+	+	+
Tannins	$FeCl_3$	Bluish green colour	+	+	+
Reducing sugars	Fehlings	Brick red ppt	+	+	+

Keys: +++ = High Presence; ++ = Moderate present; + = present; - =absent

M_E =ethyl acetate fraction

M_B= butanol fraction

M_R=residual fraction

Phytochemical screening of the partitioned fractions (M_E, M_B and M_R) the methanol extract, M from partitioning of the crude methanol extract, (M), still confirms the presence of saponins, alkaloids, flavonoids, steroids, tannins and reducing sugars present in all the fractions.

4.8 Quantitative Screening of the Partitioned Fractions

Quantitative screening the partitioned fractions using various standard methods to reveal different quantity of phytochemicals as presented in Table 4.8

Table 4.8: Quantitative phytochemical screening of the partitioned fractions.

Fractions	Flavonoids	Alkaloids	Tannins	Saponins
M_B	281.097 ± 0.779	40.982 ± 0.575	81.685 ± 1.786	314.554 ± 1.430
M_E	149.409 ± 0.748	33.170 ± 0.002	44.221 ± 0.243	179.382 ± 0.178
M_R	148.882 ± 0.246	25.153 ± 0.923	40.372 ± 0.691	350.958 ± 0.823

M_E =Ethyl acetate fraction

M_B= Butanol fraction

M_R=Residual fraction

Phytochemical screening of the partitioned fractions (M_E, M_B and M_R) the methanol extract. (M) from partitioning of the crude methanol extract, M, still confirms the presence of saponins, alkaloids, flavonoids, steroids, tannins and reducing sugars present in all the fractions as shown on Table 4.7 and 4.8 with appreciable quantity of saponins (350.958 ± 0.823) in the residual fraction >>>>flavoniods (281.097 ± 0.779) in butanol fraction>>>tannins (81.685 ± 1.786) in butanol fraction>> alkaloids (40.982 ± 0.575) also in butanol fraction. Ethylacetate also had appreciable amount of polar compounds, extracted more by partitioning using butanol.

4.9 GC-MS Analysis of Compound in Sapogenin Fraction Mss_1

The Gas chromatography/ mass spectrometry (GC-MS) result of compounds present in sapogeninfraction$Mss_1$is presented in Table 4.9 and their corresponding structures in i, ii, iii.

Table 4.9: Compounds detected in sapogenin fraction (Mss_1) of *C. glutinosum* by GC-MS

Name	Class	R.T	Area%	m/z	MF
Stigmasta-7,22-dien-3-ol, acetate,(3.β.,5.α.,22E)-	(i) Phytosterols	17.016	3.84	454	$C_{31}H_{50}O$
9,19-Cyclolanostan-3-ol,acetate, (3.β.)-	(ii) phytosterols	19.993	0.89	470	$C_{32}H_{54}O_2$
Stigmasterol	(iii) Phytosterols	20.343	6.87	412	$C_{29}H_{48}O$

KEY:

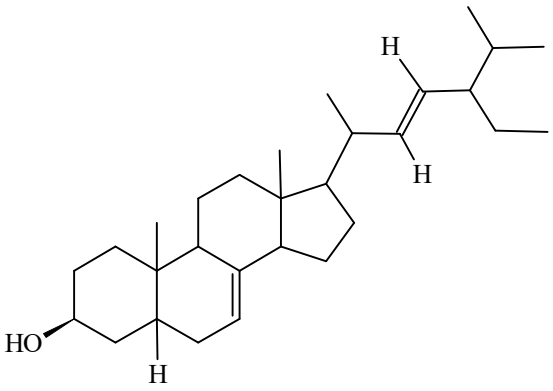
Mss = Sapogenin fraction

R.T = Retention Time

m/z = Mass/ Charge ratio

MF = Molecular Formular

GC-MS analysis of Mss_1 obtained from fractionation of the sapogenin fraction using column chromatography revealed steroidal compounds. Mss_1 showed Stigmasta-7,22-dien-3-ol, acetate, (3.β.,5.α.,22E), 9,19-Cyclolanostan-3-ol,acetate, (3.β.), Stigmasterol, which are phytosterols. Cardoso *et al*. (2019) reported cough suppressant activity on this class of compounds.



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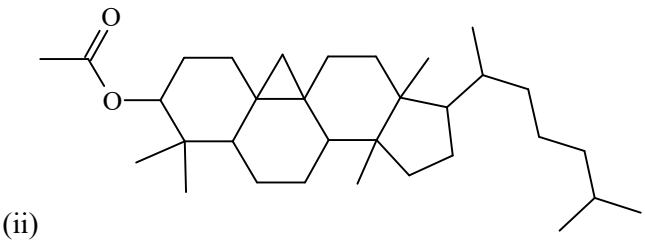

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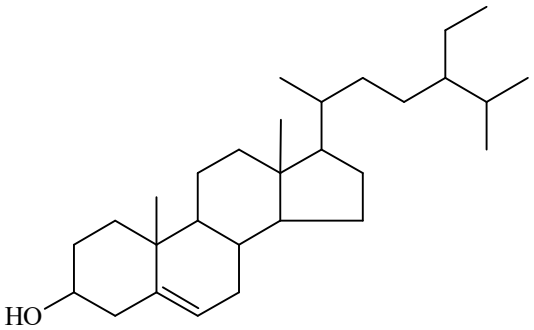

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Figure 4.1: Compounds detected in Mss_1 sapogenin portion of *C. glutinosum* by GC-MS

4.10 GC-MS Analysis of Compound in Sapogenin Fraction Mss_2

The Gas chromatography/ mass spectrometry (GC-MS) result of compounds present in sapogenin fraction Mss_2 is presented in Table 4.10.

