

**PHYTOCHEMICAL AND ANTIMICROBIAL SCREENING OF THE STEM  
BARK EXTRACT(S) OF *INDIGOFERA ARRECTA* HOCHST EX A.  
RICH (FABACEAE)**

*BY*

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## DECLARATION

I declare that work in this thesis titled phytochemical and antimicrobial screening of the stem bark extract of *Indigofera arrecta* was performed by me, in the department of Chemistry, under the supervision of Professors G.I Ndukwe and J.O Amuputan. The information derived from literature has been duly acknowledged in the text and a list of references provided. This thesis serves as a confirmation of the ethno-medicinal uses of the plant.

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## CERTIFICATION

This thesis titled phytochemical and antimicrobial screening of the stem bark extract(s) of *Indigofera arrectaby* Ebere PromiseAkabuogu,meet the regulation governing the award of Master of Science degree in Organic Chemistry of the Ahmadu Bello University, Zaria,and is approved for its contribution to knowledge and literary presentation

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## **DEDICATION**

This research work is dedicated to the glory of God for his infinite mercies, blessings, protection and guidance.

## ACKNOWLEDGEMENT

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## ABSTRACT

The Pulverized stem bark of *Indigofera arrecta* was exhaustively extracted with methanol and concentrated in vacuo using rotary evaporator at 40 °C. The extract was later subjected to solvent partitioning to yield soluble extracts of n-hexane, ethyl acetate, chloroform, and methanol. General phytochemical screening of the fractions revealed the presence of secondary metabolites such as cardiac glycoside, steroid, terpenes flavonoids and tannins. The antimicrobial activity against *S. aureus*, *S. pyogenes*, *S. feacalis*, *S. typhii*, *E. coli*, *C. ulcerans*, *P. vulgaris* and *C. albicans* was tested using the tube dilution and agar diffusion methods as outlined by the NCCLS. The results of the antimicrobial activity as indicated by the zones of inhibition of growth of microorganism ranged from 20mm to 40mm for the n-hexane extract, 16mm to 21mm for ethyl acetate extract and 20mm to 27mm for the methanol extract. The MIC result for the n-hexane, ethyl acetate and methanol extracts ranged from 7.5mg/ml to 15mg/ml. The MIC of 15mg/ml exhibited by the n-hexane extract against both gram positive and gram negative bacteria indicates broad spectrum activity of *Indigofera arrecta*. The n-hexane fractions was subjected to Column Chromatography using silica gel to yield 87 fractions, which were combined based on their thin layer chromatography analysis and recrystallized in methanol to give a pure white crystalline powder, which melts at 144°C. The structure of the isolated compound was established by spectroscopic analysis and by direct comparison of the data obtained with those reported in literature to be Stigmasterol (3 $\beta$ ,22E-Sigmasta-5,22-dien-3-ol).

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## LIST OF ABBREVIATION

cm	Centimeter
cm <sup>-1</sup>	Per centimeter
Hz	Hertz
ml	Milliliter
nm	Nanometer
ppm	Parts per million
UV	Ultra violet
IR	Infrared
$\lambda_{\max}$	Wave length of maximum absorption
TLC	Thin layer chromatography
$\delta$	Chemical shift in ppm
s	Singlet
d	Doublet
dd	Double doublet
MHz	Mega hertz
%	Percentage
R <sub>f</sub>	Retardation factor
HMBC	Hetero nuclear multiple bond correlation
HSQC	Homo nuclear single quantum coherence
COSY	Correlation spectroscopy
DEPT	Distortionless enhancement by polarization transfer
MIC	Minimum inhibitory concentration
MBC	Minimum bactericidal concentration

## CHAPTER ONE

### 1.0 INTRODUCTION

#### 1.1 Definition of a drug

A drug can be described as any chemical substance that has no nutritional value when introduced into the body but causes some physiological effects within the system (Mbah, 2000). Drugs are classified under pharmaceuticals. Pharmaceutical drug, according to Dey (2006), also refers to as medicine or medicament, can be loosely defined as any substance intended for use in the diagnosis, cure, mitigation, treatment or prevention of diseases.

Some pharmaceuticals occur naturally in plants. These can be called phyto pharmaceuticals. By the strictest definition, they are drugs or chemicals that may have health related effects but are not considered essential nutrients. Protein, carbohydrates, fats, minerals and vitamins are regarded as essential nutrients. Some pharmaceuticals found in plants include gedunin and nimbolide from *Azadirachta indica* (Neem) (Khalid and Duddeck, 1993); santonin, a sesquiterpenoid lactone is found in species of *Artemisia* which grows in Asia, quinine and alkaloid occurs in the bark of *cinchona* tree; penicillin- a beta-lactam is produced by fungi in the genus *Penicillium* (Finar, 2003) and reserpine-an alkaloid is isolated from *Rauwolfia* plant.

