PHYTOCHEMICAL AND ANTIMICROBIAL ANALYSES OF THE STEM BARK OF *Sarcocephalus latifolius* (Smith Bruce) AND AERIAL PART OF S*pillanthes acmella* (Murr)

BY

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DECLARATION

I hereby declare that the work in this dissertation titled "Phytochemical and antimicrobial analyses of the stem bark of *Sarcocephalus latifolius* (Smith Bruce) and aerial part of *Spillanthes acmella* (Murr)" was performed by me in the Department of Chemistry ABU, Zaria, Nigeria, under the supervision of Prof. G.I Ndukwe, Prof. Amupitan J.O and Assoc. Prof. R.G. Ayo. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this dissertation has been presented for another degree or diploma at any institution.

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Name of Student	Signature	Date

CERTIFICATION

This dissertation titled "Phytochemical and antimicrobial analyses of the stem bark of *Sarcocephalus latifolius* (Smith Bruce) and aerial part of *Spillanthes acmella* (Murr)" by Isah, Yinusa meets the regulations governing the award of the degree of Doctor of Philosophy of Ahmadu Bello University, Zaria, and is approved for its contribution to knowledge and literary presentation.

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DEDICATION

"To my wife, Faridat, my children (Abdul-Qudus Onoruoiza and Aishat Ahuoiza) and my beloved late Mother Hajarat Onimire"

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I wish to thank the Almighty Allah, the most Beneficent and the most Merciful who has given me the strength and guided me through the entire work and to my ever dedicated, patient, encouraging and hard working supervisors in the persons of Professor G.I Ndukwe, Professor J.O Amupitan and Dr.R.G Ayo for their unrelenting effort and their useful suggestions, guidance, encouragement and support which had made it possible for me to conclude this research work.

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ABSTRACT

Phytochemical studies and antimicrobial analyses of the root and stem bark of *Sarcocephalus latifolius* (Smith Bruce) and the aerial plant parts of *Spillanthes acmella* (Murr) were carried out. The phytochemical screening of the root and stem bark of *Sarcocephalus latifolius* revealed the presence of carbohydrate, glycoside (root), anthraquinones, cardiac glycoside, saponins, steroid and triterpenes, flavonoids (root),tannins (stem-bark) and alkaloids in plant parts studied. While the aerial plant part of *Spillanthes acmella* showed the presence of carbohydrate, glycoside, anthraquinones, cardiac glycoside, saponins, steroid triterpenes, saponins, flavonoids and alkaloids. The microbial screening of the crude extracts of the plants were carried out on the following clinical microbes; Methycilin Resistant *Staphylococcus aureus (MRSA), Staphylococcus aureus, Streptococcus pyrogenes, Bacillus subtilis, Corynebacterium ulcerans, Escherichia coli, Proteus mirabilis, Proteus vulgaris, Pseudomonas aureginosa, Salmonella typhi, Shigellia dysenteric ,Candida albicans, Candida virusei*, and *Candida tropicalis* to determine their activities. The extracts from *Sarcocephalus latifolius* showed that six (6), seven (7), ten (10) and seven (7) microorganisms were sensitive to the petroleum ether, chloroform, ethyl acetate and methanol fractions respectively. while the activities for the extracts from *Spillanthes acmella* showed that eight (8), nine (9), eight (8) and nine (9) of the microorganisms were sensitive to the petroleum ether, chloroform, ethyl acetate and methanol fractions respectively. The Minimum Inhibitory Concentrations (MIC), Minimum Bacterial Concentrations (MBC) and Minimum Fungicidal Concentrations (MFC) of the extracts from *Sarcocephalus latifolius* stem bark and aerial plant parts of *Spillanthes acmella* were carried out and the values determined respectively. An attempted

chromatographic separation of the methanol extract of *Sarcocephalus latifolius* bark yielded EFSB, SB-C1 and SB-C2 while the petroleum ether fraction of *Spillanthes acmella* afforded SPEC-1. These compounds were characterized by 1-D and 2-D NMR spectra technique and comparison made with those of literature to be a mixture of sitosterol and sitostenone, sitosterol, betunilic acid and stigmasterol respectively. The isolated and characterized compounds were subjected to microbial screening and their activities compared with standard drugs. EFSB (mixture of sitosterol and sitostenone) showed sensitivity to all tested microorganisms with the exception of MR*SA*, *Corynebacterium ulcerans, Pseudomonas aureginosa* and *Candida tropicalis* with zone of inhibition ranging from 20 to 25 mm. The MIC value for the sensitive organisms was 12.5 µg/ml while the MBC/MFC was 50 µg/ml for these sensitive microbes with exception of *Bacillus subtilis* that was found to be 25 µg/ml. When these values were compared to that of standard drug (sparfloxacin), EFSB had a lower value of MIC as compared to the drug. This showed that EFSB was comparable to this drug and had a wider broad spectrum of activities over the tested organism. It was noted that all the tested organisms against SB-C1 (sitosterol) were sensitive with the exception of Methycilin Resistant *Staphylococcus aureus*, *Escherichia coli, Proteus mirabilis* and *Candida tropicalis*. The MIC and MBC/MFC results showed that *Bacillus subtilis, Salmonella typhi* and *Shigellia dysenteric* were completely exterminated at a concentration of 25 µg/ml while *Staphylococcus aureus, Streptococcus pyrogenes, Corynebacterium ulcerans, Proteus vulgaris*, *Proteus aureginosa, Candida albicans* and *Candida virusei* were exterminated at 50 µg/ml.The result showed that SB-C1 was comparable to standard drug Sparfloxacin because both exterminated *Bacillus subtilis, Salmonella typhi* and *Shigellia dysenteric* at 25 µg/ml. Most of the tested microorganisms

were sensitive to SB-C2 (Betunilic acid) with the exception of *Corynebacterium ulcerans*. The zone of inhibition observed for the microbes range between 21 to 34 mm. It was observed that, the MIC for the sensitive organisms was 12.5 µg/ml with the exception of *Salmonella typhi and Shigellia dysenteric* that was 6.25 µg/ml. The MBC/MFC values for *Staphylococcus aureus, Streptococcus pyrogenes, Bacillus subtilis, Escherichia coli, Proteus vulgaris, Salmonella typhi, Shigellia dysenteric* and *Candida virusei was* 25 µg/ml while for MR*SA*, *Proteus mirabilis, Pseudomonas aureginosa, Candida albicans* and *Candida tropicalis* was 50 µg/ml. It was noted that SB-C2 has a wider broad spectrum of activities than that of standard drug sparfloxacin. The zone of inhibition of SPEC-1 (stigmasterol) was between 20 to 24 mm. *Bacillus subtilis* was observed to be the most sensitive organism with the largest zone of inhibition of 24 mm while the smallest zone of inhibition was recorded for *Proteus mirabilis, Candida virusei and Candida tropicalis* at 20 mm. The MIC and MBC/MFC showed that all the sensitive organisms had MIC value at 12.5 µg/ml while the MBC/MFC was observed for these microbes at a concentration higher than that of the MIC at 50 µg/ml. The MFC was equally observed at a concentration higher than that of the standard drug Fluconazole. SPEC-1 (stigmasterol) had a wider range of broad spectrum over the standard drug against the entire organisms tested.

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CHAPTER ONE

1.0 Introduction

1.1 Medicinal plants

Medicinal plants have been used virtually in all cultures as a major source of medicine. The widespread use of herbal remedies and healthcare preparations, as those described in ancient texts such as the Vedas and the Bible, are obtained from commonly used traditional herbs and medicinal plants. This usage has been traced to the occurrence of natural products present in them with medicinal properties.

Traditional medicine, bequeathed from generation to generation, is rich in domestic recipes and communal practice, encompassing concepts and methods for the protection and restoration of health. Traditional medicine has served as a fount of alternative medicine, new pharmaceutical and healthcare products. The best known examples of traditional medicine, differing in concept and protocol, are well-developed systems such as acupuncture and ayurvedic medicine that have been widely used to conserve human health in China and India (Lucy and Edgar, 1999).

Developed countries, in recent times, are turning to the use of traditional medicinal systems that involve the use of herbal drugs and remedies. About 1400 herbal preparations are used widely (Lucy and Edgar, 1999), according to a recent survey in Member States of the European Union. Herbal preparations are popular and are of significance in primary healthcare in Belgium, France, Germany and the Netherlands and of recent in Nigeria. Such popularity of healthcare plant-derived products have been traced to their increasing acceptance and use in the cosmetic industry as well as to increasing public costs in the daily maintenance of personal health and well being. Examples of such beauty-oriented

therapeuticals are skin tissue regenerators, anti-wrinkling agents and anti-age creams. Most dermaceuticals are derived from algal extracts that are rich in minerals and the vitamin B group. Skincare products such as skin creams, skin tonics, etc derived from medicinal plants are grouped together as dermaceuticals. Also, amongst the poor, cures and drugs, derived from plants, constitute the main source of healthcare products (Hoareau & Da Silve; 1999) .

Ethnobotany and ethno-medicinal studies are today, recognized as the most viable methods of identifying new medicinal plants or refocusing on those earlier reported for bioactive constituents (Adjanahoun *et al*., 1991; Farnsworth, 1966). The clinical success of quinine and quinidine isolated from the *Cinchona* tree bark and, recently artemisinin from *Artemisia annua,* in the treatment of malaria have rekindled great interest in medicinal plants as potential sources of novel drugs (Di Flumeri *et al*., 2000). Plants which are observed to be efficacious and frequently prescribed may contain compounds that are potential drug candidates and could rightly be recommended for further examination. Gorman, (1992) drew attention to the power of Chinese folk medicinal plants in treating maladies from eczema and malaria to respiratory disorders.

Scientific investigations of medicinal plants have been initiated in many countries because of their contributions to health care. The continual search for, and the interest in natural plant products, for use as medicines has acted as the catalyst for exploring methodologies involved in obtaining the required plant materials and hence probing into their constituents. In the selection of plants for pharmacological screening, five approaches are known, namely:

i. The random approach which involves the collection of all plants from the study area,
ii. Phytochemical targeting which deals with the collection of all the members of the plant's family known to be rich in bioactive compounds,
iii. The ethnobotanical survey approach, which is based on traditional medicinal uses of the plant(s),
iv. The chemotaxonomic approach which is based on plants having similar constituents which may be in different families and
v. The screening of specific parts of a plant such as the seeds, barks, roots, leaves and other plant parts (Farnsworth, 1966).

It has also been reported that plant sampling based on ethnobotanical survey approach showed greater percentage of yielding bioactive useful medicinal compounds over the other methods even though targeted and random screening of plants and their extracts for activity have also yielded excellent results (Khafagi and Dewedar, 2000).

1.1.1 Phytochemistry is the study of chemicals produced in plants, describing the isolation, purification, identification, and structure of the large number of secondary metabolites found in plants

1.1.2 Phytochemicals.

Phytochemicals are non-nutritive plant chemicals that have protective or disease preventive properties. There are thousands known phytochemicals. It is well-known that plants produce these chemicals to protect themselves from attack. But recent researches

demonstrate that they can protect humans against diseases. Some of the well-known phytochemicals are lycopene in tomatoes, isoflavones in soy and flavonoids in fruits.

There are many phytochemicals and each works differently. These are some possible actions;

Antioxidant: Most phytochemicals have antioxidant activity and protect human cells against oxidative damage and reduce the risk of developing certain types of cancer. Some of the phytochemicals with antioxidant activities are Allyl sulfides found in onions, leeks, garlic and the carotenoids found in fruits and carrots, the flavonoids can be found in fruits and vegetables, while polyphenols are present in tea and grapes (Pradeep *et al,* 2012).

Hormonal action: Isoflavones can be found in soy which helps to imitate human estrogens and help to reduce menopausal symptoms and osteoporosis.

Stimulation of enzymes: The indoles are found in cabbages and can stimulate enzymes that can make the estrogen less effective and could reduce the risk of having breast cancer. Other phytochemicals that can interfere with enzymes, are protease inhibitors found in soy and beans and terpenes found in citrus fruits and cherries.

Interference with DNA replication: Saponins found in beans can interfere with the replication of cell DNA thereby preventing the multiplication of cancer cells. Capsaicin, found in hot peppers can protect DNA from carcinogens.

Anti-bacterial effect: The phytochemical allicin from garlic has anti-bacterial properties.

Some phytochemicals bind physically to cell walls thereby preventing the adhesion of pathogens to human cell walls. Proanthocyanidins are responsible for the anti-adhesion properties of cranberry. Consumption of cranberries will reduce the risk of urinary tract infections and will improve dental health. (Ahmed and Urooj, 2010).

Food containing phytochemicals are already part of our daily diet. In fact, most food contains phytochemicals except for some refined ones such as sugar or alcohol. Some food, such as whole grains, vegetables, beans, fruits and herbs, contain many phytochemicals. The easiest way to get more phytochemicals is to eat more fruits such as blueberries, cranberries, cherries, apple, etc. and vegetables such as cauliflower, cabbage, carrots, broccoli, etc. It is recommended that the daily intake of at least 5 to 9 servings of fruits or vegetables will be sufficient for an adult. Fruits and vegetables are also rich in minerals, vitamins and fibre and low in saturated fat. Phytochemicals are naturally present in many foods but it is expected that through bio-engineering new plants will be developed, which will contain higher levels. This would make it easier to incorporate more phytochemicals into our diet.

1.2 Background to the study

Nature has served as a rich repository of medicinal plants for thousands of years and an impressive number of modern drugs have been isolated from natural sources, notably of plant origin (Cowan, 1999). Herbal medicine, based on their traditional uses in the form of powders, liquids or mixtures, has been the basis of treatment for various ailments since ancient times.

The use of herbs as complementary and alternative medicine has increased drastically in the last 20–25 years (Rios and Recio, 2005). According to World Health Organization (WHO) traditional medicines are relied upon by 65–80% of the World's population for their primary health care needs. Moreover, emergence of multiple drug resistant strains of microorganisms due to indiscriminate use of antibiotics to treat infectious diseases has

generated a renewed interest in herbal medicine (Chopra *et al*., 1997). The beneficial health effects of many plants, used for centuries as seasoning agents in food and beverages, have been claimed for preventing food deterioration and as antimicrobials against pathogenic microorganisms. Antimicrobial potentials of different medicinal plants are being extensively studied all over the world (Ahmad; 1998; Arora and Kaur., 1999., Arora *et al*., 2009, Rojas *et al*., 2006) but only a few studies have been carried out in a systematic manner. However, in the absence of any scientific proof of their effectiveness, the validity of these remedies remains questionable and their use locally restricted. Phytochemical and pharmacological investigations of several plants have already led to the isolation of some of the natural antimicrobials (Iwu *et al;* 1999).

In the present study, two medicinal plants *viz. Sarcocephalus latifolius* and *Spillanthes acmella* belonging respectively to the families of Rubiaceae and Asteraceae were selected to assess their antibacterial potentials. These plants are common household remedy for a variety of gastrointestinal disorders, e.g. diarrhea and dysentery,

This study was therefore carried out to provide scientific justification for the use of these medicinal plants in the treatment of conditions associated with pains and infections and to isolate the possible active ingredients and characterize them for possible integration into the already known antimicrobial drugs in use.

1.3 Importance of natural products to drug discovery

The use of medicinal plants for treatment of microbial diseases is well known and has been documented since ancient times. Plants synthesize many components, which act as defensive agents, helping to protect them from microbial infection and other predators.

Those compounds are bioactive and can be medicinal, intoxicating or toxic depending on circumstances. Several plant species have been tested for antimicrobial properties, but the vast majority has not yet been adequately evaluated (Ogundare and Onifade, 2009).

For many decades, synthetic chemicals as drugs have been effective in the treatment of most diseases. The pharmaceutical industry has synthesized over 3 million new chemicals in their effort to produce new drugs. Despite their success in developing drugs to treat or cure many diseases, the treatment of certain diseases such as cancer, Acquired Immunodeficiency Syndrome (AIDS), heart diseases and diabetes have not been a complete success due to the complexity of these diseases.

Over the centuries, people have been living in close association with the environment and relying on its flora and fauna as source of food and medicine. As a result, many societies have their own rich plant pharmacopeias. In developing countries, due to economic factors, nearly 80% of the population still depends on the use of plant extracts as sources of medicine. Natural products also play important role in the health care system in developed countries and below are the few examples of the numerous uses of plant metabolites in medicine:

1. The isolation of the analgesic morphine from the *Opium poppy*, (*Papaver somniferous)* in 1816 led to the development of many highly effective pain relievers.

2. The discovery of penicillin from the filamentous fungus *Penicillium notatum* by Fleming in 1929 had a great impact on the investigation of nature as a source of new bioactive agents.

3. Natural products can also be used as starting materials for semi synthetic drugs. The main examples are plant steroids, which led to the manufacture of oral contraceptives and other steroidal hormones.

Today, almost every pharmacological class of drugs contains a natural product or natural product analog. The investigation of higher plants has led to the discovery of many new drugs. So far, only a small portion of higher plants have been investigated. Consequently, there still remains a big reservoir of useful chemical compounds not only as drugs, but also as templates for synthetic analogues.

1.4 Research problem

The plant kingdom represents an enormous reservoir of biological valuable molecule to be discovered. Among the numerous plant species, only a small percentage has been used for medicinal purposes and very few of the phytochemical, biological and pharmacological activities have been investigated. From the long term use of synthetic drugs with harmful side effect stimulated the researcher to find out compounds of natural origin for a major role as drug and template for the development of new drugs, which will provide better efficacy and less toxicity.

1.5 Aims and objective

The objective of this work is to isolate the relevant metabolites of these plants, study or screen them for anti-microbial activities and characterize them using the relevant spectroscopic tools.

CHAPTER TWO

2.0 Literature review

2.1 Plant metabolite of pharmacological importance

The use of natural products as medicinal agents presumably predates the earliest recorded history as the earliest humans used various, but specific plants to treat illness. Many plant products exhibit pharmacological responses in animals and man. Such plant products include alkaloid, cardiac glycosides, flavonoids, saponins and tannins. A brief discussion of these various pharmacological products are outlined below

2.1.1 Alkaloids: Alkaloids are a chemically heterogeneous group of basic nitrogen containing substances found predominantly in higher plants and also occur in lower plants, animals, microorganisms and marine organisms. Alkaloids usually contain one or two nitrogen atoms although some like ergotamine may contain up to five nitrogen atoms. The term alkaloid was coined in 1819 by the pharmacist Meissner and meant simply alkali-like molecules.' True Alkaloids ' were defined as compounds meeting the additional four qualifications namely:

(i) Nitrogen is a part of heterocyclic ring

(ii) The occurrence of compound is restricted to plant kingdom

(iii) The compound has complex molecular structure

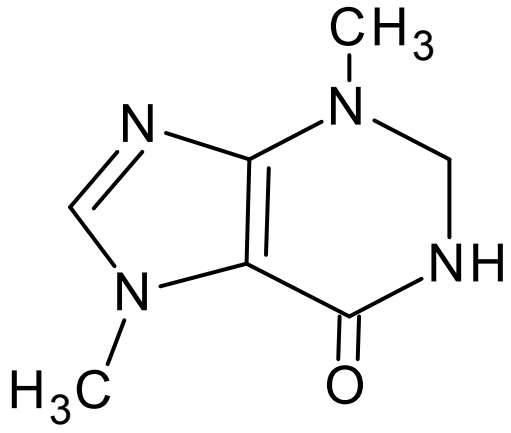
(iv) The compound manifests significant physiological activity

Many of the earliest isolated compounds with biological activity were alkaloids. This may be due to the ease of isolation. In the extraction of these alkaloids, knowledge of their solubility and salts are very important, since the difference in solubility between the two

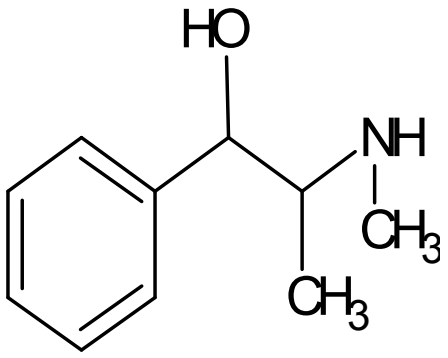
forms really provide methods for the isolation of the alkaloid from the plant. Generally the free bases are sparingly soluble in water but soluble in organic solvents while the salts show a reverse order of solubility. There is no one particular definition of the term 'alkaloid' that is completely satisfactory and no boundary exists between alkaloids and naturally occurring complex amines. However alkaloids can generally be said to be 'those basic substances which contain one or more nitrogen atoms, usually in combination as part of a cyclic system (Harbone, 1973) as shown in structures I and II.

Their manifold pharmacological activities have always excited man's interest and since early times selected plant products have been used as poisons, euphoriants, psychedelics, stimulants or as medicines. Many of our modern drugs now contain the same compounds or synthetic analogues and the pharmacological and toxicological properties of these compounds are thus of immense interest and importance.

2.1.2 Glycosides: Glycosides are general word that embraces all the many and varied combinations of sugars and a glycose or genins. The usual linkage between the sugar and aglycone is an oxygen linkage between the reducing group of a sugar and an alcoholic or phenolic hydroxyl group of the aglycone. They are characterized by the fact that on hydrolysis, either by acid or enzyme they yield one or more sugar moiety, the aglycone or genins.



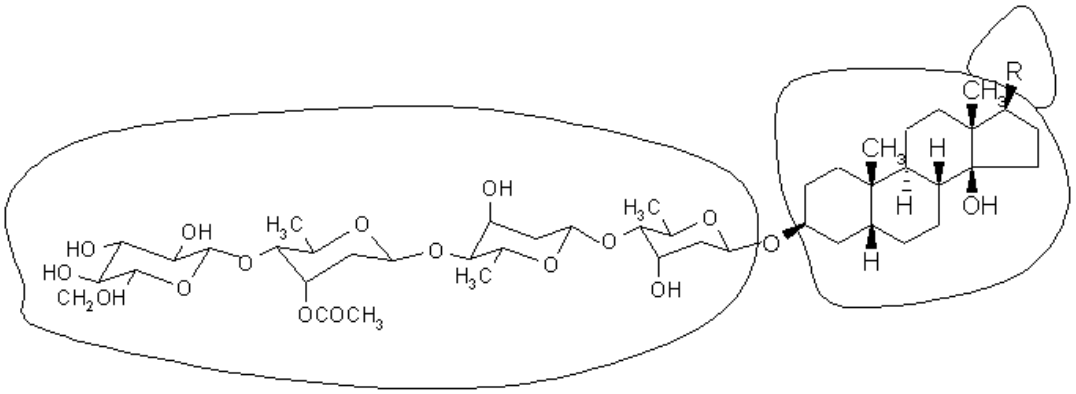
Caffein I



Ephedrine II

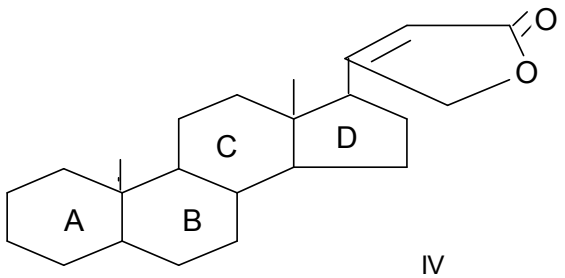
(Phenethylamine alkaloid)

2.1.3 Cardiac glycoside: The cardiac glycosides are an important class of naturally occurring drugs whose actions include both beneficial and toxic effects on the heart. Plants containing cardiac glycosides have been used as poisons and heart drugs at least since 1500 B.C. Throughout history these plants or their extracts have been variously used as arrow poisons, emetics, diuretics, and heart tonics. Cardiac glycosides are widely used in the modern treatment of congestive heart failure and for treatment of arterial fibrillation and flutter. Yet their toxicity remains a serious problem. Cardiac glycosides are composed of two structural features: the sugar (glycoside) and the non-sugar (aglycone - steroid) moieties. (Structure III).



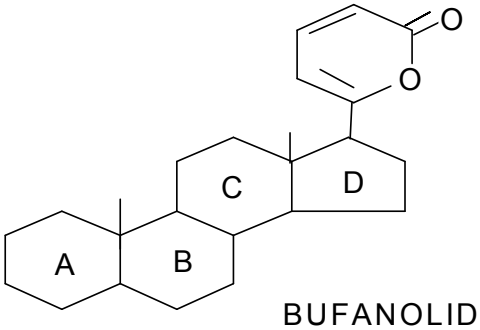
III

The R group at the 17-position defines the class of cardiac glycoside.



IV

CADENOLIDE

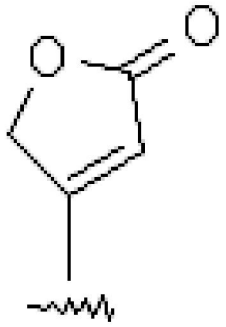


V

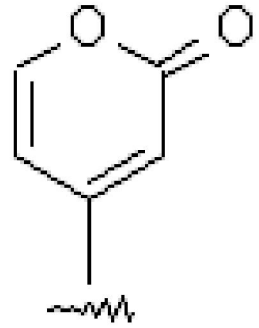
BUFANOLIDE

Two types of aglycone can be distinguished based on the presence of five or six membered ring. These are known as cardenolides (IV) of which, digitoxigenin is an example and bufanolides (V) which has scillarenin as an example. The two classes have been observed in nature - the cardenolides and the bufadienolides (VI and VII). The cardenolides have an unsaturated butyrolactone ring while the bufadienolides have α-pyrone ring.

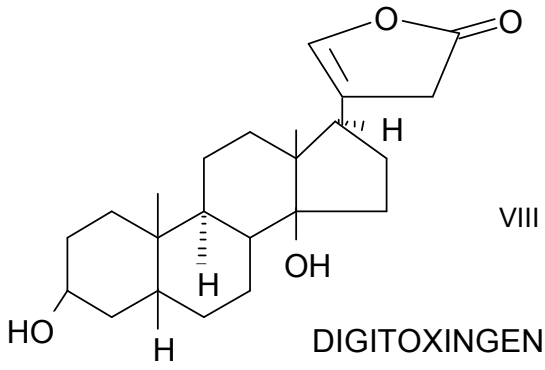
Cardiac glycosides are cardenolide group and are the most important medicinally. They have long been employed as important ingredient for arrow poisoning and drugs. They act directly on the heart muscles. They are used pharmaceutically to strengthen weakened hearts and show some important effect in some cardiac arrhythmias. The heart-arresting properties of these glycosides make them most effective as arrow poisons. Many glycosides of various digitalis species have been employed in western medicine. Digitoxigenin (VIII) and Gitoxigenin (IX) are major aglycones of *Digitalis purperea* cardio-active glycoside. Evans, (1989) reported that the bufanolides have found use as drugs.

R =  **cardenolides**

VI

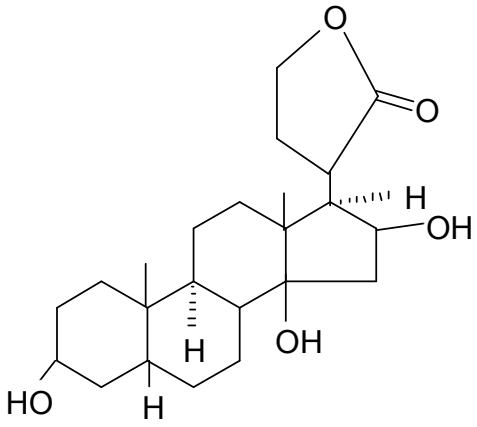
R =  **bufadienolides**

VII



VIII

DIGITOXINGENIN



IX

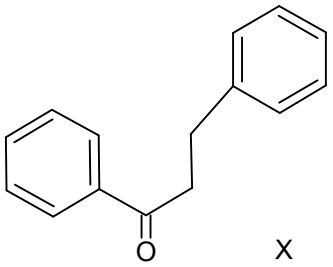
GITOXIGENIN

2.1.4 Flavonoids: Flavonoids are a group of naturally occurring polyphenolic compounds that are widely found in the plant kingdom. They occur naturally as plant pigments in a broad range of fruits and vegetables as well as beverages such as tea, red wine, coffee and beer (Joule and Smith, 1972). Many of the known four thousand different flavonoids are part of our regular diet (Middleton and Kandaswami, 1994).

Flavonoids are Carbon 15 compounds composed of two aromatic rings linked through three carbon bridges with a carbonyl functional group located at one end of the bridge. They can be subdivided into several classes of flavonoids and flavonoid related compounds such as dihydrochalcone (X), chalcone (XI), flavanones (XII), Isoflavones (XIII) and aurones (XIV) (Geissman and Crout, 1969). Structure XV is the skeleton of a large class of flavonoids known as flavone.

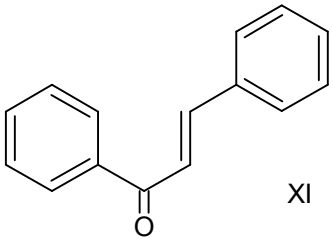
Flavonoids have gained recent interest because of their broad biological and pharmacological activities. Flavonoids have been reported to exert multiple biological effects including antimicrobial (Proestos *et al*, 2005), cytotoxicity (Yenjai *et al*, 2004), anti-inflammatory (Furuta *et al*, 2004) as well as anti- tumor activities (Xia *et al*, 2000). Hertog and co-workers (1993) showed the inverse correlation between flavonoids intake and coronary heart disease mortality. Flavonoids have attracted the interest of researchers because they showed promise of being powerful antioxidants which can protect the human body from free radicals (Melou *et al*; 2005; Rusak and Gutzeit, 2005; Zhao *et al*; 2003). Many flavonoids such as quercetin (XVI), luteolin (XVII) and cathechins (XVIII) are better antioxidants than the nutrient antioxidants such as vitamin C, vitamin E and β-carotene (Rice *et al*, 1997). The function of an antioxidant is to intercept and react with free radicals at a rate faster than the substrate. Since free radicals are able to attack at a

variety of targets including lipids, fats and proteins, it is believed that they may damage organisms, leading to disease, poisoning and including aging (Wright *et al*; 2001).



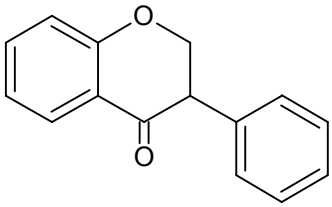
X

Dihydrochalcone



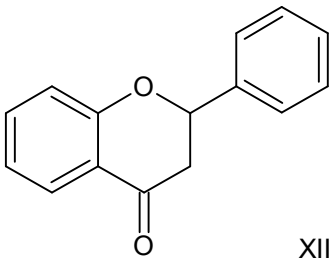
XI

Chalcone



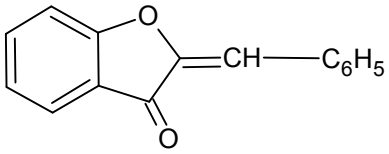
XIII

Isoflavone



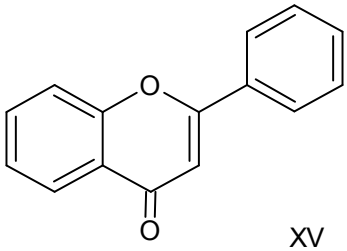
XII

Flavanones



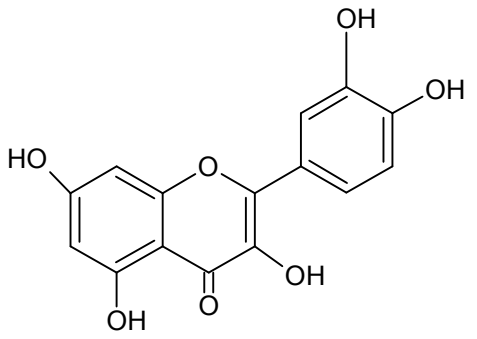
XIV

Aurones



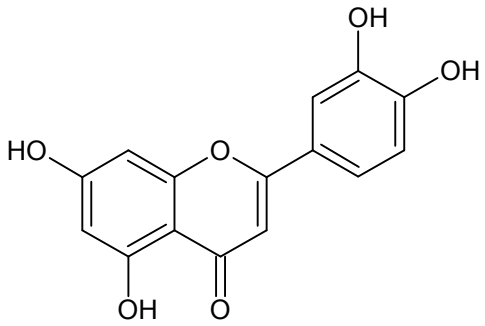
XV

Flavone

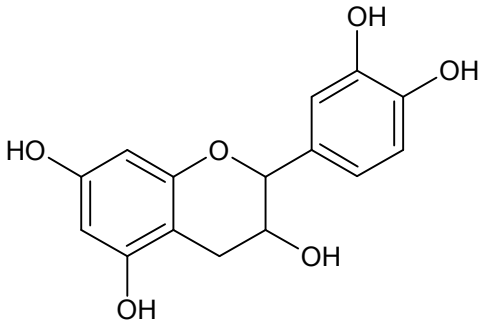


XVI

Quercetin



Luteolin XVII



Catechin XVIII

2.1.5 Saponin and steroidal sapogenin: Saponins are glycosidic compounds often referred to as "natural detergent" because of their foaming property. Saponins are mainly of the triterpenoidal type, oleanolic acid and the hedagenin being the main constituents. Saponins are glycosidic compounds composed of a steroid (C-27) or triterpenoids (C-30) saponins nucleus with one or more carbohydrate branches.

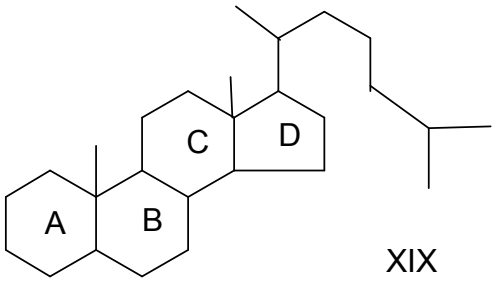
Plant materials containing saponins have long been used in many parts of the world due to their detergent property. For example, in Europe, the roots of *Saponaria officianalis* (caryophyllaceae) and also in South America the bark of *Quaillaia saponaria* (Rosaceae) have all been used. These types of plants contain high percentage of glycosides known as saponins which word is derived from the Latin word 'Sapo' meaning soap. They are characterized by their property of producing a frothing in aqueous solution which is characteristic test for saponins. Also, they have heamolytic properties hence when injected into the blood stream they are highly toxic.

As glycosides they are hydrolyzed by mineral acids to give an aglycone saprogenic and various sugars and related uronic acid. According to the structure of the aglycone or sapogenin, two kinds of sapogenins are observed viz;

i. Steroidal tetracyclic triterpenoids (XIX)

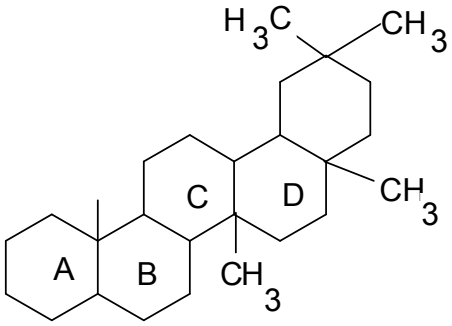
ii. Pentacyclic triterpenoids (XX)

iii. Steroidal saponins are of great importance and of interest due to their relationship with compounds such as the sex hormones, cortisone, diuretic steroid, vitamin D and cardiac glycosides. Some are used as starting materials for the syntheses of certain compounds. Steroidal saponins are less widely distributed in nature.



XIX

STEROIDAL SKELETON

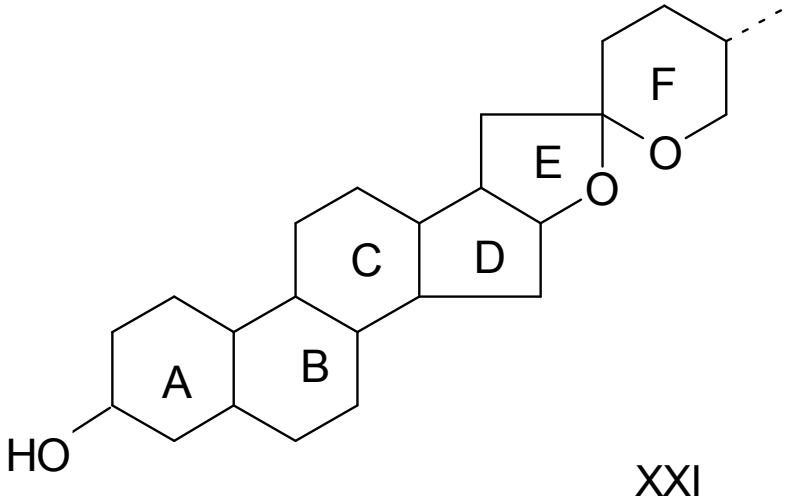


XX

PENTACYCLIC TRITERPENOID

Phytochemical survey has shown that they are present in plant families such as Dioscoreacea (Dioscorea species), Amaryllidacea (Agava species), Leguminosae

(Fenugreek). Solanacea (Solanum), etc. Diosqenin (XXI) is the major sapogenin which is isolated from tubers of many Dioscorea species (Amako, 1999).



XXI

DIOSGENIN

2.1.6 Tannins: Tannins are a group of plant polyphenols which possess unique properties, such as precipitating proteins and heavy metals from solutions. It is also an old term denoting substances present in plant extracts that were able to combine with protein of animal hides, prevent their putrefaction, and convert them into leather. This definition excluded simpler phenolic substance present with tannins such as gallic acid (XXII), catechin (XIII), chlorogenic, and chlorogenic acid etc (Karamali and Teunis; 2001).

These may under certain condition give precipitates with gelatin and be partly retained by hide powder. This class of compounds is usually referred to as "pseudotannins". Two groups of tannins are usually recognized which are:

2.1.6.1 Hydrolysable tannins: Acid or enzymes hydrolyze these and their solutions turn blue with iron salt. This consists of two main types. Gallitannins and elagic acid, example are glucogalin (XXIV), elagic acid (XXV), hexahydroxy diphenoic acid (XXVI) etc (Karamali and Teunis; 2001).

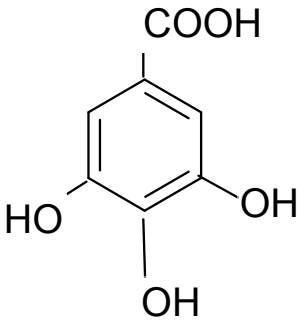
Hydrolysable tannins are of pharmacological interest because of antiviral and antitoxic properties.

2.1.6.2 Non-Hydrolysable or Condensed Tannins: These include all the true tannins and they are more resistant to hydrolysis. They are related to flavonoid pigment and their solutions turn green with iron chloride. They have been used in medicine to aid the healing of wounds and burns. They are also thought to have some protective properties against toxins when taken internally while in ruminants they minimize the effect of bloat. Example of this is catechin (XXIII).

Although recent biochemical research revealed the pharmacological importance of tannins contained in crude drugs and food, the relationship between structure and function of tannins has not yet been understood. Tannins are distributed all over the plant kingdom. They are commonly found in both gymnosperms as well as angiosperms. In terms of location of the tannins in a plant, they are mainly located in the vacuoles or surface wax of

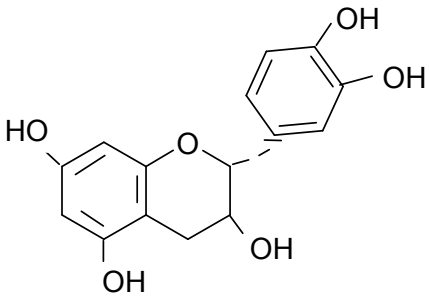
the plants (Simon Mole; 1993)). These sites are where tannins do not interfere with plant metabolism and it is only after cell breakdown and death that the tannins are active in metabolic effects. Tannins are found in leaf tissues, bud tissues, seed tissues, root tissues and stem tissues. An example of the location of the tannins in the stem tissue is that they are often found in the growth areas of trees, such as the secondary phloem and xylem and the layer between the cortex and epidermis. Tannins may help regulate the growth of tissues. They are also found in the heartwood of conifers and may play a role in inhibiting microbial activity, thus resulting in the natural durability of the wood (Branham *et al*; 1989). However, there may be a loss in the bioavailability of tannins in plants due to birds, pests, and other pathogens (Kadam *et al;* 1990).