Table 4.10: Compounds detected in sapogenin fraction (Mss_2) of *C. glutinosum* by GC-MS

Name	Class	R.Time	Area %	M/Z	Molecular formular
ϒ.-Sitosterol	(iv)Phytosterols	20.427	4.36	414	$C_{29}H_{50}O$
Androstane-17-carboxylicacid,3,11-bis(acetyloxy)-12-hydroxy	(v)Phytosterols	17.698	3.26	450	$C_{25}H_{38}O_7$
Isophytol, acetate	(vi)Diterpenoid	15.151	0.28	296	$C_{20}H_{50}O_2$
Dodecane,2,6,10-trimethyl	(vii)sesquiterpeniod	11.831	0.50	212	$C_{15}H_{32}$

KEY:

Mss = sapogenin fraction

R.T = Retention Time

m/z = Mass/ Charge ratio

M/F = Molecular Formular

GC-MS analysis of Mss_2 obtained from fractionation of the sapogenin fraction using column chromatography revealed steroidal and triterpenoidal compounds. Mss_2 revealed ϒ.-Sitosterol, Androstane-17-carboxylic acid,3,11-bis (acetyloxy)-12-hydroxy,

Isophytol, acetate, Dodecane,2,6,10-trimethyl which belong to phytosterols, diterpenoid and sesquiterpenoid class of compounds respectively.

Sesquiterpenes have some pharmacological properties which include anti-infamatory, antibacterial , expectorant and antitussive activities (Hu *et al*., 2017), which might be responsible for the antitussive activity exhibited by *C.glutinosum.* Phytosterols have been reported to posses antibacterial and antitussive activities (Gariola *et al*., 2018).

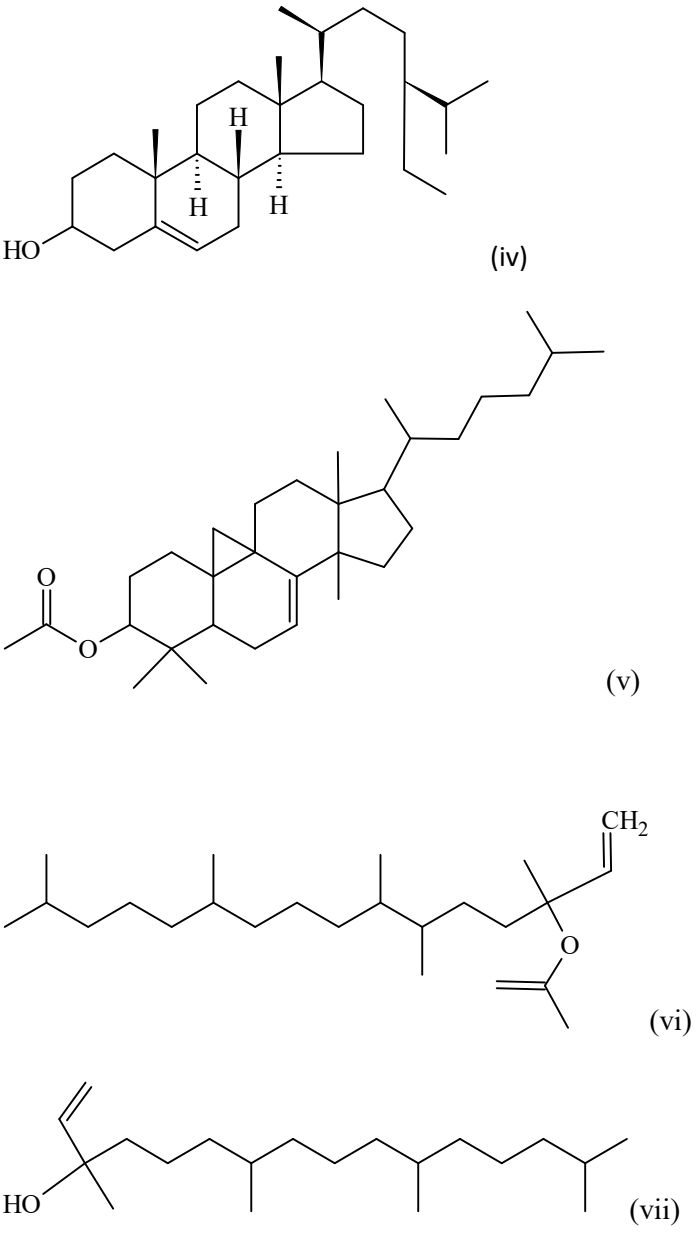


Figure 4.2: Compounds detected in Mss_2 sapogenin portion of *C. glutinosum* by GC-MS

4.11 GC-MS Analysis of Compound in Sapogenin Fraction Mss_3

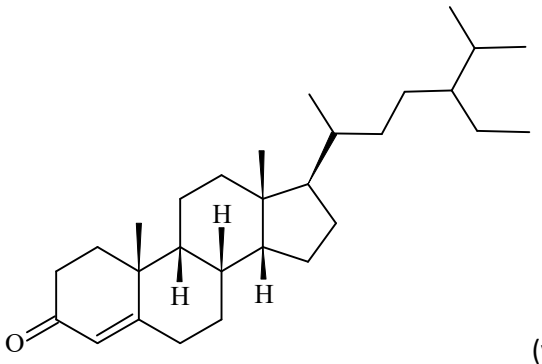
The Gas chromatography/ mass spectrometry (GC-MS) result of compounds present in sapogenin fraction Mss_3 is presented in Table 4.11.