#### 1.2 Medicinal plants

According to biblical records, Prophet Ezekiel reported that the fruits will serve as food and their leaves for healing (Ezekiel 47:12). Thus, the use of plants for medicinal purposes dates back to thousands of years ago as the earliest humans used various plants to treat illness

(Dey, 2006). Medicinal plants or healing herbs, as they are called, are used in treating and preventing specific ailments and diseases and as such are considered to play a beneficial role in health care.

Srivastava *et al.*, (1996) earlier stated that hundreds of plant species are recognized as having medicinal values and four out of every five of those plants are collected from the wild forest while most are from the flora of developing countries. The medicinal properties or values may be present in one or all the plants parts like roots, stem, bark, leaves, flower, fruit or seeds.

In fact, with all the progress in synthetic chemistry and biotechnology, plants are still indispensable source of drugs and natural products on the basis of their therapeutics (Lawn, 1993).

Some common medicinal plants that occur in our locality include *Azadirachta indica* (Neem), *Ocimum gratissimum*, *Persea americana*, *Vernonica amygdaline*, *Astonia boonei*, *Zanthoxylum gilleti* and *Bucchoslozia coreaca* among others.

### **1.3 Medicinal plant research**

The efficacy of these medicinal plants is based on the presence of secondary metabolites which belong to a group of compounds called natural products. Natural products are those chemical compounds derived from living organisms, plants, animals, insects and the study of natural products is the investigation of their structure formation, applications and purpose in the organisms. The drugs or active ingredients derived from natural products are usually secondary metabolites, for example, terpenes, flavonoids, saponins and alkaloids and their derivatives. Today, these compounds must be pure and highly characterized compounds through scientific research.

Medicinal plant research starts by people carrying out general screening of plants which are collected either randomly or based on local reputations as medicinal plants after botanically identified by a reputable authority or plant taxonomist. This screening consists mainly of solvent extraction and standard tests of the extracts for the presence of such class of compounds or secondary metabolites as alkaloids, saponins and phenolic compounds. This in itself may not lead to the discovery of any new biologically active compound if carried out competently and consistently but it could provide data from which plants with potential biological activity could be selected for further detailed study (Adjanooun et al., 1991, Farnsworth, 1996).

The extracts are then fractionated with a view of isolating pure compounds. Modern isolation techniques include all types of chromatography often guided by bioassays to isolate the active compounds. The chromatographic procedures include absorption and partition chromatography in columns, thin layer and recently high performance liquid Chromatography. The structures of the isolate in modern times are elucidated primarily by spectroscopic techniques. The compounds can be identified as already known compounds or entirely new compounds.

### **1.3.1 AIM**

1. The aim of this work was to justify or otherwise the claimed ethnomedicinal uses of the plant

### **1.3.2 OBJECTIVES**

- I. Collection, proper botanical identification, drying and pulverizing of the plant.

- II. Extraction of the powdered plant material using different solvents based on the elutropic series i.e from non-polar to most polar.
- III. The extract of this plant would be subjected to phytochemical and antimicrobial screening
- IV. Analytical separations involving several consecutive steps of chromatographic techniques.
- V. Structural elucidation of the isolated compound(s) using spectral techniques

#### **1.4:SCOPE AND LIMITATION OF THE RESEARCH**

The scope of this research work would be:

1. The phytochemical screening, antibacterial, antifungal screening, isolation, characterization and structural elucidation of the active principles of *Indigofera arrecta*.

#### **1.5 JUSTIFICATION OF THE RESEARCH**

The need to know the active ingredients in the stem of *Indigofera arrecta* which are responsible for the cure/treatment of various ailments such as epilepsy, sores treatment, diarrhea and ulcer and to have scientific evidence of the claims of the ethnomedicinal practices of the stem of *Indigofera arrecta*.