During World War II, liver damage was seen in humans treated with tannic acid for burns. Subsequently experience in rat by Korpassy show that repeated sub-cutaneous injection of hydrolysable tannin' gallotannic acid led to hepatoxicity' cirrhosis' and liver tumors in this species. He reviewed this work extensively again (Korpassy, 1961) similarly, three condensed non-hydrolysable tannins were found to produce liver tumors and sarcomas at the site of injection following repeated sub-cutaneuos injection in mice. High oral doses of gallotannic acid in rats were claimed to result in some precirrhotic lesions in the liver, but no report has been made of the induction of tumors by orally administered tannins (James; 1973). Beverages such as coffee, tea, and claret contains small varying amount of tannins.



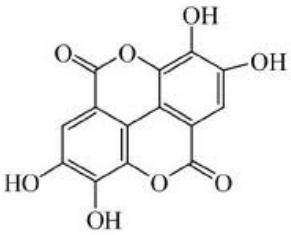
GALLIC ACID

XXII

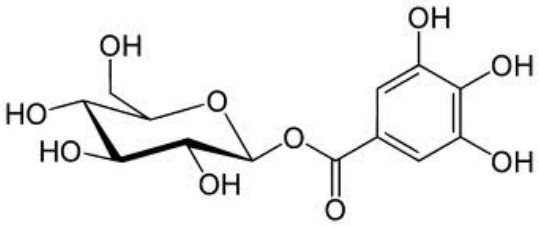


[+] — CATECHIN

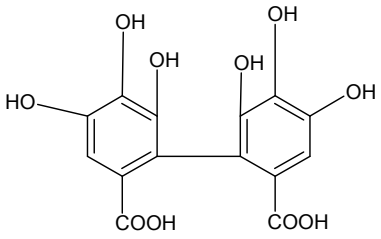
XXIII



ELLAGIC ACID XXIV



GLUCOGALLIN XXV



HEXAHYDROXYDIPHENOIC ACID XXVI

2.2 General Botanical characteristic of *Rubiaceae*

Rubiaceae is a family of flowering plants, variously called the madder family, bedstraw family or coffee family. The family got its name from the word madder genus *Rubia*. Some other plants, such as the *naucleaceae, cinchona, ixora* and *theligonaceae* have been included in the family. There are about 630 genera and over 13000 species. The Rubiaceae is one of the six most abundant angiosperm families including *Asteraceae, Ochidaceae, Fabaceae, Poaceae* and *Euphorbiaceae* in terms of number of genera and species. The *Rubiaceae* species can easily be found in warm and tropical climates around the world (Dalziel, 1957). Members of this family are usually shrubs while some are trees or herbs. A large number of this family grows in sub-Saharan Africa.

2.2.1 The plant *Sarcocephalus latifolius* (Smith Bruce)

Family: *Rubiaceae*

Genus: *Sarcocephalus*

Specie*: latifolius*

Hausa*: Tafashiya, Tuwon biri*

Igbo: *Ubuluinu*

Synonym

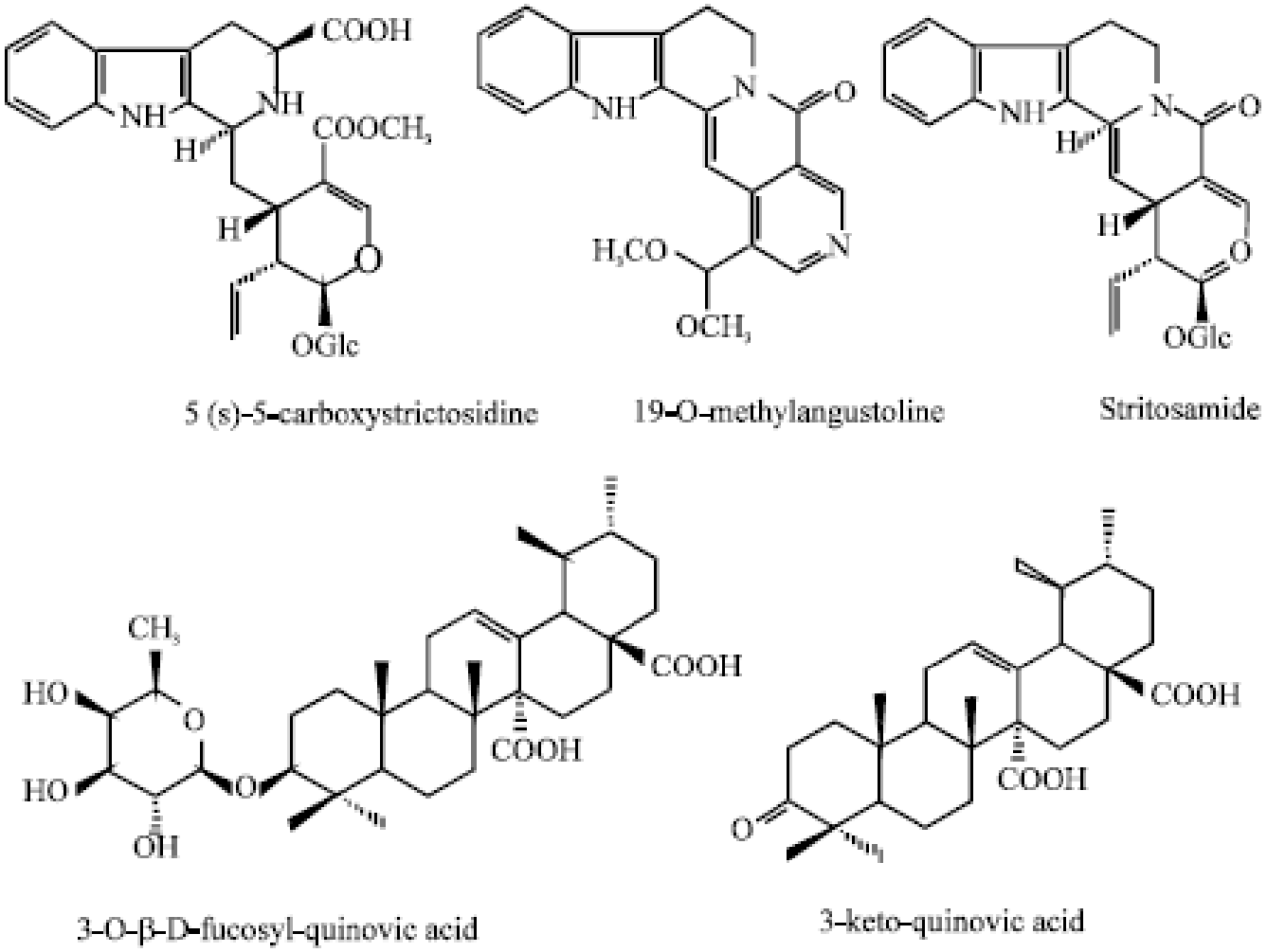
Nauclea latifolia Sm

Sarcocephalus latifolius is of the Rubiaceae family, of genus *Sarcocephalus* and species *latifolius*. The Hausas call it *Tafashiya* or *Tuwon biri*, Igbo's call it *Ubuluinu. Sarcocephalus* is a genus of tropical evergreen trees and shrubs in the family Rubiaceae

(Appendix I). *Sarcocephalus latifolius* (Smith Bruce) is one of the members and often used by traditional healers in Sierra Leone and neighboring countries as tonic and fever medicine, chewing stick for dental health, toothaches, dental cures, septic mouth, malaria, diarrhea and in the treatment of dysentery (Etkin *et al;* 1990 and Lamidi *et al;* 1995). The leaves are used in the treatment of fever, while the roots and bark are used for the treatment of venereal disease, wounds and as odontalgic remedy (Pedro and Antonio, 1998). It is a shrub or small spreading tree that is widely distributed in the Savanna and commonly found in tropical Africa.

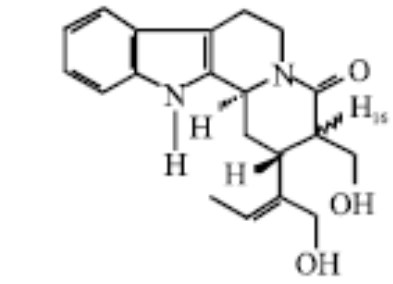
2.2.2 Chemical constituent of *Sarcocephalus species*

A wide range of monoterpene indole alkaloids including Nauclefine 1 and 2 have been reported to be isolated from Nauclea species. Strictosamide, carboxystrictosidine, methylaugustoline and 3-keto-quinovic acid were the major alkaloids from *Nauclea pobequinii* (XXVII).*Nauclea latifolia* contains several phytochemicals like alkaloids, flavonoids, steroids and glycosides. Several alkaloids like naucleafoline, nauclechine and naufoline have been isolated from the leaves while. naucletine, nauclefine, naucledidinal, epinaucleidinal and augustine (Hotellier *et al*., 1975, 1979) have also been isolated. Naucleidal and epinaucleidal have been isolated from an antiviral preparation produced by roasting *Nauclea latifolia* fruits (Morah, 1994). Again five monoterpene indole alkaloids, naucleamides A to E (XXVIII) were isolated from the bark and wood of the plant. Naucleamide E was said to be the unique monoterpene indole alkaloid possessing a pentacyclic ring system with an amino acetal bridge (Shigemori *et al*., 2003)

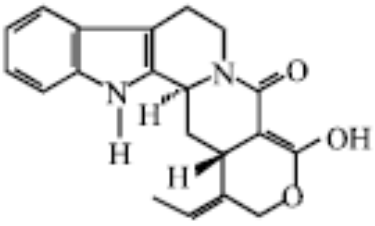


Structure XXVII: Chemical structure of some compounds isolated from *Nauclea pobequinii*

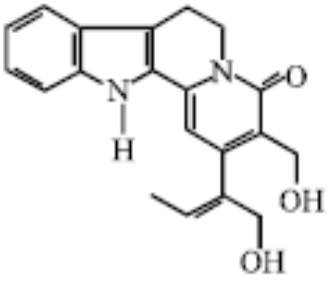
Structure XXVIII: Chemical structure of some compounds isolated from *Nauclea latifolia*



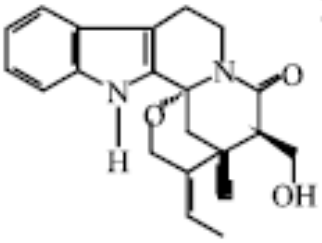
Nauleamide A: H16 = α
Naucleamide B: ZH16 = β



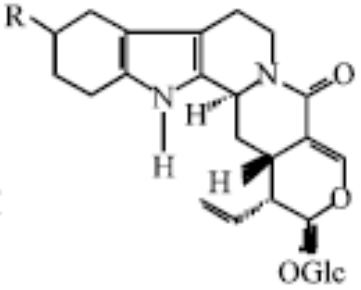
Naucleamide C



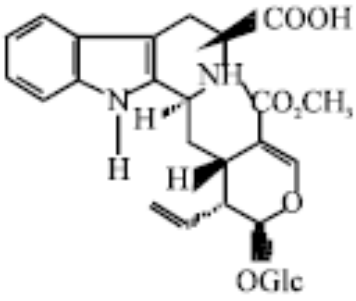
Naucleamide D



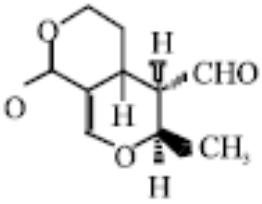
Naucleamide E



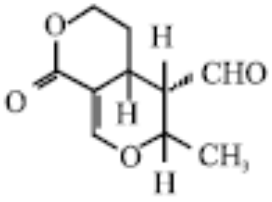
Strictosamide: R = H
10- hydroxystritosamide: R = OH



Tetrahydeodesoxycoridfoline



Naucledial



Epinaucleidal

2.3 General Botanical characteristics of Asteraceae

Asteraceae or Compositae are commonly referred to as the aster, daisy, or sunflower family. They are exceedingly large and widespread family of Angiospermae (Stevens, 2001 and Jeffrey, 2007). The group has more than 23.000 currently accepted species

which spread across 1620 genera and 12 subfamilies. Most members of Asteraceae are herbaceous, but a significant number are also shrubs, vines and trees. The family has a worldwide distribution, and mostly common in the arid and semi-arid regions of subtropical and lower temperate latitudes. (Barkely *et al*; 2006). They are mostly herbaceous plants, but some are shrubs, trees and climbers. They are generally easy to distinguish from other plants, mainly because of their characteristic inflorescence and other shared characteristics (Judd *et al*; 2007).

Asteraceae is an economically important plant family. Some members provide products including cooking oils, lettuce, sunflower seeds, artichokes, sweetening agents, coffee substitutes and teas. Several genera are popular with the horticultural community, including marigold, pot marigold (calendula), cone flowers, various daisies, *fleabane erigeron, chrysanthemums, dahlias, zinnias*, and *heleniums*. A number of species have become weeds, including the most famous dandelion *Taraxacum officinale* in North America,

This family generally store energy in the form of inulin. They produce iso/chlorogenic acid, sesquiterpene lactones, pentacyclic triterpene alcohols, various alkaloids, acetylenes (cyclic, aromatic, with vinyl end groups), tannins. They have terpenoid essential oils which never contain iridoids (Stevens, 2001). Plants in Asteraceae are medicinally important in areas that don't have access to Western medicine. They are also commonly featured in medical and phytochemical journals because the sesquiterpene lactone compounds contained within them are an important cause of allergic contact dermatitis. Allergy to these compounds is the leading cause of allergic contact dermatitis in florists in the US (Odom *et al*; 2000).

2.3.1 The plant *Spillanthes acmella* (Murr)

Family: Asteraceae

Genus: *Spillanthes*

Specie*: Acmella Murr*

Hausa: Parpehi

Igbo: Ilulonoica

Yoruba: Awere pepe

Synonym: *Spillanthes filicaulis*

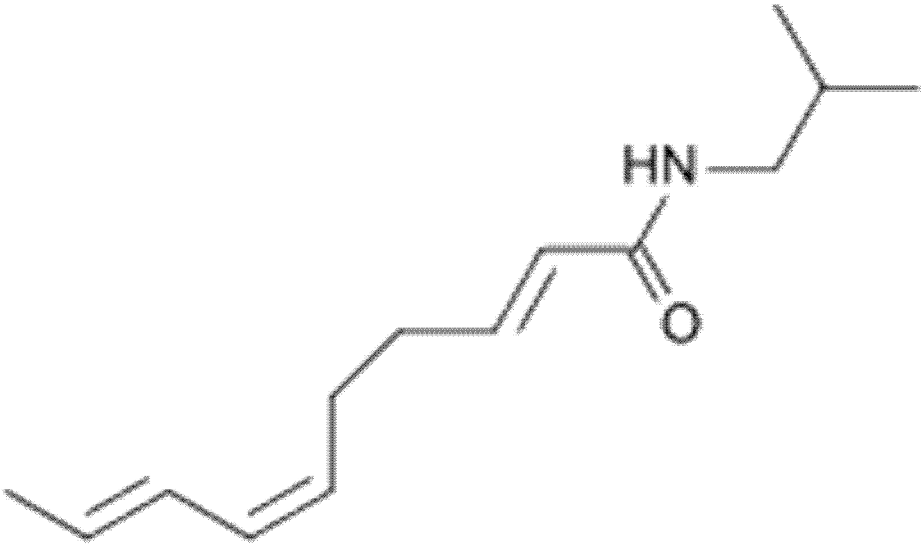
Spilanthes acmella Murr is an indigenous herb growing as an annual throughout the tropics (Appendix II). The whole plant is claimed to possess medicinal properties. The flowers are chewed to relieve toothache and the crushed plant is used in rheumatism (Balian *et al*; 2006, Kirtikar and Basu, 1935). The plant is generally known as tooth ache plant (Sinha, 1996). The fresh and cooked leaves are combined with chilies and garlic to add flavour and vitamins to foods (Miyazawa *et al*; 2006). The leaves, along with alum, are recommended as emetic (Gill, 1992). Chakraborty *et al*; (2004) evaluated the anti-inflammatory and analgesic activity of the aqueous extract of s*pillanthes acmella*.

The most important taste-active molecules present are fatty acid amides (Structures XXIX, XXX and XXXI) such as spilanthol, which is responsible for the trigeminal and saliva-inducing effects of products such as *Jambu oleoresin*, a concentrated extract from paracress (Ramsewak *et al*; 1999, Gokhale and Bhide, 1945). Its properties provide relief to dry mouth by enhancing saliva production. Spilanthol proved effective at killing mosquitoes, with an LD_{100} (at 24 hours) at a concentration of 12.5 µg/ml and showed 50% mortality at 6.25 µg/ml. The mixture of isomers of spilanthol showed a 66% weight

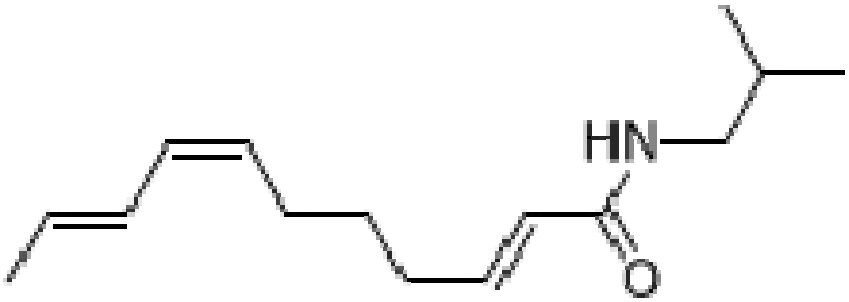
reduction of *H. zea* neonate larvae at 250 µg/mL concentration after 6 days. (Ramsewak *et al*; 1999).

Besides the main active ingredient spilanthol, *Acmella* also contains stigmasteryl-3-*O*-β-D-glucopyranoside and a mixture of triterpenes. The isolation and total synthesis of the active ingredients have been reported. (Ley *et al*; 2006)

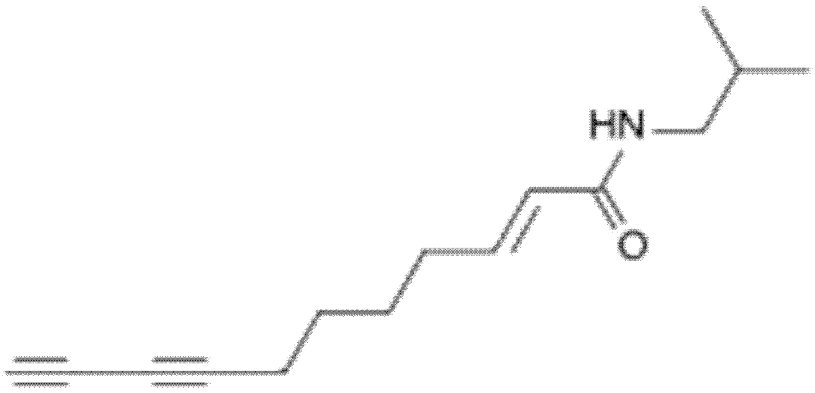
Ramsewak *et al*, 1999 equally reported the presence of flavanoids in the aqueous extract which was responsible for the significant anti-inflammatory and analgesic property of the plant. The extract produced dose dependent and significant inhibition of prostaglandins which involved in the late phase of acute inflammation and pain perception (Usher, 1984). Detailed studies using the extract may reveal the exact mechanism of action of the flavanoids responsible for the anti-inflammatory and analgesic activity. *Spillanthes acmella* extract has been tested against various yeasts and bacteria and was essentially inactive. (Holetz *et al*; 2002). *Spillanthes acmella* has been shown to have a strong diuretic action in rats. (Ratnasooriya *et al*; 2004). This observation and the regular uses of this plant by the natives prompt this research if there could be any other metabolite that could be of any pharmacological importance.



XXIX: Spilanthol, (2*E*, 6Z, 8E)-deca-2, 6, 8-trienoic acid isobutyl amide



XXX: (2E, 7Z, 9E)-Undeca-2, 7, 9-trienoic acid isobutyl amide, another alkyl amide from *Acmella oleracea*



XXXI: (2*E*)-Undeca-2-en-8, 10-diynoic acid isobutyl amide

CHAPTER THREE

3.0 Materials and method

3.1 Chemicals /Materials

3.1.2 Materials for Phytochemical Analysis:

(i) 10% Ammonium solution

(ii) methanol

(iii) chloroform

(iv) resorcinol crystal

(v) phloroglucinol

(vi) 5% KOH and 20% KOH

(vii) Phenyl hydrazine

(viii) 95% Ethanol

(ix) Lead acetate solution

(x) 1% 3, 5- dinitrobenzoic acid

(xi) Magnesium turning.

(xii) 10% Hydrochloric Acid

(xiii) 20% Sodium hydroxide solution

(xiv) 1% Ferric chloride solution

(xv) Dilute and concentrated tetraoxosulphate vi Acid

(xvi) 10% sodium Nitrate

(xvii) Chloroform

(xviii) Pyridine

(xix) Acetic anhydride

(xx) Concentrated Nitric acid

(xxi) Glacial acetic acid

(xxii) 1% Picric acid solution

(xxiii) 10% Tannic acid solution

3.1.3 Reagent Used:

3.1.4 Reagents for phytochemical Screening

3.1.4.1 Ammoniacal Silver Nitrate Solution*:* Silver nitrate (3.0g) was dissolved in 50ml of distilled water and ammonia solution was added dropwise until the initial precipitate of silver oxide was dissolve. This resulting solution was made up to 100ml in a volumetric flask.

3.1.4.2 Molish's Reagent: (10% solution of β- naphthol in alcohol).

3.1.4.3 Wagner's Reagent: Iodine (2.0g) and potassium iodide (3.0g) were dissolved in distilled water and sufficient amount of water was added and made up to 100ml mark.

3.1.4.4 Dragendorff's Reagent: Bismuth nitrate (0.85g) was dissolve in 10ml of acetic acid and 40ml distilled water was added to give a stock solution (A). Potassium iodide (8.0g) was equally dissolved in 20ml of distilled water to give a stock solution (B). 5ml of each of the stock solution (A) and (B) were mixed with 20ml of 33% acetic acid followed by 100ml distilled water to give the Dragendorff's reagent.

3.1.4.5 Meyers Reagent: Mercuric chloride (1.36g) was added into 60ml of distilled water to give solution A. Potassium iodide (5.0g) to give solution B. Both solution A and B were mixed together and made up to 100ml mark.

3.1.4.6 Fehling Solution: Solution A was prepared by dissolving copper sulphate (7.0g) and 0.1ml of tetraoxosulphate (vi) acid with sufficient distilled water and made up to 100ml mark. Solution B was equally prepared by dissolving sodium potassium tartrate (35.2g) and sodium hydroxide (15.4g) in sufficient distilled water and made up to 100ml mark. Equal volumes of solution (A) and (B) were mixed immediately before use.

3.1.4.7 Barfoeds reagent: Crystallized neutral copper acetate (13.3g) was dissolved in 200ml of 1% acetic acid solution.

3.1.4.8 Iodine solution: Iodine crystal (2.5g) was added to potassium iodide (KI) (5.0g) and 25ml of distilled water was added to the mixture to give iodine solution.

3.1.5 Solvents for Extraction:

(i) Petroleum ether 60-80^{o}C (Aldrich)

(ii) Chloroform (Aldrich)

(iii) Ethyl Acetate (Aldrich)

(iv) Methanol (Aldrich)

3.1.6 Purification of Solvent for Extraction: All solvents used were distilled by simple distillation.

3.1.7 Materials for TLC:

(i) Chromatographic Tank

(ii) 10 (20 x 20 cm) glass plates

(iii) Gallenkamp Spreader

(iv) Ultraviolet lamp (254 and 366mm)

(v) Silica gel (Park Scientific Limited, Northampton,UK)

3.1.8 Material for Spectroscopic analysis

(i). Bruker-Advance 400 MHz FT-NMR Spectrometer

(ii). Perkin Elmer Spectrum 100 FTIR Spectrometer

(v) UV-Visible Lamp

(iv) Agilent Technologies 1200 Series Binary SL (LC-MS)

3.1.9 Materials for Antimicrobial Test:

(i) Microbiological media (nutrient broth): Mueller Hinton agar.

Test Organism:

Methycilin Resistant Staphylococcus Aureus (MRSA)

Staphylococcus aureus

Streptococcus pyrogenes

Bacillus subtilis

Corynebacterium ulcerans

Escherichia coli

Proteus mirabilis

Proteus vulgaris

Pseudomonas aureginosa

Salmonella typhi

Shigellia dysenteric

Candida albicans

Candida virusei,

Candida tropicalis,

(ii) Petri-dishes

(iii) Sterile pipette

(iv) 6mm cork borer

(v) Incubator

(vi) Autoclave

(vii) Dimethylsulphoxide (DMSO) 10%

3.2 Plant Materials:

The aerial part of *Spillanthes acmella,* root and stem bark of *Sarcocephalus latifolius* were separately collected from Okene in Okene local Government Area of Kogi state, Nigeria in March 2010. These were identified at the Herbarium of the Faculty of Sciences, Ahmadu Bello University, Zaria, Nigeria. The voucher numbers of the specimens were 534 and 1268 and were deposited at the Herbarium. The samples were air-dried and pulverized

using wooden pestle and mortar. Finally, they were stored separately in air-tight polythene bags and kept away from moisture until needed for analysis.

3.3 Extraction procedure

The pulverized plant material of *Spillanthes acmella* (212.60g) and (221.80g) of *Sarcocephalus latifolius* stem bark were weighed respectively and extracted separately and exhaustively with 2 litres of various solvents for 72hrs in a soxhlet extractor. Extraction was with petroleum ether (60-80^{o}C), followed by chloroform, ethyl acetate and methanol respectively. Various extracts were concentrated at 40^{o}C using the rotary evaporator.

3.4 Phytochemical analysis

The pulverized root, stem bark of *Sarcocephalus latifolius* and aerial plant parts of *Spillanthes acmella* were screened for plant metabolites. Standard techniques of Brain and Turner, (1975) were employed in the phytochemical screening. The presence or absence of typical metabolites such as carbohydrates, glycosides, anthraquinones, cardiac glycoside, saponins, flavonoids tannins, saponins and alkaloids were determined.

3.4.1 Test for Carbohydrates:

3.4.1.1 Molish's Test: (Standard Test for Carbohydrate).

About 1.0 g of the plant material was extracted with distilled water by heating in a water-bath. It was then filtered. To the filtrate was added few drops of molish's reagent carefully and a few volume of concentrated tetraoxosuphate (VI) acid was added from the side of the test-tube to form a lower layer.

Reddish coloured ring was observed at the interphase. This showed that carbohydrate was present in the various plant materials.

3.4.1.2 Test for Sugar (Barfoeds Test)

To 1ml of aqueous solutions of the plants samples in a separate test-tube, 1ml of Barfoed reagent was added and heated in a water-bath. A red precipitate was observed in the plant parts of *Sarcocephalus latifolious* and *Spillanthes acmella* samples showing the presence of Monosacharides.

3.4.1.3 Test for Free Reducing Sugar (Fehling's Test)

2ml of aqueous solution of the plant samples was shaken separately in a test tube and 5ml of equal mixture of fehling solution I and II (heated) was added stepwise and boiled for two minutes. Green precipitate was formed showing the presence of free reducing sugar in all the samples.

3.4.1.4 Test for Ketoses (Selivanoff's Test)

Few crystals of resorcinol was equally added to 2ml of aqueous solutions of the plant samples and the crystals dissolved resulting to a faint yellow solution which indicated the presence of ketoses in the samples. Also, 2ml of Conc. HCl was added and the solution heated in a water bath. Orange colour was equally observed showing the presence of ketoses sugar.

3.4.1.5 Test for Pentoses:

2ml of plant samples solution in a test tube and 2ml of Conc. HCl containing phloroglucinol was added and heated. A red colour was formed in all the sample solution showing the presence of pentose sugar.

3.4.1.6 Test for Starches:

(i) Fehling Test:

2ml aqueous solutions of the plant samples in a test-tube were taken and 5ml of equal mixture of fehling solution I and II was added.

The solutions was then boiled and hydrolysed. Blue colour was initially observed and after hydrolysis a brick-red precipitate was formed showing the presence of starch in plant samples.

Test for Soluble Starch:

5ml of 5% K0H was added to 5ml of aqueous solutions of the plant samples in a test-tube separately. This was then shaken and heated in a boiling water bath. Yellow colour indicating the presence of soluble starch was observed.

3.4.1.7 Test for Osazones:

Phenylhydrazine in acetic acid was added to about 2ml of water solution of the crude plant sample and shaken. Fine yellow needle like crystals were observed showing the presence of osazone in plant samples.

3.4.2 General Test for Tannins (Plant Phenol):

Plant material (5.0g) was added 10ml of distilled water and shaken. This was filtered and to the filtrated was added a solution of ferric chloride ($FeCl_3$). A blue – black precipitate was observed.

3.4.3 Test for Cardiac Glycosides:

3.4.3.1 Extraction of the Glycoside from Plant Material:

Powdered plant material (2.0g) was placed in a test-tube and 10% ethanol in water was added. This was placed on a water-bath and heated for 3 minutes. The solution was cooled to room temperature and filtered. The filtrate was then evaporated in an evaporating dish on water-bath and was allowed to cool to room temperature. Petroleum ether (5ml) was added to the dish and stirred for a minute. The petroleum ether portion was discarded to remove the chlorophyll, pigments and fats. Petroleum ether (5ml) was then added again and stirred thoroughly for 5 minutes. This was decanted into a test-tube and anhydrous sodium sulphate (5mg) was added. This was shaken gently and filtered into a clean dry test-tube which was later divided into three portions and labeled I, II, III. The Lieberman-Burchard and Salkowski test were carried out on the test tubes labeled I and II while the test tube III served as a control. The extraction and the tests were carried out on all the samples as follows:

3.4.3.2 The Lieberman-Burchard Test: Test for unsaturated sterol and triterpenoids):

Three drops of acetic anhydride were added gently to test tube I containing the sample mixture. Also, one drop of concentrated tetraoxosulphate VI acid was added and mixed gently. A brownish-yellow colouration was observed which finally changed to green.

3.4.3.3 The Salkowski Test (Ring Test):

The second test-tube (II) was held at an angle 45^{o} and 3 drops of concentrated tetraoxosulphate (VI) acid was added from the side of the test-tube. A reddished-brown colour was observed at the interphase.

3.4.3.4 Keller-Killiani Test for Cardiac Glycosides:

Plant material (0.5g) was dissolved in 2ml of glacial acetic acid containing one drop of ferric chloride ($FeCl_3$) solution. This was then treated with 1ml of concentrated tetraoxosulphate (VI) acid.

A brown ring was obtained at the interphase between glacial acetic acid layer and the tetraoxosulphate (VI) acid layer. A violet ring appeared below the brown ring.

3.4.3.5 Legal Test for Cardenolides:

Plant material (1g) was mixed with pyridine in a fume cupboard. This was filtered and to the filtrate was added few drops of 2% sodium nitroprusside together with a few drops of 2% sodium hydroxide solution. A red colour, which faded to brownish yellow, was observed.

3.4.4 Froth test for Saponins:

Powdered plant material (1g) was shaken with 10ml of distilled water warmed and allowed to stand for 30 minutes. Honeycomb was observed which persisted after 30 minutes.

3.4.5 GENERAL CHEMICAL TEST FOR ALKALOIDS:

3.4.5.1 Extraction of the Alkaloid from the Plant Material:

Powdered plant material (1g) was placed in a small beaker and four drops of ammonium solution was added and mixed thoroughly. This was allowed to stand for 10 minutes so that the liquid can penetrate the material. Sufficient quantity of a mixture of chloroform and ethanol (ratio 1:1) was added just to soak and suspend the powder. This was allowed to stand for another 20 minutes with occasional stirring with a glass rod. The mixture was filtered through a plug of cotton wool. The filtrate was evaporated to dryness using water bath. It was cooled and the residue dissolved in 5ml of chloroform. Solution was then transferred to a small separatory funnel and shaken with 3ml dilute tetraoxosulphate (VI) Acid. This was allowed to separate into two layers. The chloroform layer was discarded. The acid layer in the funnel was completely made alkaline by the addition of concentrated ammonia solution and alkalinity was established by testing with indicator paper. This was shaken with 3ml chloroform and the layers were allowed to separate in a separatory funnel. The chloroform layer was collected. This process was repeated and the two chloroform extracts were combined and evaporated just to dryness. The following tests were carried out on the residue of the extracts.

The residue from above was dissolved in 3ml ethanol and divided into unequal portions of 1ml and 2ml respectively.

3.4.5.2 Test for Alkaloids in the respective Extracts from *Sarcocephalus latifolius* and *Spillanthes acmella*

The respective residues obtain from above was dissolved in 3ml ethanol and divided into two unequal portions 1ml and 2ml. The 2ml – portion was neutralized with dilute tetraoxosulphate (VI) acid. Few drops of this solution were treated with the following alkaloid reagents namely Meyer's reagent, Dragendoff's reagent, 1% picric acid solution, Wagner's reagent and 10% tannic acid solution. There was formation of precipitate from the *Sarcocephalus latifolius* residue. The test with aerial plant part of *Spillanthes acmella* residues with Wagner's reagent gave reddish-brown precipitate, Dragendoff's reagent gave blue-black precipitate; tannic acid gave orange precipitate and finally Meyer's reagent gave light yellow coloration.

3.4.5.3 Vitalis – Morin Test for Tropane Alkaloid:

The 1ml portion of the extract from above was evaporated to dryness in a porcelain basin using a hot plate. The residue was dissolved in a few drops of glacial acetic acid and evaporated to dryness and cooled. Three drops of fuming nitric acid was added to this residue making sure that the acid came in contact with the whole of the residue. This was again evaporated to dryness and cooled. The final residue was dissolved in few drops of acetone-free water and two drops of 3% potassium hydroxide solution in methanol was quickly added. A purple color was not observed.

3.4.6 Tests for Anthracene Derivatives:

3.4.6.1 Bornttrager's Test:

1ml aqueous solution of powdered plant materials were respectively (*Sarcocephalus latifolius* and *Spillanthes acmella*) boiled with 5ml of 10% hydrochloric acid for 3 minutes to hydrolyze the glycoside. The hot solution was filtered in a boiling tube and cooled. This was gently extracted with 5ml benzene. The upper benzene layer was pipetted off and the benzene solution shaken gently with half of the volume of 10% ammonium hydroxide. No colour change observed. The solution remained brownish in colour.

3.4.7 Tests for Flavonoids:

3.4.7.1 Ammoniacal Silver Nitrate Test:

Plant material (1g) was dissolved in 5ml of distilled water and few drops of ammoniacal silver nitrate solution added and the color changes before and after heating for about 5 minutes was observed. Brown colored solution with brown precipitate was observed before heating and this turned brownish black after heating.

3.4.8 Tests for Resin:

Small quantity of the plant material was dissolved in few drops of acetic anhydride and one drop of concentrated tetraoxosulphate (VI) acid was added. No colour change was observed and the solution remained brownish in colour.

3.5 ANTIMICROBIAL SCREENING

3.5.1 Media preparation

The Mueller Hinton agar was the medium used as the growth medium for the microbes. The medium was prepared according to the manufacturer's instruction. It was boiled to dissolve and was sterilized at 121^0C for 15minutes. This medium was cooled to 45^0C and was then dispensed into sterilized Petri dishes; the plates were covered and allowed to cool and solidify.

3.5.2 Preparation of Microbial Culture

The following clinical microbes used were *Methycilin Resistant Staphylococcus Aureus (MRSA), Staphylococcus aureus, Streptococcus pyrogenes, Bacillus subtilis, Corynebacterium ulcerans, Escherichia coli, Proteus mirabilis, Proteus vulgaris, Pseudomonas aureginosa, Salmonella typhi, Shigellia dysenteric, Candida albicans, Candida virusei, and Candida tropicalis*. They were all obtained from the Department of Medical Microbiology, Ahmadu Bello University Teaching Hospital, Shika, Zaria. All the microbes were checked for purity.

3.5.3 Preparation of solutions of crude plant extracts and isolated pure compounds for antimicrobial screening tests.

The extracts were made into solutions of various concentrations using distilled water. Stock solutions of the plant extracts were prepared by initially dissolving 0.4 g of crude extracts and 0.005 g of isolated pure compounds in 10ml of DMSO to obtain an initial concentration of 40 mg/ml for the crude extracts and 50 µg/ml of the isolated pure

substance respectively. From these stock solutions concentrations of 40-, 20-, 10-, 5-, and 2.5mg/ml and 50-, 25-, 12.5-, 6.25- and 3.125µg/ml for the crude extracts and pure compound were prepared respectively by serial dilution. The well diffusion method was used to determine the anti-microbial activities of the substances respectively (Bauer et al; 1966).

3.5.4 Determination of anti-microbial activities of the plant extracts using the well diffusion method.

The cork and bore diffusion method of Bauer et al; (1966) and Barry and Thornsberry, (1985) were used in the anti-microbial screening. Inoculation of the prepared plates with the organism was done using a wire loop to transfer a strand of the organism into the plate followed by cross-streaking with the same wire loop to achieve uniform spread on the plate. A control was set up alongside using pure DMS0 for each strain of organism. The plates were incubated at 37^0C for 24 h after which they were examined for zones of inhibition of growth with a transparent ruler and a pair of divider and the result recorded in millimeters.

3.5.5 Determination of Minimum Inhibitory Concentration (MIC)

The Minimum Inhibitory Concentration (MIC) was carried out on the test-organisms and were done by the nutrient broth dilution method .Mueller Hinton broth was prepared according to the manufacturer's standard of 28 g/1000 ml. In this case double strength was prepared by dissolving 28 g in 500 ml of distilled water which was swirled and mixed thoroughly by heating to allow uniform dissolution after which 5 ml of it was dispensed

into 30 sets of universal bottles and sterilized in an autoclave at 121^0C for 15 min. The agar was allowed to cool to 45^0C and each graded solution was then mixed gently with molten double strength nutrient agar in a Petri-dish and allowed to solidify for one hour.

Two-fold serial dilution of the extracts and pure compound in the sterilized broth were made to obtained the concentrations of 40-, 20-, 10-, 5-, 2.5mg/ml and 50-, 25-, 12.5-, 6.25- 3.125µg/ml respectively. The initial concentrations of the extract were obtained by dissolving 0.4g and 0.005g of each extract in 10 ml of the sterile broth. Having obtained different concentrations of the extracts and pure compound in the broth, 0.1ml of the standard inoculums of the micro-organism in the normal saline was then inoculated into the different concentrations of the extracts in the test-tubes and the test-tubes were then incubated at 37^0C for 24hours and 30^0C for 1-7days for the fungi. The lowest concentration of the extract in the test-tubes which showed no turbidity was recorded as the minimum inhibitory concentration (MIC).