Table 4.11: Compounds detected in sapogenin fraction (Mss_3) of *C. glutinosum* by GC-MS

Name	Class	R.T	Area%	m/z	MF
β.-Sitosterol	(viii) Phytosterols	20.427	4.36	414	$C_{29}H_{50}O$
Stigmast-4-en-3-one	(ix) Phytosterols	17.698	3.26	450	$C_{25}H_{38}O_7$
Cholest-4-ene,3.β.-(methoxymethoxy)-	(x) Phytosterols	15.151	0.28	296	$C_{20}H_{50}O_2$
Docosane-1,2-diol,isopropylidenederivative	(xi) Sesquiterpenoid	11.831	0.50	212	$C_{15}H_{32}$

KEY: Mss = sapogenin fraction,
R.T = Retention Time
m/z = Mass/ Charge ratio
M/F = Molecular Formular

GC-MS analysis of Mss_3 obtained from fractionation of the sapogenin fraction using column chromatography revealed steroidal and triterpenoidal compounds, showed β.-Sitosterol, Stigmast-4-en-3-one, Cholest-4-ene,3.β.-(methoxymethoxy), Docosane-1,2-diol, isopropylidene derivative which are phytosterol and sesquiterpenoid class of compounds, which according to literature has been effective in the treatment of cough (Ajuru *et al.*, 2017). El-Souod *et al.,* (2018) reported antitussive and expectorant activity, which revealed phytosterols and sesquiterpenoid exhibit antitussive, antibacterial and expectorant activities.



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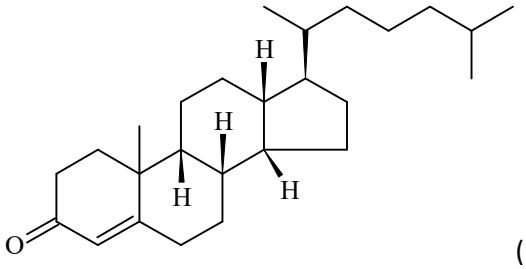

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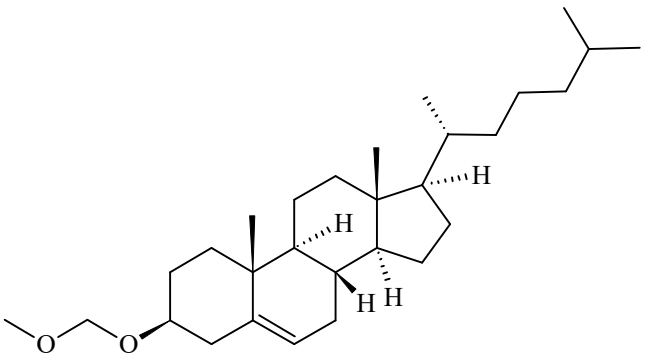

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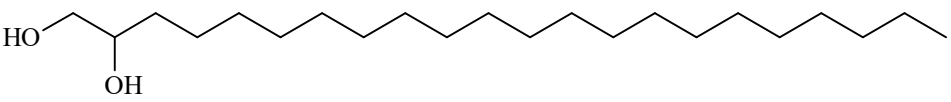


(xi)

Figure 4.3. Compounds detected in Mss_3 sapogenin portion of *C. glutinosum* by GC-MS

4.12 C-MS Analysis of Compound in Alkaloidal Fraction M_A

The Gas chromatography/ mass spectrometry (GC-MS) result of compounds present in alkaloidal fraction M_A is presented in Table 4.12 .

Table 4.12: Compounds detected in alkaloidal fraction M_A of *C. glutinosum* by GC-MS

Name	Class	R.T	Area%	m/z	MF
Pyrrolidin-2-one, 5-pentyl-	(xii) Pyrolidine	11.047	0.62	155	$C_{10}H_{21}N$
Bis(2-ethylhexyl) phthalate	(xiii)Alkaloid amine	19.127	8.18	273	$C_{16}H_{35}NO_2$
4-Piperidinone,2,2,6,6 tetramethyl-	(xiv) Piperidines	7.008	0.20	155	$C_9H_{17}NO$

KEY:

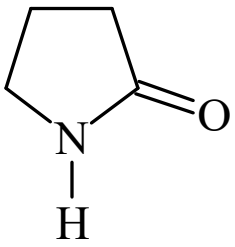
Mss = sapogenin fraction

R.T = Retention Time

m/z = Mass/ Charge ratio

M/F = Molecular Formular

GC-MS of M_A revealed different class of alkaloids which are Pyrolidine alkaliods, Alkaloidial amine, and Piperidines alkaloids. This class of compounds has been effective in the treatment of cough (Ojima *et al*., 2018), Alkaloid shows a range of biological activities which include antin-flammatory, analgesic, antitussive and expectorant activities (Saadat *et al*., 2018). This also might be responsible for the antitussive activity exhibited by stem bark of *C. glutinosum*