## CHAPTER TWO

### 2.0 LITERATURE REVIEW

#### 2.1 BOTANICAL DESCRIPTION OF THE GENUS *INDIGOFERA ARRECTA*

Annual to perennial herbs or subshrubs up to 2(–3) m tall; Erect stem, copiously branched, slightly ridged, covered with appressed, whitish or brownish and 2-branched hairs. Leaves are arranged spirally, imparipinnate; stipules subulate to bristle-like, 2–9 mm long; petiole up to 1.5 cm long, thickened at base, rachis up to 6 cm long; stipels subulate, up to 1 mm long; petiolules c. 1 mm long; leaflets 7–21, narrowly elliptical-oblong, up to 20 mm × 7 mm, usually glabrous above, appressed hairy below. Inflorescence a usually sessile, many flowered axillary raceme up to 5 cm long but usually much shorter; bracts lanceolate, c. 1 mm long, caducous. Flowers bisexual, papilionaceous; pedicel c. 1 mm long, strongly reflexed in fruit; calyx c. 1.5 mm long, the tube about as long as the 5 triangular lobes, brownish appressed hairy; corolla c. 5 mm long, pinkish or reddish, standard obovate, narrowed gradually to the base, wings with very short claws, keel laterally spurred; stamens 10, 3–4 mm long, upper one free, the other 9 united into a tube; ovary superior, 1-celled, with long style. Fruit a linear pod 12–17 mm long and c. 2 mm wide, straight, slightly tetragonal, brown when ripe, 4–6-seeded with slight constrictions between the seeds. Seeds shortly oblong, c. 2 mm × 1.5 mm, rhombic in cross-section. Seedling with epigeal germination cotyledons thick, short-lasting.

##### 2.1.1 Other botanical information

*Indigofera* is a very large genus comprising approximately 700 species and is distributed throughout the tropics and subtropics of Africa, Asia and the Americas. In Africa and the

southern Himalayas are the richest in species. Over 300 species have been recorded for tropical Africa.

For indigo production several *Indigofera* species are used but there are 3 closely related major ones: *Indigofera arrecta*, *Indigofera tinctoria* L., which probably originates from tropical Asia but is now distributed pantropically, and *Indigofera suffruticosa* Mill., originating from tropical America and now locally cultivated elsewhere in the tropics, including Africa and Madagascar but not in tropical East Africa. The origin and identity of *Indigofera* plants cultivated for dye production is often obscure as a result of introduction, selection and the close affinity of species. *Indigofera arrecta* is sometimes difficult to separate from *Indigofera tinctoria*. The latter usually differs in its larger and less numerous leaflets and longer fruits containing more seeds. In East Africa, but not in West Africa, *Indigofera arrecta* generally occurs at higher altitudes (1000–2000 m) than *Indigofera tinctoria* (below 1000 m). *Indigofera suffruticosa* differs from *Indigofera arrecta* by its short, 10–15 mm long, red-brown pods. Intermediate specimens between these 3 species have been found, possibly of hybrid origin.

### **2.1.2 ORIGIN AND GEOGRAPHIC DISTRIBUTION**

Natal indigo originates from Africa. It occurs almost throughout tropical Africa, and also in northern and eastern South Africa, Swaziland and southern Arabia. Its range has probably been extended by its cultivation for indigo and subsequent naturalization. It is widely planted in India and south-eastern Asia.

The use of indigo extracted from *Indigofera* species has a long history. Indigo plants and the dyestuff were already mentioned in the oldest Sanskrit records. The blue dye used for textiles of Egyptian mummies may, in some cases, come from an *Indigofera* species but woad (*Isatis tinctoria* L.) is another indigo-blue producing plant known to ancient Egyptians. The most ancient archaeological textiles discovered in West Africa, the ‘Tellem textiles’, were found in funeral caves in the Bandiagara cliff in the Dogon area of Mali. Some of them date back to the 11<sup>th</sup> or 12<sup>th</sup> centuries and already include stripes, checks and tie-and-dye patterns done with indigo from *Indigofera* or *Philenoptera* species. An early description of the local extraction process of indigo from an *Indigofera* species in Guinea was left by the Portuguese explorer André Alvares de Almada in 1566. In Africa Natal indigo has been the most important species for the production of indigo since the beginning of the 20<sup>th</sup> century.



**Figure 1: THE PLANT *INDIGOFERA ARRECTA***

## 2.2 CHEMICAL CONSTITUENTES OF *INDIGOFERA ARRECTA*

The need to identify active chemical constituents in plant extracts requires phytochemical and analytical techniques. Different phytoconstituents have different degrees of solubility in different types of solvents depending upon their polarity and structure (El-Mahamood and Doughari 2008). Phytochemical surveys are being seen as the first step towards the discovery and structural elucidation of useful natural organic constituents for textile or medicinal applications (Hostettmann et al., 2000). Many plants are chemically very variable depending on the locality where they are found with some of the constituents occurring only at certain seasons of the year (Adelani, 2007).