3.5.6 Determination of Minimum Bactericidal Concentration (MBC) and Minimum Fungicidal Concentration (MFC) of the crude extract

The minimum bactericidal and fungicidal concentrations of the extracts was carried out in order to determined whether the test micro-organisms were killed or only their growths were inhibited. Mueller Hinton agar was prepared according to manufacturer's instructions and sterilized at 121^0C for 15 minutes and poured into sterile Petri dishes to cool and solidify. The content of each MIC recorded was sub-cultured onto the prepared medium. Incubation was made at 37^0C for 24hours after which the plates were observed for colony

growth. The MBC/MFC was taken as the plate with the lowest concentration of the extract or pure compound without colony growth.

3.5.7 Chromatographic separation of crude extracts.

3.5.8 The vacuum liquid chromatography (VLC)

This technique was basically a preparative layer chromatographic (PLC) separation that was ran as a column. The solvent flow was aided by means of a vacuum with a stepwise gradient elution in which the polarity of the system was increased gradually during the elution by increasing the proportion of the more polar solvent. The column was monitored by collecting 20 ml each and spotting on silica Thin Layer Chromatographic paper; this was developed and viewed for similarity. Similar fractions were pooled together for further purification. The column was always allowed to run dry after which each fraction was collected.

The set-up comprises of a sintered glass funnel of an appropriate size that was filled with approximately 6–7 cm of loose silica and tapped to give a level surface. The setup is as shown in appendix iii. Vacuum was applied at the base and the surface was pressed firmly to give a completely level, well compacted bed of approximately 4.5–5.5 cm high. The column was checked for voids and channels by pouring petroleum ether onto the silica bed column while applying suction. The column was seen to be packed properly, the solvent descended in a uniform horizontal line.

3.5.9 Isolation of EFSB from the methanol fraction,

The methanol fraction was column chromatographed under vacuum and eluted with Pet Ether and ethyl acetate respectively based on the preliminary TLC carried out. The

fractions collected were pooled together based on similarities to yield four fractions viz; ETCI, ETC2, ETC3 and ETC4 respectively. The ETC1 was further cleaned up using VLC to obtain 30 different fractions coded ETCC1 to ETCC30.

From the TLC result it was found that fractions ETCC 6, 7, 8 and 9 had the same R_f value of 0.9 using TLC and the solvent mixture Pet. Ether: Ethyl acetate (1:9) and they were pooled together to yield sample **EFSB.**

3.6.0 Isolation of SB-C1 and SB-C2 from methanol fraction

Methanol bark extract (3.0 g) of the bark of *Sarcocephalus latifolius* was dissolved in methanol and filtered to eliminate any undissolved lump. This was pre absorbed in sufficient celite. This was packed with 30 g silica gel and subjected to vacuum liquid chromatography (VLC) using an isocratic solvent of petroleum ether: ethyl acetate (1:9). By collecting 20 ml each forty five different fractions were collected. Based on their thin layer chromatographic (TLC) analysis, fractions 6, 7, 8 and 9 were combined to give SB-C which was later subjected to VLC and eluted isocratically using petroleum ether: ethyl acetate (1:9) to obtain eight fractions coded SB-C-1 to SB-C-8. Fractions 5 and 6 showed prominent single yellow and blue spots which were further cleaned using PTLC to obtain **SB-C1** and **SB-C2** respectively.

3.6.1 Isolation of SPEC-1 from the Petroleum ether fraction

The methanol crude extracts was later subjected to solvent partitioning using petroleum-ether, chloroform, ethyl acetate respectively to obtain various crude fractions. The petroleum ether fraction was subjected to vacuum liquid chromatography using petroleum

ether and ethyl acetate for the gradient elution. Various fractions (SPE1 to SPE54) were obtained and in each case 25 ml of eluent was collected.

All the fractions obtained were examined for purity using thin layer chromatographic technique and the solvent mixture used was petroleum ether /ethyl acetate in the ratio 1:9. From this similar fractions were combined to give SPEC. This was further purified using by preparatory thin layer chromatography (PTLC) with petroleum ether: ethyl acetate (1:9) to obtain SPEC-1 which was a white crystalline substance.

The isolated compounds; EFSB, SB-C1, SB-C2 and SPEC-1 were check for purity under the UV- light. These shows single spot confirming their purity. These were subsequently subjected to spectroscopic analyses using the Infrared, 1D and 2D NMR spectroscopy.

CHAPTER FOUR

4.0 Results and Discussion

4.1 Extraction

Continuous extraction method was used to extract the various plant parts used in this work. The various weights of the plants' parts extracted and the accompanying extractive values are as recorded in Tables 4.1 and 4.2 respectively. Methanol extract of stem bark of *Sarcocephalus latifolius* gave the highest extractive and percentage value followed by chloroform, petroleum ether and ethyl acetate. While it was highest for the methanol extract of *Spillanthes acmella* followed by the chloroform, petroleum ether and finally ethyl acetate fraction

4.2 Phytochemical Screening

Phytochemical screening of the root and stem bark of *Sarcocephalus latifolius* (Smith Bruce) and aerial part of *Spillanthes acmella (*Murr*)* were carried out as earlier recorded and the following phytochemicals were identified. For the root and stem bark of *Sarcocephalus latifolius* revealed the presence of carbohydrates, glycosides (root) anthraquinones, cardiac glycoside,saponnins,steroid and triterpenes, flavonoids in the root, tannins (stem-bark) and alkaloids respectively as shown in Table 4.3. Likewise the aerial parts of *Spillanthes acmella* revealed the presence of carbohydrates, glycosides, anthraquinones, cardiac glycosides, saponins, steroids, triterpenes, saponins, flavonoids and alkaloids as recorded in Table 4.4.

From the phytochemical identification of carbohydrate carried out using the Molish's test gave positive identification of carbohydrate as been present in the plant materials (Table 4.5). Further tests showed the presence of combined/reducing sugar.

An aqueous extract of the plant material gave a positive response to the 1% ferric chloride ($FeCl_3$) test. This was an indicative of the presence of tannins. Generally, condensed tannins would give this result.

Cardiac glycoside is an important plant metabolite because of its remarkable uses therapeutically to strengthen a weakened heart (Table 4.6). Lieberman-Burchardt test showed a positive response to the plant material. A brownish-yellow colour which finally changed to green was observed. This revealed the presence of steroidal Nucleus as the aglycone in the cardiac glycoside. However, the plant materials were positive to Salkowski test by the formation of reddish – brown at the interphase hence showed the presence of steroidal ring as the aglycone portion of the cardiac glycoside. This supported the result obtained from the Liberman-Burchardt test.

Both the Keller-Killiani and legal test were both positive. The former showed the presence of deoxy sugar, which was a characteristic of cardenolide while the later was an indicative of the presence of cardenolide. In general, the plant material contained cardiac glycoside as depicted by Table 4-3 below.

The solutions of aqueous extract produced a honeycomb which persists for 10-30 minutes. This was an evidence of the presence of saponins in the plant materials.

Preliminary identification for the presence of alkaloids was evaluated using the following: Meyer reagent, Dragendoff's reagent, 1% picric acid solution, Wagner's reagent and 10% tannic acid solution (Table 4.7). There was formation of precipitate from the

Sarcocephalus latifolius residue. The test with aerial plant part of *Spillanthes acmella* residues with Wagner's reagent gave reddish-brown precipitate, Dragendoff's reagent gave blue-black precipitate; tannic acid gave orange precipitate and finally with Meyer's reagent gave light yellow coloration. These tests confirmed the presence of alkaloids in the plant parts used. The Vitalis-Morin tests did not give purple colorations to the extract, which was an indication of the absence of tropane alkaloids.

The ammoniacal silver nitrate test showed a brown colored solution with brown precipitate before heating which later turned to brownish black after heating. This was an indication of the presence of flavonol glycoside (Amako, 1999). Flavonoids are condensed tannins. This test was a confirmation to the test for condensed tannins earlier carried out.

The presence of these plant metabolites called for an in-depth research into these plants. These metabolites are of great pharmacological importance. Many triterpenes and their aglycones have been reported to have various uses as antiulcerogenic, anti-inflammatory, fibrinolytic, antipyretic, analgesic and anti-edematous in action (Ndukwe *et al*; 2005). Tannins have a healing property in that they act, as protective agents against toxins and as antiviral agents (Harbone and Baxter, 1993).

Table 4.1: Amounts of extracts from 212.60g powdered aerial parts of *Spillanthes acmella*

Solvent medium	Weight of extract(g)	Percentage extract (%)
Petroleum ether	2.80	1.32
Chloroform	4.70	2.21
Ethyl acetate	2.50	1.18
Methanol	14.10	6.63

Table 4.2: Amounts of extracts from 221.80 g powdered stem bark of *Sarcocephalus latifolius*

Solvent medium	Weight of extract(g)	Percentage extract (%)
Petroleum ether	2.40	1.08
Chloroform	0.47	0.21
Ethyl acetate	0.90	0.41
Methanol	22.70	10.23

Table 4.3: Result of the preliminary phytochemical screening of the root and stem bark of *Sarcocephalus latifolius*

Property tested	Root	Stem
Carbohydrate	+	+
Glycoside	+	_
Anthraquinones	+	+
Cardiac glycoside	+	+
Saponins	+	+
Steroidal triterpenes	+	+
Flavonoids	+	_
Tannins	_	+
Alkaloids	+	+

KEY: + → Present – → Absent

Table 4.4: Result of the preliminary phytochemical screening of aerial parts of *Spillanthes acmella*

Property tested	Remark
Carbohydrate	+
Glycoside	+
Anthraquinones	+
Cardiac glycoside	+
Saponins	+
Steroidal triterpenes	+
Flavonoids	+
Tannins	_
Alkaloids	+

KEY: + → Present – → Absent

Table 4.5: Detection of Sugar in Plant Material (aerial part of *Spillanthes acmella*, root and stem bark of *Sarcocephalus latifolius*)

Test	Observation	Inference
Molish's test	Reddish coloured at interphase	Carbohydrate present
Fehling test	Brick-red precipitate	Combined/reducing sugar present

Table 4.6: Identification Test result for Cardiac Glycosides (aerial part of *Spillanthes acmella*, root and stem bark of *Sarcocephalus latifolius*)

Sample	Test	Observation	Inference
Powered plant material	Lieberman-Burchardt	Brownish-yellow colour to green	Cardiac glycoside present
	Salkowski	Reddish-brown at the interphase	Cardiac glycoside present
	Keller-Killiani	Brownish ring at the interphase	Cardiac glycoside presence
	Legal	Red fades to brownish yellow	Cardiac glycoside presence

4.3 Antimicrobial Screening

To ascertain the biological activities of the various extracts antimicrobial screening were carried out using; Methicillin Resistant *Staphylococcus Aureus (*MR*SA), Staphylococcus aureus, Streptococcus pyrogenes, Bacillus subtilis, Corynebacterium ulcerans, Escherichia coli, Proteus mirabilis, Proteus vulgaris, Pseudomonas aureginosa, Salmonella typhi, Shigellia dysenteric, Candida albicans, Candida virusei,* and *Candida tropicalis* clinical isolates.

4.3.1 Antimicrobial Screening of *Sarcocephalus latifolius*

All the crude extracts from the *Sarcocephalus latifolius* stem bark were active on *Streptococcus pyrogenes, Bacillus subtilis, Escherichia coli, Salmonella typhi, Shigellia dysenteric and Candida albicans* but not active on *Methycilin resistant Staphylococcus aureus, Candida tropicalis, Proteus mirabilis and Pseudomonas aureginosa.* However, the petroleum ether fraction was not active on the following, *Staphylococcus aureus, Corynebacterium ulcerans, Proteus vulgaris, Pseudomonas aureginosa and Candida virusei.* The chloroform extract was not active on Corynebacterium *ulcerans, Proteus* vulgaris, *and Candida virusei*. The methanol extract fraction was not active on *Staphylococcus aureus, Proteus vulgaris*, *and Candida virusei.* The antimicrobial activities and their zones of inhibition of these extract are shown in Tables 4.8 and 4.9. These activities could be due to the presence of some very active bioactive substances in the plant.

Table 4.7: General Identification Test for Alkaloids in *Sarcocephalus latifolius* and *Spillanthes acmella*

Test	Observation	Inference
Meyer's reagent	Precipitate	Alkaloid present
Dragendoff's reagent	Precipitate	Alkaloid present
1% picric Acid solutions	Precipitate	Alkaloid present
Wagner's reagent	Precipitate	Alkaloid present
10% Tannic acid solution	Precipitate	Alkaloid present

Table 4.8: Antimicrobial activities of the fractions from the stem bark of *Sarcocephalus latifolius*

Test Organism	SBPE	SBCF	SBEa	SBMe
MR*SA*	R	R	R	R
Staphylococcus aureus	R	S	S	R
Streptococcus pyrogenes	S	S	S	S
Bacillus subtilis	S	S	S	S
Corynebacterium ulcerans	R	R	S	S
Escherichia coli	S	S	S	S
Proteus mirabilis	R	R	R	R
Proteus vulgaris	R	R	S	R
Pseudomonas aureginosa	R	R	R	R
Salmonella typhi	S	S	S	S
Shigellia dysenteric	S	S	S	S

Candida albicans	S	S	S	S
Candida virusei	R	R	S	R
Candida tropicalis	R	R	R	R

Key = S=Sensitive R= Resistant

SBPE=*Sarcocephalus latifolius* petroleum ether extract

SBCF= *Sarcocephalus latifolius* chloroform extract

SBEa= *Sarcocephalus latifolius* ethyl acetate extract

SBMe= *Sarcocephalus latifolius* methanol extract

Table 4.9: Zone of inhibitions (mm) of the crude fractions of the bark of *Sarcocephalus latifolius*

Test Organism	SBPE	SBCF	SBEa	SBMe
MRSA	0	0	0	0
Staphylococcus aureus	0	16	18	0
Streptococcus pyrogenes	17	18	17	18
Bacillus subtilis	20	21	20	22
Corynebacterium ulcerans	0	0	14	16
Escherichia coli	17	17	16	17
Proteus mirabilis	0	0	0	0
Proteus vulgaris	0	0	16	0
Pseudomonas aureginosa	0	0	0	0
Salmonella typhi	17	18	16	17

Shigellia dysenteric	17	19	17	16
Candida albicans	15	14	15	15
Candida virusei	0	0	16	0
Candida tropicalis	0	0	0	0

SBPE=*Sarcocephalus latifolius* petroleum ether extract

SBCF= *Sarcocephalus latifolius* chloroform extract

SBEa= *Sarcocephalus latifolius* ethyl acetate extract

SBMe= *Sarcocephalus latifolius* methanol extract

4.3.2: Minimum Inhibition Concentration (MIC) of the extracts from *Sarcocephalus latifolius.*

To determine further the activities of the extracts, minimum inhibitory concentration experiment were carried on the organisms that proved sensitive to the extracts. MIC values were obtained thus; the Petroleum ether fraction of the stem bark of *Sarcocephalus latifolius* had MIC value on B.c*oli, Salmonella typhi, Shigellia dysenteric* and *Candida albicans at* 20 mg/ml *and on Bacillus subtilis* at 10 mg/ml. The chloroform extract had MIC values for *Staphylococcus aureus, Streptococcus pyrogenes, Escherichia coli, Salmonella typhi, Shigellia dysenteric and Candida albicans at* 20 mg/ml while for *Bacillus subtilis* was 10 mg/ml. The MIC of the ethyl acetate fraction was observed at 20 mg/ml for *Staphylococcus aureus, Streptococcus pyrogenes, Corynebacterium ulcerans, Escherichia coli, Proteus vulgaris, , Salmonella typhi, Shigellia dysenteric ,Candida albicans and Candida virusei* while it was observed for *Bacillus Subtilis* at 10mg/ml.The

methanol fraction showed MIC for *Streptococcus pyrogenes, Corynebacterium ulcerans, Escherichia coli, Salmonella typhi, Shigellia dysenteric and Candida albicans at* 20 mg/ml and at10 mg/ml for *Bacillus subtilis.* These results are as presented in Tables 4.10, 4.11, 4.12 and 4.13.

4.3.3: MBC/MFC for *Sarcocephalus latifolius* stem bark extracts

This was determined so as to know if the extracts can go further to kill the microorganisms which were sensitive to them. The MBC/MFC results was observed to be a little above the MIC values. The MBC/MFC was observed to be lowest in the Chloroform and methanol extracts on *Bacillus subtilis* at 20mg/ml while it was highest at 40mg/ml for this extract. This is an indication that there are bioactive substances in the chloroform and methanol extracts that are active on *Bacillus subtilis*. The MBC/MFC results are as shown in Tables 4.14, 4.15, 4.16 and 4.17.

Table 4.10: Minimum Inhibitory Concentrations (mg/ml) of crude petroleum ether extract of *Sarcocephalus latifolius* on the test microbes.

Test Organism	40	20	10	5	2.5
MR*SA*					
Staphylococcus aureus					
Streptococcus pyrogenes	_	**O***	+	++	+++
Bacillus subtilis	_	_	***O****	+	++
Corynebacterium ulcerans					
Escherichia coli	_	**O***	+	++	+++
Proteus mirabilis					
Proteus vulgaris					
Pseudomonas aureginosa					
Salmonella typhi	_	**O***	+	++	+++
Shigellia dysenteric	_	**O***	+	++	+++
Candida albicans	_	**O***	+	++	+++
Candida virusei					
Candida tropicalis					

Key: **-** *=No turbidity (No growth),* ***O**** *= MIC,* **+** *= Turbid (Light growth),* **++** *= Moderate turbidity and* **+++** *= High turbidity.*

Table 4.11: Minimum Inhibitory Concentrations (mg/ml) of crude chloroform extract of *Sarcocephalus latifolius* on the test microbes.

Test Organism	40	20	10	5	2.5
MR*SA*					
Staphylococcus aureus	_	**O***	+	++	+++
Streptococcus Pyrogenes	_	**O***	+	++	+++
Bacillus subtilis	_	_	***O****	+	++
Corynebacterium ulcerans					
Escherichia coli	_	**O***	+	++	+++
Proteus mirabilis					
Proteus vulgaris					
Pseudomonas aureginosa					
Salmonella typhi	_	**O***	+	++	+++
Shigellia dysenteric	_	**O***	+	++	+++
Candida albicans	_	**O***	+	++	+++
Candida virusei					
Candida tropicalis					

Key: **-** *=No turbidity (No growth),* ***O**** *= MIC,* **+** *= Turbid (Light growth),* **++** *= Moderate turbidity and* **+++** *= High turbidity.*

Table 4.12: Minimum Inhibitory Concentrations (mg/ml) of crude ethyl acetate extract of *Sarcocephalus latifolius* on the test microbes.

Test Organism	40	20	10	5	2.5
MR*SA*					
Staphylococcus aureus	_	**O***	+	++	+++
Streptococcus pyrogenes	_	**O***	+	++	+++
Bacillus subtilis	_	_	***O****	+	++
Corynebacterium ulcerans	_	**O***	+	++	+++
Escherichia coli	_	**O***	+	++	+++
Proteus mirabilis					
Proteus vulgaris	_	**O***	+	++	+++
Pseudomonas aureginosa					
Salmonella typhi	_	**O***	+	++	+++
Shigellia dysenteric	_	**O***	+	++	+++
Candida albicans	_	**O***	+	++	+++
Candida virusei	_	**O***	+	++	+++
Candida tropicalis	_	**O***	+	++	+++

Key: **-** *=No turbidity (No growth),* ***O**** *= MIC,* **+** *= Turbid (Light growth),* **++** *= Moderate turbidity and* **+++** *= High turbidity.*

Table 4.13: Minimum Inhibition Concentration (mg/ml) of crude methanol extract of *Sarcocephalus latifolius* on the test microbes

Test Organism	40	20	10	5	2.5
MR*SA*					
Staphylococcus aureus					
Streptococcus pyrogenes	_	**O***	+	++	+++
Bacillus subtilis	_	_	***O****	+	++
Corynebacterium ulcerans	_	**O***	+	++	+++
Escherichia coli	_	**O***	+	++	+++
Proteus mirabilis					
Proteus vulgaris					
Pseudomonas aureginosa					
Salmonella typhi	_	**O***	+	++	+++
Shigellia dysenteric	_	**O***	+	++	+++
Candida albicans	_	**O***	+	++	+++
Candida virusei					
Candida tropicalis					

Key: **-** *=No turbidity (No growth),* ***O**** *= MIC,* **+** *= Turbid (Light growth),* **++** *= Moderate turbidity and* **+++** *= High turbidity.*

Table 4.14: Minimum Bactericidal/Fungicidal Concentrations (mg/ml) of crude petroleum extract of the bark of *Sarcocephalus latifolius* on the test microbes

Test Organism	40	20	10	5	2.5
MR*SA*					
Staphylococcus aureus					
Streptococcus pyrogenes	**O***	+	++	+++	++++
Bacillus subtilis	**O***	+	++	+++	++++
Corynebacterium ulcerans					
Escherichia coli	**O***	+	++	+++	++++
Proteus mirabilis					
Proteus vulgaris					
Pseudomonas aureginosa					
Salmonella typhi	**O***	+	++	+++	++++
Shigellia dysenteric	**O***	+	++	+++	++++
Candida albicans	**O***	+	++	+++	++++
Candida virusei					
Candida tropicalis					

*Key: **-** =No colony growth), **O*** = MBC/MFC, **+** =Scanty colonies growth, **++** = Moderate colonies growth and **+++** = Heavy Colonies growth.*

Table 4.15: Minimum Bactericidal/Fungicidal Concentrations (mg/ml) of crude chloroform extract of the bark of *Sarcocephalus latifolius* on the test microbes

Test Organism	40	20	10	5	2.5
MRS*A*					
Staphylococcus aureus	**O***	+	++	+++	++++
Streptococcus pyrogenes	**O***	+	++	+++	++++
Bacillus subtilis	_	**O***	+	++	+++
Corynebacterium ulcerans					
Escherichia coli	**O***	+	++	+++	++++
Proteus mirabilis					
Proteus vulgaris					
Pseudomonas aureginosa					
Salmonella typhi	**O***	+	++	+++	++++
Shigellia dysenteric	**O***	+	++	+++	++++
Candida albicans	**O***	+	++	+++	++++
Candida virusei					
Candida tropicalis					

*Key: **-** =No colony growth), **O*** = MBC/MFC, **+** =Scanty colonies growth, **++** = Moderate colonies growth and **+++** = Heavy Colonies growth.*

Table 4.16: Minimum Bactericidal/Fungicidal Concentrations (mg/ml) of crude ethyl acetate extract of the stem bark of *Sarcocephalus latifolius* on the test microbes

Test Organism	40	20	10	5	2.5
MR*SA*					
Staphylococcus aureus	**O***	+	++	+++	++++
Streptococcus pyrogenes	**O***	+	++	+++	++++
Bacillus subtilis	**O***	+	++	+++	++++
Corynebacterium ulcerans	**O***	+	++	+++	++++
Escherichia coli	**O***	+	++	+++	++++
Proteus mirabilis					
Proteus vulgaris	**O***	+	++	+++	++++
Pseudomonas aeruginosa					
Salmonella typhi	**O***	+	++	+++	++++
Shigellia dysenteric	**O***	+	++	+++	++++
Candida albicans	**O***	+	++	+++	++++
Candida virusei	**O***	+	++	+++	++++
Candida tropicalis					

*Key: **-** =No colony growth), **O*** = MBC/MFC, **+** =Scanty colonies growth, **++** = Moderate colonies growth and **+++** = Heavy Colonies growth.*

Table 4.17: Minimum Bactericidal/Fungicidal Concentrations (mg/ml) of crude methanol extract of the bark of Sarcocephalus latifolius on the test microbes.

Test Organism	40	20	10	5	2.5
MRS*A*					
Staphylococcus aureus					
Streptococcus pyrogenes	**O***	+	++	+++	++++
Bacillus subtilis	_	**O***	+	++	+++
Corynebacterium ulcerans	**O***	+	++	+++	++++
Escherichia coli	**O***	+	++	+++	++++
Proteus mirabilis					
Proteus vulgaris					
Pseudomonas aureginosa					
Salmonella typhi	**O***	+	++	+++	++++
Shigellia dysenteric	**O***	+	++	+++	++++
Candida albicans	**O***	+	++	+++	++++
Candida virusei					
Candida tropicalis					

*Key: **-** =No colony growth), **O*** = MBC/MFC, **+** =Scanty colonies growth, **++** = Moderate colonies growth and **+++** = Heavy Colonies growth.*

4.3.4 Antimicrobial screening of aerial parts of *Spillanthes acmella* (Murr)

The antimicrobial screening of the crude extracts fractions of *Spillanthes acmella* was performed to know which of the microorganisms was sensitive or resistant to the fractions. It was observed that the following microorganisms; *Streptococcus pyrogenes, Bacillus subtilis, Escherichia coli, Shigellia dysenteric* and *Candida albicans* were sensitive to the extracts, while Methycilin resistant *Staphylococcus aureus, Pseudomonas aureginosa* and *Candida tropicalis* were all resistant to the extracts. *Proteus mirabilis, Salmonella typhi* and *Candida virusei* were all resistant to the crude petroleum ether fraction and *Staphylococcus aureus, Streptococcus pyrogenes, Bacillus subtilis, Corynebacterium ulcerans, Escherichia coli, Proteus vulgaris, Shigellia dysenteric and Candida albicans* were sensitive to petroleum ether fraction. *Proteus mirabilis* and *Proteus vulgaris* were resistant to the crude chloroform fraction and *Staphylococcus aureus, Streptococcus pyrogenes, Bacillus subtilis, Corynebacterium ulcerans, Escherichia coli, Salmonella typhi*. *Shigellia dysenteric, Candida albicans* and *Candida virusei* were all sensitive to crude chloroform extract.

Staphylococcus aureus, Corynebacterium ulcerans and Salmonella typhi were resistant to the crude ethyl acetate fraction while Streptococcus *pyrogenes, Bacillus subtilis, Escherichia coli, Proteus mirabilis, Proteus vulgaris, Shigellia dysenteric, Candida albicans* and *Candida virusei* were all sensitive to crude ethyl acetate extract. *Streptococcus pyrogenes, Bacillus subtilis, Corynebacterium ulcerans, Escherichia coli, Proteus mirabilis, Proteus vulgaris, Salmonella typhi, Shigellia dysenteric* and *Candida albicans* were all sensitive to the methanol extracts while *Staphylococcus aureus, Corynebacterium ulcerans, Salmonella typhi* and *Candida virusei* were resistant to the

crude methanol fraction. These results are as presented in Table 4.18. The zones of inhibition for the extracts were observed for petroleum ether to be between 15 to 22, chloroform extract 16 to 20, ethyl acetate 16 to 2t is3 and for methanol extract was 16 to 24 (Table 4.19)

Table 4.18: Antimicrobial activities of the crude fractions from the aerial part of *Spillanthes acmella*

Test Organism	SAPE	SACF	SAEa	SAMe
MR*SA*	R	R	R	R
Staphylococcus aureus	S	S	R	R
Streptococcus pyrogenes	S	S	S	S
Bacillus subtilis	S	S	S	S
Corynebacterium ulcerans	S	S	R	S
Escherichia coli	S	S	S	S
Proteus mirabilis	R	R	S	S
Proteus vulgaris	S	R	S	S
Pseudomonas aureginosa	R	R	R	R
Salmonella typhi	R	S	R	S
Shigellia dysenteric	S	S	S	S
Candida albicans	S	S	S	S
Candida virusei	R	S	S	R
Candida tropicalis	R	R	R	R

KEY: SFPE= *Spillanthes acmella* petroleum ether extract

SACF= *Spillanthes acmella* chloroform extract

SAEa= *Spillanthes acmella* ethyl acetate extract

SAMe= *Spillanthes acmella* methanol extract

Table 4.19: Zone of inhibitions of the crude fractions from the aerial part of *Spillanthes acmella* Murr

Test Organism	SAPE	SACF	SAEa	SAMe
MRS*A*	0	0	0	0
Staphylococcus aureus	19	17	0	0
Streptococcus pyrogenes	17	18	19	21
Bacillus subtilis	22	20	23	24
Corynebacterium ulcerans	17	19	0	19
Escherichia coli	17	18	17	18
Proteus mirabilis	0	0	18	19
Proteus vulgaris	16	0	16	17
Pseudomonas aureginosa	0	0	0	0
Salmonella typhi	0	17	0	16
Shigellia dysenteric	19	17	18	16
Candida albicans	15	16	16	17
Candida virusei	0	17	16	0
Candida tropicalis	0	0	0	0

KEY: SFPE= *Spillanthes acmella* petroleum ether extract

SACF= *Spillanthes acmella* chloroform extract

SAEa= *Spillanthes acmella* ethyl acetate extract

SAMe= *Spillanthes acmella* methanol extract

4.3.5 Minimum Inhibitory Concentration of the extracts from *Spillanthes acmella*

The Minimum Inhibitory Concentrations (MIC) experiment of the crude extracts was equally determined further to ascertain the activities of the extracts. MIC values of the crude petroleum ether fraction (Table 4.20) of *Spillanthes acmella* was observed to inhibit the growth of *Staphylococcus aureus, Staphylococcus pyrogenes, Corynebacterium ulcerans, Escherichia coli, Proteus vulgaris, Shigellia dysenteric* and *Candida albicans* at concentration 20 mg/ml and inhibit *Bacillus subtilis at* 10 mg/ml. The crude chloroform extract fraction (Table 4.21) had MIC value at 20 mg/ml for *Staphylococcus aureus, Staphylococcus pyrogenes, Corynebacterium ulcerans, Escherichia coli, Salmonella typhi, Shigellia dysenteric* and *Candida albicans and Candida virusei* while it inhibited the growth of *Bacillus subtilis* at 10mg/ml. These are indication that these fractions contained a very active compound.

Ethyl acetate fraction inhibited the growths of *Staphylococcus pyrogenes, Escherichia coli, Proteus mirabilis, Proteus vulgaris, Shigellia dysenteric, Candida albicans* and *Candida virusei* at 20 mg/ml while *Bacillus subtilis* had MIC value at 10 mg/ml (Table 4.22). The crude methanol fraction had MIC values (Table 4.23) of 20 mg/ml for *Corynebacterium ulcerans, Escherichia coli, Proteus mirabilis, Proteus vulgaris, Salmonella typhi, Shigellia dysenteric* and *Candida albicans* 10 mg/ml for *Staphylococcus pyrogenes* and *Bacillus subtilis respectively*.

Table 4.20: Minimum Inhibition Concentrations (mg/ml) of crude petroleum extracts of the aerial part of *Spillanthes acmella* Murr on the test microbes

Test Organism	40	20	10	5	2.5
MR*SA*					
Staphylococcus aureus	_	**O***	+	++	+++
Streptococcus Pyrogenes	_	**O***	+	++	+++
Bacillus Subtilis	_	_	***O****	+	++
Corynebacterium	_	**O***	+	++	+++
Ulcerans					
Escherichia Coli	_	**O***	+	++	+++
Proteus Mirabilis					
Proteus Vulgaris	_	**O***	+	++	+++
Pseudomonas aureginosa					
Salmonella typhi					
Shigellia dysenteric	_	**O***	+	++	+++
Candida albicans	_	**O***	+	++	+++
Candida Virusei					
Candida Tropicalis					

*Key: **-** =No turbidity (No growth), **O*** = MIC, **+** = Turbid (Light growth), **++** = Moderate turbidity and **+++** = High turbidity.*

Table 4.21: Minimum Inhibition Concentrations (mg/ml) of crude chloroform extracts of the aerial part of *Spillanthes acmella* Murr on the test microbes.

Test Organism	40	20	10	5	2.5
MR*SA*					
Staphylococcus aureus	_	**O***	+	++	+++
Streptococcus pyrogenes	_	**O***	+	++	+++
Bacillus subtilis	_	_	***O****	+	++
Corynebacterium ulcerans	_	**O***	+	++	+++
Escherichia coli	_	**O***	+	++	+++
Proteus mirabilis					
Proteus vulgaris					
Pseudomonas aureginosa					
Salmonella typhi	_	**O***	+	++	+++
Shigellia dysenteric	_	**O***	+	++	+++
Candida albicans	_	**O***	+	++	+++
Candida virusei	_	**O***	+	++	+++
Candida tropicalis					

Key: **-** *=No turbidity (No growth),* ***O**** *= MIC,* **+** *= Turbid (Light growth),* **++** *= Moderate turbidity and* **+++** *= High turbidity.*

Table 4.22: Minimum Inhibition Concentrations (mg/ml) of crude ethyl acetate extracts of the aerial part of *Spillanthes acmella* Murr on the test microbes

Test Organism	40	20	10	5	2.5
MR*SA*					
Staphylococcus aureus					
Streptococcus pyrogenes	_	**O***	+	++	+++
Bacillus subtilis	_	_	***O****	+	++
Corynebacterium ulcerans					
Escherichia coli	_	**O***	+	++	+++
Proteus mirabilis	_	**O***	+	++	+++
Proteus vulgaris	_	**O***	+	++	+++
Pseudomonas aeruginosa					
Salmonella typhi					
Shigellia dysenteric	_	**O***	+	++	+++
Candida albicans	_	**O***	+	++	+++
Candida virusei	_	**O***	+	++	+++
Candida tropicalis					

Key: **-** *=No turbidity (No growth),* ***O**** *= MIC,* **+** *= Turbid (Light growth),* **++** *= Moderate turbidity and* **+++** *= High turbidity.*

Table 4.23: Minimum Inhibition Concentrations (mg/ml) of crude methanol extracts of the aerial part of *Spillanthes acmella* Murr on the test microbes

Test Organism	40	20	10	5	2.5
MR*SA*					
Staphylococcus aureus					
Streptococcus pyrogenes	_	_	***O****	+	++
Bacillus subtilis	_	_	***O****	+	++
Corynebacterium ulcerans	_	**O***	+	++	+++
Escherichia coli	_	**O***	+	++	+++
Proteus mirabilis	_	**O***	+	++	+++
Proteus vulgaris	_	**O***	+	++	+++
Pseudomonas aeruginosa					
Salmonella typhi	_	**O***	+	++	+++
Shigellia dysenteric	_	**O***	+	++	+++
Candida albicans	_	**O***	+	++	+++
Candida virusei					
Candida tropicalis					

Key: **-** *=No turbidity (No growth),* ***O**** *= MIC,* **+** *= Turbid (Light growth),* **++** *= Moderate turbidity and* **+++** *= High turbidity.*

4.3.6 Minimum Bactericidal Concentration (MBC) / Minimum Fungicidal Concentration (MFC) for *Spillanthes acmella*

This was determined to ascertain whether the microorganisms were only inhibited or are completely exterminated by the extracts. It was observed that *Staphylococcus aureus, staphylococcus pyrogenes, Corynebacterium ulcerans, Escherichia coli, Proteus vulgaris, Shigellia dysenteric and Candida albicans* were exterminated at 40 mg/ml and *Bacillus subtilis* at 20 mg/ml (Table 4.24). the chloroform extract completely exterminate *Staphylococcus aureus, staphylococcus pyrogenes, Bacillus subtilis, Corynebacterium ulcerans, Escherichia coli, Salmonella typhi, Shigellia dysenteric, Candida albicans and Candida virusei* at 40 mg/ml (Table 4.25). *Staphylococcus pyrogenes, Escherichia coli, Proteus mirabilis*, *Proteus vulgaris, Shigellia dysenteric, Candida albicans* and *Candida virusei were exterminated at* 40 mg/ml *and for Bacillus subtilis* at 20 mg/ml by the ethyl acetate fraction of this plant (Table 4.26).

The MBC/MFC for the methanol extract were observed for the following organism, *staphylococcus Pyrogenes*, and *Bacillus Subtilis* at 20 mg/ml while for *Corynebacterium ulcerans, Escherichia coli, Proteus mirabilis*, *Proteus vulgaris, salmonella typhi, Shigellia dysenteric* and *Candida albicans.* Generally the MBC/MFC for the methanol fraction of the crude aerial part of *Spillanthes acmella* had a value a little above that of the MIC values observed (Table 4.27).

Table 4.24: Minimum Bactericidal/Fungicidal Concentrations (mg/ml) of crude petroleum ether extract from the aerial part of *Spillanthes acmella* on the test microbes

Test Organism	40	20	10	5	2.5
MR*SA*					
Staphylococcus aureus	**O***	+	++	+++	++++
Streptococcus pyrogenes	**O***	+	++	+++	++++
Bacillus subtilis	_	**O***	+	++	+++
Corynebacterium ulcerans	**O***	+	++	+++	++++
Escherichia coli	**O***	+	++	+++	++++
Proteus mirabilis					
Proteus vulgaris	**O***	+	++	+++	++++
Pseudomonas aureginosa					
Salmonella typhi					
Shigellia dysenteric	**O***	+	++	+++	++++
Candida albicans	**O***	+	++	+++	++++
Candida virusei					
Candida tropicalis					

*Key: **-** =No colony growth), **O*** = MBC/MFC, **+** =Scanty colonies growth, **++** = Moderate colonies growth and **+++** = Heavy Colonies growth.*

Table4.25: Minimum Bactericidal/Fungicidal Concentrations (mg/ml) of crude chloroform extract of the aerial part of *Spillanthes acmella* on the test microbes.

Test Organism	40	20	10	5	2.5
MR*SA*					
Staphylococcus aureus	**O***	+	++	+++	++++
Streptococcus pyrogenes	**O***	+	++	+++	++++
Bacillus subtilis	**O***	+	++	+++	++++
Corynebacterium ulcerans	**O***	+	++	+++	++++
Escherichia coli	**O***	+	++	+++	++++
Proteus mirabilis					
Proteus vulgaris					
Pseudomonas aureginosa					
Salmonella typhi	**O***	+	++	+++	++++
Shigellia dysenteric	**O***	+	++	+++	++++
Candida albicans	**O***	+	++	+++	++++
Candida virusei	**O***	+	++	+++	++++
Candida tropicalis					

*Key: **-** =No colony growth), **O*** = MBC/MFC, **+** =Scanty colonies growth, **++** = Moderate colonies growth and **+++** = Heavy Colonies growth.*

Table 4.26: Minimum Bactericidal/Fungicidal Concentrations (mg/ml) of crude ethyl acetate extract of the aerial part of *Spillanthes acmella* on the test microbes.

Test Organism	40	20	10	5	2.5
MRS*A*					
Staphylococcus aureus					
Streptococcus pyrogenes	**O***	+	++	+++	++++
Bacillus subtilis	_	**O***	+	++	+++
Corynebacterium ulcerans					
Escherichia Coli	**O***	+	++	+++	++++
Proteus mirabilis	**O***	+	++	+++	++++
Proteus vulgaris	**O***	+	++	+++	++++
Pseudomonas aureginosa					
Salmonella typhi					
Shigellia dysenteric	**O***	+	++	+++	++++
Candida albicans	**O***	+	++	+++	++++
Candida virusei	**O***	+	++	+++	++++
Candida tropicalis					

*Key: **-** =No colony growth), **O*** = MBC/MFC, **+** =Scanty colonies growth, **++** = Moderate colonies growth and **+++** = Heavy Colonies growth.*

Table 4.27: Minimum Bactericidal/Fungicidal Concentrations (mg/ml) of crude methanol extract of the aerial part of *Spillanthes acmella* on the test microbes.