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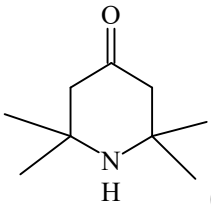

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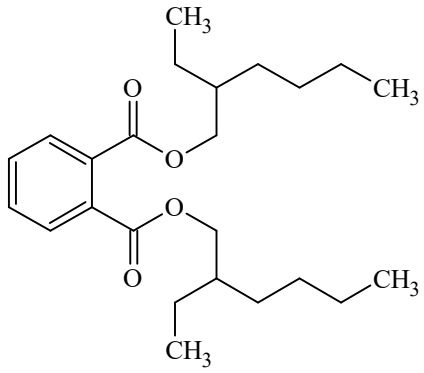

(xiv)

Figure 4.4: Compounds detected in Alkaloidal fraction M_A of *C. glutinosum* by GC-MS

4.13 GC-MS Analysis of Hexane Fraction (M_H)

The Gas chromatography/ mass spectrometry (GC-MS) result of compounds present in hexane fraction M_H is presented in Table 4.13.

Table 4.13: Compounds detected in hexane fraction (M_H) of *C. glutinosum* by GC-MS.

Name	Class	Area%	r. Time	Formula	Mass
Benzene, 1,2-dichloro-	Dichlorobenzene	0.51	5.712	$C_6H_4Cl_2$	146
Silane, cyclohexyldımethoxymethyl-	Organometallic	0.87	7.901	$C_9H_{20}O_2Si$	188
Eicosane	Alkane	1.85	12.225	$C_{20}H_{42}$	282
Heneicosane		1.04	12.263	$C_{21}H_{44}$	296
Benzene, 1,1'-(3-methyl-1-propene-1.3-diyl		1.48	13.115	$C_{16}H_{16}$	208
Nonane, 4,5-dimethyl-		0.52	6.525	$C_{11}H_{24}$	156
1-Heneicosanol		1.58	14.122	$C_{21}H_{44}O$	312
Dodecane		0.33	8.583	$C_{12}H_{26}$	170
Tetradecane, 5-methyl-		0.43	9.512	$C_{15}H_{32}$	212
Octadecanoic acid. 2.3-dihydroxypropyl		4.09	18.206	$C_{24}H_{38}O_4$	390
Tetradecane		1.10	11.139	$C_{14}H_{30}$	198
2-methylhexacosane	Alcohol	2.04	15.141	$C_{27}H_{56}$	380
1-(+)-Ascorbic acid 2.6-dihexadecanoate		1.44	15.372	$C_{38}H_{68}O_8$	652
Pentadecanoic acıd		1.61	16.057	$C_{25}H_{50}O_2$	382
Sulfurous acid, octadecyl 2-propyl ester		1.63	16.427	$C_{24}H_{50}O$	354
17-Pentatriacontene		1.74	16.562	$C_{15}H_{30}O_2$	242
Methyl 2-hydroxy-heptadecanoate		4.95	16.898	$C_{21}H_{94}O_3S$	376
Ethanol, 2.2-(dodecylimino)bis-		4.49	17.057	$C_{35}H_{70}$	490
Phenol, 2,4-bis(1,1-dimethylethyl)-		8.06	12.132	$C_{14}H_{22}O$	206
Bis(2-ethylhexyl) phthalate	Ester	1.83	17.709	$C_{16}H_{35}NO_2$	273
Tetrapentacontane, 1.54-dibromm-		6.80	18.916	$C_{54}H_{108}Br_2$	914
Dibutyl phthalate		0.78	14.973	$C_{16}H_{22}O_4$	278
n-Tetracosanal-1		11.15	15.651	$C_{24}H_{50}O$	354
		100			

GC-MS analysis of hexane fraction (M_H) obtained from partitioning of methanol extract of *C. glutinosum* stem bark revealed the presence of alkane, alcohol, esters,

dichlorobenzene and organometallic compounds. Mostly alkanes, alcohols, and essential oils were present in hexane fraction of *C. glutinosum*, as reported by Alowanou *et al* (2015). Antitussive activity was not carried out on hexane fraction (M_H) due to quantity restrains.