The mode of action of plants producing dyeing effects on selected textile materials can be better investigated if the active ingredients are identified and characterized. Leafy twigs of *Indigofera arrecta* and some closely related *Indigofera* species are the main sources of the indigo dye used since very ancient times for dyeing textiles blue. The leaves and twigs do not contain indigo but colourless precursors that must be extracted and processed to produce the indigo dye. Because of its fascinating deep blue colour, its great colour fastness to light and the wide range of colours obtained by combining it with other natural dyes, indigo has been called 'the king of dyes'. No other dye plants have had such a prominent place in as many civilizations as *Indigofera* species.

In western Africa indigo is by far the most important dye of plant origin. It plays an important role in traditional cultures of many people, whether only wearers of indigo-dyed textiles like the Tuareg in the Sahara and Sahel region (Niger, Mali), or renowned indigo dyers, among which the Soninke (Sarakole) and Wolof in Senegal, the Marka women of the Djenné region in Mali, the Dyula and Baule in Côte d'Ivoire, the Yoruba and Hausa in Nigeria and the

Bamoum and Bamileke in Cameroon. This part of the world is a major centre of textile decoration techniques based on the concept of ‘ resist-dyeing’ and linked with indigo dyeing: intricate patterns are made on the cloth that will resist being dyed. This is done by tying, sewing or plaiting parts of the cloth or by covering them with starch pastes or wax. The piece of cloth is then plunged into the indigo vat to dye the untreated parts. After the resist threads or pastes have been removed, white patterns on a blue ground appear. Light blue patterns on a blue-black ground are formed if a last indigo bath is given after undoing the resist. In Madagascar too, indigo dyeing is very important, for instance in raffia ikats, beautiful textiles in which the ‘resist’ patterns are formed by tying parts of the warp yarns with thick threads before dyeing them with indigo and putting them onto the weaving loom, which again creates white designs on a blue ground. However, the use of natural indigo is rapidly declining and nowadays, synthetic indigo is almost exclusively used, not only in industrialized processes but also at craft level. *Indigofera arrecta* is grown as a cover crop and for green manure, especially in tea, coffee and rubber plantations. It gives shade and protection, suppresses weeds and improves the soil. The residue remaining after indigo extraction is also applied as manure. In Malawi the young leaves are eaten as a vegetable. The plants are grazed by live stock.

### **2.2.1 TRADITIONAL MEDICINAL USES OF *INDIGOFERA ARRECTA***

Numerous applications in traditional medicine have been reported: leaves and roots are used externally to treat itching and in an infusion or decoction as an antispasmodic, sedative, febrifuge, vermifuge, abortive, diuretic and purgative, e.g. to treat gum infections, snakebites, gonorrhoea, epilepsy and jaundice; the fruits and seeds are used to treat ophthalmia. (Gills, 1992). In Ghana an aqueous extract of leaves from young shoots is administered orally to patients with *Diabetes mellitus*. A medicine for the management of peptic ulcer and methods of its preparation

and use have been patented. In several regions in Africa it is believed that the indigo in clothes prevents skin complaints. In East Africa the twigs are used for cleaning teeth.

### 2.2.2 USES OF SOME OF THE GENUS

Several of them and especially *indigofera tinctoria* and *indigofera suffruticosa* are used to produce the dye indigo. *Indigofera arrecta*, *Justicia betonica* and *Rubia corrdifolia* potential dye-yielding plants were screened to determine the characteristic chemical constituents present in their crude plant extracts. Screening procedures and standard methods of qualitative chemical analysis of plant extracts reported by various authors (Faraz et al., 2003; Laity et al., 2002, Ndukwe et al., 2007, Venkatesan et al., 2009, El-Mahmood and Doughari, 2008) were used in the study, with the aim of further isolation and characterization. Colonial planters in the caribbean grew indigo and transported its cultivation when they settled in the colony of South Carolina. Exports of the crops did not expand until the mid-to late 18<sup>th</sup> century. When Eliza Lucas Pinckney and enslaved Africans successfully cultivated new strains near Charleston it became the second most important cash crop in the colony (after rice) before the American Revolution. It comprised more than one-third the value of all exports.

The chemical, aniline, from which many important dyes are derived, was first synthesized from *indigofera suffruticos*.