Test Organism	40	20	10	5	2.5
MRS*A*					
Staphylococcus aureus					
Streptococcus pyrogenes	_	**O***	+	++	+++
Bacillus subtilis	_	**O***	+	++	+++
Corynebacterium ulcerans	**O***	+	++	+++	++++
Escherichia coli	**O***	+	++	+++	++++
Proteus mirabilis	**O***	+	++	+++	++++
Proteus vulgaris	**O***	+	++	+++	++++
Pseudomonas aureginosa					
Salmonella typhi	**O***	+	++	+++	++++
Shigellia dysenteric	**O***	+	++	+++	++++
Candida albicans	**O***	+	++	+++	++++
Candida virusei					
Candida tropicalis					

Key: **-** *=No colony growth),* ***O**** *= MBC/MFC,* **+** *=Scanty colonies growth,* **++** *= Moderate colonies growth and* **+++** *= Heavy Colonies growth.*

4.4.0 Spectra results

The methanol extract of the stem bark of *Sarcocephalus latifolius* gave EFSB, SB-C1 and SB-C2 while petroleum ether extract fraction of *Spillanthes acmella* gave SPEC-1 after several purifications. However these components were subjected to spectroscopic analyses using infrared (IR), Nuclear Magnetic Resonance (NMR) and Gas Chromatography-mass Spectrometer (GC-MS).

4.4.1 EFSB Spectral data

Compound **EFSB** was obtained as a white amorphous solid. The IR (KBr γ_{max} cm^{-1}) of EFSB (Fig 1) showed absorption peaks at 3381.77 typical of hydroxyl group (OH), 2866.22 is characteristics of carbon-carbon single bond (C-C). The peak at 1710.90cm^{-1} is typical of carbonyl signal (C=O). 1460.40 is due to the methylene groups while the signal at 1378.06 cm^{-1} is associated with carbon-carbon double bond (C=C).

The ^{1}H NMR of this compound (Fig 2) revealed signals for two methyl singlet at δ1.0169 and 0.6890; four doublet of methyl at δ 1.0256 , 0.8585 , 0.8058 and 0.8013 .There were three (3) recognizable vinylic proton signals observed at δ 5.7009 , 5.3369 and 5.3238. The proton signal observed at δ 3.53 could be attributable to hydroxyl functional group (-OH).

The ^{13}C NMR spectrum (Fig 3) showed a total of fifty-five (55) recognizable carbon signals. The signals observed at δ 140.7743, δ 123.7451, δ 121.7273, and δ 77.2202 are typical of olefinic carbon signals while the signal peak at 71.7615ppm was characteristic of C-O. The peak observed at 171.7615ppm was typical of the presence of carbonyl (C=O) group .The remaining carbon signals with chemical shifts of 11.8681 and 56.7799 ppm were also recorded.

Based on these spectroscopic data EFSB was considered to be a mixture of β - Sitosterol (Structure XXXII) and β- sitostenone (Structure XXXIII) and a literature search on those spectra tend to lend more credence to the compound as recorded by Wen-Hsin Li *et al*., 2008 (Table 4.29 for IR and 4.30 for NMR), Rubinstein *et al*., 1976; sakakibara *et al*., 1983; Kulsum *et al*., 2009. The spectra results are as shown in figures 1, 2, 3, 4, 5, 6 and 7. Literature has shown that β- sitosterol is difficult to be obtained in pure form. (Pollock and Stevens., 1965, Fieser and Fieser., 1962). β - Sitosterol have been isolated from various plant species including the root of this particular plant. However, this is the first time it will be isolated from the stem bark of this plant. β-Sitosterol is a common constituent of herbal plants which has been found to have biological activity, such as anti hypercholestrolaemic and estrogenic effect (Buckingham, 1998). It has equally been shown recently to have gastro protective activity in several experimental ulcer models in rats (Navarrete *et al*., 2002). Antimicrobial, anti-inflammatory, analgesic and anti-pyretic activity of β -sitosterol have been reported previously (Gupta *et al*., 1980; Salvador *et al*., 2004). It inhibited neutrophil migration into the inflamed tissue, as in acute topic inflammation it showed decreased myeloperoxidase activity (Gómez *et al*; 1999). Also, β-sitosterol has been reported to have quite strong antioxidant activity (Weng and Wang, 2000).So, the use of this plant in venereal disease, wound, diarrhea and dysentery treatment by the native has been really justified.

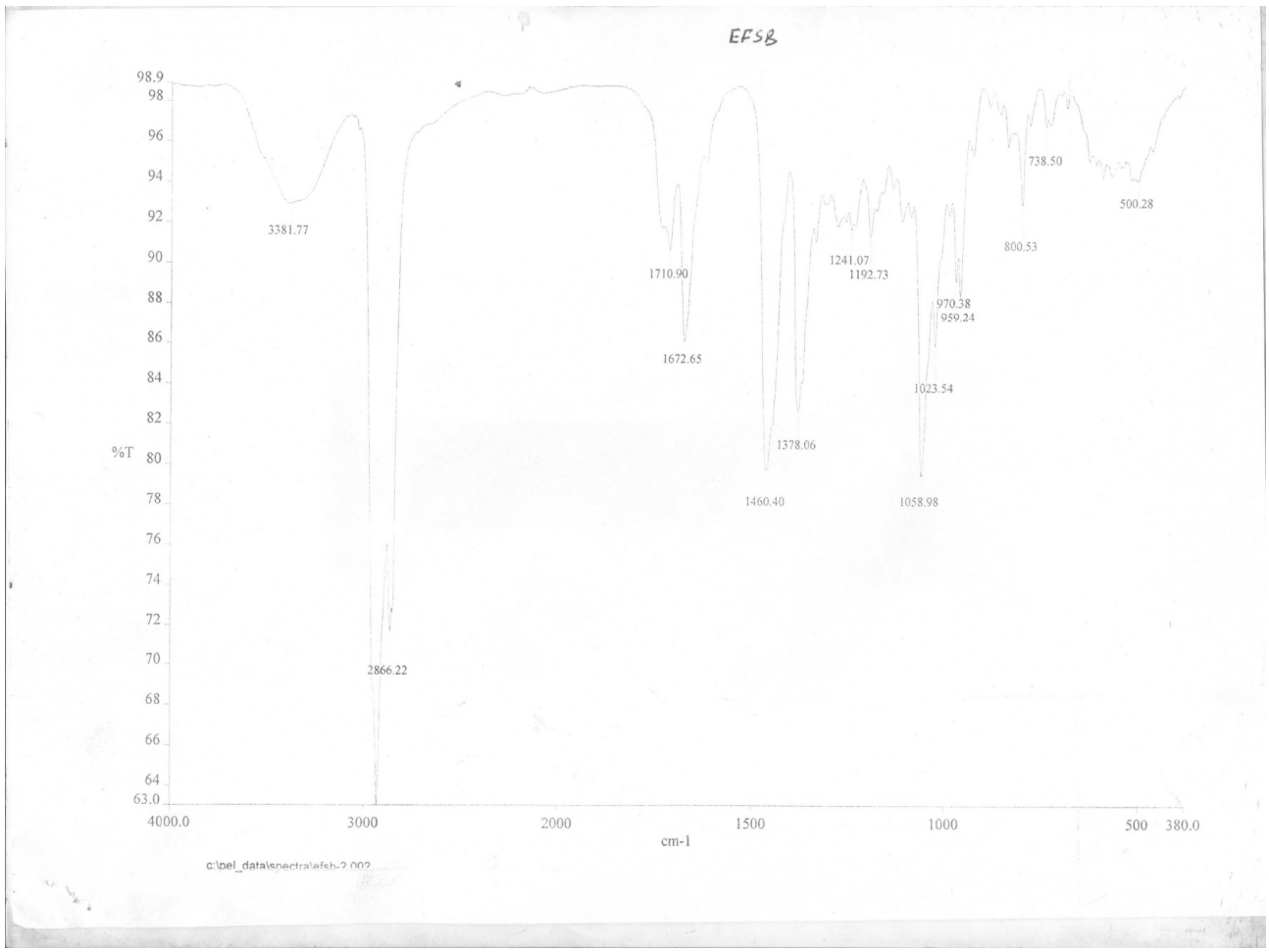


Figure 1: Infrared spectroscopy of EFSB

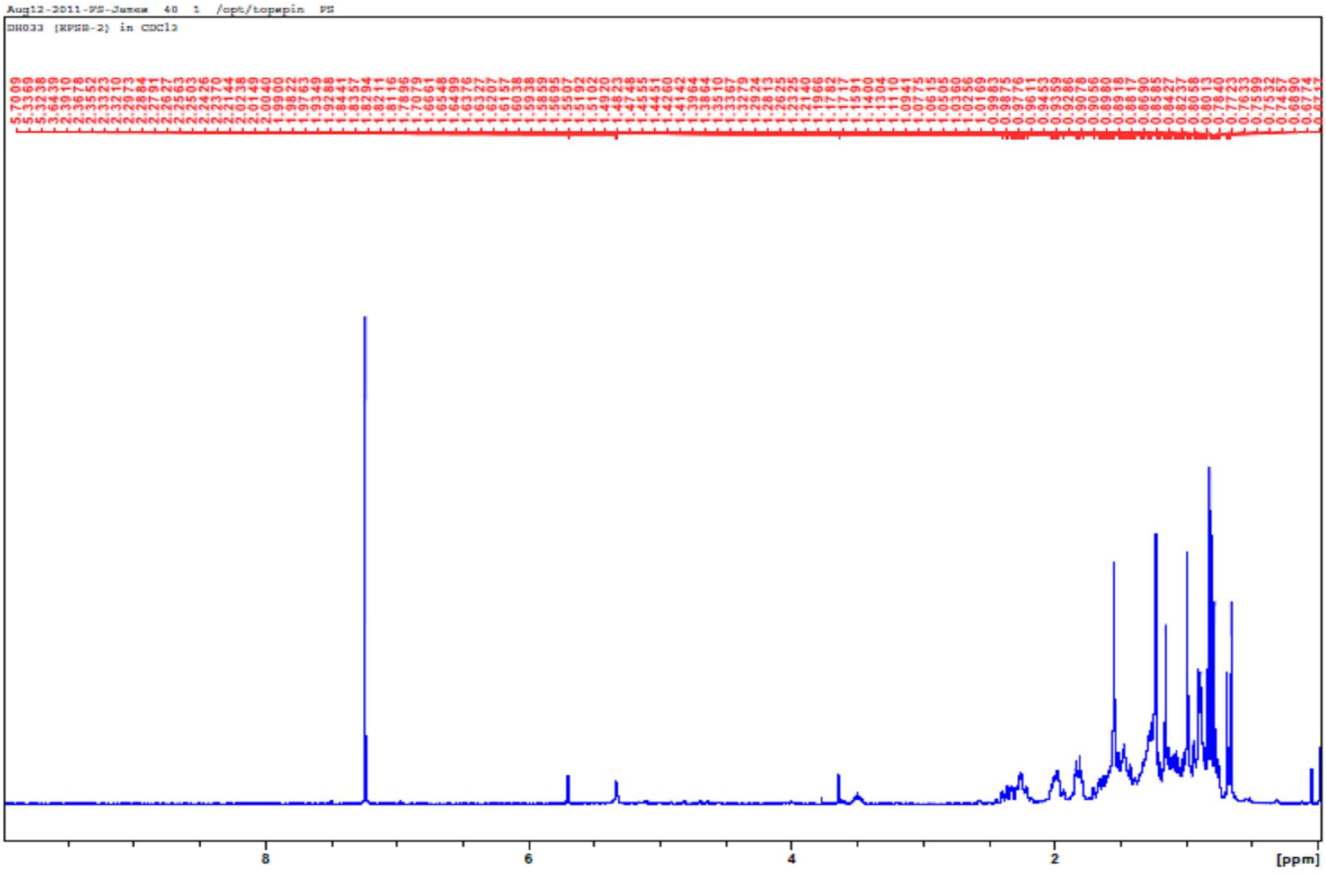


Figure 2: ^{1}H NMR spectral for compound EFSB

Table 4.28: 13C NMR spectra data of EFSB from experiment

Carbon	β-Sitosterol	B-Sitostenone
1	37.28	56.78
2	31.68	32.06
3	71.82	199.71
4	37.26	123.75
5	140.77	171.76
6	121.73	3297
7	31.92	35.70
8	31.92	35.70
9	56.78	56.78
10	36.52	37.26
11	21.09	21.03
12	37.26	37.26
13	56.78	56.78
14	56.78	56.78
15	24.31	24.19
16	28.26	24.18
17	56.78	56.78
18	11.87	11.99
19	19.41	19.03
20	36.15	36.12
21	19.04	18.71
22	33.96	33.96
23	29.16	29.16
24	56.78	56.78
25	26.08	26.08
26	18.79	17.40
27	19.82	19.82
28	23.06	23.06
29	11.87	12.0

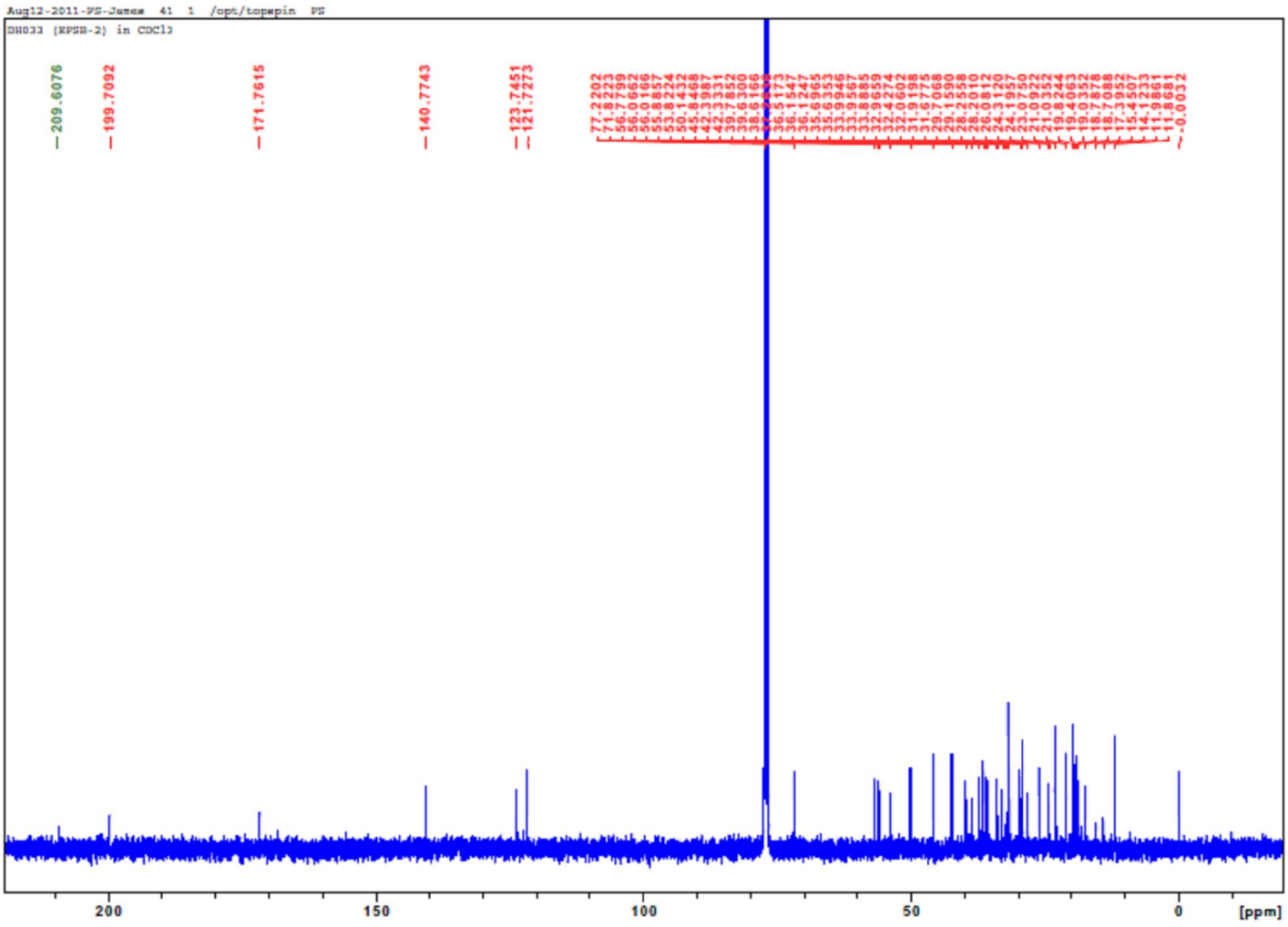


Figure 3: ^{13}C NMR spectral for compound EFSB

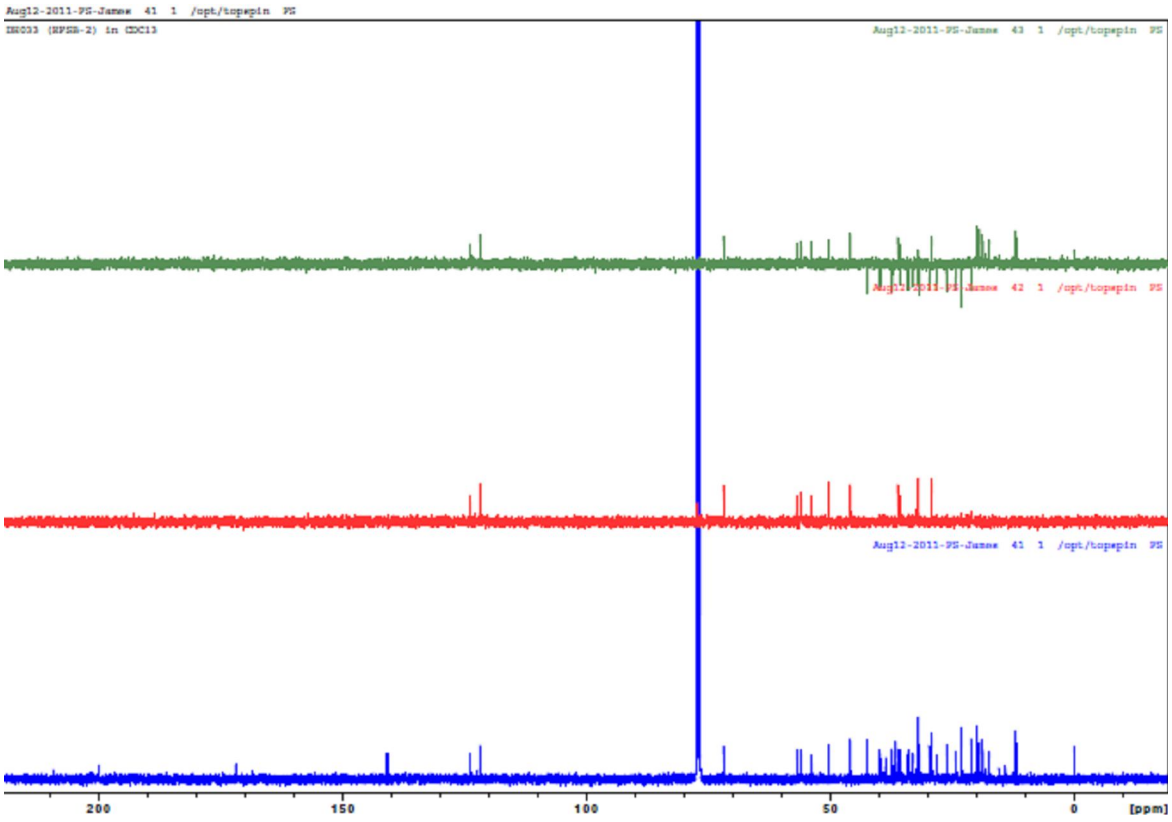


Figure 4: DEPT -135 experiment for EFSB

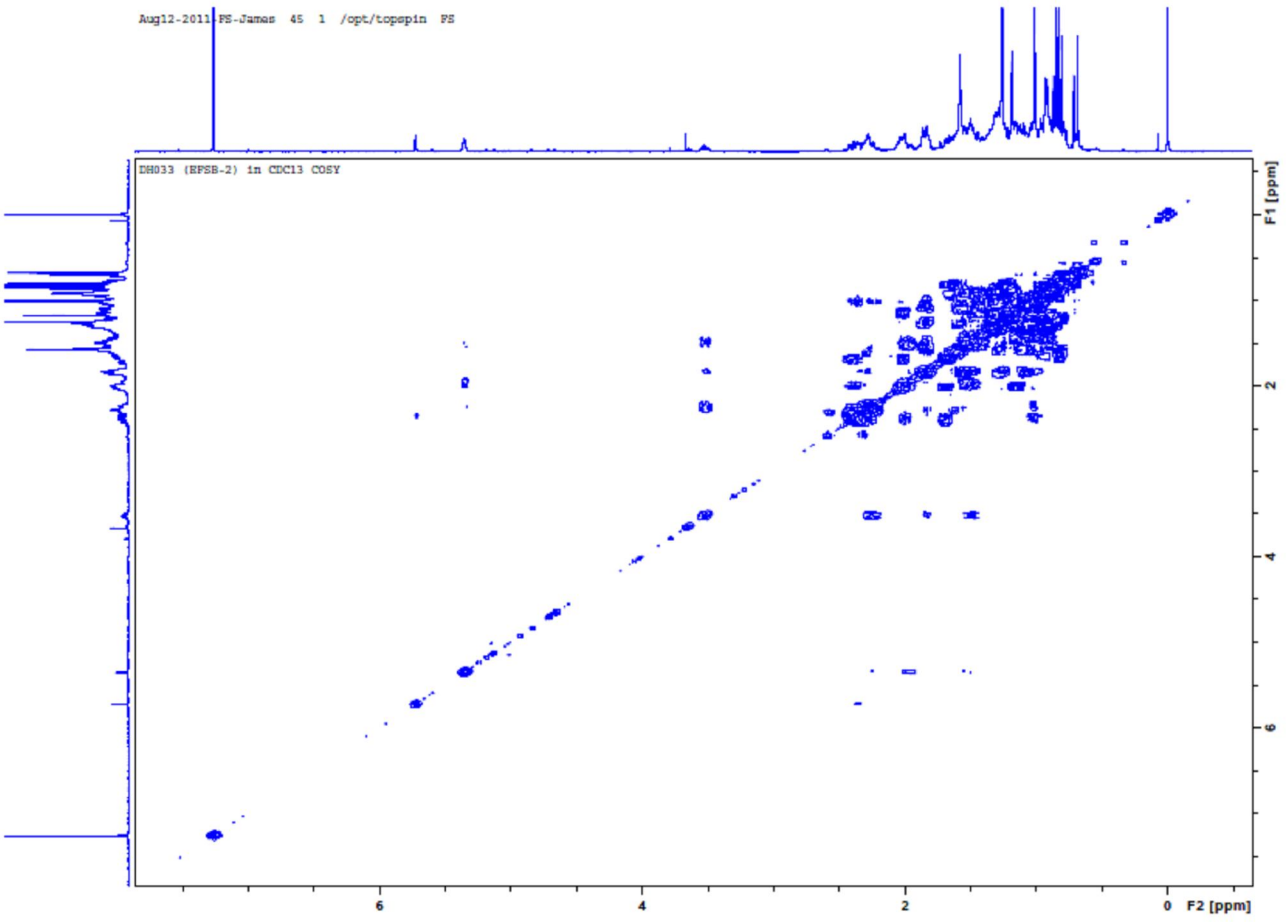


Figure 5: COSY experiment of EFSB

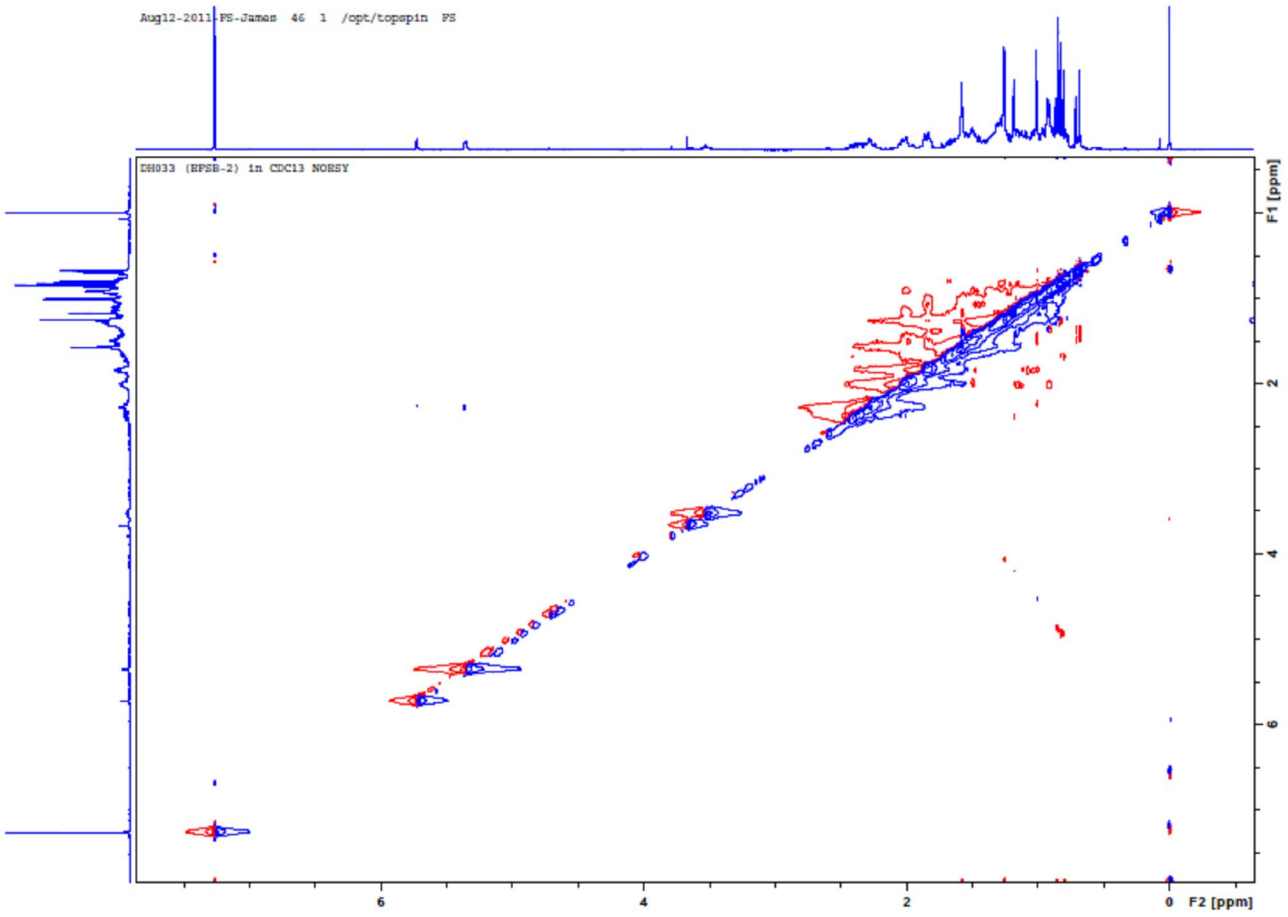


Figure 6: NOESY experiment of EFSB

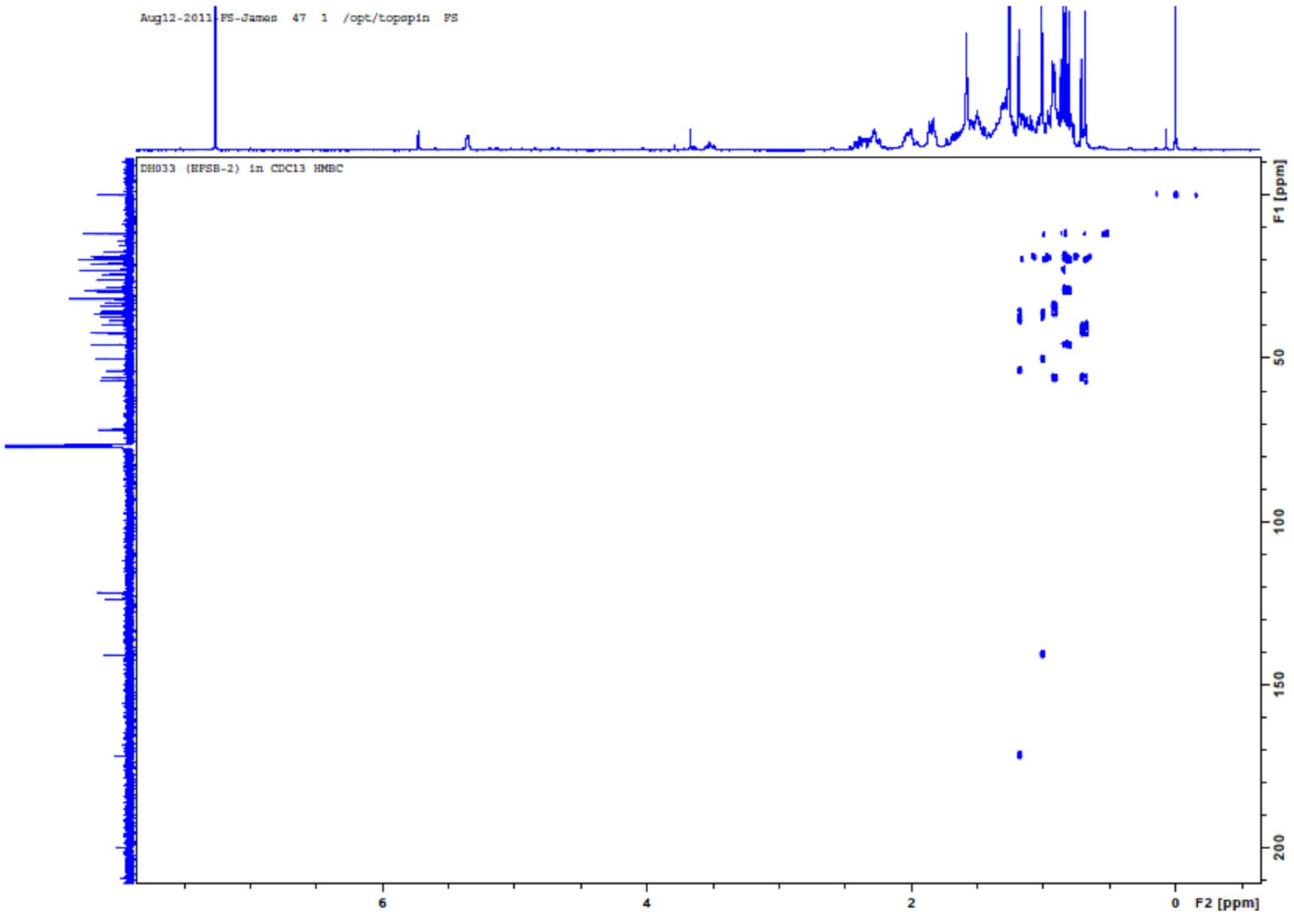
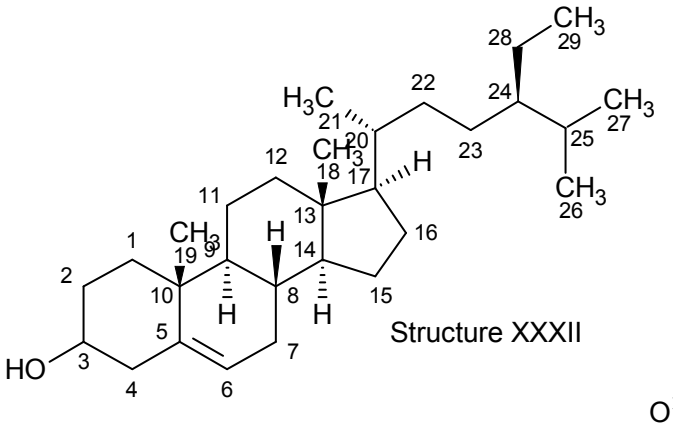
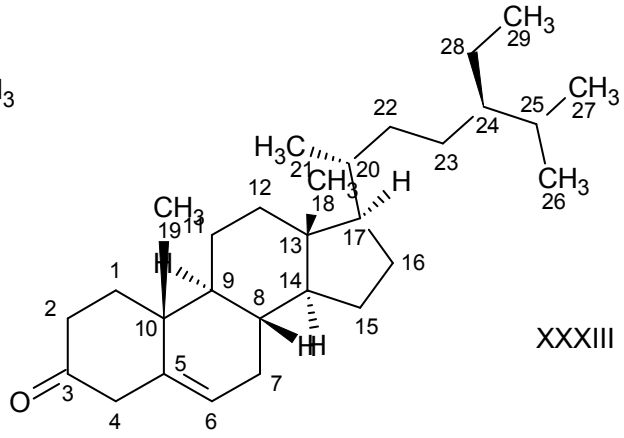


Figure 7: HMBC experiment of EFSB



Structure XXXII

Sitosterol



XXXIII

Sitostenone

Table 4.29: IR values for β-sitosterol and β-sitostenone

β-sitosterol	**β-sitostenone**
3428 (OH)	2930(C-H)
2930(C-H)	2871(C-H)
2852(C-H)	1686(C=O)
1657(C=C)	1620(C=C)
1465(CH_2)	1465(CH_2)

*Wen-Hsin Li *et al.,* 2008

Table 4.30: 13C NMR spectra data of β-sitosterol and β-sitostenone (δ ppm)

Carbon	β-sitosterol	β-sitostenone
1	37.24	56.61
2	31.65	32.04
3	71.80	199.71
4	42.29	123.71
5	140.75	171.78
6	121.71	32.94
7	31.90	33.87
8	31.90	35.70
9	50.12	53.80
10	36.49	38.59
11	21.07	21.01
12	39.76	39.61
13	42.31	42.37
14	56.76	56.0
15	24.29	24.17
16	28.24	28.18
17	56.05	55.86
18	11.97	12.0
19	19.39	19.01
20	36.14	36.10
21	19.02	18.68
22	33.93	33.96
23	29.14	29.13
24	45.82	45.81
25	26.06	26.05
26	18.79	17.34
27	19.81	19.80
28	23.06	23.05
29	11.85	12.0

*Wen-Hsin Li *et al*., 2008

4.4.2 Antimicrobial activities of EFSB

The antimicrobial activity experiment was carried out on EFSB to ascertain its biological activities. The well- in-plate diffusion method was used in the test. EFSB was found to be sensitive to *Staphylococcus aureus, Streptococcus Pyrogenes, Bacillus Subtilis, Escherichia Coli, Proteus Mirabilis, Proteus vulgaris, Salmonella Typhi, Shigellia Dysenteric, Candida albicans and Candida virusei and not sensitive to* MR*SA, Corynebacterium ulcerans, pseudomonas aureginosa* and *Candida tropicalis.* The zone of inhibition was between 20 to 25 mm (Table 4.31 and 4.32).

4.4.3 Minimum Inhibition Concentration of EFSB

The MIC was determined to ascertain whether the microorganism were only inhibited or exterminated. EFSB was found to exterminate *Staphylococcus aureus, Streptococcus pyrogenes, Bacillus subtilis, Escherichia coli, Proteus mirabilis, Proteus vulgaris, Salmonella Typhi, Shigellia dysenteric, Candida albicans and Candida virusei* at concentration of 12.5 µg/ml (Table 4.33).

4.4.4 Minimum Bactericidal Concentration (MBC)/Minimum Fungicidal Concentration (MFC) for EFSB

The MBC/MFC (Table 4.34) was found to be 50 µg/ml for *Staphylococcus aureus, Streptococcus pyrogenes, Escherichia coli, Proteus mirabilis, Proteus vulgaris, Salmonella Typhi, Shigellia dysenteric, Candida albicans and Candida virusei* with exception of *Bacillus subtilis* that was recorded at 25 µg/ml.

Comparing these biological activities values with those obtained for a standard drug (Sparfloxacin), EFSB was found to have a lower MIC value as compared to the drug. This showed that EFSB was comparable to this drug and had a wider broad spectrum of activities over the tested organism (Table 4.35).

Table 4.31: Antimicrobial activities of pure isolated compounds

Test Organism	SB-C1	SB-C2	EFSB	SPEC-1
MR*SA*	R	S	R	R
Staphylococcus aureus	S	S	S	S
Streptococcus pyrogenes	S	S	S	S
Bacillus subtilis	S	S	S	S
Corynebacterium ulcerans	S	R	R	R
Escherichia coli	R	S	S	S
Proteus mirabilis	R	S	S	S
Proteus vulgaris	S	S	S	S
Pseudomonas aureginosa	S	S	R	R
Salmonella typhi	S	S	S	S
Shigellia dysenteric	S	S	S	S
Candida albicans	S	S	S	S
Candida virusei	S	S	S	S
Candida tropicalis	R	S	R	S

Key: SB-C1= *Sarcocephalus latifolius* stem bark combination 1

SB-C 2= *Sarcocephalus latifolius* stem bark combination 2

EFSB=Methanol fraction *of Sarcocephalus latifolius* stem bark

SPEC-1=*Spillanthes filicaulis* pet-ether combination

Table 4.32: Zone of inhibitions (mm) of the pure isolated compounds on the test microorganism

Test Organism	SB-C1	SB-C2	EFSB	SPEC-1
MRS*A*	0	21	0	0
Staphylococcus aureus	20	27	21	21
Streptococcus pyrogenes	24	29	20	22
Bacillus subtilis	27	34	25	24
Corynebacterium ulcerans	22	0	0	0
Escherichia coli	0	27	20	21
Proteus mirabilis	0	24	21	20
Proteus vulgaris	23	27	20	21
Pseudomonas aureginosa	20	24	0	0
Salmonella typhi	27	32	23	21
Shigellia dysenteric	25	30	20	21
Candida albicans	20	24	20	21
Candida virusei	21	26	20	20
Candida tropicalis	0	21	0	20

Key: SB-C1= Sarcocephalus latifolius stem bark combination 1

SB-C 2= Sarcocephalus latifolius stem bark combination 2

EFSB= Methanol fraction of Sarcocephalus latifolius stem bark

SPEC-1= Spillanthes filicaulis pet-ether combination

Table 4.33: Minimum inhibition Concentrations (µg/ml) of EFSB on the test microbes

Test Organism	50	25.5	12.5	6.25	3.125
MR*SA*					
Staphylococcus aureus	_	_	**O***	+	++
Streptococcus pyrogenes	_	_	**O***	+	++
Bacillus subtilis	_	_	**O***	+	++
Corynebacterium ulcerans					
Escherichia coli	_	_	**O***	+	++
Proteus mirabilis	_	_	**O***	+	++
Proteus vulgaris	_	_	**O***	+	++
Pseudomonas aureginosa					
Salmonella typhi	_	_	**O***	+	++
Shigellia dysenteric	_	_	**O***	+	++
Candida albicans	_	_	**O***	+	++
Candida virusei	_	_	**O***	+	++
Candida tropicalis					

Key: **-** *=No colony growth,* ***O**** *=MIC,* **+** *=light growth,* **++** *= Moderate colonies growth*

Table 4.34: Minimum Bactericidal/Fungicidal Concentrations (µg/ml) of EFSB on the test microbes

Test Organism	50	25.5	12.5	6.25	3.125
MR*SA*					
Staphylococcus aureus	**O***	+	++	+++	++++
Streptococcus pyrogenes	**O***	+	++	+++	++++
Bacillus subtilis	_	**O***	+	++	+++
Corynebacterium ulcerans					
Escherichia coli	**O***	+	++	+++	++++
Proteus mirabilis	**O***	+	++	+++	++++
Proteus vulgaris	**O***	+	++	+++	++++
Pseudomonas aureginosa					
Salmonella typhi	**O***	+	++	+++	++++
Shigellia dysenteric	**O***	+	++	+++	++++
Candida albicans	**O***	+	++	+++	++++
Candida virusei	**O***	+	++	+++	++++
Candida tropicalis					

Key: **-** *=No colony growth,* ***O**** *=MBC/MFC,* **+** *= scanty colonies,* **++** *= Moderate colonies growth,* **+++***= Heavy colonies growth*

Table 4.35: Minimum Inhibition Concentrations (µg/ml) of Sparfloxacin on the test microbes

Test Organism	50	25.5	12.5	6.25	3.125
MRS*A*	_	**O***	+	++	+++
Staphylococcus aureus	_	_	**O***	+	++
Streptococcus pyrogenes	_	_	**O***	+	++
Bacillus subtilis	_	_	**O***	+	++
Corynebacterium ulcerans	_*	**O***	+	++	+++
Escherichia coli	_	**O***	+	++	+++
Proteus mirabilis	_	**O***	+	++	+++
Proteus vulgaris	_	**O***	+	++	+++
Pseudomonas aureginosa	_	_	_	_	_
Salmonella typhi	_	**O***	+	++	+++
Shigellia dysenteric	_	_	**O***	+	++
Candida albicans	□	□	□	□	□
Candida virusei	□	□	□	□	□
Candida tropicalis	□	□	□	□	□

Key: **-** *=No colony growth,* ***O**** *=MIC,* **+** *= scanty colonies growth,* **++** *= Moderate colonies growth,* **+++** *= Heavy colonies growth*

4.4.5 SB-C1 spectral data

SB-C1 was isolated as colorless crystalline needle shape compound. The IR cm^{-1} spectrum showed absorption peaks at 3414.36 cm^{-1} which was characteristic of OH stretching. The peak at 2930.75 was attributed to CH_2; likewise the value at 2851.66 was typical of CH stretching. 1709.29 was typical peak for C=C bond. The peak at 1053 could be due to the presence of a cycloalkane (Figure 8).