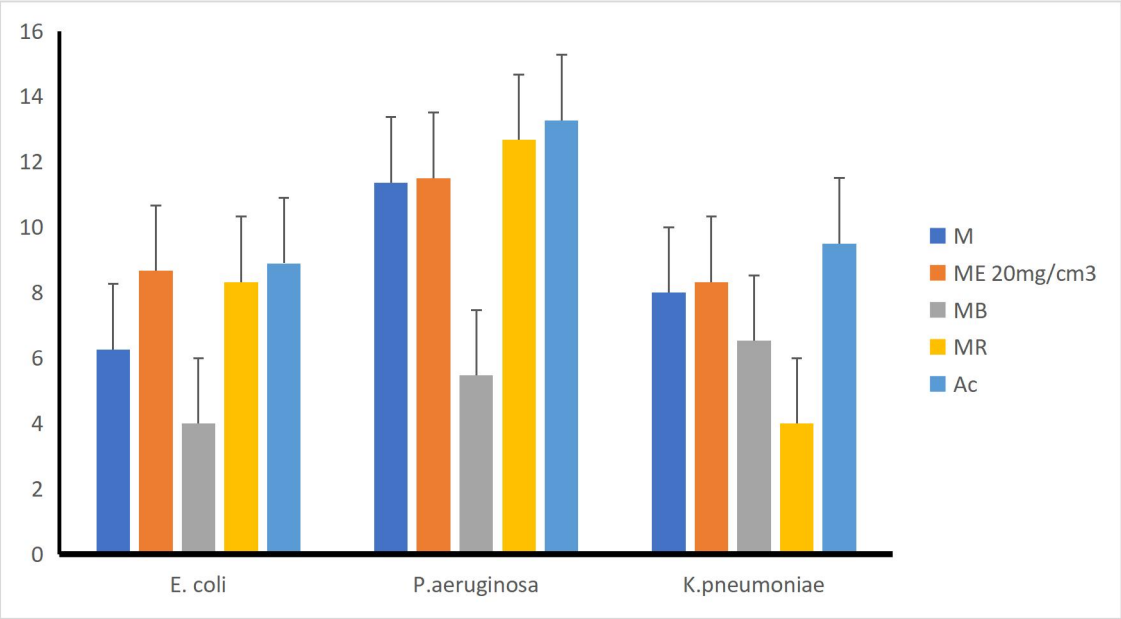

Figure 4.5: Diameter of zones of inhibition of Crude Methanol extract of *C. glutinosum* Stem Bark and its Partitioned Fractions against some test organism.

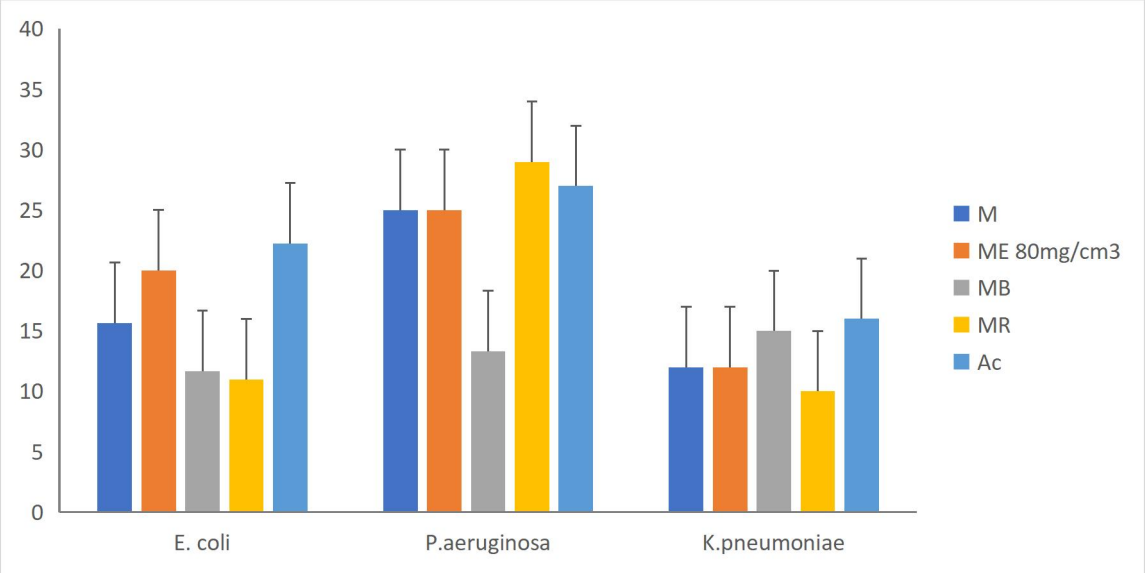

Figure 4.6: Diameter of zones of inhibition of Crude Methanol extract of *C. glutinosum* Stem Bark and its Partitioned Fractions against some test organism

It shows that *Pseudomonas aeruginosa* had the highest susceptibility to ethylacetate fraction having 25.00±1.40 mm and *Escherichia coli* which had the lowest susceptibility to be 15.67±0.90. Zones of inhibition greater than 14 mm were considered as good antimicrobial activity. All the isolate were susceptible to potassium cluvalunate used as control.

4.14 The Minimum Inhibitory Concentration of Crude Methanol Extract of *C. glutinosum* Stem Bark and its Partitioned Fractions

The minimum inhibitory concentration result of organisms tested on the extract/fraction at different concentration in mg/cm^3 is shown in Table 4.15.

Table 4.14: The minimum inhibitory concentration of crude methanol extract of *C. glutinosum* stem bark and its partitioned fractions against test organism

Organism	Extract/ fraction	Concentration (mg/cm^3)						
		100	50	25	12.5	6.25	3.13	1.56
E. coli	M	+	+	+	++	-	-S	-
P. aeruginosa		+	+	+	++	-	-	-
E. coli	M_E	+	+	+	++	-	-	-
P. aeruginosa		+	+	+	++	-	-	-
K. pneumonia	M_B	+	+	+	++	-	-	-
P. aeruginosa	M_R	+	+	+	++	-	-	-

mg/cm^3; milligrams per centimeter ; ++; minimum inbibitory concentration(mic); + = present; - =absent; M = Methanol extract, M_E =Ethyl acetate fraction, M_B= Butanol fraction, M_R=Residual fraction

The methanol extract and its partitioned portion of *C. glutinosum* stem bark was active against the growth of *Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumonia* with zones of inhibition ranging from 25.00±1.40-15.67±0.90.

The minimum bactericidal concentration result of organisms tested on the extract/fraction at different concentration in mg/cm^3 is shown in Table 4.16.