Several species of this group are used to alleviate pain. The herbs are generally regarded as analgesic with anti-inflammatory activities, rather than an anodyne. *Indigofera articulate* Gouan (Arabic khedaish) was used for toothache and *indigofera oblongifolia* forsskal (Arabic Hasr) was used as an anti-inflammatory for insect stings, snakebites, and swellings. (Okwu and Ekoaduchi, 2004).

*Indigofera suffruticosa* and *indigofera aspalthoides* have also been used as anti-inflammatories. The Maasai people of Kenya use parts of *Indigofera brevicalyx* and *Indigofera swaziensis* as toothbrushes.

In Indonesia, especially Sundanese ethnic traditionally used *Indigofera tinctoria* L called a” tarum” as dye for batik. The use of”tarum”had faded away since Dutch colonialism introduces artificial color.

### **2.3.0 PRODUCTION AND INTERNATIONAL TRADE**

An antiquity indigo was traded from India to the Mediterranean region, but this became more important during the Middle Ages. Large-scale cultivation of *Indigofera* started in the 16<sup>th</sup> century in India and south-eastern Asia. Later, large plantations were established in Central America and in the southern United States. The large-scale export of indigo from Asia to Europe started at about 1600 and had to compete with dye from wood which was cultivated mainly in France, Italy, Germany and Great Britain. By the end of the 17<sup>th</sup> century, indigo had almost completely replaced wood. Synthetic indigo, which came into commercial production in 1897, proved catastrophic to the production of natural indigo, and by 1914 only 4% of the total world production was of plant origin. Then followed a period in which synthetic indigo lost some of its importance due to competition from new synthetic dyes, but the ever growing popularity of blue jeans enormously contributed to a revival of interest in indigo. At present, the crop is still cultivated for dye production on a small scale in India and in some parts of Africa, southern Arabia (Yemen), Central America and Indonesia. The most important present-day centre of indigo production from *Indigofera* is probably the northern part of Karnataka state in India. In India, annual production of *Indigofera* dye gradually decreased from 3000 t from 600,000 ha in

1890, to 50 t from 4000 ha in the 1950s, to an annual export fluctuating between 2t and 20t in the 1990s.

### 2.3.1 PHARMACOLOGICAL PROPERTIES

*Indigofera* plants contain the glucoside indican(Samantha and Agarwal.,2009). After soaking the plants in water, enzymic hydrolysis transforms indican into indoxyl and glucose. Indigotin (often also called indigo or indigo-blue) formation results from the grouping of two molecules of indoxyl in the presence of oxygen. Indigotin is insoluble in water, so to dye textiles it must be reduced to a soluble form (colourless 'leuco' indigo or indigo-white) by a fermentation process under alkaline conditions or by a chemical reducing agent such as sodium dithionite. Subsequent oxidation by airing of the textile after its removal from the dye bath, results in the regeneration of indigotin and fixation of the blue colour onto the textile. Natural indigo can contain varying proportions of a chemically related red dye called indirubin and of the minor isomeric compounds isoindirubin (red) and isoindigo (brown). The leaves of *Indigofera arrecta* contain 4.5% N, 0.02% P<sub>2</sub>O<sub>5</sub>, 1.95% K<sub>2</sub>O and 4.5% CaO. Whole plants of Natal indigo showed good palatability in sheep. An aqueous extract prevented the development of hyperglycaemia in genetically obese diabetic mice. In tests with rats, an intraperitoneal administration of a hot water extract of dried leaves decreased the plasma glucose levels of fasting normoglycaemic rats, but did not prevent the rise in plasma glucose after an oral glucose load. It was suggested that the extract is insulinotropic and may require functional β-cells to be active. In tests with healthy, non-diabetic, young adult male volunteers in Ghana, an extract of Natal indigo increased the erythrocyte sedimentation rate and decreased lymphocyte concentration in the blood. It did not alter the mean systolic and diastolic pressures, nor did it

change fasting blood glucose, whereas serum marker enzymes and metabolites for hepatic and renal functions remained normal. These data suggest that the species may not have overt toxic reactions but could affect the immune status of users. The extract also showed no acute and subchronic toxic effects in tests with mice.

### **2.3.2 ADULTERATIONS AND SUBSTITUTES**

Several other, often unrelated, plant species are sources of indigo dyes. Examples include woad from Europe, *Polygonum tinctorium* Aiton from China, Korea and Japan, Assam indigo (*Strobilanthes cusia* (Nees) Kuntze) from Indo-China and Thailand and *Marsdenia tinctoria* R.Br. from tropical. Other natural sources of indigo are a mutant of the fungus *Schizophyllum commune* and the purple snail *Hexaplex trunculus*, which mostly contains precursors of indigo and only minor proportions of the bromine derivatives of indigo of which the famous Tyrian purple dye of antiquity was composed. The most important substitute of natural indigo, however, is the synthetic industrial product.