The ^{1}H NMR of SB-C1 (Figure 9) revealed signals for two methyl at δ0.67 , δ 1.01 and three secondary methyl at δ0.85, δ 0.82 and δ 0.80.

The ^{13}C NMR (Figure 10) showed recognizable absorption peaks at 140.8 and 121.7 which are characteristic signal for a double bond respectively. The value at 71.8 was a typical characteristic signal for a beta-hydroxyl functional group. In addition to these values, twenty nine carbon signals was observed including six methyl, nine methylenes, eleven methane and three quaternary carbons from the Dept experiment (Fig 11). Base on these data coupled with comparison made with data obtained from literature (Agrawal *et al*; 1985, Pretsch *et al*; 2000 and Arjun patra *et al*; 2010 (Table 4.36). The compound was proposed to be β-sitosterol (Structure XXXIV). Other spectra obtained for this compound are as contained in Figures 12, 13, 14 and 15.

Figure 8: Infrared spectra of experiment of SB-C1

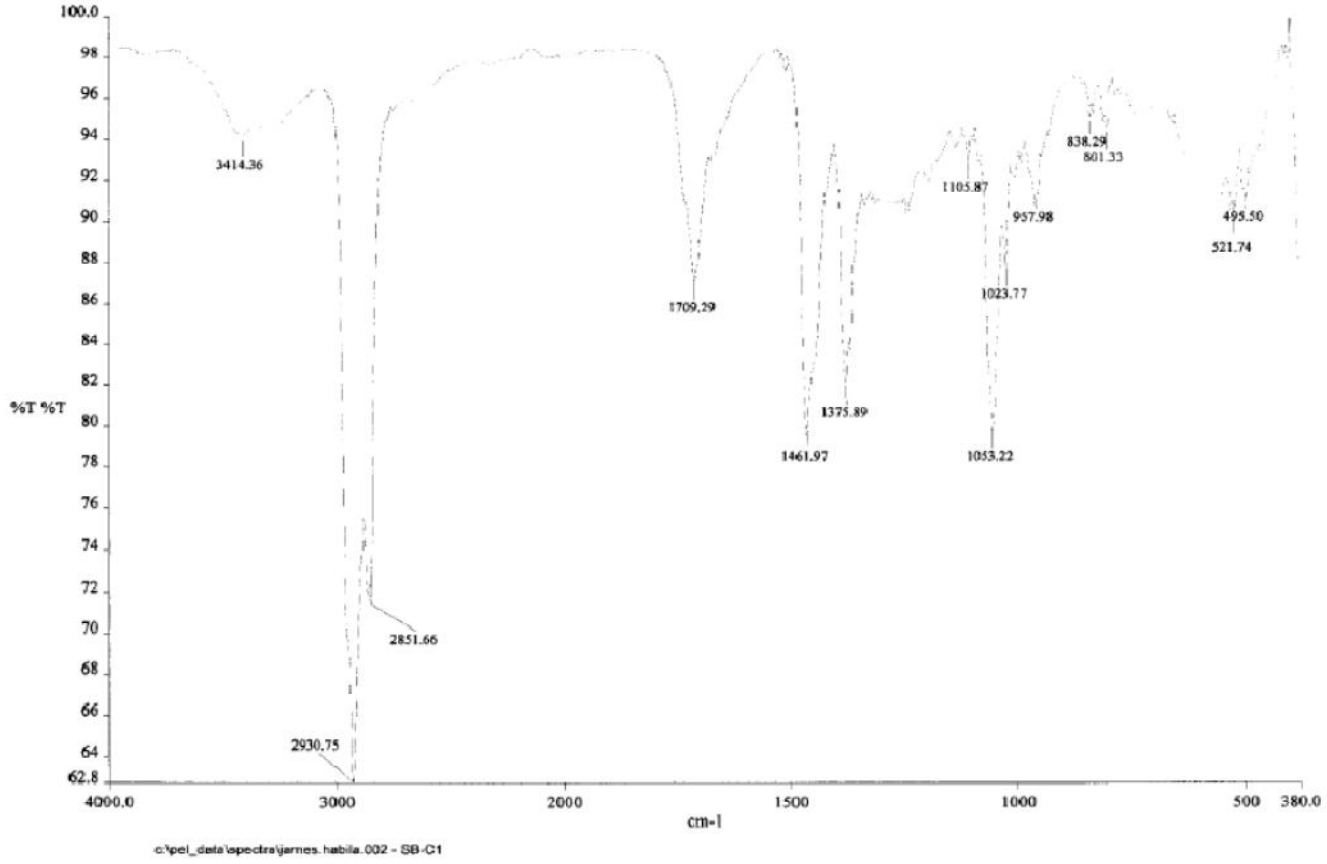


Figure 9: ^{1}H NMR Spectra of SB-C1

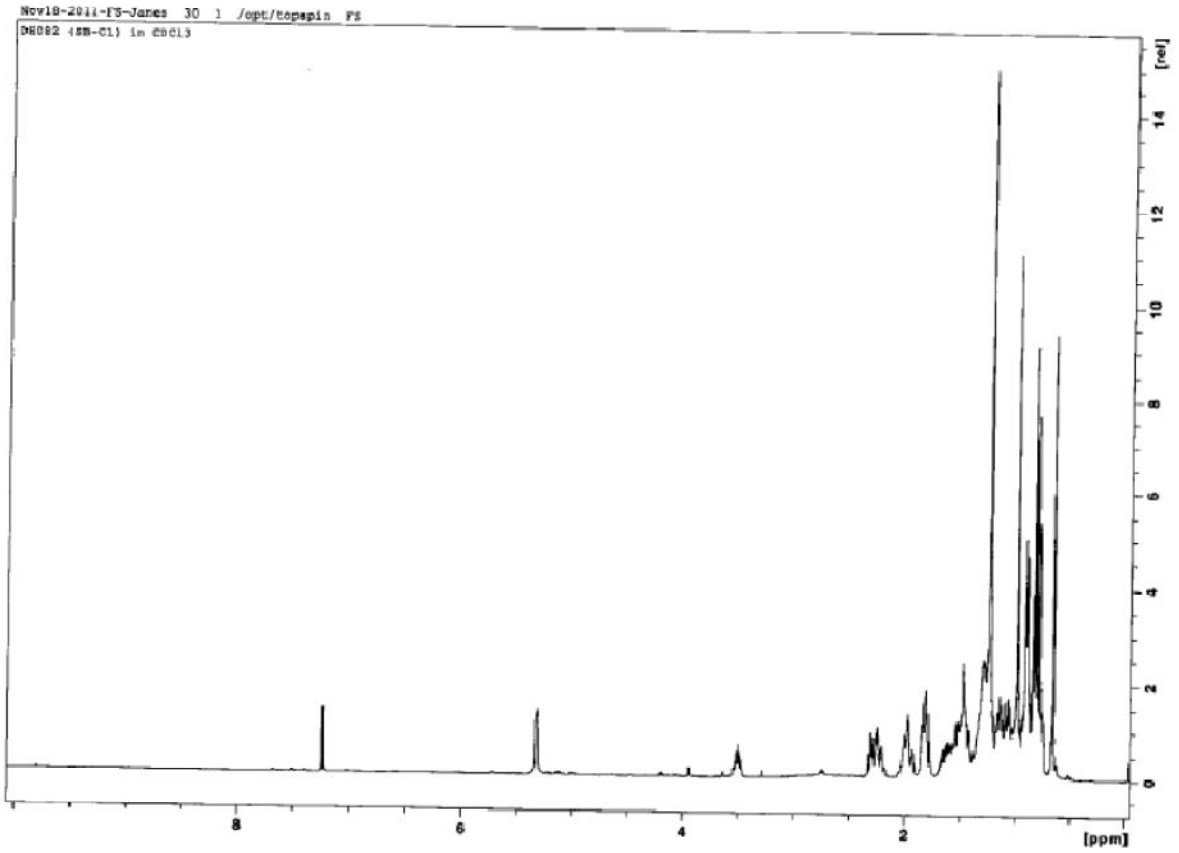


Figure 10: ^{13}C NMR Spectra of SB-C1

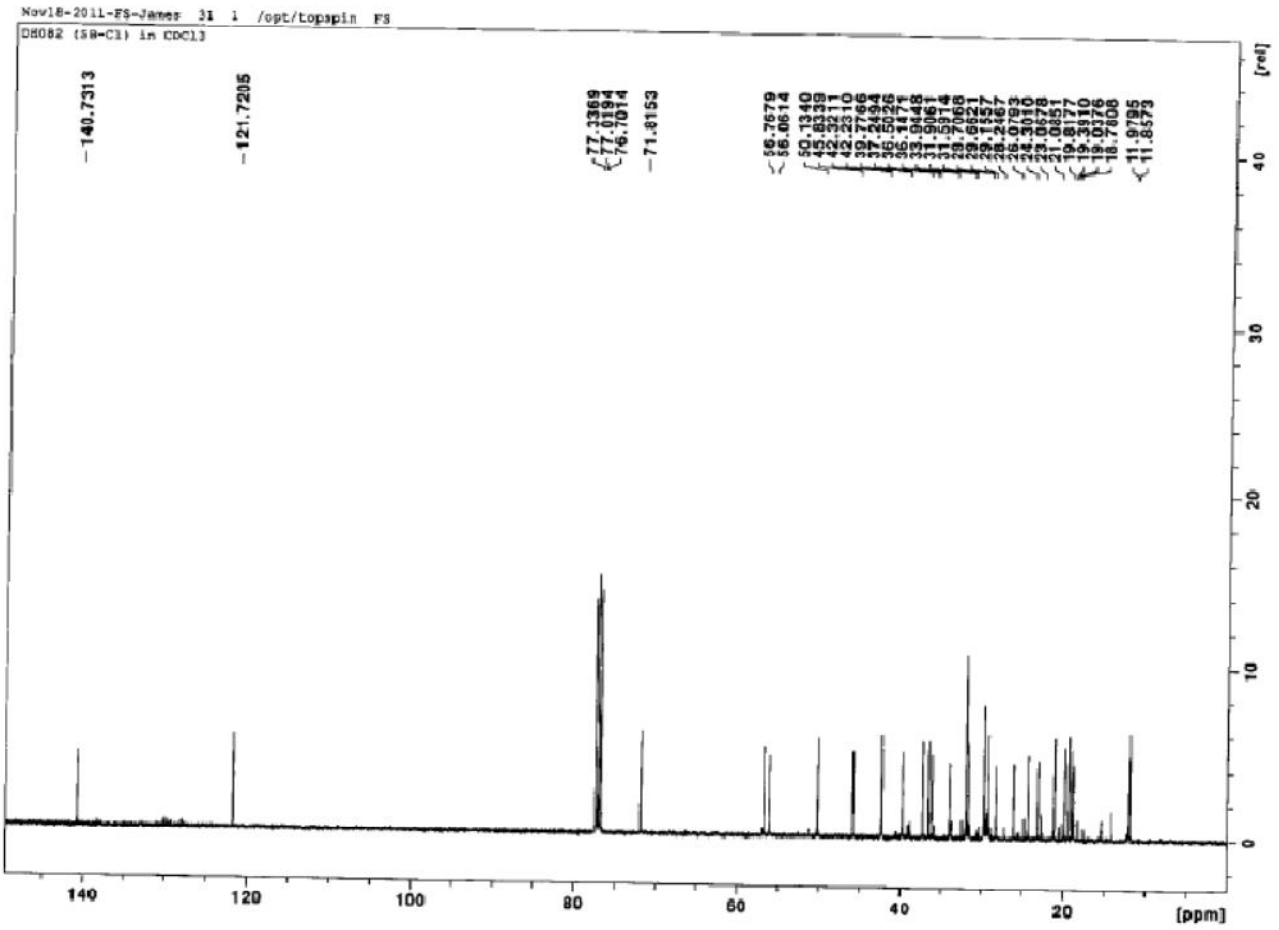


Figure 11: DEPT-135 Spectra of SB-C1

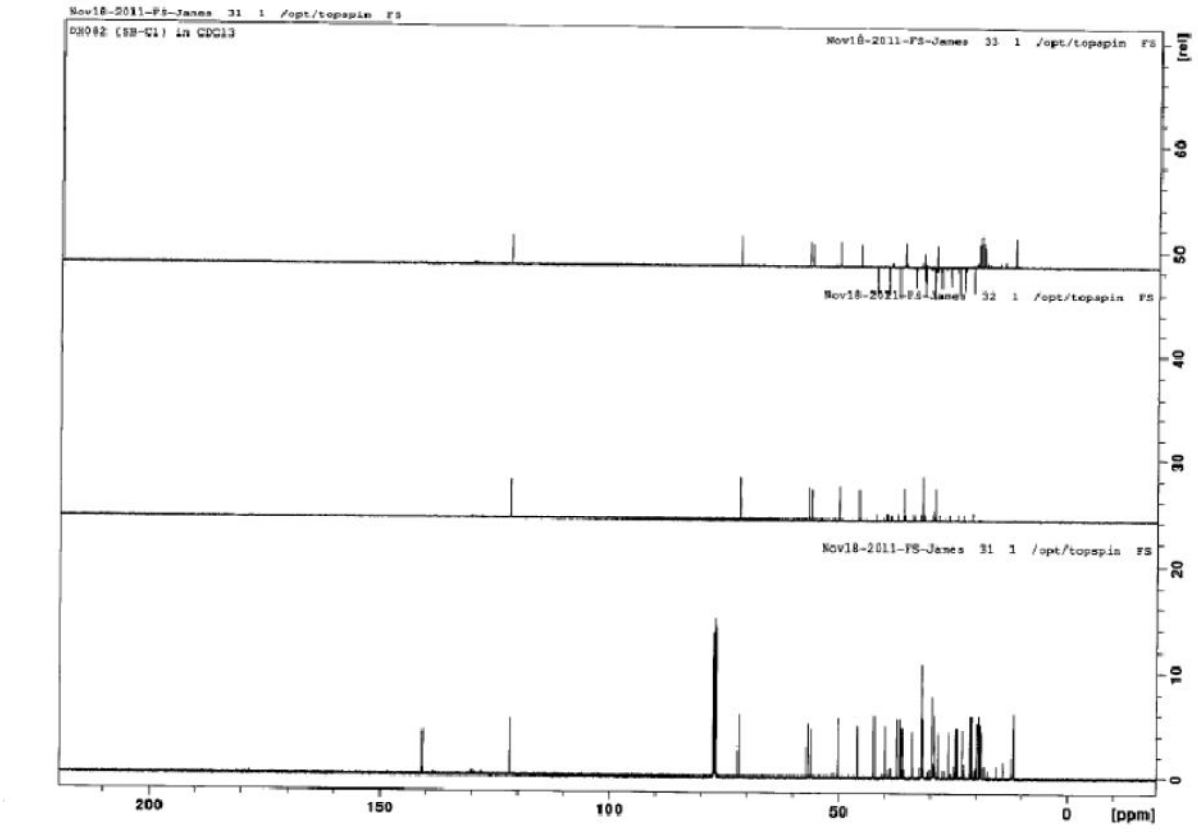


Figure 12: COSY Spectra of SB-C1

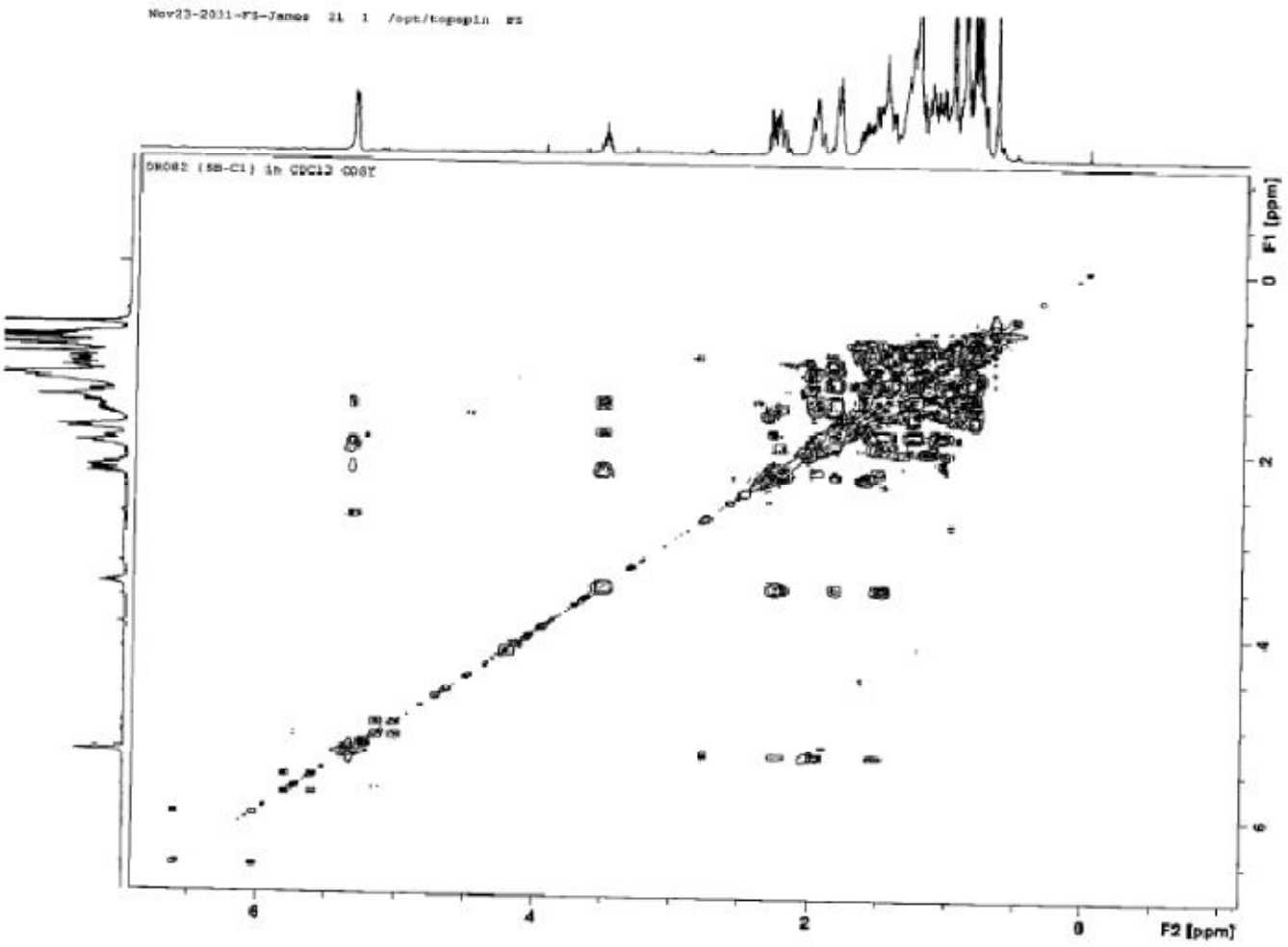
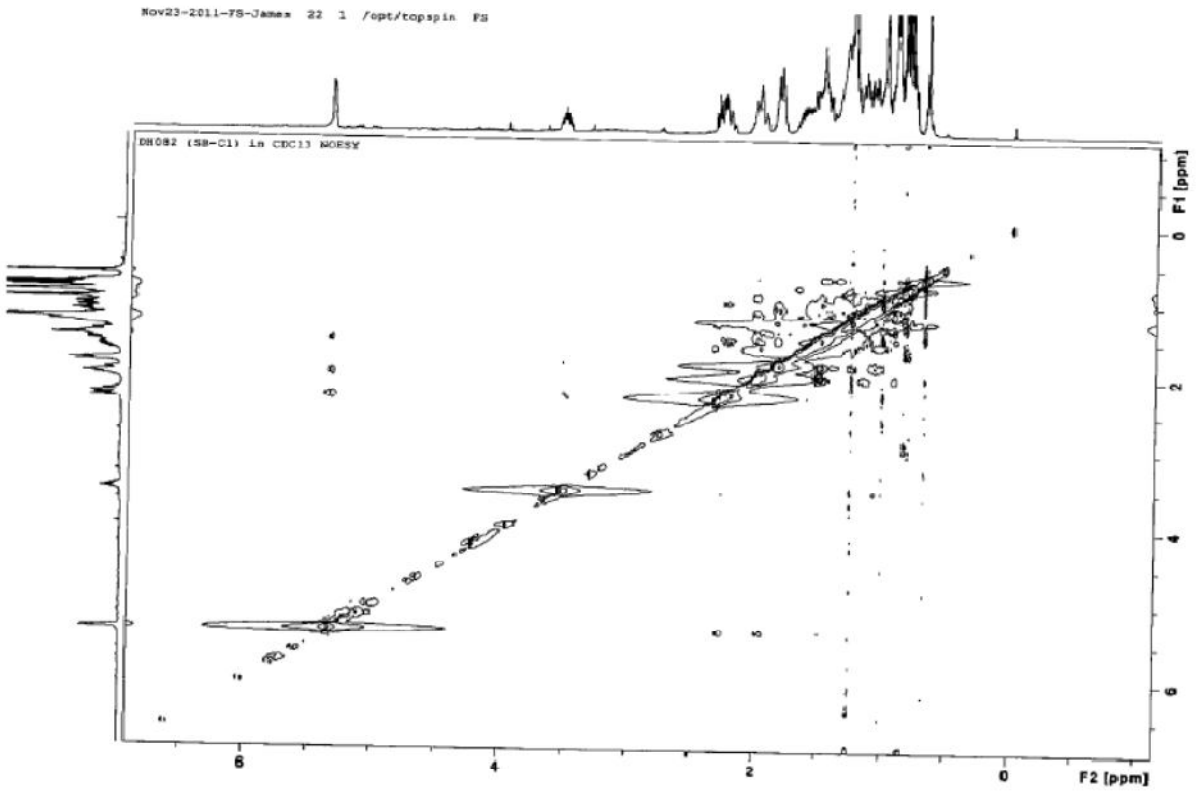


Figure 13: NOESY Spectra of SB-C1



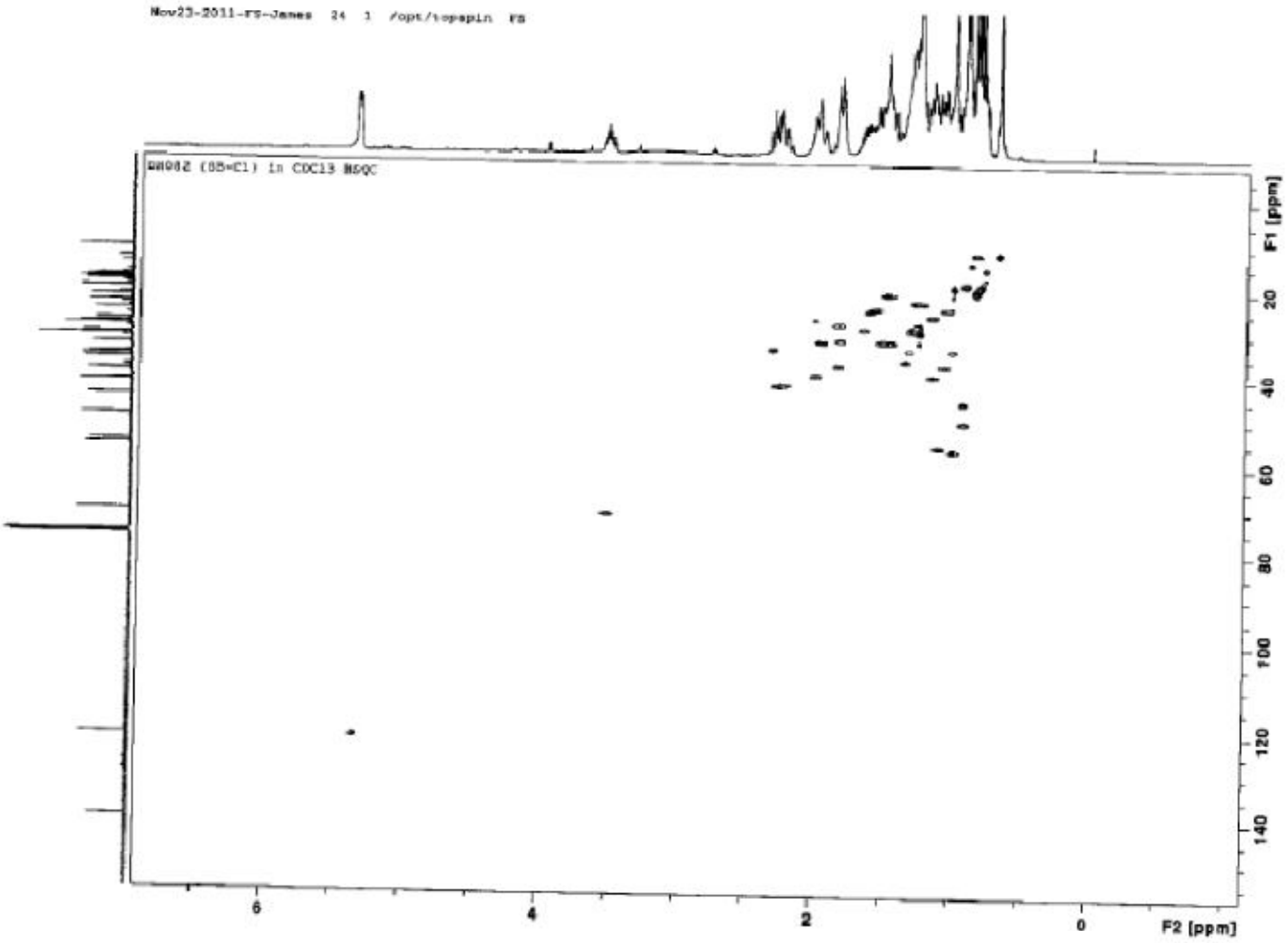


Figure 14: HSQC Spectra of SB-C1

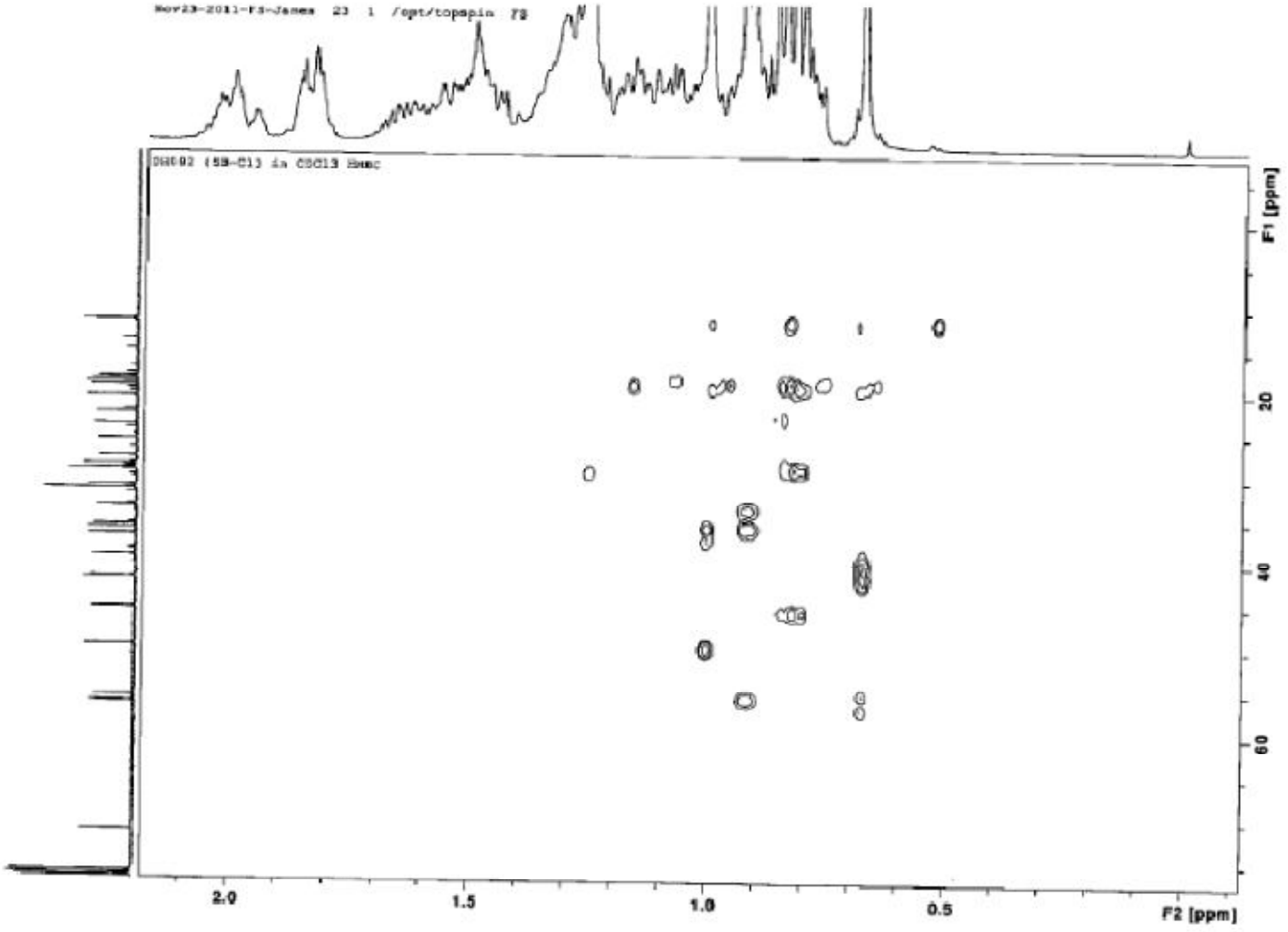
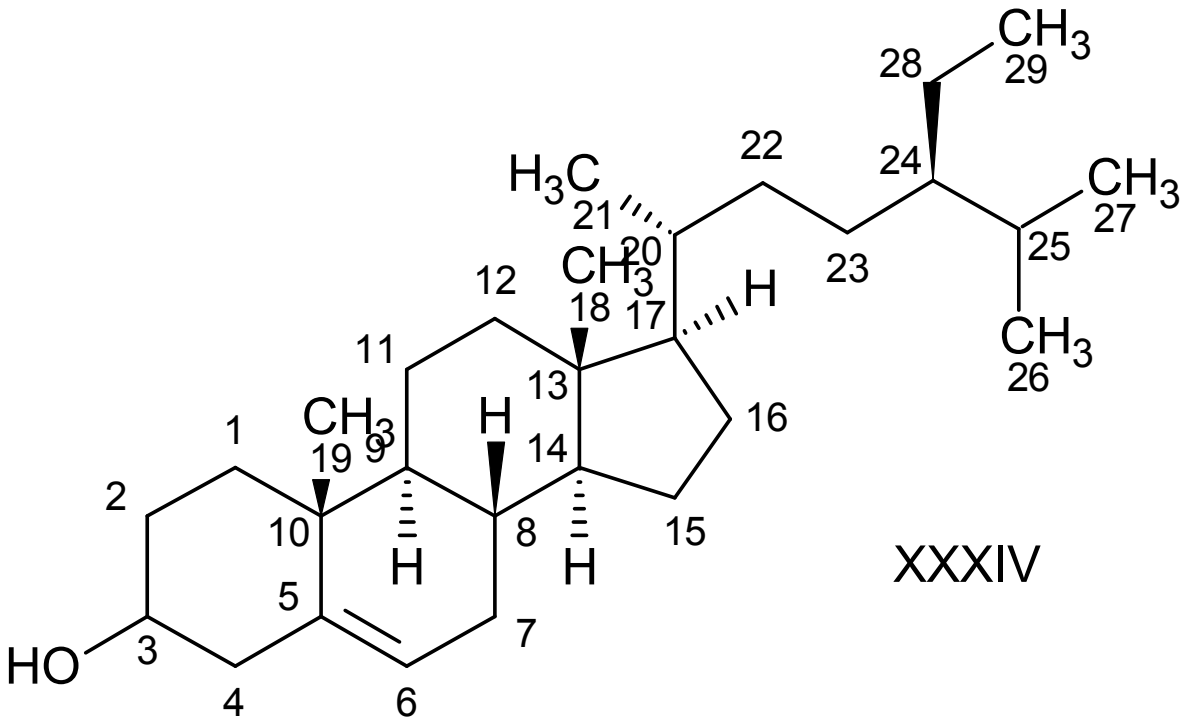


Figure 15: HMBC Spectra of SB-C1

Table: 4.36: ^{13}C NMR Assignment of SB-C1 (β-Sitosterol)

Carbon position	*Lit. value	Exp. Value	CHn
1	37.28	37.25	CH_2
2	31.69	31.59	CH_2
3	71.82	71.82	CH
4	42.33	42.23	CH_2
5	140.70	140.73	C
6	121.72	121.72	CH
7	31.69	31.59	CH_2
8	31.93	31.91	CH
9	50.17	50.13	CH
10	36.52	36.50	C
11	21.10	21.09	CH_2
12	39.80	39.77	CH_2
13	42.33	42.32	C
14	56.79	56.77	CH
15	24.37	24.30	CH_2
16	28.25	28.25	CH_2
17	56.09	56.06	CH
18	11.86	11.86	CH_3
19	19.40	19.40	CH_3
20	36.52	36.14	CH
21	18.79	19.03	CH_3
22	33.98	33.94	CH_2
23	26.14	26.08	CH_3
24	45.88	45.83	CH
25	28.91	29.70	CH
26	19.80	19.82	CH_3
27	18.79	18.78	CH_3
28	23.10	23.07	CH_2
29	11.99	11.98	CH_3

***Arjun Patra *et al* (2010)**


CH3
28
29
H3C
22
24
CH3
21
27
20
25
CH3
12
18
17
H
23
11
CH3
26
13
1
CH3
H
16
19
9
14
2
10
8
15
H
H
XXXIV
5
7
HO
3
4
6

Sitosterol

4.4.6 Antimicrobial activities of SB-C1

The antimicrobial activities of SB-C1 were carried out in order to know if it had biological activities. The well-in-plate diffusion method was used in the test. It was observed that SB-C1 was active on *Staphylococcus aureus, Streptococcus pyrogenes, Bacillus subtilis, Corynebacterium ulcerans, vulgaris, Pseudomonas aureginosa, Salmonella typhi, Shigellia dysenteric, Candida albicans, and Candida virusei* and not active on Methycilin Resistant *staphylococcus aureus, Escherichia coli, Proteus mirabilis* and *Candida tropicalis* (Table 4.31). The zone of inhibition was between 20 to 27 mm, with *Bacillus subtilis* and *Salmonella typhi* to be the most sensitive organism with the highest zones of inhibition of 27 mm (Table 4.32).

4.4.7 Minimum Inhibition Concentration (MIC) of SB-C1

The MIC was determined to ascertain the lowest concentration level at which this compound could inhibit the growth of these organisms. SB-C1 was found to inhibit *Staphylococcus aureus, Streptococcus pyrogenes, Bacillus subtilis, Corynebacterium ulcerans, vulgaris, Pseudomonas aureginosa, Salmonella typhi, Shigellia dysenteric, Candida albicans and Candida virusei at 12.5* µg/ml (Table 4.37). This result confirmed the activities of the compound earlier determined.

4.4.8 Minimum Bacterial Concentration (MBC) / Minimum Fungicidal Concentration (MFC) of SB-C1

The result from the MBC/MFC (Table 4.38) showed that *Bacillus subtilis, Salmonella typhi* and *Shigellia dysenteric* were completely exterminated at a concentration of 25

µg/ml, while *Staphylococcus aureus, Streptococcus pyrogene, Corynebacterium ulcerans, Proteus vulgaris, Proteus aureginosa, Candida albicans* and *Candida virusei* were exterminated at 50 µg/ml.

This result was compared to a standard drug (sparfloxacin) (Table 4.35) and was discovered that SB-C1 could be compared with the standard drug. This was because they both exterminated *Bacillus subtilis, Salmonella typhi* and *Shigellia dysenteric* at 25 µg/ml showing that they could be used to treat such related ailment that could be caused by these micro organisms.

The anticarcinogenic property of β-sitosterol in DMH-induced colon carcinogenesis has been studied to be due to its antioxidant property and its ability to suppress the altered β-catenin and PCNA expression in colonic mucosa of DMH-treated rats in short-term colon carcinogenesis. Hence the study suggested that β-sitosterol exerts a chemo preventive effect in DMH-induced experimental carcinogenesis, indicating its potential as an anticancer drug. (Albert *et al*; 2010)

Table 4.37: Minimum Inhibition Concentrations (µg/ml) of SB-C1 on the test microbes

Test Organism	50	25.5	12.5	6.25	3.125
MR*SA*					
Staphylococcus aureus	_	_	**O***	+	++
Streptococcus pyrogenes	_	_	**O***	+	++
Bacillus subtilis	_	_	**O***	+	++
Corynebacterium ulcerans	_	_	**O***	+	++
Escherichia coli					
Proteus mirabilis					
Proteus vulgaris	_	_	**O***	+	++
Pseudomonas aureginosa	_	_	**O***	+	
Salmonella typhi	_	_	**O***	+	++
Shigellia dysenteric	_	_	**O***	+	++
Candida albicans	_	_	**O***	+	++
Candida virusei	_	_	**O***	+	++
Candida tropicalis					

Key: **-** *=No colony growth,* ***O**** *=MIC,* **+** *=light growth,* **++** *= Moderate colonies growth*

Table 4.38: Minimum bactericidal/fungicidal Concentrations (µg/ml) of SB-C1 on the test microbes

Test Organism	50	25.5	12.5	6.25	3.125
MR*SA*					
Staphylococcus aureus	**O***	+	++	+++	+++
Streptococcus pyrogenes	**O***	+	++	+++	+++
Bacillus subtilis	_	**O***	++	+++	+++
Corynebacterium ulcerans	**O***	+	++	+++	+++
Escherichia coli					
Proteus mirabilis					
Proteus vulgaris	**O***	+	++	+++	+++
Pseudomonas aeruginosa	**O***	+	++	+++	+++
Salmonella typhi	_	**O***	++	+++	+++
Shigellia dysenteric	_	**O***	++	+++	+++
Candida albicans	**O***	+	++	+++	+++
Candida virusei	**O***	+	++	+++	+++
Candida tropicalis					

Key: **-** *=No colony growth,* ***O**** *=MBC/MFC,* **+** *= scanty colonies growth,* **++** *= Moderate colonies growth,* **+++** *= Heavy colonies growth*

4.4.9 SB-C2 spectral data

The IR (cm^{-1}) Spectrum (Figure 16) result showed main absorption band at 2931.42 cm^{-1} which might be characteristic OH stretching in an alcohol, while a strong absorption peak observed at 1713.93 cm^{-1} was typical of carbonyl functional group (C=O) of the carboxylic acid. While signal at 521.91 was typical for C=C olefinic.