Table 4.15: Minimum bactericidal concentration of crude methanol extracts and its Partitioned Fractions of *Combretum glutinosum* Stem Bark

Organism	Extract/ fraction	Concentration (mg/cm^3)						
		100	50	25	12.5	6.25	3.13	1.56
E. coli	M	+	+mbc	+	+	-	-	-
P.aeruginosa		+	+mbc	+	+	-	-	-
E. coli	M_E	+	+mbc	+	+	-	-	-
P.aeruginosa		+	+mbc	+	+	-	-	-
K. pneumonia	M_B	+	+	+	+	-	-	-
P.aeruginosa	M_R	+	+	+	+	-	-	-

mg/cm^3; milligrams per centimeter;+ = present; - =absent;mbc= minimum bactericidal concentration; M =Methanol Extract, M_E =Ethyl acetate fraction, M_B= Butanol fraction, M_R=Residual fraction

The antimicrobial activity of the extract and fraction might be due to the presence of steroidal, triterprnoidal and alkaloidal compounds present in stem bark of *C. glutinosum.*

CHAPTER FIVE

5.0 CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

Combretum glutinosum have been reported by the traditional medicine healers in Nigeria to have antittusive activities. The scientific evaluation of *C. glutinosum* exhibited antitussive activity against selected test organisms. The results confirms the traditional use of *C. glutinosum* as an antitussive. Quantitative and qualitative screening revealed the presence of various bioactive compounds. Investigation of total (crude) saponins content of crude methanol extract (M) revealed that *C. glutinosum* is rich in sapogenins and the determination of total (crude) alkaloidal content revealed alkaloidal compounds as identified using various chromatographic techniques. This study suggests the relevance of *C. glutinosum* in the treatment of microbial infections and its uses in medicine.

5.2 Recommendations

From the result, it is therefore recommended that:

1. The compounds present in sapogenin fractions Mss_1-Mss_3 of *C. glutinosum* stem bark should be isolated and characterized.
2. The compounds present in alkaloidal fraction M_{A1} of *C. glutinosum* stem bark should also be isolated and characterized
3. The Isolated compounds, Mss_1-Mss_3 and M_{A1} should be used for antitussive activity against selected test organisms.

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APPENDICES

Hit#:3 Entry:191096 Library:NIST11.lib
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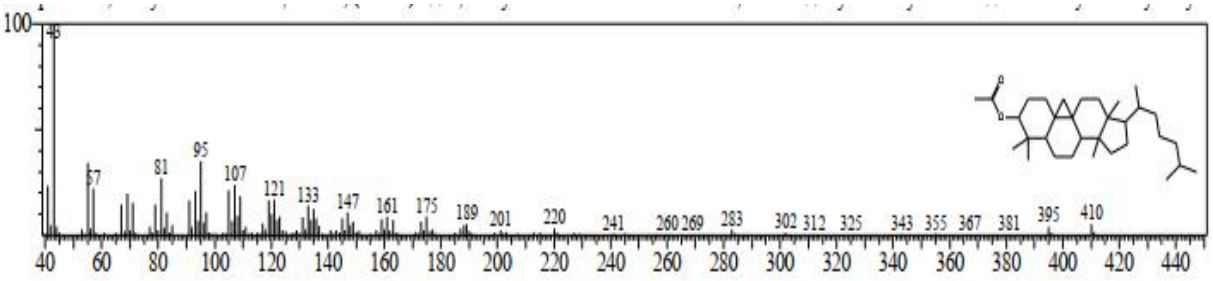

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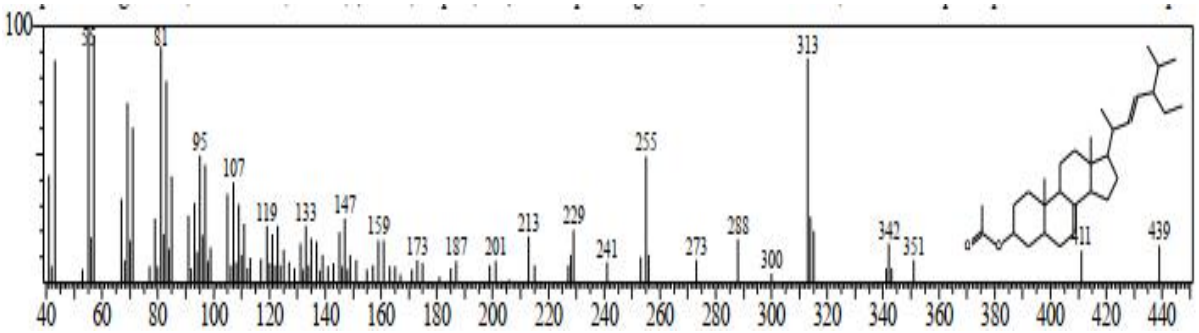