The ^{1}H NMR (Figure 17) spectra revealed signals at δ4.58 and δ3.93 which appeared as one proton doublet indicating an exomethylene group. While those signals observed at δ3.16 and δ2.98 are attributed to secondary carbinol. Also, the broad spectrum at δ 1.63 was an indicative of a vinyl methyl group. The resonance signals at δ 0.66, 0.73, 0.90, 0.94 and 0.99 are for five tertiary methyl groups (Shumaia; 2011)

The ^{13}C NMR spectrum (Figure 18) showed thirty (30) major recognizable signals in a broadband decoupled spectrum. The peaks at δ 27.99 , 15.35 , 16.02 , 16.13 , 14.69 , 19.37 are attributed to six methyl group and peaks at 150.41 , 109.70 are typical for an exomethylene groups and the peak observed at 79.01 was characteristic of a secondary hydroxyl bearing carbon while the peak at 179,20 could be due to a carbonyl functional group (Table: 4.39). In addition twelve methylene, six methane and six quaternary carbon atoms were observed from the DEPT experiment (Figure 19).

It was observed from literature that, the chemical shifts at δ150.41, 179.20 and 109.70 were characteristic peaks for Betunilic type of skeleton respectively (Ahmad, 1994). Based on these data with other 2D NMR i.e COSY, NOESY,HSQC and HMBC (Figure 20, 21, 22 and 23) coupled with literature ([a]Mahato and Kundu 1994, Sharma *et al; 2010*) the structure of the compound was proposed to be betunilic acid (Structure XXXV)

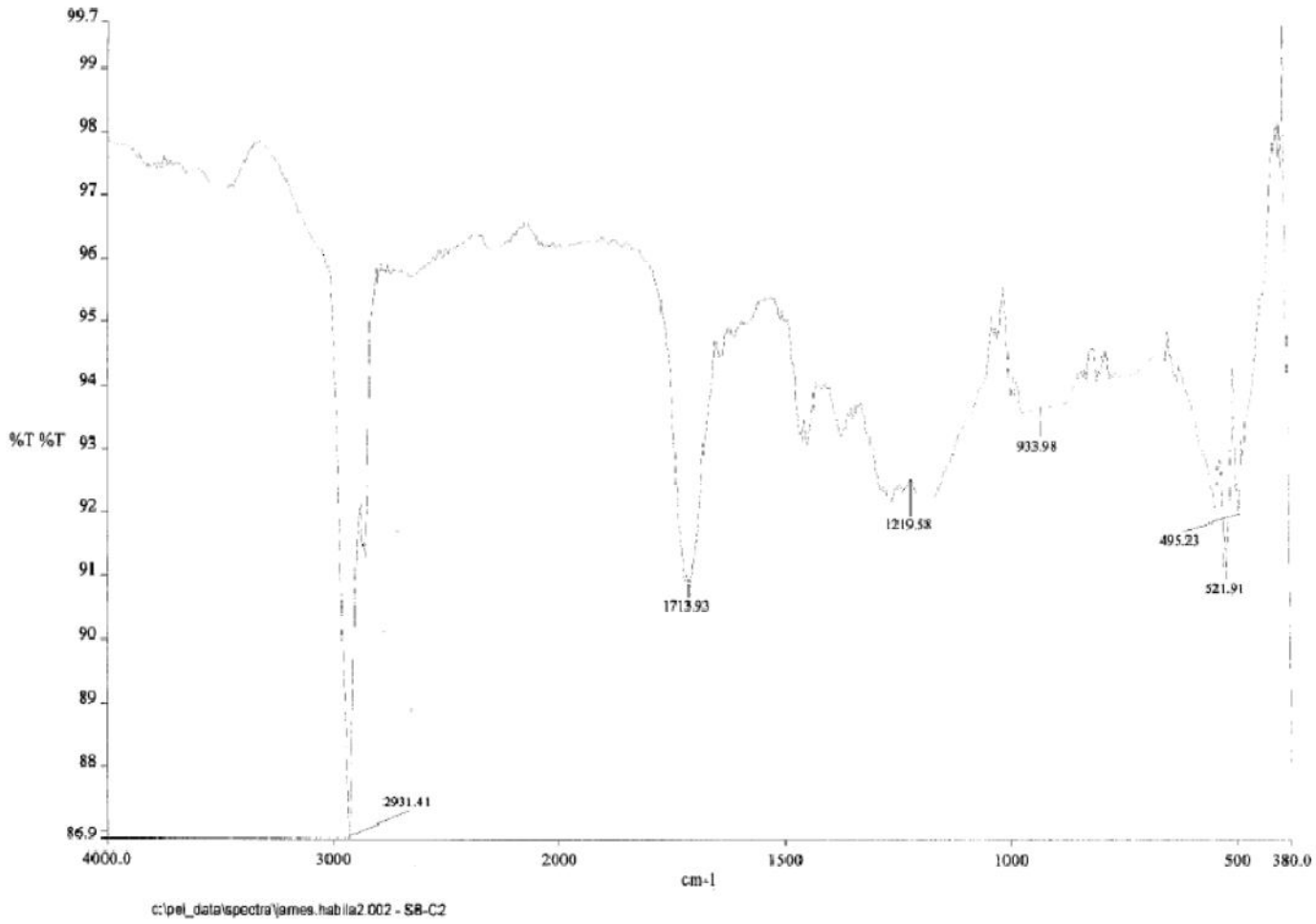


Figure 16: Infrared spectra of SB-C2

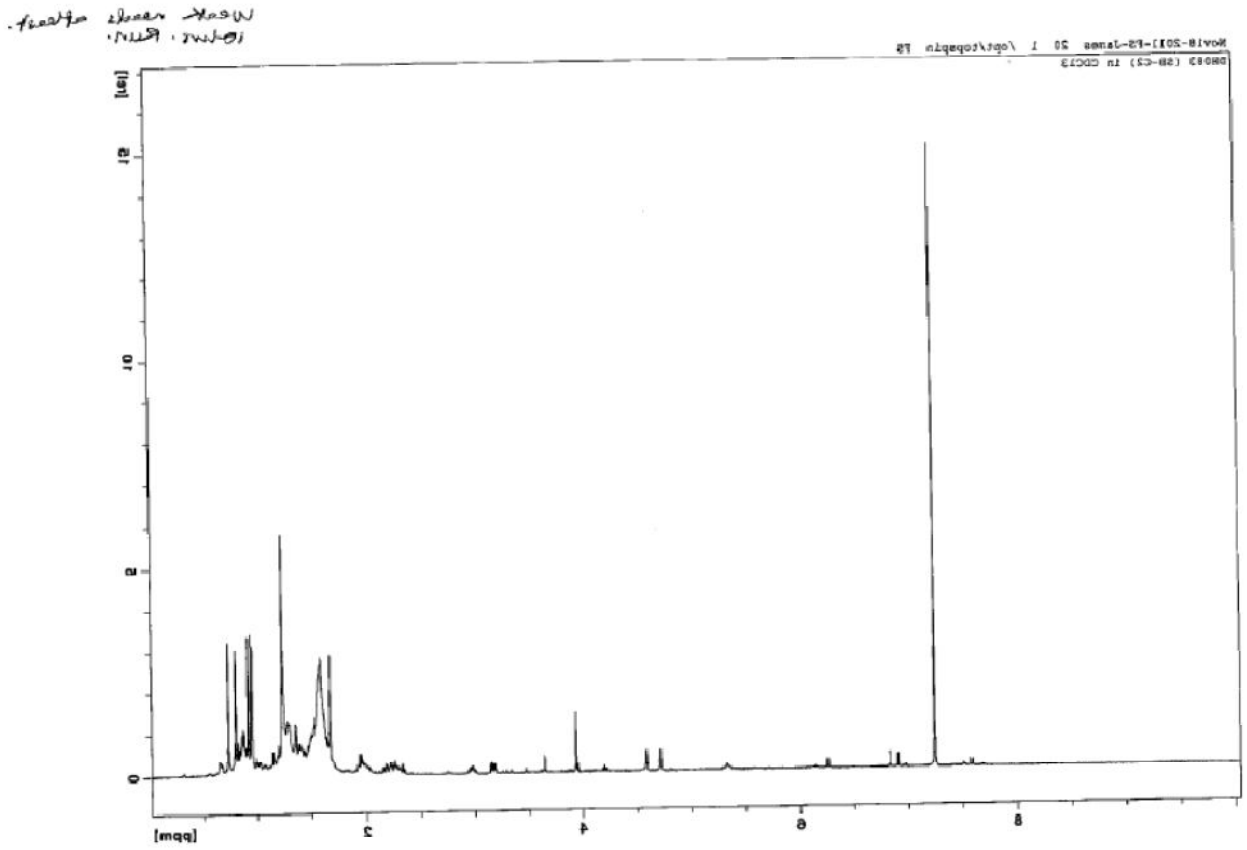


Figure 17: ^{1}H NMR spectra of SB-C2

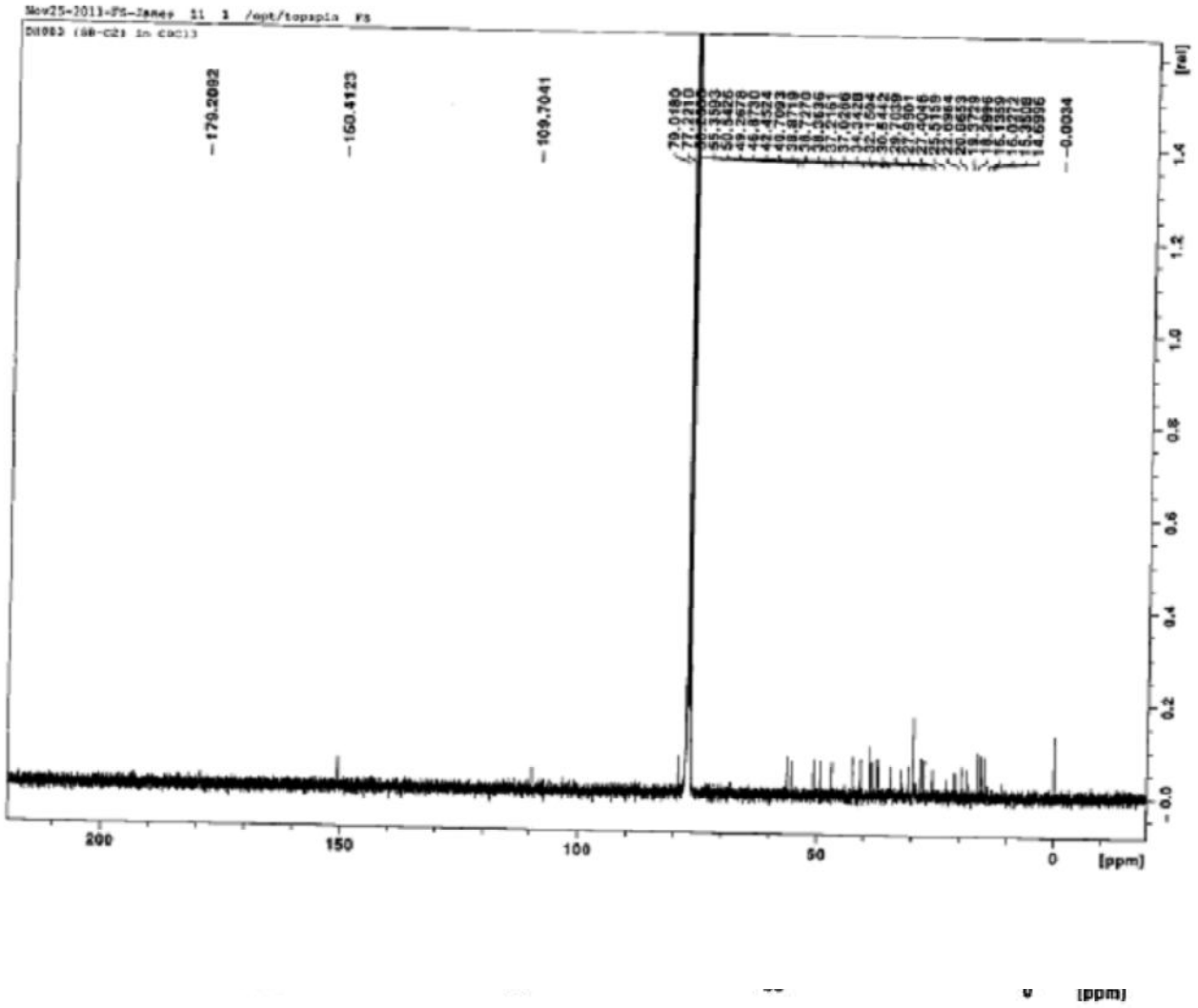


Figure 18: ^{13}C NMR spectra of SB-C2

Figure 19: DEPT-135 spectra of SB-C2

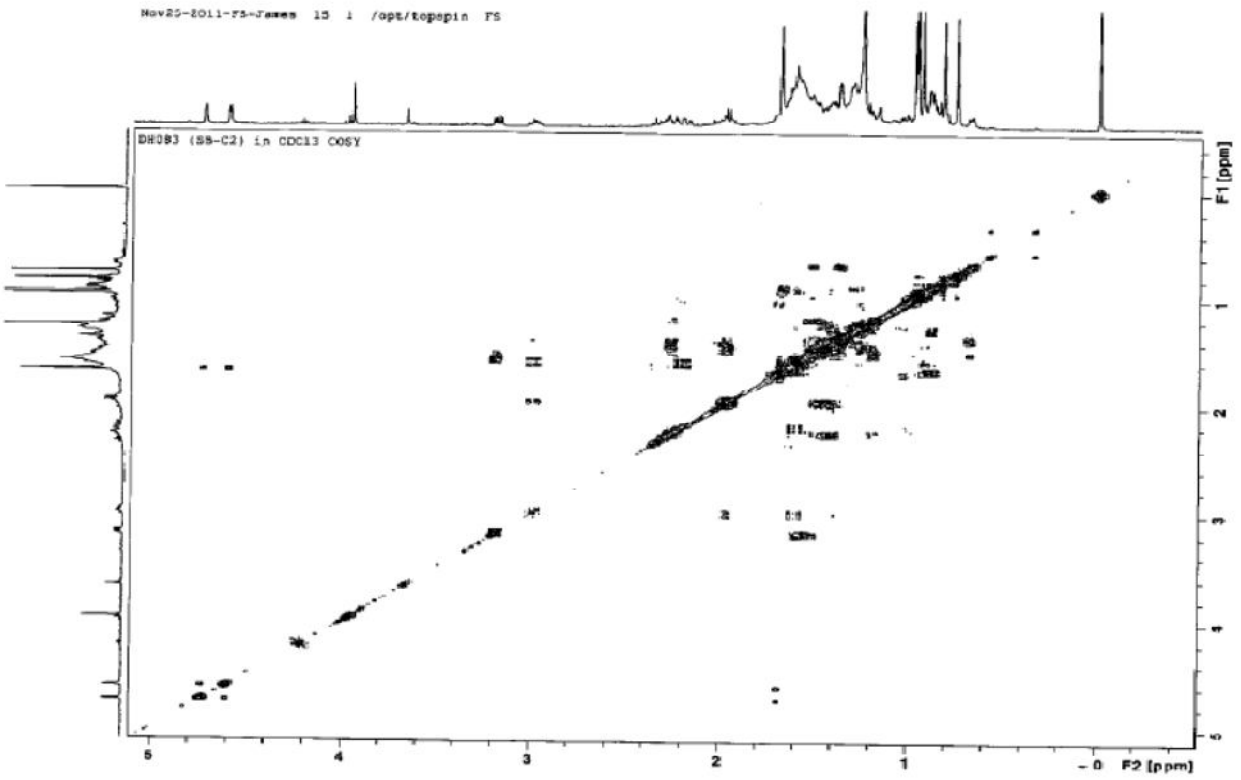


Figure 20: COSY spectra of SB-C2

Figure 21: NOESY spectra of SB-C2

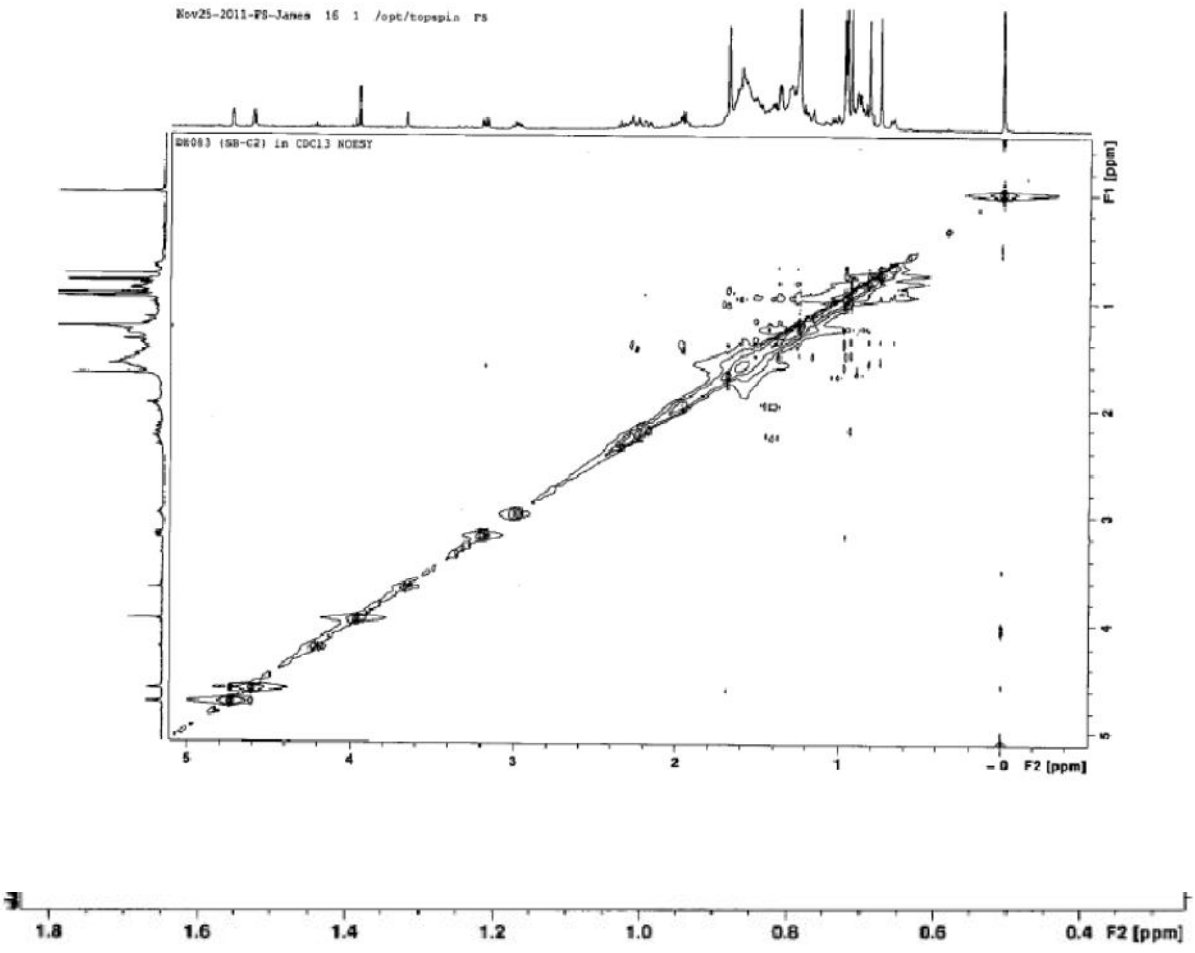


Figure 22: HSQC spectra of SB-C2

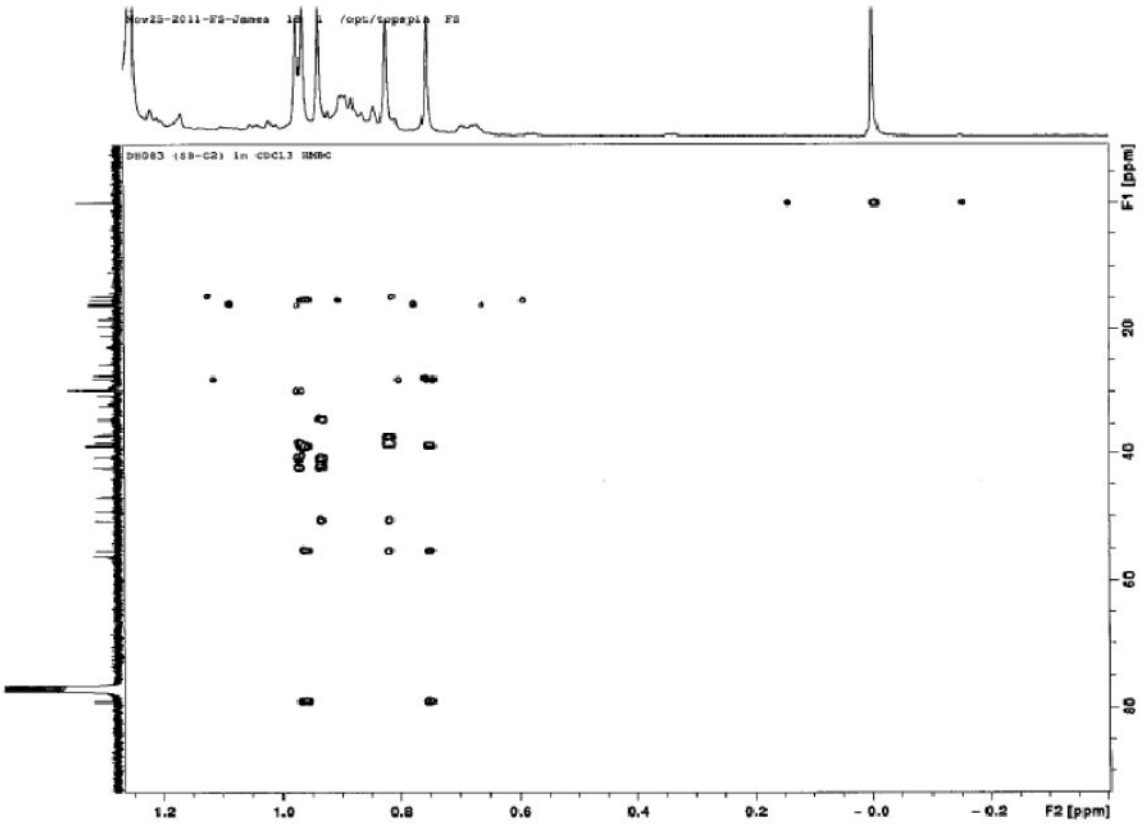
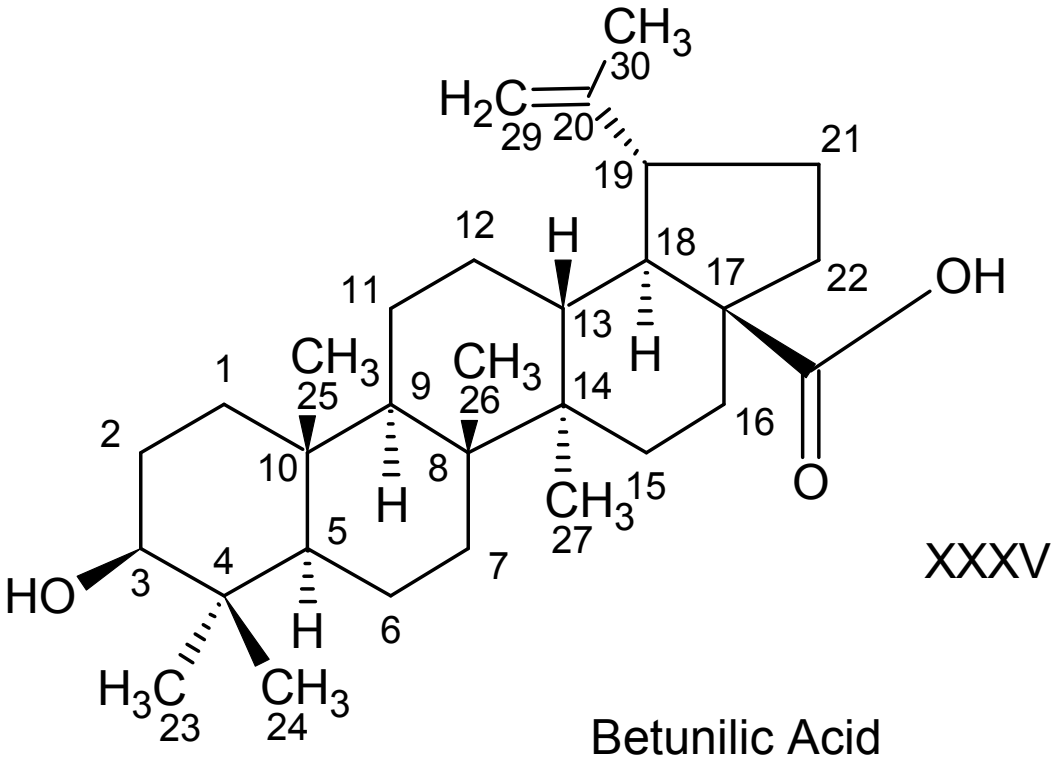


Figure 23: HMBC spectra of SB-C2

Table 4.39: ^{13}C NMR data assignment for SB-C2

Carbon Position	^{13}C Shift ppm [a]Lit. Value	^{13}C Shift ppm [b]Lit. Value	^{13}C Shift Experimental	CH_n
1	38.70	39.0	38.36	CH_2
2	27.40	27.6	27.40	CH_2
3	78.90	78.2	79.01	CH
4	38.80	39.1	38.87	C
5	55.30	55.5	55.35	CH
6	18.30	18.4	18.29	CH_2
7	34.30	34.5	34.34	CH_2
8	40.70	40.8	40.70	C
9	50.50	50.7	50.54	CH
10	37.20	37.3	37.21	C
11	20.80	21.0	20.86	CH_2
12	25.50	25.7	25.51	CH_2
13	38.80	38.1	38.72	CH
14	42.40	42.5	42.45	C
15	30.50	30.2	30.54	CH_2
16	32.10	32.9	32.15	CH_2
17	56.30	47.1	56.23	C
18	46.80	48.1	46.87	CH
19	49.20	49.2	49.26	CH_2
20	150.30	150.1	150.41	C
21	29.70	30.6	29.70	CH_2
22	37.00	37.0	37.02	CH_2
23	27.90	27.9	27.99	CH_3
24	15.30	15.5	15.35	CH_3
25	16.00	16.4	16.02	CH_3
26	16.10	16.7	16.13	CH_3
27	14.70	15.0	14.69	CH_3
28	180.50	180.3	179.20	C00H
29	109.60	108.9	109.70	CH_2
30	19.40	19.6	19.37	CH_3

^{13}C NMR Litt. Values ([a]Mahato and Kundu 1994, [b] Prince P. Sharma *et al, 2010*)



Betunilic Acid

4.5.0 Antimicrobial activities of SB-C2

The microbial activities of SB-C1 was carried out on some selected fourteen microorganisms and found that it was sensitive to Methicillin Resistant *Staphylococcus aureus (MRSA), Staphylococcus aureus, Streptococcus pyrogenes, Bacillus subtilis, Escherichia coli, Proteus mirabilis, Proteus vulgaris, Pseudomonas aureginosa, Salmonella typhi, Shigellia dysenteric, Candida albicans, Candida virusei, and Candida tropicalis* and not sensitive to *Corynebacterium ulcerans*. The zone of inhibition was observed to be between 21 to 34 mm (Table 4.32).

4.5.1 Minimum Inhibitory Concentration (MIC) of SB-C2

The inhibitory concentration of this compound was carried out on the selected microorganisms and discovered that Methicillin Resistant *Staphylococcus aureus (MRSA), Staphylococcus aureus, Streptococcus pyrogenes, Escherichia coli, Proteus mirabilis, Proteus vulgaris, Pseudomonas aureginosa, Candida albicans, Candida virusei, and Candida tropicalis* had MIC value at 12.5 µg/ml, while Salmonella *typhi* and *Shigellia dysenteric* had values at 6.25 µg/ml (Table 4.40).

4.5.2 Minimum Bacterial Concentration (MIC)/Minimum Fungicidal Concentration (MFC) of SB-C2

The MBC/MFC of SB-C1 was recorded for *Staphylococcus aureus, Streptococcus pyrogenes, Bacillus subtilis, Escherichia coli, Proteus vulgaris, Salmonella typhi, Shigellia dysenteric* and *Candida virusei* at a concentration of 25 µg/ml while it was observed for

MR*SA*, *Proteus mirabilis, Pseudomonas aureginosa, Candida albicans* and *Candida tropicalis* at a concentration of 50 µg/ml (Table 4.41). Comparing this activity with that obtained for a standard drug sparfloxacin, it indicates that SB-C1 has a broad spectrum of activities to that of the standard drug as shown in Table 4.35.

Table 4.40: Minimum inhibition Concentrations (µg/ml) of SB-C2 on the test microbes

Test Organism	50	25.5	12.5	6.25	3.125
MR*SA*	_	_	**O***	+	++
Staphylococcus aureus	_	_	**O***	+	++
Streptococcus pyrogenes	_	_	**O***	+	++
Bacillus subtilis	_	_	_	**O***	++
Corynebacterium ulcerans					
Escherichia coli	_	_	**O***	+	++
Proteus mirabilis	_	_	**O***	+	++
Proteus vulgaris	_	_	**O***	+	++
Pseudomonas aureginosa	_	_	**O***	+	
Salmonella typhi	_	_	_	**O***	+
Shigellia dysenteric	_	_	_	**O***	+
Candida albicans	_	_	**O***	+	++
Candida virusei	_	_	**O***	+	++
Candida tropicalis	_	_	**O***	+	++

Key: **-** *=No colony growth,* ***O**** *=MIC,* **+** *=light growth,* **++** *= Moderate colonies growth*

Table 4.41: Minimum Bactericidal/Fungicidal Concentrations (µg/ml) of SB-C2 on the test microbes

Test Organism	50	25.5	12.5	6.25	3.125
MR*SA*	**O***	+	++	+++	++++
Staphylococcus aureus	_	**O***	+	++	+++
Streptococcus pyrogenes	_	**O***	+	++	+++
Bacillus subtilis	_	**O***	+	++	+++
Corynebacterium ulcerans					
Escherichia coli	_	**O***	+	++	+++
Proteus mirabilis	**O***	+	++	+++	++++
Proteus vulgaris	_	**O***	+	++	+++
Pseudomonas aureginosa	**O***	+	++	+++	++++
Salmonella typhi	_	**O***	+	++	+++
Shigellia dysenteric	_	**O***	+	++	+++
Candida albicans	**O***	+	++	+++	++++
Candida virusei	_	**O***	+	++	+++
Candida tropicalis	**O***	+	++	+++	++++

Key: **-** *=No colony growth,* ***O**** *=MIC,* **+** *=light growth,* **++** *= Moderate colonies growth*

4.5.3 Spectral data of SPEC-1

SPEC-1 was obtained as a white solid crystal. The IR (cm^{-1}) absorption band values (Figure 24) observed at 3421.78cm^{-1} was characteristic of O-H stretching. The peak at 2960.78 cm^{-1} was typical of cyclic olefinic (–HC=CH-) stretching, while the absorption frequency at 1504.47 cm^{-1} was typical of carbon to carbon double bond (C=C) absorption. However, this signal was weak (Pretsch *et al*., 2000). The absorption at 1432.61 cm^{-1} was assignable to cyclic $(CH_2)_n$ bending absorption frequency. While the peak at 1342.20 cm^{-1} was attributable to OH deforming absorption (Grasseli, 1973). The absorption at 1016.92 cm^{-1} was typical of cycloalkane moieties.

The ^{1}H NMR spectrum (Figure 25) of SPEC-1 showed peaks at δ 0.8438 and 0.8337 which are typical for tertiary methyl groups (angular methyl proton) respectively and another two multiplet at δ 5.3358 (1H) and 5.1632 (2H) for three olefinic protons and another multiplet at δ 3.4983 for carbinylic protons (Pateh, *et al*; 2009).

The ^{13}C NMR (Figure 26) showed some recognizable signals at 140.7669 and 121.7248 ppm (Table 4.42) which was typical of double bond (Agrawal, *et al*., 1985).The value observed at 71.8260 ppm was characteristic of a β-hydroxyl group (Prestch, *et al*., 2000). While the signals observed at 21.2215 and 12.0547 ppm (Smith, 1978) are typical of angular carbon atoms respectively. From these spectra data coupled with the DEPT-135 experiment, COSY, NOESY, HSQC and HMBC (Figure 27, 28, 29, 30 and 31) respectively and the values obtained from literature (Pateh *et al*., 2009) the compound was proposed to be stigmasterol (Structure XXXVI).