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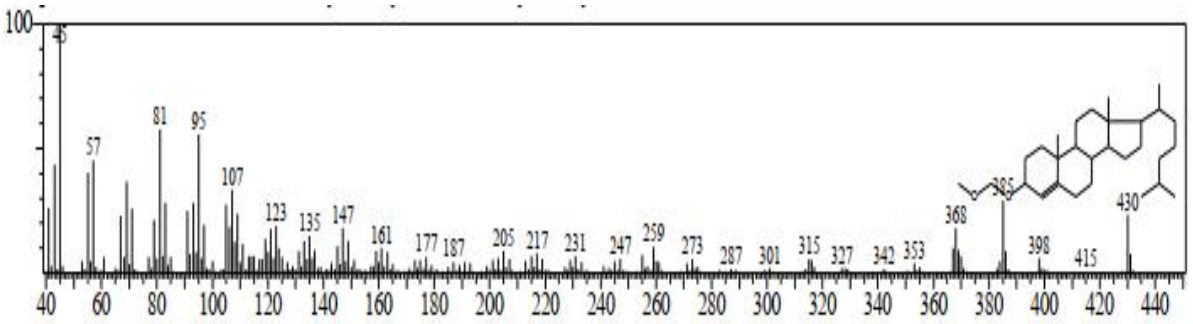

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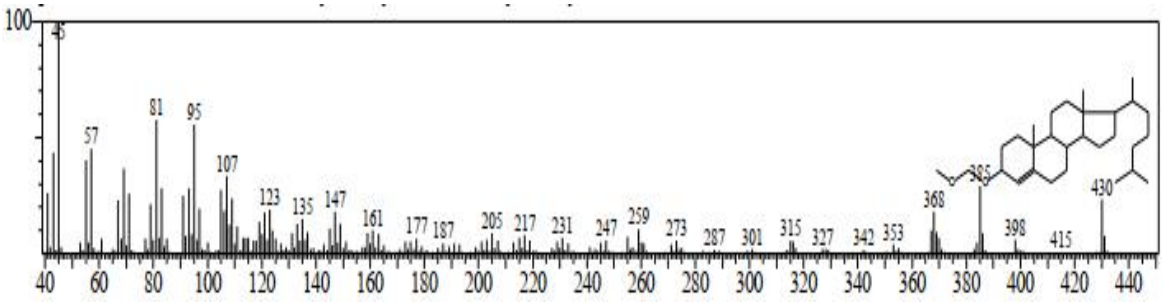

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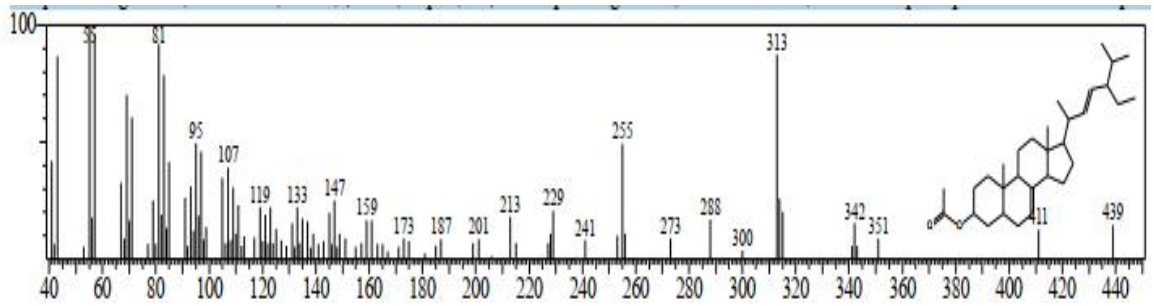

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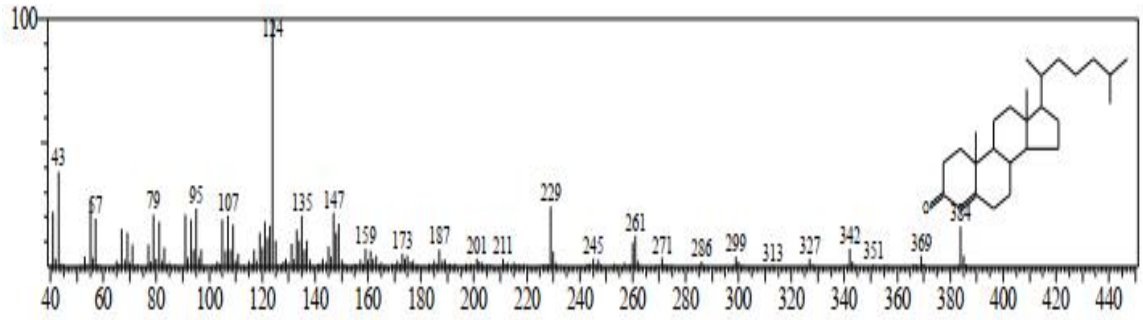
CompName:Cholest-4-en-3-one $$ 3-Oxocholest-4-ene $$ Cholestenone $$ .delta.4-Cholesten-3-one $$ .delta.-4-Cholesten-3-one $$ 4-Cholestenone $$ 4-Cho



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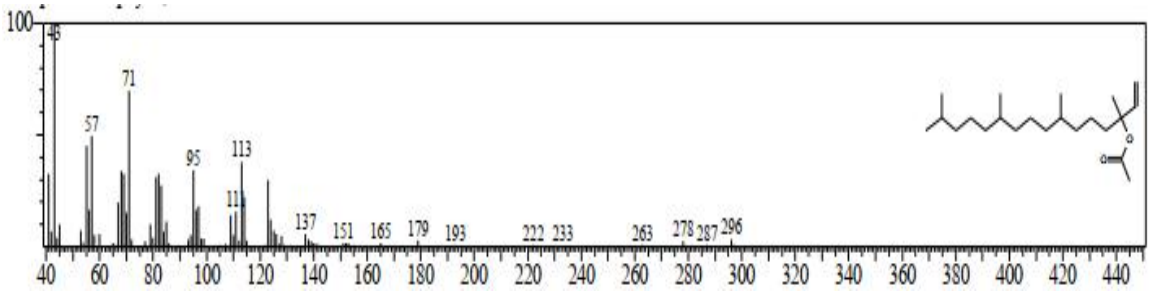

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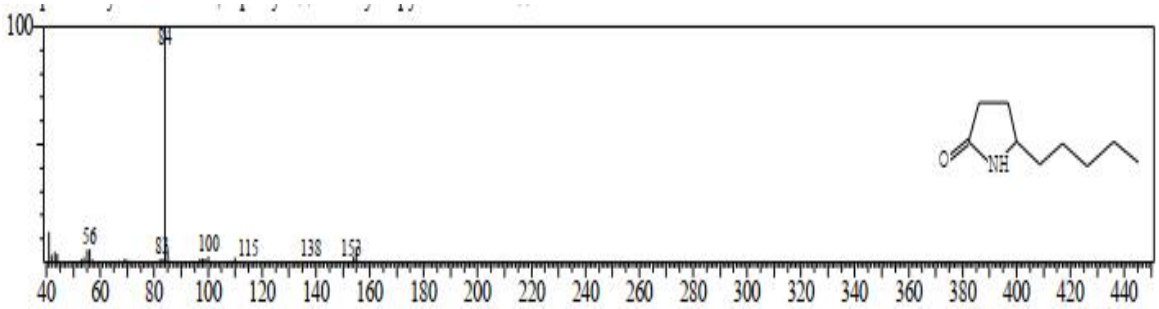

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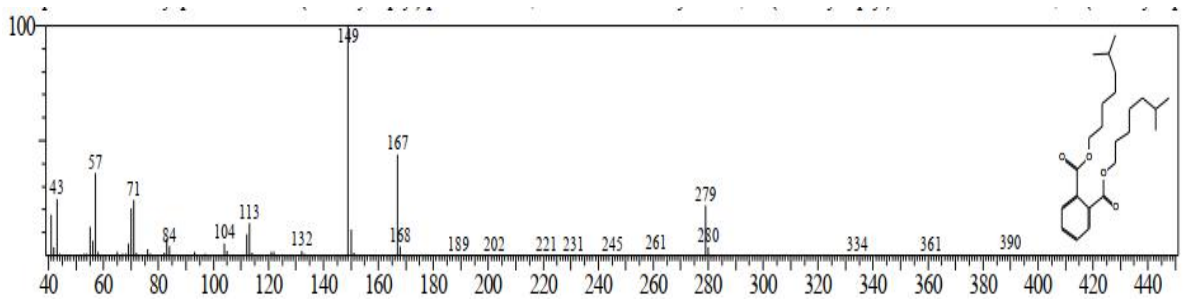

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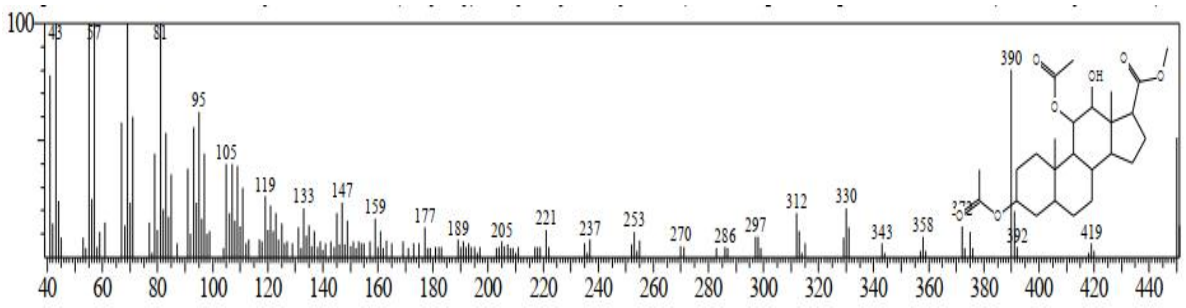

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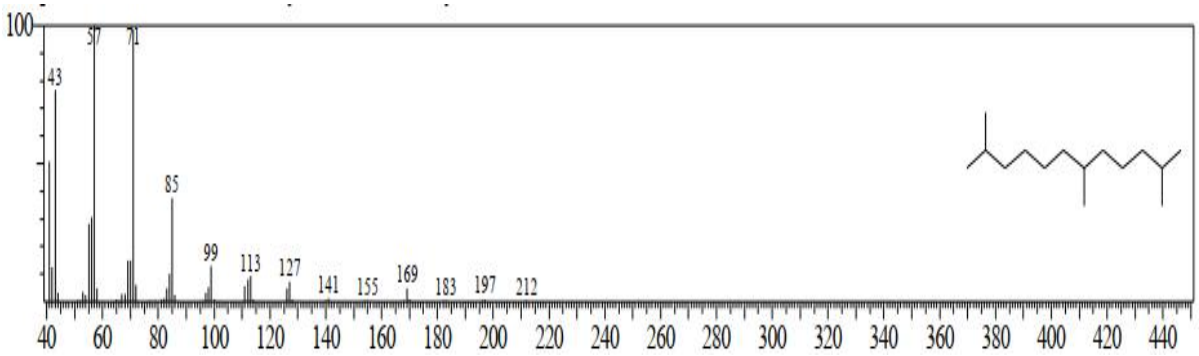

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