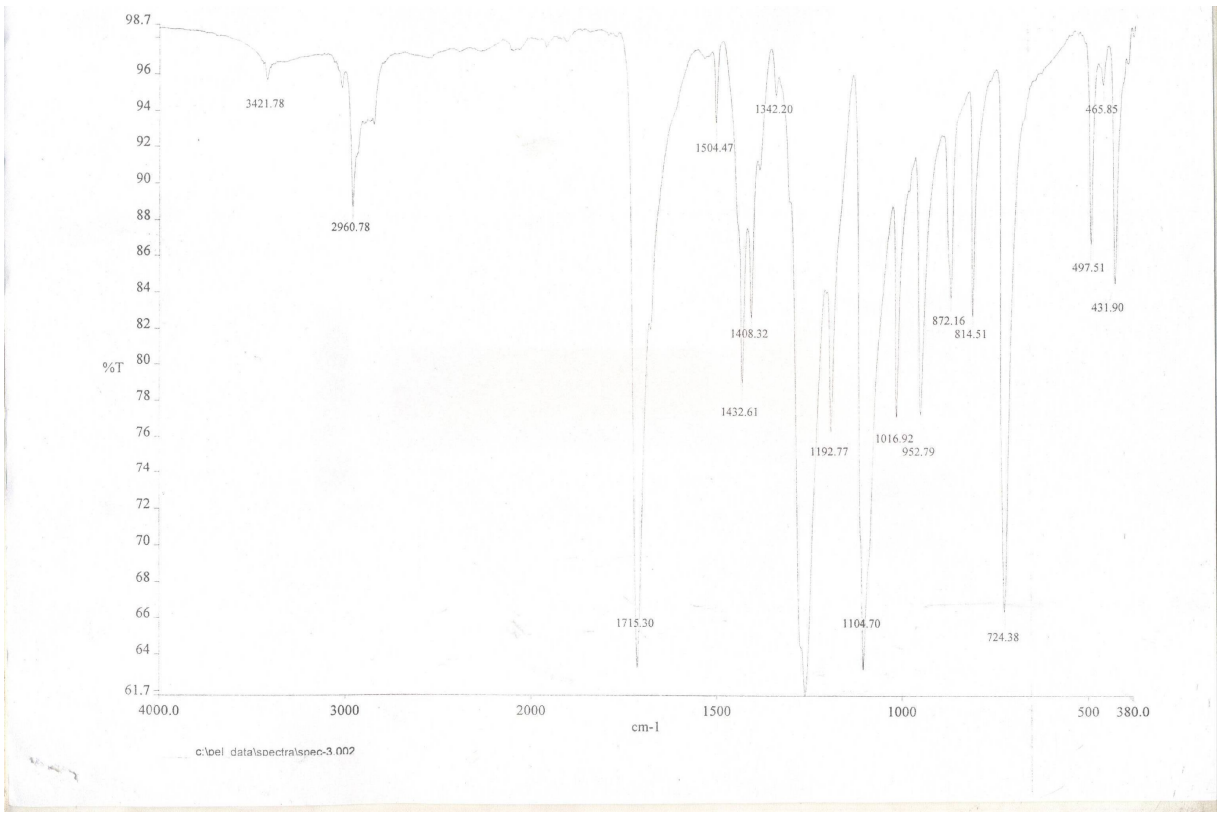


Figure 24: Infrared spectra of SPEC-1

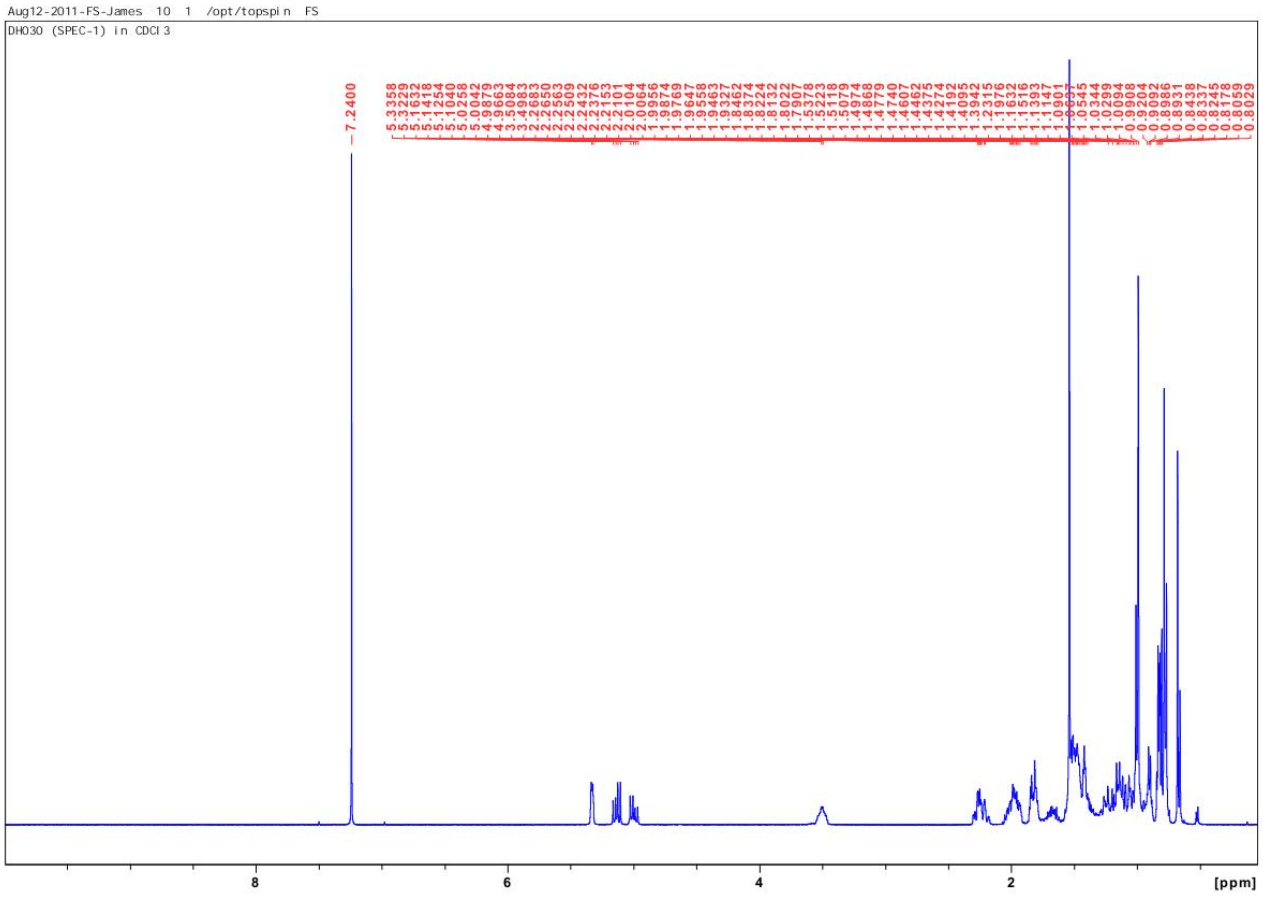


Figure 25: ^{1}H NMR spectra of SPEC-1

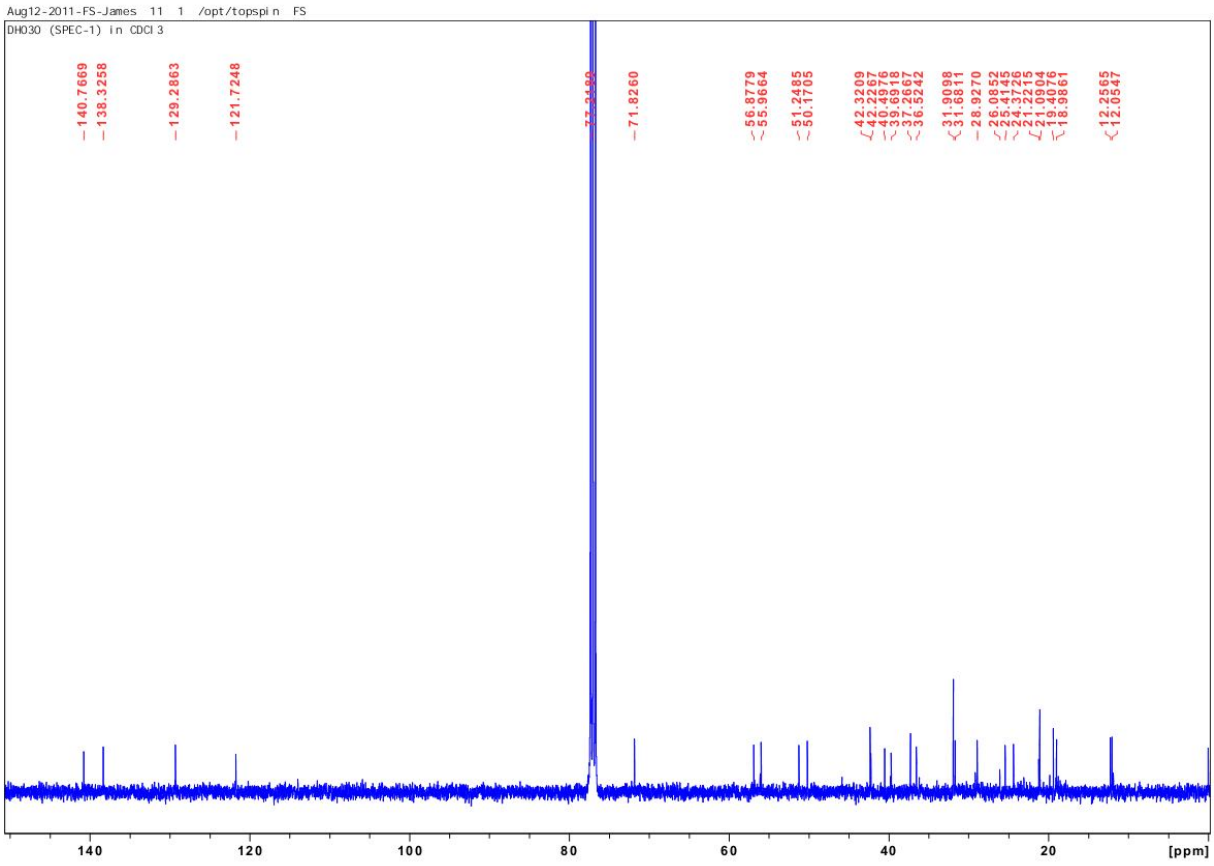


Figure 26: The ^{13}C NMR spectra of SPEC-1

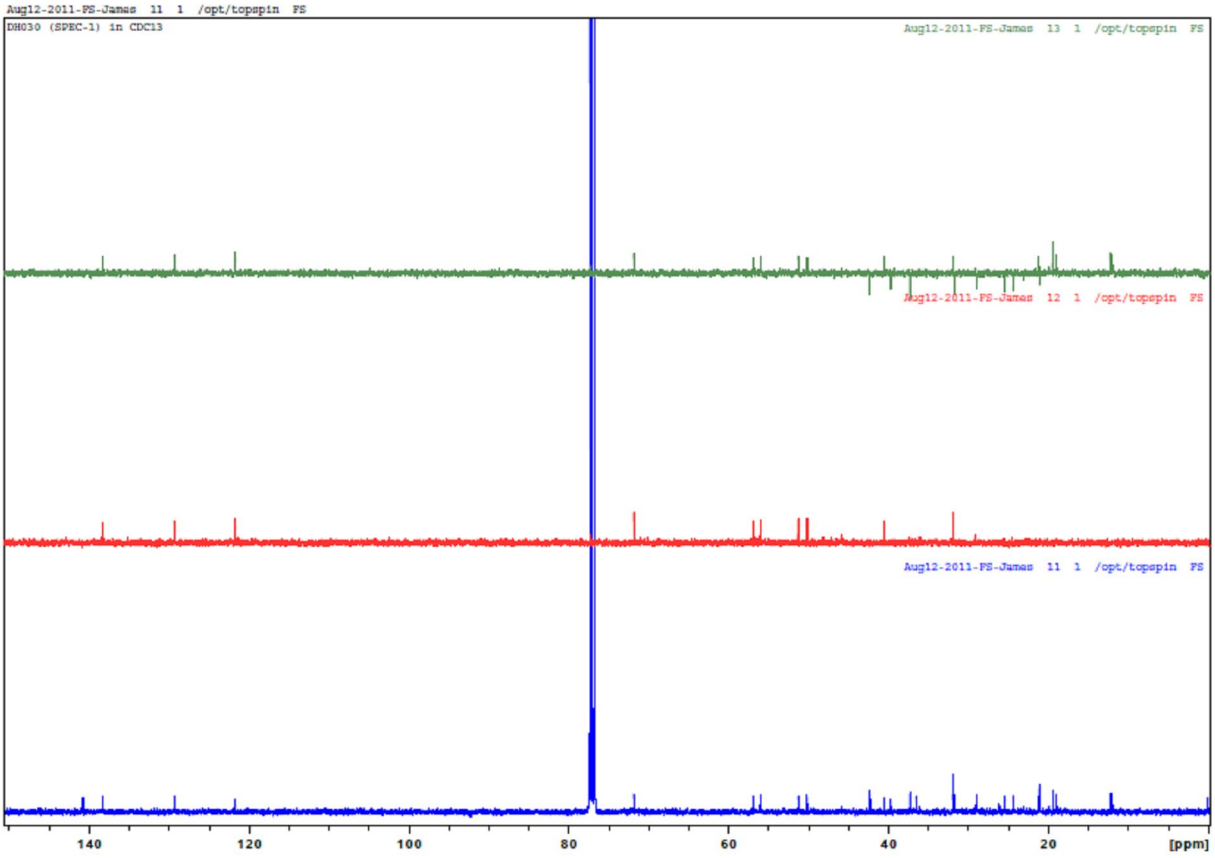


Figure 27: The DEPT-135 experiment spectra of SPEC-1

Figure 28: The COSY spectra of SPEC-1

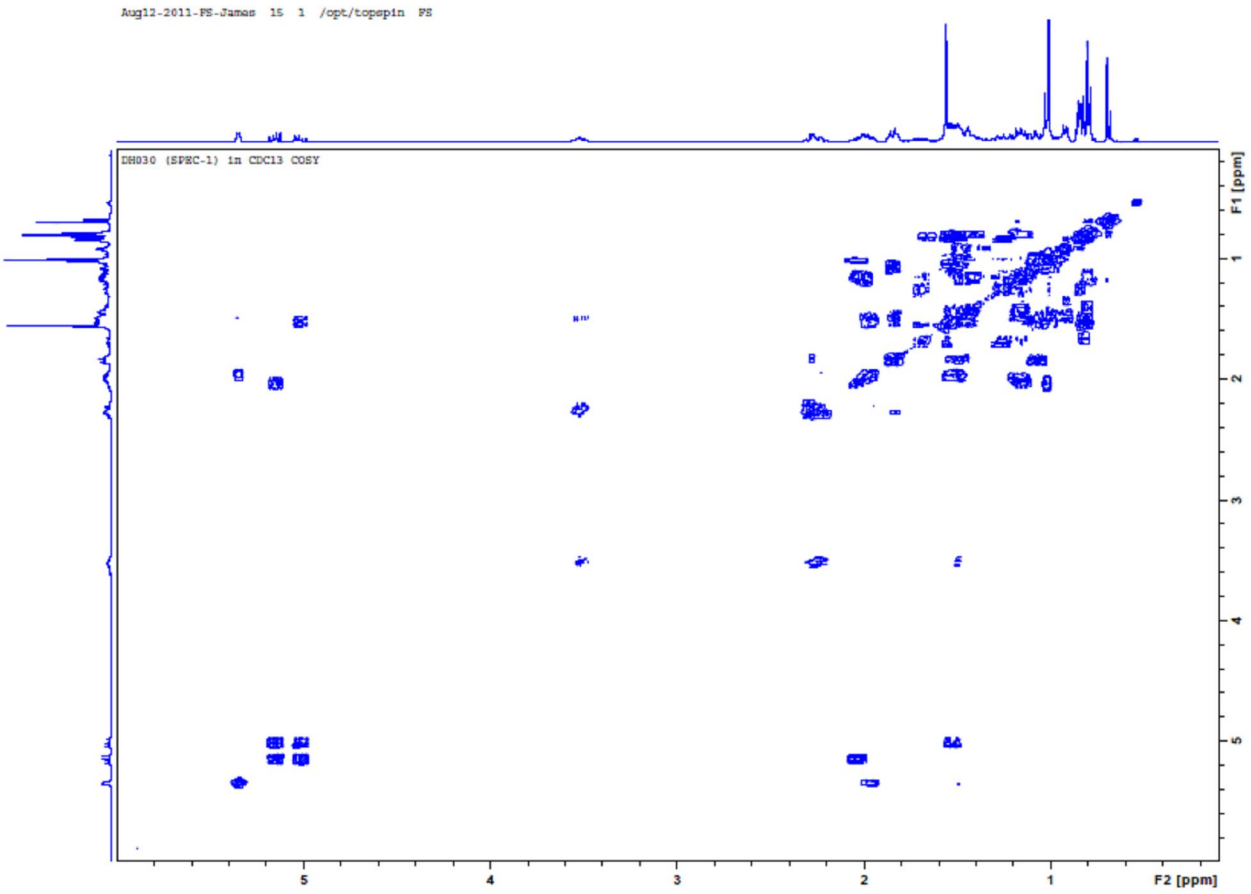


Figure 29: The NOESY spectra of SPEC-1

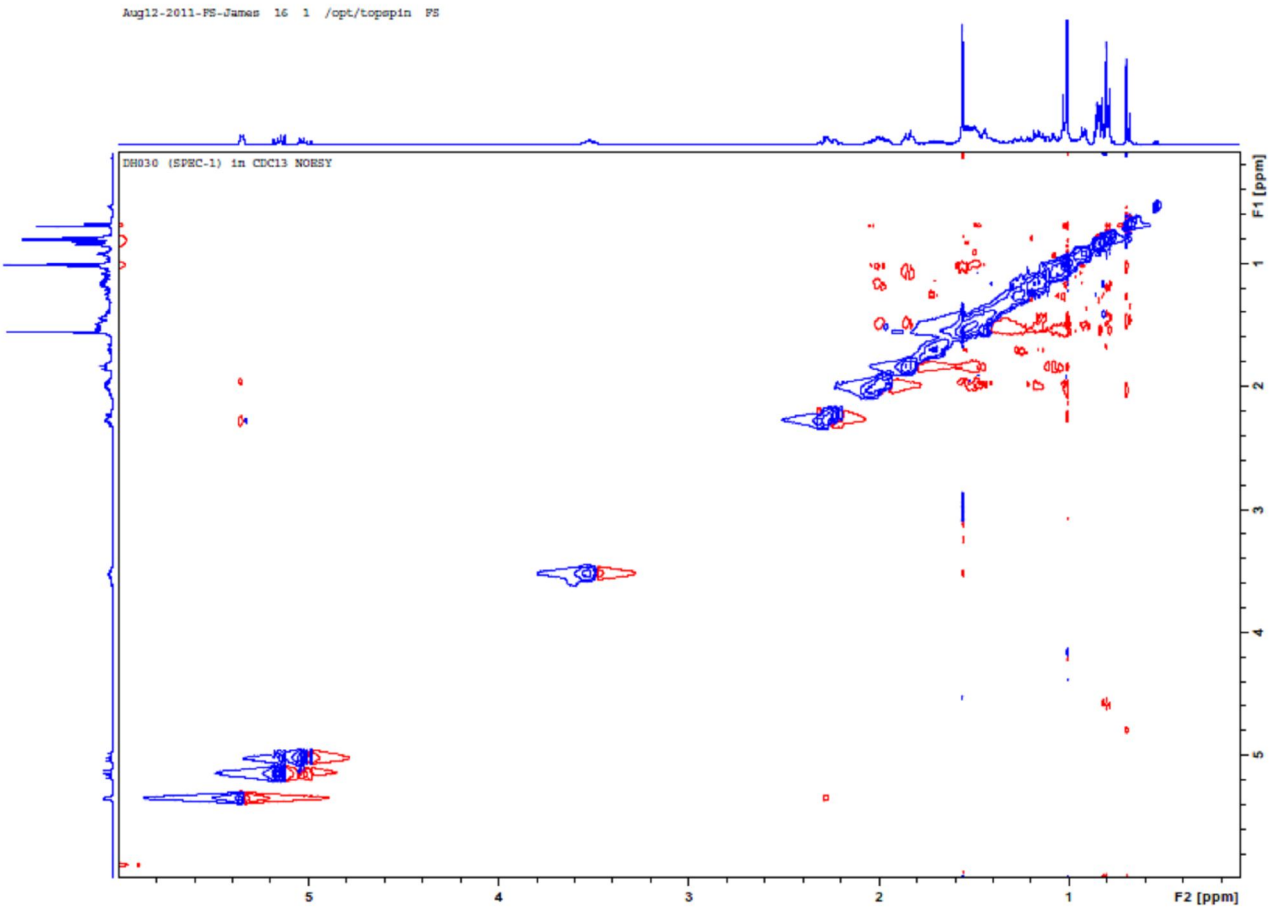


Figure 30: The HSQC spectra of SPEC-1

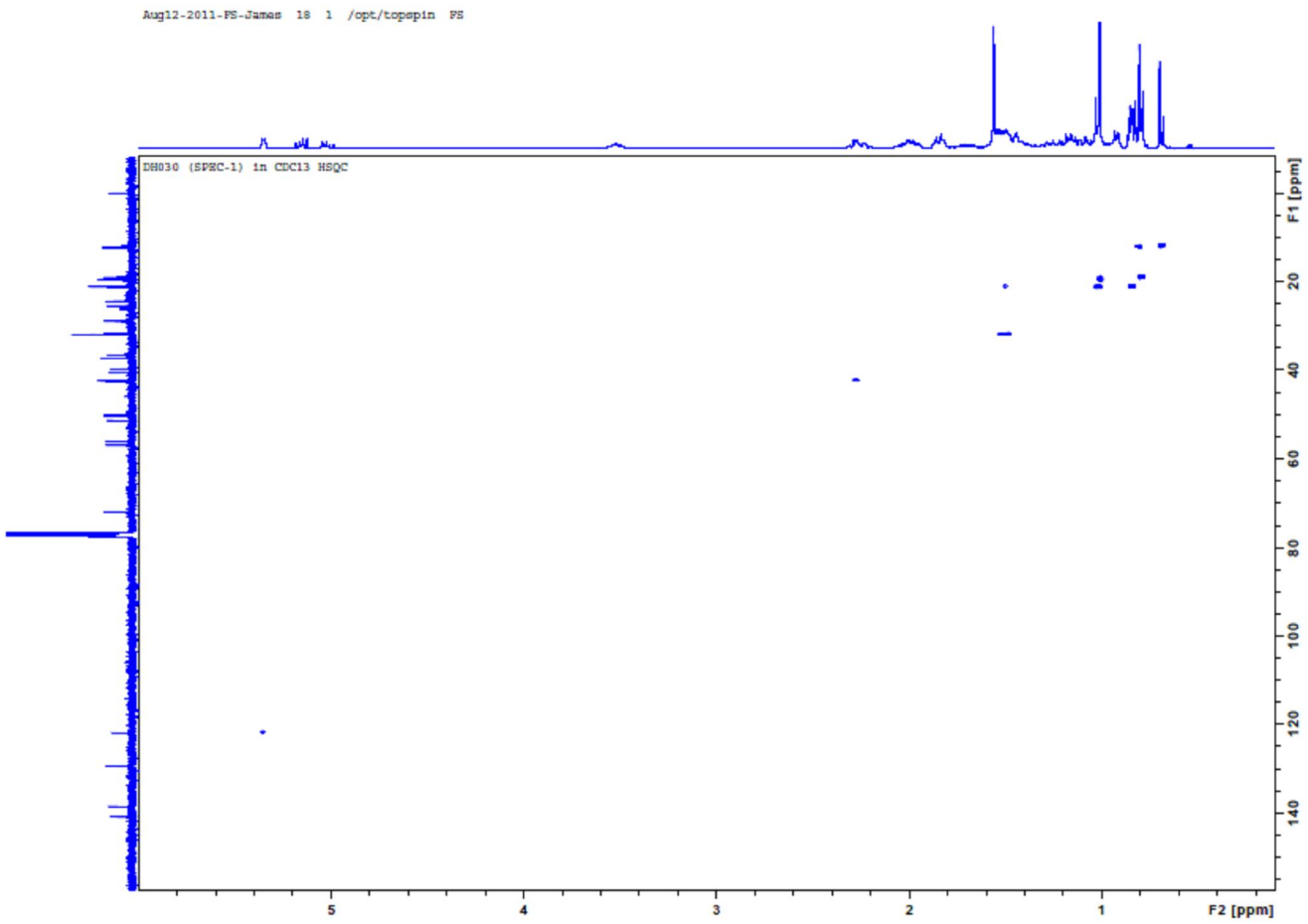


Figure 31: The HMBC spectra of SPEC-1

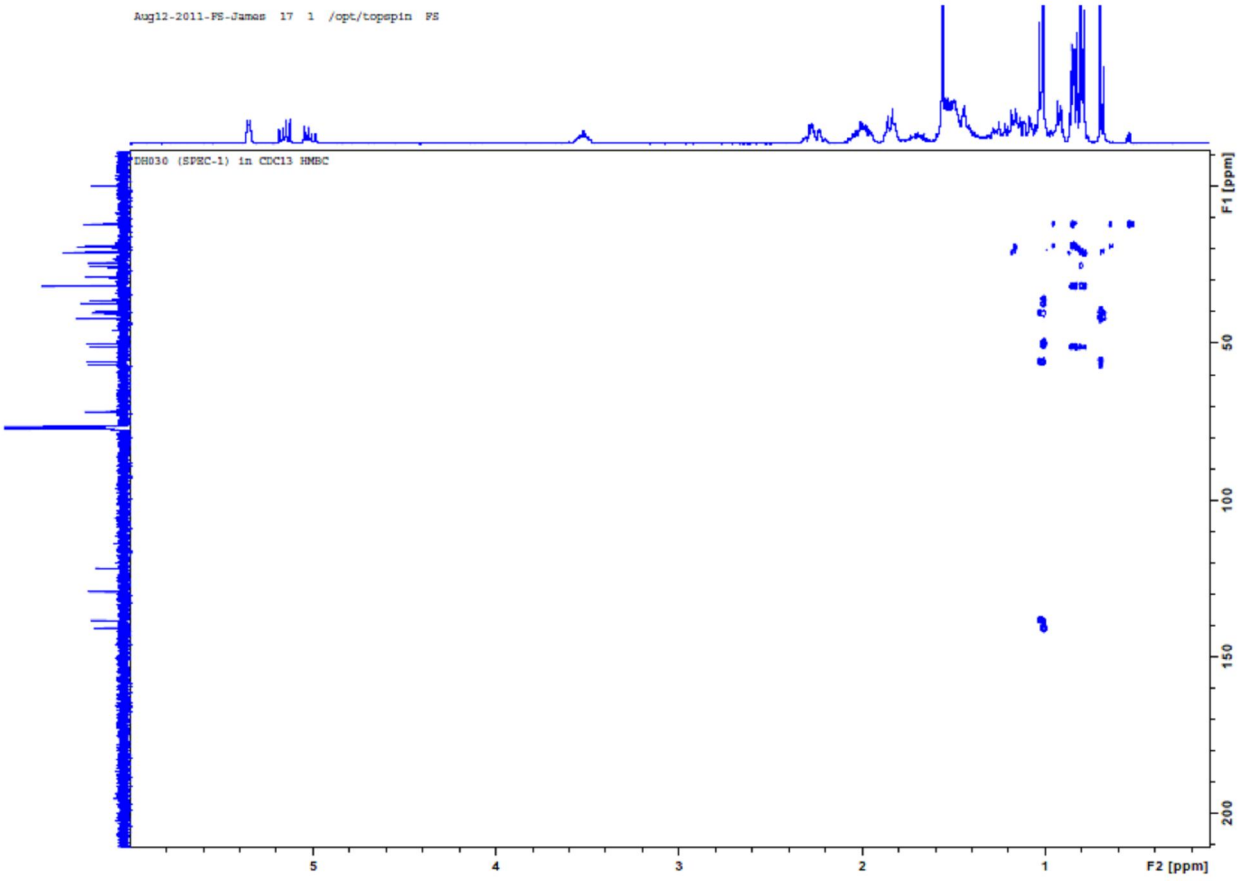
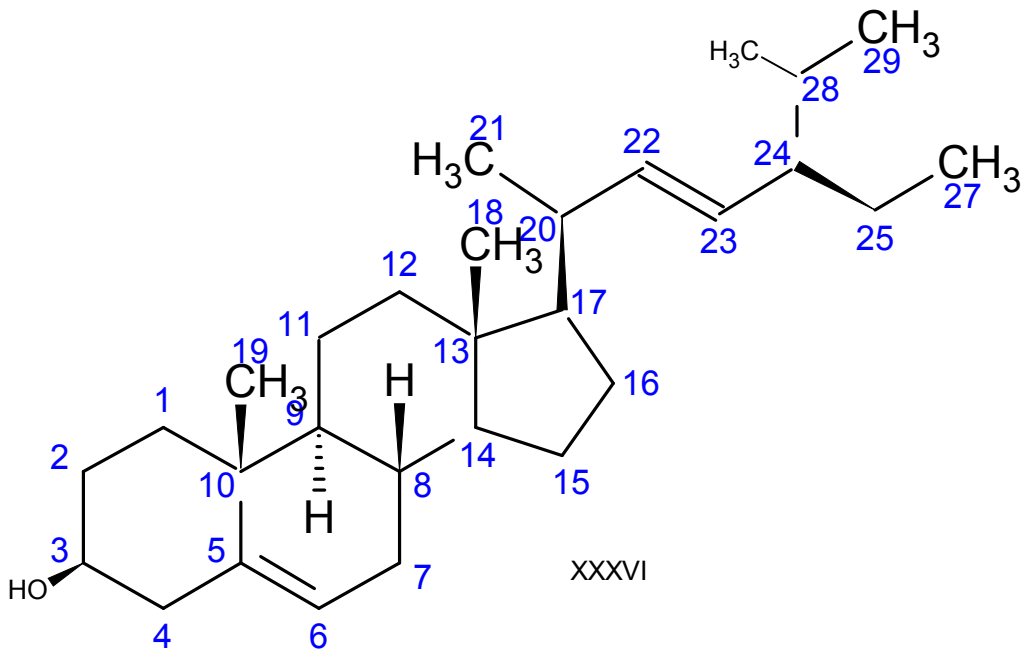


Table 4.42: ^{13}NMR data of Stigmasterol

S/No	Litt. Value *β-Stigmasterol	Experimental value β-Stigmasterol
	^{13}C	^{13}C
1	37.3	37.2667
2	31.6	31.6811
3	71.8	71.8260
4	42.3	42.3209
5	140.8	140.7669
6	121.7	121.7248
7	31.9	31.9098
8	31.9	31.9098
9	51.2	51.2485
10	36.5	36.5242
11	21.1	21.0904
12	39.7	39.6918
13	42.3	42.3209
14	56.9	56.8779
15	24.4	24.3726
16	28.4	28.9270
17	56.1	55.9664
18	11	12.0547
19	21.2	21.2215
20	40.5	40.4976
21	21.2	21.2215
22	138.3	138.3258
23	129.3	129.2863
24	51.2	50.1705
25	31.9	31.6811
26	21.2	21.2215
27	19	19.4076
28	25.4	25.4145
29	12.1	12.2565

*Pateh *et al*., 2009



XXXVI

Stigmasterol

4.5.4 Antimicrobial activities of SPEC-1

The biological activities of SPEC-1 was carried out and found that it was sensitive to *Staphylococcus aureus, Streptococcus Pyrogenes, Bacillus Subtilis, Escherichia Coli, Proteus mirabilis, Proteus vulgaris, Salmonella Typhi, Shigellia Dysenteric ,Candida albicans, Candida virusei, Candida tropicalis* and not sensitive to Methicillin Resistant *Staphylococcus aureus(MRSA), Corynebacterium ulcerans and Pseudomonas aureginosa*. The zone of inhibition was observed to be between 20 to 24 mm (Table 4.32). *Bacillus subtilis* was the most sensitive organism with the largest zone of inhibition of 24 mm while the smallest zone of inhibition was *Proteus mirabilis, Candida virusei* and *Candida tropicalis* at 20 mm.

4.5.5 Minimum Inhibitory Concentration (MIC) of SPEC-1

SPEC-1 had MIC on the following organisms; *Staphylococcus aureus, Streptococcus pyrogenes, Bacillus subtilis, Escherichia coli, Proteus mirabilis, Proteus vulgaris, Salmonella Typhi, Shigellia dysenteric ,Candida albicans, Candida virusei, Candida tropicalis* at 12.5 µg/ml (Table 4.43).

4.5.6 Minimum Bacterial Concentration (MBC)/Minimum Fungicidal Concentration (MFC) of SPEC-1

The MBC/MFC (Table 4.44) observed for *Staphylococcus aureus, Streptococcus pyrogenes, Bacillus subtilis, Escherichia coli, Proteus mirabilis, Proteus vulgaris, Salmonella typhi, Shigellia dysenteric, Candida albicans, Candida virusei, and Candida tropicalis* was 50µg/ml which was a little higher than the MIC. The MFC of this

compound was observed at a higher concentration than that of the standard drug fluconazole (Table 4.45).

Despite that SPEC-1 had a higher value of MFC; it had a wider range of broad spectrum over the standard drug against the entire microorganisms tested. It was generally noted that SPEC-1 could be used as anti bacteria and as antifungal agent in the treatment of fungal related diseases.

Table 4.43: Minimum Inhibition Concentrations (µg/ml) of SPEC-1 on the test microbes

Test Organism	50	25.5	12.5	6.25	3.125
MR*SA*					
Staphylococcus aureus	_	_	**O***	+	++
Streptococcus pyrogenes	_	_	**O***	+	++
Bacillus subtilis	_	_	**O***	+	++
Corynebacterium ulcerans					
Escherichia coli	_	_	**O***	+	++
Proteus mirabilis	_	_	**O***	+	++
Proteus vulgaris	_	_	**O***	+	++
Pseudomonas aureginosa					
Salmonella typhi	_	_	**O***	+	++
Shigellia dysenteric	_	_	**O***	+	++
Candida albicans	_	_	**O***	+	++
Candida virusei	_	_	**O***	+	++
Candida tropicalis	_	_	**O***	+	++

Key: **-** *=No colony growth,* ***O**** *=MIC,* **+** *=light growth,* **++** *= Moderate colonies growth*

Table 4.44: Minimum Bactericidal/Fungicidal Concentrations (µg/ml) of SPEC-1 on the test microbes

Test Organism	50	25.5	12.5	6.25	3.125l
MR*SA*					
Staphylococcus aureus	**O***	+	++	+++	++++
Streptococcus pyrogenes	**O***	+	++	+++	++++
Bacillus subtilis	**O***	+	++	+++	++++
Corynebacterium ulcerans					
Escherichia coli	**O***	+	++	+++	++++
Proteus mirabilis	**O***	+	++	+++	++++
Proteus vulgaris	**O***	+	++	+++	++++
Pseudomonas aureginosa					
Salmonella typhi	**O***	+	++	+++	++++
Shigellia dysenteric	**O***	+	++	+++	++++
Candida albicans	**O***	+	++	+++	++++
Candida virusei	**O***	+	++	+++	++++
Candida tropicalis	**O***	+	++	+++	++++

Key: **-** *=No colony growth,* ***O**** *=MBC/MFC,* **+** *=Scanty colonies growth,* **++** *= Moderate colonies growth,* **+++***= Heavy colonies growth*

Table 4.45: Minimum Inhibition Concentrations (µg/ml) of Fluconazole on the test microbes

Test Organism	50	25.5	12.5	6.25	3.125
MRSA	□	□	□	□	□
Staphylococcus aureus	□	□	□	□	□
Streptococcus pyrogenes	□	□	□	□	□
Bacillus subtilis	□	□	□	□	□
Corynebacterium ulcerans	□	□	□	□	□
Escherichia coli	□	□	□	□	□
Proteus mirabilis	□	□	□	□	□
Proteus vulgaris	□	□	□	□	□
Pseudomonas aureginosa	□	□	□	□	□
Salmonella typhi	□	□	□	□	□
Shigellia dysenteric	□	□	□	□	□
Candida albicans	_	_	**O***	+	++
Candida virusei	_	_	**O***	+	++
Candida tropicalis	_	_	**O***	+	++

Key: **-** *=No colony growth,* ***O**** *=MIC,* **+** *=light growth,* **++** *= Moderate colonies growth,* **ɵ***=Resistant.*

CHAPTER FIVE

5.0 Summary, Conclusion and Recommendation

5.1 Summary

This work describes the phytochemical screening, isolation, characterization and antimicrobial activities of various fractions and the isolated compounds obtained from *Sarcocephalus latifolius* (Smith Bruce) and *Spillanthes acmella* (Murr), which are two plants variously used in Ethnomedicinal practices in Nigeria. The extraction was done by the continuous extraction method to yield the various plant extracts. Preliminary phytochemical analysis was carried out on the stem bark and root of *Sarcocephalus latifolius*, while the aerial plant parts of *Spillanthes acmella* was used for the analyses. The screening of the root and stem bark of *Sarcocephalus latifolius* revealed the presence of carbohydrate, glycoside (root), anthraquinones, cardiac glycoside, saponins, steroid and triterpenes, flavonoids (root),tannins (stem-bark) and alkaloids in plant parts studied. The aerial plant parts of *Spillanthes acmella* showed the presence of carbohydrate, glycoside, anthraquinones, cardiac glycoside, saponins, steroid triterpenes, saponins, flavonoids and alkaloids.

The antimicrobial examination of the plants extracts were carried out in vitro using the well-in-plate dilution method on the following clinical microorganisms; Methycilin Resistant *Staphylococcus aureus (MRSA), Staphylococcus aureus, Streptococcus Pyrogenes, Bacillus Subtilis, Corynebacterium ulcerans, Escherichia coli, Proteus mirabilis, Proteus vulgaris, Pseudomonas aureginosa, Salmonella typhi, Shigellia dysenteric ,Candida albicans, Candida virusei, and Candida tropicalis* to determine their biological activities.

The microbial screening of the fractions obtained from *Sarcocephalus latifolius* (petroleum ether, chloroform, ethyl acetate and methanol fractions) were found to be sensitive to most of the microorganism tested with the exception of *Methycilin resistant staphylococcus aureus, Candida tropicalis, Proteus mirabilis* and *Pseudomonas aureginosa*. The zone of inhibition for these fraction ranged between 14 and 22mm. Most of the extracts showed MIC values to the sensitive organism at 20mg/ml except *Bacillus subtilis* that was observed to be 10 mg/ml for ethyl acetate and methanol fraction of *Sarcocephalus latifolius*. The MBC/MFC showed result that was a little above that of the MIC. This value was lowest for chloroform and methanol against *Bacillus subtilis* at 20 mg/ml and highest for all other extract at 40 mg/ml.

The antimicrobial screening of all the extracts from *Spillanthes acmella* were found to be resistant to Methycilin resistant *staphylococcus aureus, Pseudomonas aureginosa* and *Candida tropicalis* and the zone of inhibition was found to be between 15 and 19 mm. The MIC results for the fractions were observed at 20 mg/ml, while it was observed for *Bacillus subtilis* at 10 mg/ml for the fractions and observed for *Streptococcus pyrogenes* in the methanol fraction 10 mg/ml.

The MBC/MFC values for *Spillanthes acmella* gave values that were a little above that of the MIC. The MBC/MFC for petroleum ether against sensitive microorganism was observed at 40 mg/ml, while it was recorded for *Bacillus subtilis* at 20 mg/ml. Also, the chloroform, ethyl acetate fractions were observed at 40 mg/ml and 20 mg/ml for *Bacillus subtilis* for the extracts respectively. But, the MBC/MFC for methanol extract were recorded for the sensitive microbes at a concentration of 20 mg/ml which was a little above that of the MIC.

Isolation of the pure active component of the extracts was done based on the sensitivity of the extracts to the microorganism. The isolation was done using the Vacuum Liquid Chromatography (VLC) (appendix III). While the purification was done using the convectional Thin Layer Chromatography (TLC) to obtain EFSB (combination of Sitosterol and Sitostenone), SB-C1 (Sitosterol), SB-C2 (Betunilic Acid) from the methanol fraction of the stem bark of *Sarcocephalus latifolius* and SPEC-1 (Stigmasterol) from the petroleum ether fraction of the aerial plant part of *Spillanthes acmella*.

The isolated pure compounds were characterized and identified using spectroscopic method of analyses such as; Nuclear Magnetic Resonance (1-D and 2-D NMR) spectroscopy, Fourier Transform Infrared spectroscopy (FTIR) and also by comparing these data obtained with that of the literature to established the structure of the compound.

The isolated and characterized pure compounds were subjected to antimicrobial screening against some clinical selected microorganisms and comparison of their potency with standard drug was carried out using the well-in-plate diffusion method. **EFSB** (Sitosterol and Sitostenone) isolated from the bark of *Sarcocephalus latifolius* was found to be sensitive to most of the tested microbes and gave zone of inhibition range between 20 and 25 mm and the MIC value for the sensitive organism was recorded at concentration of 12.2 µg/ml while the MBC/MFC was recorded at 50 µg/ml with the exception of *Bacillus subtilis* that was recorded at 25 µg/ml. The EFSB had a lower value of MIC as compared to the standard drug (Sparfloxacin) showing that, it had a broad spectrum of activities over the tested organism. Antimicrobial result for **SB-C1** (Sitosterol) from the stem bark of *Sarcocephalus latifolius* showed that, most of the organisms were sensitive with the exception of MR*SA, Escherichia coli, Proteus mirabilis and Candida tropicalis*. The zone

of inhibitions for the sensitive organisms weres found to be between 20 to 27 mm with *Bacillus subtilis* and *Salmonella typhi* giving the largest value at 27 mm. The MIC and MBC/MFC indicated that *Bacillus subtilis, Salmonella typhi* and *Shigellia dysenteric* were exterminated at 25 µg/ml while *Staphylococcus aureus, Streptococcus pyrogene, Corynebacterium ulcerans, Proteus vulgaris, Proteus aureginosa, Candida albicans* and *Candida virusei* were recorded at 50 µg/ml. The results showed that, SB-C1 (sitosterol) was comparable to standard drug Sparfloxacin because the drug equally had MIC value recorded at 25 µg/ml showing that they could be used to treat related ailment.

SB-C2 (Betunilic acid) from *Sarcocephalus latifolius* was found to be sensitive to the entire tested organisms with the exception of *Corynebacterium ulcerans*. The zone of inhibition was observed to be between 21 to 34 mm. while, the MIC value for the sensitive organisms was found to be 12.5 µg/ml with the exception of *Salmonella typhi* and *Shigellia dysenteric* that showed value at 6.5 µg/ml. The MBC/MFC for the sensitive organisms was recorded at of 25 µg/ml for *Staphylococcus aureus, Streptococcus pyrogene, Bacillus subtilis, Escherichia coli, Proteus vulgaris, Salmonella typhi, Shigellia dysenteric* and *Candida virusei*, while it was observed for MR*SA, Proteus mirabilis, Pseudomonas aureginosa, Candida albicans* and *Candida tropicalis* at a concentration of 50 µg/ml. Comparing these results with that of standard drug, it was noted that, it had a wide broad spectrum of activities as the drug (Sparfloxacin).

SPEC-1(Stigmasterol) isolated from the aerial plant parts of *Spillanthes acmella* (Murr) showed zone of inhibition that range between 20 to 24 mm with *Bacillus subtilis* having the highest at 24 mm. The MIC value for sensitive microbes was recorded at 12.5 µg/ml while the MBC/MFC had a value higher than that of MIC at 50µg/ml. The MFC was

observed to be comparable to that of the standard drug Fluconazole. This was an indication that SPEC-1 (Stigmasterol) had a wide range of broad spectrum of activities over the standard drug and could be used as an antifungal agent in the treatment of fungal related diseases.

5.2 Conclusion

Medicinal plants used in the folk medicine may be an interesting and largely unexplored source for the development of potential new compounds. But it is necessary to isolate the active principles and characterize their constituents for the benefit of human being. It was our attempt to identify the new compounds in these plants that revealed three compounds (Mixture of sitosterol and Sitostenone, sitosterol and Betunilic acid) from *Sarcocephalus latifolius* and one (Stigmasterol) from *Spillanthes acmella.* All these isolated compounds had previously been isolated and established. But, this is the first time Betunilic acid will be isolated from the stem bark of *Sarcocephalus latifolius*. Stigmasterol had previously been isolated from *Spillanthes acmella.*

From the antimicrobial activities of the isolated compounds the use of these plants in various diseases related ailment by the natives has been really justified. These plants have shown to have very high active component in them hence the high microbial activities recorded for the isolated compounds.

5.3 Recommendation

It is based on this fact that we are suggesting further work to be carried out on the isolation of these compounds and their activities in vivo for possible drug development.

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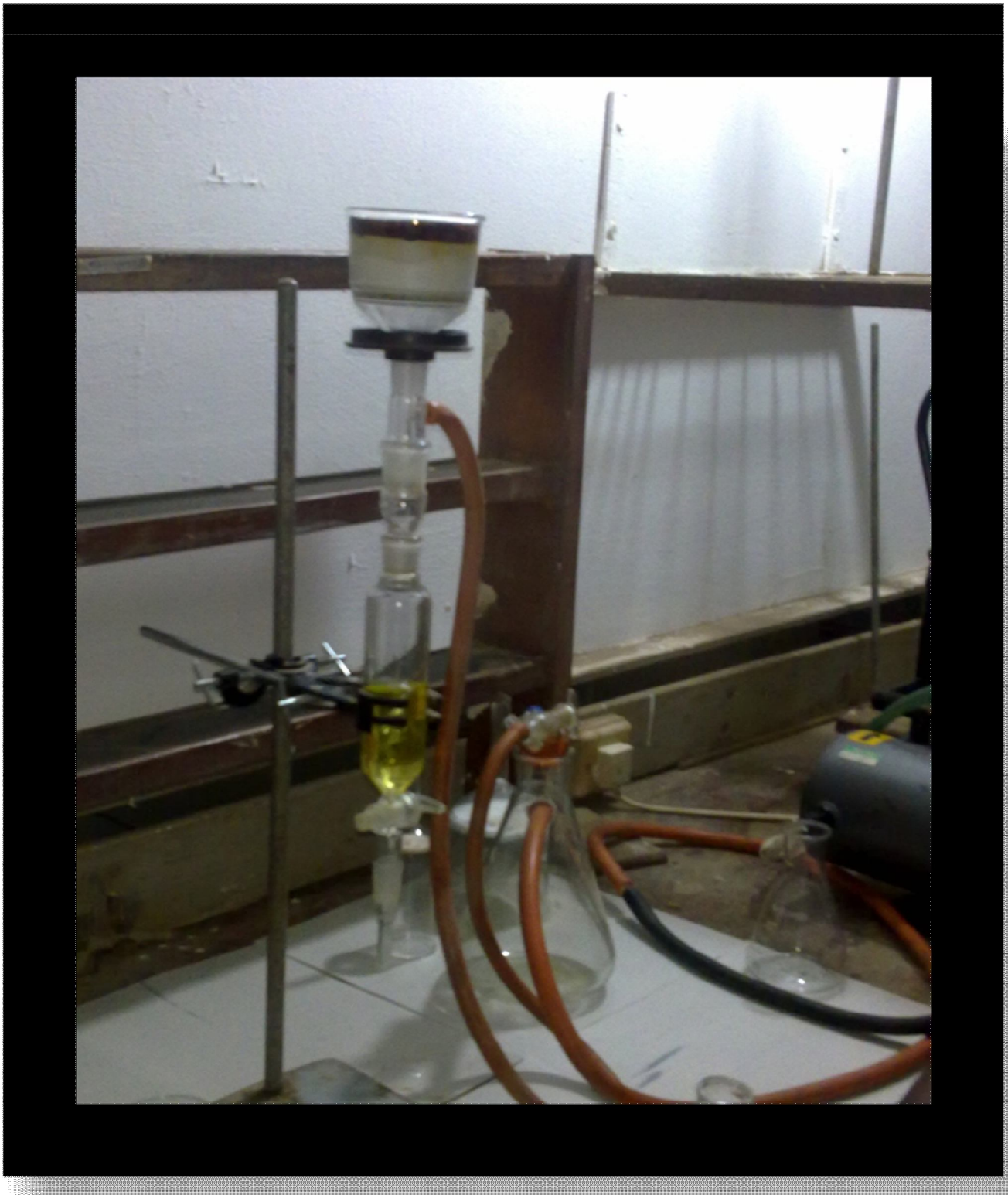
Appendix 1: Sarcocephalus latifolius (smith Bruce)



Appendix II: Spillanthes acmella Murr



Appendix III: Vacuum Liquid Chromatography



Appendix IV

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Isah et al., 2012. Isolation of Stigmasterol from Aerial Plant Part of Spillanthes acmella Murr
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Original Article
Life Science

Isolation of Stigmasterol from Aerial Plant Part of *Spillanthes Acmella* Murr

Yinusa ISAH [1,*], IlogbulemG NDUKWE [2], Joseph O AMUPITAN [2]

ABSTRACT [ENGLISH/ANGLAIS]

Pulverized aerial plant material (300 g) of *Spillanthes acmella* was exchaustively extracted with methanol and concentrated in vacuo using rotary evaporator at 40oC.the extract was later subjected to solvent partitioning to yield the following extracts petroleum ether, chloroform, Ethyl acetate and methanol. The petroleum ether crude extract was subjected to Vacuum Liquid Chromatographic (VLC) technique to yield various fractions which were combined based on their thin layer chromatography analysis to give SPEC. This was further purified by the Preparatory Thin Layer Chromatography (PTLC) to give SPEC-1.The structure of the isolated compound was established by 1D and 2D NMR spectroscopic analysis and by direct comparison of the data obtained with those reported in literature to be Stigmasterol.

Keywords: Spillanthes,toothache,vacuum liquid chromatography,stigmasterol

RÉSUMÉ [FRANÇAIS/FRENCH]

Pulvérisé matériel végétal aérienne (300 g) de Spillanthes acmella a été extraite avec du méthanol exchaustively et concentrée sous vide en utilisant un évaporateur rotatif àl'extrait a ensuite été soumis 40oC.the à un cloisonnement solvant pour donner l'éther de pétrole extraits ci-dessous, le chloroforme, l'acétate d'éthyle et de méthanol. L'extrait brutd'éther de pétrole a été soumis à un vide chromatographie en phase liquide (VLC)technique pour obtenir différentes fractions qui ont été réunies sur la base de leur analyse chromatographie sur couche mince de donner SPEC. Ceci a été purifiée par lachromatographie sur couche mince préparatoire (PTLC) pour donner SPEC-1.Lestructure du composé isolé a été établi par l'analyse RMN 1D et 2D spectroscopique etpar comparaison directe des données obtenues avec celles rapportées dans la littérature pour être Stigmastérol.

Mots-clés: Spillanthes, les maux de dents, la chromatographie liquide sous vide, le stigmastérol

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INTRODUCTION

Spillanthes acmella Murr is a flowering herb in the family Asteraceae. It is known as toothache plant or pancress as the leaves and the flower heads contain analgesic agent spilanthol that is used to numb toothache. The fresh and cooked leaves are combined with chilies and garlic to add flavour and vitamins to foods [1]. Some chemical constituents have been isolated from the plant such as spilanthol an alkyl amide [2]. This is responsible for the saliva inducing effect of the plant, and some triterpenoids [3]. The leaves along with alum are recommended as emetic [4].Because of this observation and the regular uses of the plant by the natives prompted this research possibly to find out if there could be more metabolites that can be of pharmacological relevance.

MATERIALS AND METHODS

Collection of plant material

The aerial plant part of *Spillanthes acmella* was collected from Okene, in Okene Local Government Area of Kogi State, Nigeria in March 2010. This was identified at the Herbarium of the Department of Biological Sciences, Faculty of Science, Ahmadu Bello University, Zaria Nigeria. The voucher specimen with number 534 was deposited at the Herbarium. The sample was air-dried, pulverized using wooden pestle and mortar. Finally, this was stored in an air-tight polythene bag and kept away from moisture until when needed for extraction.

Extraction Procedure

The pulverized plant material (300 g) was weighed and extracted exchaustively with redistilled methanol (2 liters) for 72 hours in a soxhlet extractor. Concentration of the extract was done *in vacuo* at 40 °C using rotary evaporator (*Rota vapor*) to give (43.7 g) or (15 %) of the crude extract. The methanol crude extracts was later subjected to solvent partitioning using petroleum-ether, chloroform, ethyl acetate to obtain various crude fractions. The petroleum ether fraction was subjected to vacuum liquid chromatography (VLC) using petroleum ether and ethyl

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acetate for the gradient elution to obtain various fractions (SPE1 to SPE 54) in each case 25 ml of eluent was collected.

Purification of the Pure Compound

All the fractions obtained were studied by the process of thin layer chromatographic (TLC) technique using petroleum ether and ethyl acetate in the ratio of 1:9. From the outcome similar fractions were combined to give SPEC. This was further cleaned by preparatory thin layer chromatography (PTLC) using petroleum ether: ethyl acetate (1:9) to obtain SPEC-1 which was a white crystalline substance

RESULTS AND DISCUSSION

The **IR** spectral values for **SPEC-1** are: 3421.78, 2960.78, 1504.47, 1432.61, 1408.32, 1342.20, 1192.77, 1104.70, 1016.92, 952.79, 872.16, 814.51, 724.38, 497.51, 465.85, and 431.90.

^{1}H NMR: 5.3358, 5.3229, 5.1632, 5.1418, 5.1254, 5.1040, 5.0258, 5.0042, 4.9879, 4.9663, 3.5084, 3.4983, 2.2888, 2.2837, 2.2683, 2.2650, 2.2563, 2.2509, 2.2432, 2.2376, 2.2153, 2.2101, 2.0285, 2.0104, 2.0064, 1.9956, 1.9874, 1.9769, 1.9647, 1.9558, 1.9463, 1.9327, 1.8544, 1.8462, 1.8374, 1.8224, 1.8132, 1.8022, 1.7907, 1.7098, 1.6954, 1.6865, 1.6763, 1.6620, 1.6535, 1.6390, 1.5713, 1.5223, 1.5118, 1.5079, 1.4974, 1.4868, 1.4779, 1.4740, 1.4607, 1.4462, 1.4375, 1.4274, 1.4192, 1.4095, 1.3997, 1.3942, 1.3856, 1.3755, 1.3663, 1.3569, 1.3479, 1.3257, 1.2942, 1.2826, 1.2630, 1.2571, 1.2483, 1.2438, 1.2315, 1.2159, 1.2053, 1.1976, 1.1817, 1.1632, 1.1516, 1.1393, 1.1147, 1.0901, 1.0637, 1.0545, 1.0344, 1.0299, 1.0094, 0.9908, 0.9615, 0.9476, 0.9353, 0.9293, 0.9204, 0.9092, 0.8986, 0.8931, 0.8821, 0.8438, 0.8337, 0.8245, 0.8178, 0.8059, 0.8029, 0.7846, 0.7671, 0.7440, 0.6782, 0.6599, 0.5306 and 0.5160.

^{13}C NMR: 140.7669, 138.3258, 129.2863, 121.7248, 77.2189, 71.8260, 56.8779, 55.9664, 51.2485, 50.1705, 42.3209, 42.2267, 40.4976, 39.6918, 37.2667, 36.5242, 31.9098, 31.6811, 28.9270, 26.0852, 25.4145, 24.3726, 21.2215, 21.0904, 19.4076, 18.9861, 12.2565 and 12.0547.

SPEC-1 was obtained as a white solid crystal. The IR (Figure 1) signal absorption band observed at 3421.78 cm^{-1} is characteristic of O-H stretching. Absorption at 2960.78 cm^{-1} is typical of cyclic olefinic –HC=CH- stretching while the absorption at frequencies such as 1504.47 cm^{-1} is as a result of C=C absorption. However, this signal is weak .the absorption at 1432.6 cm^{-1} was assignable to cyclic $(CH_2)_n$ bending absorption frequency. While the one at 1342.20 cm^{-1} is attributable to OH deforming absorption. The absorption at 1016.92 cm^{-1} is typical of cycloalkane moieties. These absorption frequencies were in very close agreement with the ones observed for Stigmasterol [5].

The ^{1}HNMR spectrum (Figure 2) of SPEC-1 completely corresponds to the data for Stigmasterol [6].It showed peaks for two tertiary methyl groups (angular methyl proton) at 0.8438 and 0.8337 and further had two multiplet at 5.3358 (1H) and 5.1632 (2H) for three olefinic protons and another multiplet at 3.4983 for carbinylic protons. The signal at 1.0094 corresponds to C-18 and C-19 protons respectiveiy of Stigmasterol.

The ^{13}C NMR (Figure 3) shows some recognizable signals at 140.7669 and 121.7248 ppm which is assignable to the double bond at C-5 and C-6 [7].The δ value observed at 71.8260 ppm is due to C-3 β-hydroxyl group [8]. Again the signals observed at 21.2215 and 12.0547 ppm correspond to angular carbon atoms at C-19 and C-18 respectively (Table 1). It had been reported that the lower value for C-18 is attributable to γ-gauche interaction that increases the screening of C-18 thereby causing lower chemical shift.

Figure 1: This figure shows infra-red spectra for SPEC-1

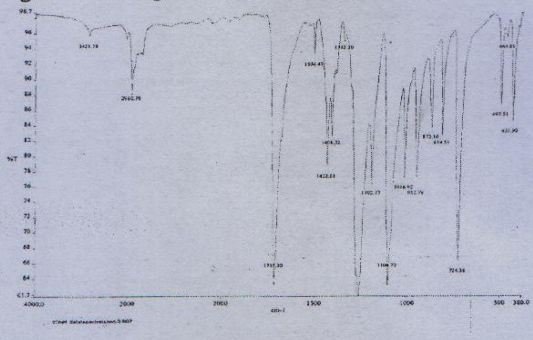
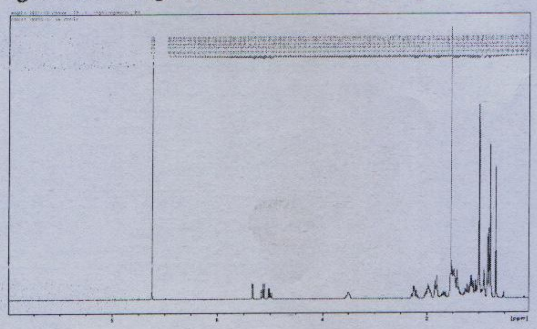


Figure 2: This figure shows ^{1}H NMR for SPEC-1



Likewise the loss of H in C-6 results in decrease in screening of C-19 leading to an increase in ^{13}C chemical shift to higher frequency [9].This explanation is also accepted for 21.2215 and 12.0547 ppm (for C-19 andC-18 respectively). From the spectra above coupled with the dept-135 (Figure 4) and with the comparison made so far it was concluded that sample SPEC-1 is β- Stigmasterol (Figure 5).

Figure 3: This figure shows ^{13}C NMR for SPEC-1

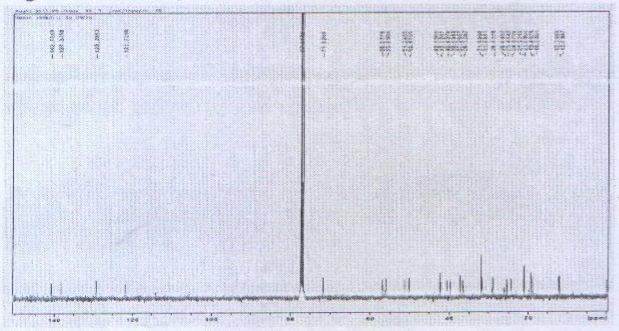


Figure 4: This figure shows dept – 135 for SPEC-1

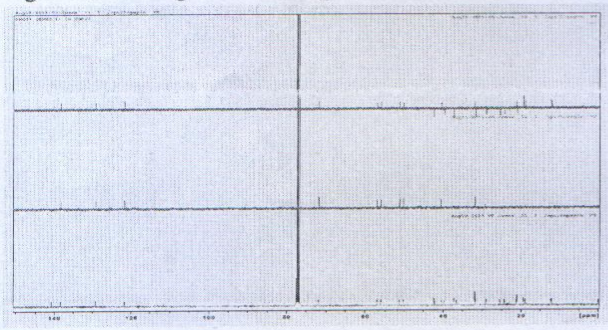


Figure 5: This figure shows β-Stigmasterol

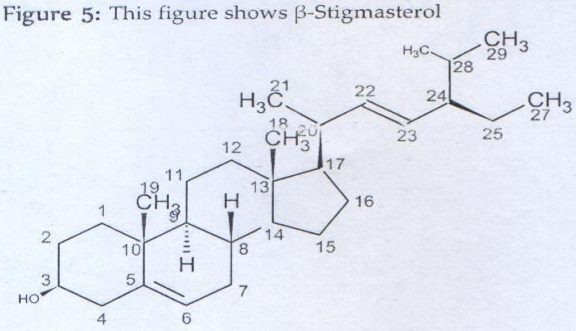


Table 1: This table shows ^{13}NMR data of Stigmasterol

S/No	β-Stigmasterol[6] Literature Value ^{13}C	β-Stigmasterol Experimental Value ^{13}C
1	37.3	37.2667
2	31.6	31.6811
3	71.8	71.8260
4	42.3	42.3209
5	140.8	140.7669
6	121.7	121.7248
7	31.9	31.9098
8	31.9	31.9098
9	51.2	51.2485
10	36.5	36.5242
11	21.1	21.0904
12	39.7	39.6918
13	42.3	42.3209
14	56.9	56.8779
15	24.4	24.3726
16	28.4	28.9270
17	56.1	55.9664
18	11	12.0547
19	21.2	21.2215
20	40.5	40.4976
21	21.2	21.2215
22	138.3	138.3258
23	129.3	129.2863
24	51.2	50.1705
25	31.9	31.6811
26	21.2	21.2215
27	19	19.4076
28	25.4	25.4145
29	12.1	12.2565

Source: Pateh et al [6]

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CONFLICT OF INTEREST

No conflict of interests was declared by authors

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Appendix V

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Isolation and bioactivity of pentacyclic triterpenoid (Betunilic acid) from the bark of *Sarcocephalus latifolius* (Smith Bruce)

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Abstract
Sarcocephalus latifolius is a plant variously used in Ethnomedicinal practices in Africa. The dried stem bark of this plant was subjected to continuous extraction using various solvents. The methanol fraction was subjected to vacuum liquid chromatography (VLC) to obtain SB-C2. The structure was established by various spectroscopic studies and comparison of the available data and seen to be Betunilic acid. The bioactivity of this compound was carried out using some clinical pathogens and the activity compared with a standard drug. It was discovered that the compound is comparable to the standard drug.
Keywords: Sarcocephalus latifolius, Methanol stem bark, Rubiaceae, vlc, SB-C2, Betunilic acid

1. Introduction

Sarcocephalus is a genus of tropical evergreen trees and shrubs belonging to the Rubiaceae family. This plant family is one of those frequently used by Ethnomedicinal practitioners in Sierra Leone and neighboring countries. It is a shrub or small spreading tree that is widely distributed in the Savannah and tropical Africa. It is known by the Hausa as *Tafashiya* or *tuwon biri,* known and called *Ubuluinu* in Igbo language in Nigeria. The current folk medicinal applications of *Sarcocephalus latifolius are* varied and numerous, for example the bark and the root extracts are said to be useful in malaria treatment (Akubue and Mittal 1982, *Oye 1990).* It is used as a tonic and remedy for treating fever, toothaches, dental cures, septic mouth, and diarrhea and in dysentery treatment and also used as chewing stick (Etkin et al 1990, Lamidi et al 1995).The bark is said to be useful in the treatment of wounds, cough and gonorrhea in Nigeria (Madubunyi, 1995).
The leaves are claimed to be useful in the treatment of fever while the roots and bark are claimed to be useful in the treatment of venereal disease, wounds and as odontalgic remedy. *(Pedro and Antonio, 1998).*
The crude root extract had been reported to have antihypertensive effect (Nworgu, 2009).New Indole alkaloids, 21-O-methylstrictosamideaglycone, Augustine, nauclifine, augustidine, 19-0-ethylaugustoline, naucleidinal, 19-epi-naucleidinal, quinovic acid-3β –O- β-D-fucopyranoside, quinovic acid-3β –O- β-D-rhamnopyranoside, scopoletin, and β-sitosterol had been isolated from the root. The strictosamide isolated from it was reported to show moderate antiplasmodial activity against plasmodium falciparium (Pedro and Antonio, 2001)

2. Plant Materials:

The stem bark of *Sarcocephalus latifolius* were collected from Okene local Government Area of Kogi state, Nigeria in March 2010.These were identified at the Herbarium of the Department of Biological Sciences of the Faculty of Sciences, Ahmadu Bello University, Zaria, Nigeria. A voucher number was deposited at the herbarium. The sample was air-dried and pulverized using wooden pestle and mortar. Finally, these were stored in an air-tight polythene bag and kept away from moisture until needed for analysis.

3. Phytochemical analysis

The plant part was screened for plant metabolites using the pulverized materials. Standard techniques of Brain and Turner (1975) were employed in the phytochemical screening. The presence or absence of the following metabolites were screened; carbohydrates, glycosides, Anthraquinones, cardiac glycoside, saponins, Flavonoids tannins, saponins and alkaloids

4. Extraction

Continuous extraction was used for 221.80g of the pounded stem bark of the plant material using the soxhlet extractor. Petroleum ether was initially used to defat it followed by chloroform, ethyl acetate and finally methanol. Each of the extracts was concentrated *in vacuo* using rotary evaporator at 40 ^{0}C and the resultant crude samples were air-dried until constant weight were obtained to afford; Petroleum ether extract (2.40g or 1.08%) ,Chloroform extract

(0.47g or 0.21%),Ethyl acetate extract(0.9g or 0.41%) and Methanol extract (22.7g or 10.23%) of crude products respectively.

5. Isolation of SB-C2 from methanol fraction

3g of the methanol extract of the bark of *Sarcocephalus latifolius* was dissolved in methanol and filtered to remove any undissolved lump. This was pre-absorbed in sufficient celite. This was packed with 30g silica gel and subjected to Vacuum Liquid Chromatography (VLC) using an isocratic solvent of petroleum ether: ethyl acetate (1:9) and by collecting 20ml fractions to obtain 45 fractions. Based on thin layer chromatographic (TLC) analysis, fraction 6, 7, 8 and 9 were combined to give SB-C which was later subjected to VLC and eluted isocratically using petroleum ether: ethyl acetate (1:9) to obtain eight fractions namely SB-C-1 to SB-C-8 .Fraction 6 showed a prominent single blue spot which was further cleaned using PTLC to obtain **SB-C2.**

6. Spectra result for compound SB-C2 (Betunilic Acid)

The Structure of the component was determined spectroscopically using Nuclear Magnetic Resonance Spectroscopy (NMR), 1D-NMR and 2D-NMR, Fourier Transform Infrared Spectroscopy (FTIR) and also by comparing the obtained data with already existing literature. The results obtained are as shown in the tables below.

IR (cm^{-1}): 2931.42, 1713.93, 1219.58, 933.98, 521.91, 495.23

^{1}H NMR(δ) 7.5865, 7.5628, 6.8985, 6.8244, 6.2612, 6.2375, 4.7135, 4.5837, 3.9553, 3.9335, 3.6449, 3.1852, 3.1726, 3.1571, 3.1447, 2.9769, 2.3280, 2.3089, 2.2778, 2.2565, 2.2483, 2.2249, 2.2178, 2.2089, 2.1858, 2.1796, 2.1564, 2.1490, 2.0229, 2.0142, 1.9879, 1.9775, 1.9622, 1.9495, 1.9338, 1.9202, 1.6665, 1.6334, 1.6118, 1.5830, 1.5707, 1.4983, 1.4894, 1.4757, 1.4648, 1.4352, 1.4271, 1.4171, 1.3948, 1.3807, 1.3705, 1.3509, 1.2786, 1.2308, 1.1971, 1.1849, 1.1460, 1.1140, 1.0777, 1.0691, 1.0603, 1.0505, 1.0278, 1.0166, 0.9969, 0.9853, 0.9515, 0.9418, 0.9152, 0.8985, 0.8755, 0.8687, 0.8576, 0.8403, 0.8210, 0.8001, 0.7847, 0.7316, 0.6710, 0.6560, 0.6477

^{13}C NMR(δ): 150.4123, 109.7041, 79.0180, 77.2210, 56.2366, 55.3593, 50.5426, 49.2678, 46.8730, 42.4524, 40.7093, 38.8719, 38.7270, 38.3636, 37.2161, 37.0266, 34.3428, 32.1504, 30.5442, 29.7039, 27.9901, 27.4046, 25.5159, 22.6964, 20.8653, 19.3729, 18.2996, 16.1359, 16.0272, 15.3508, 14.6996, -0.0034, 179.2092

7. Results and Discussion

The phytochemical screening of the stem bark of *Sarcocephalus latifolius* revealed the presence of carbohydrate, anthraquinones, cardiac glycoside, saponins, steroid triterpenes, tannins and alkaloids (Table 1)

The IR Spectrum (cm^{-1})of SB-C2 shows main absorption band at 2931.42 cm^{-1} which is typical of OH stretching vibration in an alcohol while another strong absorption observed at 1713.93 cm^{-1} may be due to a carbonyl (C=O) of the carboxylic acid in the molecule. The signal at 521.91 is typical for C=C olefinic. (Figure 1)

The 1H NMR spectrum (δ) of SB-C2 (Fig 2) revealed the presence of lupine type carbon skeleton. The signals at δ4.58 and δ3.93 appear as a proton doublet which indicates an exomethylene group while the signals observed at δ3.16 and δ2.98 could be due to secondary carbinol. Again, the broad spectrum at δ 1.63 is indicative of a vinyl methyl group. The signals at δ 0.66, 0.73, 0.90, 0.94 and 0.99 are due to five tertiary methyl groups. These data are typical of a pentacyclic triterpenoid of the Betunilic acid and comparison with published data suggested it to be Betunilic acid (Robert and Samir (2004)

The ^{13}C NMR spectrum (Figure 3) showed thirty (30) major recognizable carbon signals. Six methyl groups at δ 27.99 (C-23), 15.35 (C-24), 16.02 (C-25), 16.13 (C-26), 14.69 (C-27), 19.37 (C-30) and an exomethylene groups at 150.41 (C-20), 109.70 (C-29) and a secondary hydroxyl bearing carbon 79.01 (C-3) and a carbonyl group at 179.20 (C-28) (Table 2). In addition, twelve methylene groups, six methyl and six quaternary carbon atoms from the DEPT experiment were observed (Figure 4).The chemical shifts at δ150.41, 179.20 and 109.70 were characteristic peaks for Betunilic type of skeleton, which are due to C-20, C-28 and C-29 respectively (Ahmad, 1994).Other spectra from the 2D NMR really correlated this compound to be a Betunilic acid (Figure 5).

8. Bioactivity of SB-C2 (Betunilic acid)

The compound was found to be active on all micro organisms used in the work with the exception of Corynebacterium ulcerans. The zones of inhibition observed for the pathogens range between 21 to 34 mm .From Tables 3 and 4, it was observed that the MIC for the organisms were all 12.5 µg/ml with the exception of *Salmonella*

typhi and *Shigellia dysenteric* that had MIC values of 6.25 µg/ml respectively. The MBC/MFC values were recorded for *Staphylococcus aureus, Streptococcus pyrogenes, Bacillus subtilis, Escherichia coli, proteus vulgaris, salmonella typhi, Shigellia dysenteric* and *Candida virusei* were all 25 µg/ml but it was observed for *Methicillin Resistant Staphylococcus Aureus (MRSA), Proteus mirabilis, Pseudomonas aeruginosa, Candida albicans* and *Candida tropicalis* to be 50 µg/ml respectively. From Table 5, SB-C2 has a wider broad spectrum of activities when compared to the standard drug Sparfloxacin used in this study.

9. Conclusion

The isolation of Betunilic acid from the plant whose bioactivity was established from this work to be more active as a drug than Sparfloxacin more than justifies why the plant really serves as a general purpose antibiotic in traditional medicine in our society.

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Table 1: Preliminary phytochemical screening of the stem bark of *Sarcocephalus latifolius*

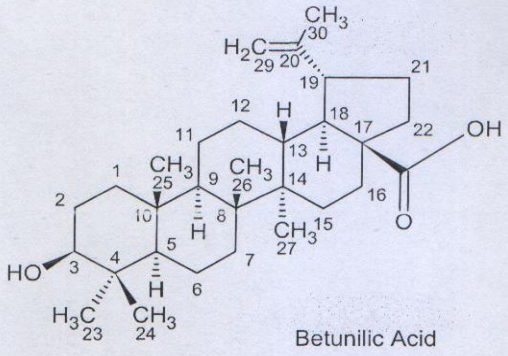
Property tested	Stem
Carbohydrate	+
Glycoside	–
Anthraquinones	+
Cardiac glycoside	+
Saponins	+
Steroidal triterpenes	+
Flavonoids	–
Tannins	+
Alkaloids	+

KEYS: + → Present – → Absent

Table 2: ^{13}C NMR data assignment for SB-C2 (Betunilic acid)

Carbon Position	^{13}C Shift ppm [a]Lit. Value	^{13}C Shift ppm [b]Lit. Value	^{13}C Shift Experimental	CH_n
1	38.70	39.0	38.36	CH_2
2	27.40	27.6	27.40	CH_2
3	78.90	78.2	79.01	CH
4	38.80	39.1	38.87	C
5	55.30	55.5	55.35	CH
6	18.30	18.4	18.29	CH_2
7	34.30	34.5	34.34	CH_2
8	40.70	40.8	40.70	C
9	50.50	50.7	50.54	CH
10	37.20	37.3	37.21	C
11	20.80	21.0	20.86	CH_2
12	25.50	25.7	25.51	CH_2
13	38.80	38.1	38.72	CH
14	42.40	42.5	42.45	C
15	30.50	30.2	30.54	CH_2
16	32.10	32.9	32.15	CH_2
17	56.30	47.1	56.23	C
18	46.80	48.1	46.87	CH
19	49.20	49.2	49.26	CH_2
20	150.30	150.1	150.41	C
21	29.70	30.6	29.70	CH_2
22	37.00	37.0	37.02	CH_2
23	27.90	27.9	27.99	CH_3
24	15.30	15.5	15.35	CH_3
25	16.00	16.4	16.02	CH_3
26	16.10	16.7	16.13	CH_3
27	14.70	15.0	14.69	CH_3
28	180.50	180.3	179.20	C00H
29	109.60	108.9	109.70	CH_2
30	19.40	19.6	19.37	CH_3

^{13}C NMR Litt. Values ([a]Mahato and Kundu 1994, [b]Prince P. Sharma *et al, 2010*)



Betunilic Acid

Fig.5

Table 3: Minimum inhibition Concentration of SB-C2 against the test microbes

Test Organism	50µg/ml	25.5µg/ml	12.5µg/ml	6.25µg/ml	3.125µg/ml
MRSA	–	–	O*	+	++
Staphylococcus aureus	–	–	O*	+	++
Streptococcus Pyrogenes	–	–	O*	+	++
Bacillus Subtilis	–	–	–	O*	++
Corynebacterium Ulcerans					
Escherichia Coli	–	–	O*	+	++
Proteus Mirabilis	–	–	O*	+	++
Proteus Vulgaris	–	–	O*	+	++
Pseudomonas aeruginosa	–	–	O*	+	
Salmonella typhi	–	–	–	O*	+
Shigellia aysenteric	–	–	–	O*	+
Candida albicans	–	–	O*	+	++
Candida Virusei	–	–	O*	+	++
Candida Tropicalis	–	–	O*	+	++

*Key: - =No colony growth, **O*** =MIC, + =light growth, ++ = Moderate colonies growth*

Table 4: Minimum Bactericidal/Fungicidal Concentration of SB-C2 against the test microbes

Test Organism	50µg/ml	25.5µg/ml	12.5µg/ml	6.25µg/ml	3.125µg/m
MRSA	O*	+	++	+++	++++
Staphylococcus aureus	–	O*	+	++	+++
Streptococcus Pyrogenes	–	O*	+	++	+++
Bacillus Subtilis	–	O*	+	++	+++
Corynebacterium Ulcerans					
Escherichia Coli	–	O*	+	++	+++
Proteus Mirabilis	O*	+	++	+++	++++
Proteus Vulgaris	–	O*	+	++	+++
Pseudomonas aeruginosa	O*	+	++	+++	++++
Salmonella typhi	–	O*	+	++	+++
Shigellia dysenteric	–	O*	+	++	+++
Candida albicans	O*	+	++	+++	++++
Candida Virusei	–	O*	+	++	+++
Candida Tropicalis	O*	+	++	+++	++++

*Key: - =No colony growth, **O*** =MIC, + =light growth, ++ = Moderate colonies growth*

Table 5: Minimum Inhibition Concentration of Spartloxacin against the test microbes

Test Organism	50µg/ml	25.5µg/ml	12.5µg/ml	6.25µg/ml	3.125µg/m
MRSA	–	O*	+	++	+++
Staphylococcus aureus	–	–	O*	+	++
Streptococcus Pyrogenes	–	–	O*	+	++
Bacillus Subtilis	–	–	O*	+	++
Corynebacterium ulcerans	–*	O*	+	++	+++
Escherichia Coli	–	O*	+	++	+++
Proteus Mirabilis	–	O*	+	++	+++
Proteus Vulgaris	–	O*	+	++	+++
Pseudomonas aeruginosa	–	–	–	–	–
Salmonella typhi	–	O*	+	++	+++
Shigellia dysenteric	–	–	O*	+	++
Candida albicans	Θ	Θ	Θ	Θ	Θ
Candida Virusei	Θ	Θ	Θ	Θ	Θ
Candida Tropicalis	Θ	Θ	Θ	Θ	Θ

Key: - =No colony growth, O =MIC, + = scanty colonies growth, ++ = Moderate colonies growth, +++ = Heavy colonies growth*

FIGURE 1: Infrared spectra of SB-C2 (Betunilic acid)

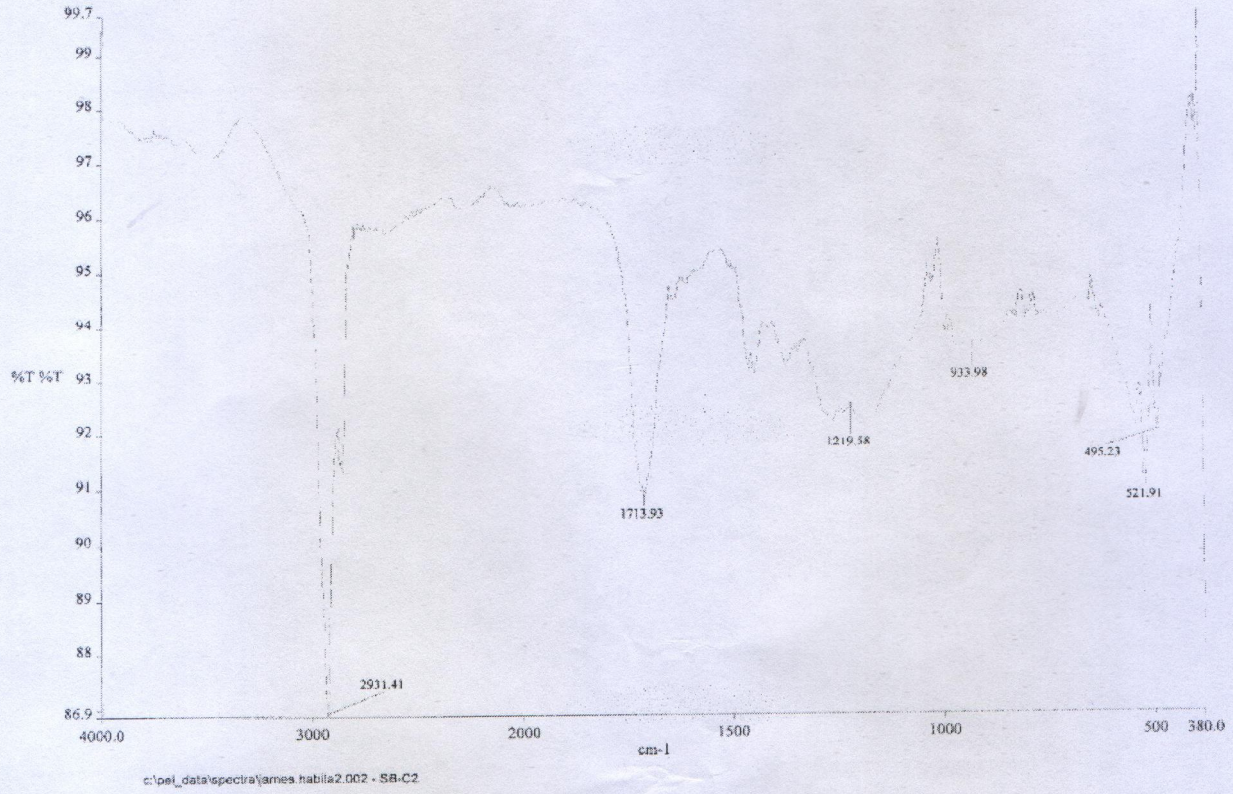


Figure 2: ^{1}H NMR spectra of SB-C2

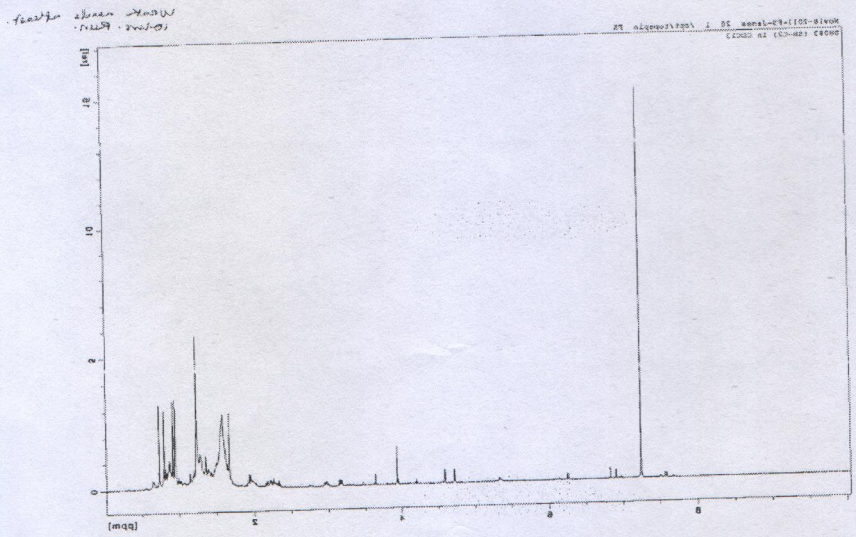


Figure 3: ^{13}C NMR spectra of SB-C2

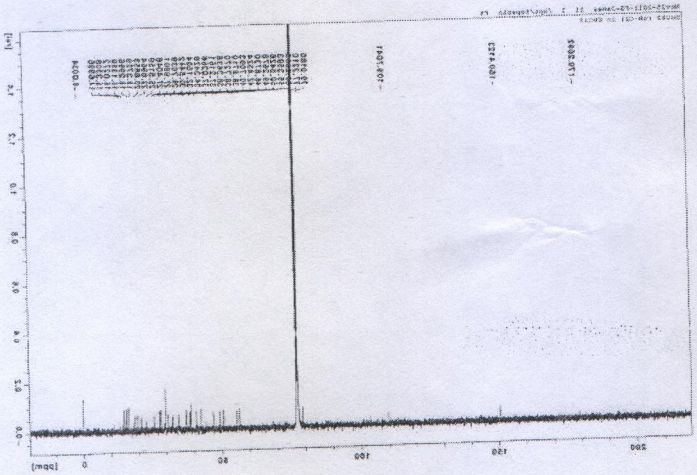
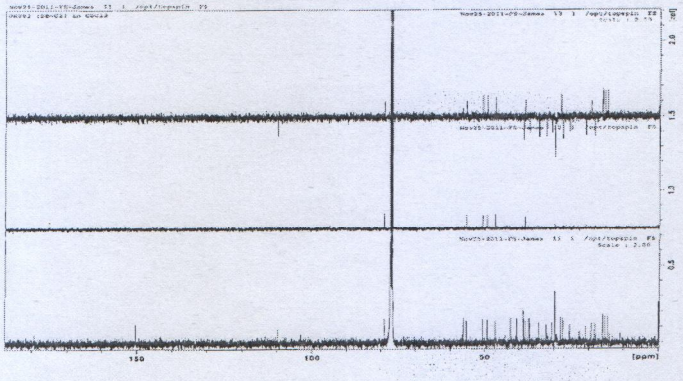


Figure 4: DEPT-135 spectra of SB-C2



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