### **2.3.3 GROWTH AND DEVELOPMENT**

Seeds germinate in about 4 days. Plants may start to flower 3 months after sowing. Like many other leguminous plants, *Indigofera arrecta* forms root nodules with nitrogen-fixing capacity with e.g. *Rhizobium indigoferae*. The total lifespan for dye crops is 2–3 years when grown as a ratoon crop.

#### **2.3.4 ECOLOGY**

*Indigofera arrecta* occurs in open deciduous forest, upland evergreen bushland, often in forest margins, and secondary regrowth. It occurs at 200–2700 m altitude, in regions with an annual rainfall of 400–1800 mm. The plant is deep-rooting and withstands drought well. When used as a cover crop, Natal indigo can only be grown in gardens or plantations with little or no shade. An established crop can withstand very wet soil for up to 2 months.

#### **2.3.5 MANAGEMENT**

The crop normally requires little attention after sowing. Weeding is done when needed. As a cover crop, Natal indigo is slashed at regular intervals. Production of seed is usually poor in plants that have been cut. Seed production therefore requires plants to be grown specifically for this purpose.

#### **2.3.6 PROPAGATION AND PLANTING**

Propagation is usually by seed. Seed yields of 675–1200 kg/ha have been reported for India. The seeds have a hard seed coat and soaking overnight in water or scarification with sulphuric acid is needed before sowing. Fields are prepared by hoeing or by one or two ploughings after showers followed by light harrowing before and after broadcast sowing. Sowing in a nursery and transplanting into the field may also be practised.

#### **2.3.7 DISEASES AND PESTS**

Natal indigo is attacked by *Ralstonia solanacearum* (synonym: *Bacillus solanacearum*), several fungi and nematodes.

Indigofera species are used as food plants by the larvae of some Lepidoptera specie including Turnip moth.

### **2.3.8 HARVESTING**

Branches are harvested when the plants are 4–5 months old and have formed a closed stand, usually at the flowering stage. The plants can be cut again 2–4 months later. Up to 3 harvests are possible per year when it is grown as a ratoon crop.

### **2.3.9 YIELD**

The dye yield from Natal indigo is higher than from any other *Indigofera* species. Annual yields of 22–100 t green matter per ha have been reported in India; the recorded output of indigo cake is 135–325 kg/ha per year.

### **2.4.0 HANDLING AFTER HARVEST**

In small-scale cultivation in Africa, harvested branches are often pounded to a soft pulp and made into balls, which are sold on the market after drying. This is the method described by André Alvares de Almada in 1566 and in all early reports. In large-scale cultivation the branches are put in a water containing tank or pit immediately after harvesting. After some hours of fermentation, during which enzymic hydrolysis leads to the formation of indoxyl, the liquid is transferred to another pit or tank and stirred continuously for several hours to stimulate oxidation of the indoxyl to indigotin. Afterwards, the solution is left to rest and the insoluble indigotin settles down to the bottom as a bluish sludge. The water is drained off, and after the indigotin has

been washed to get rid of impurities, it is pressed, dried and usually cut into cubes which can be packed and sold.

To dye textiles, indigotin must be reduced to a soluble form under alkaline conditions. In traditional processes (indigo vats), the reduction of indigotin into soluble indigo-white is achieved through a bacterial fermentation. The reducing bacteria are obtained in the bath by adding vegetable matter such as the crushed indigo balls, or, when indigo powder is used, crushed balls of *Philenoptera* leaves, or, according to some recipes, molasses, coconut-milk, banana or guava leaves. An alkaline pH of around 8.2 is adequate and is maintained by adding a potash lye prepared from the ashes of different calcinated plants specially selected for this purpose in each region

#### **2.4.1 GENERAL DESCRIPTION OF *INDIGOFERA ARRECTA* (HOCHST EX. A. RICH )**

**The general description of *Indigofera arrecta* is given as follows:**

- a) Taxonomy (Hochst ex A. Rich)

The scientific classification is:

**Kingdom:** *Plantae*

**Phylum:** *Anthocerotophyta*

**Class:** *Magnoliopsida*

**Order:** *Fabales*

**Super order:** *Rosanae*

**Family:** *Fabaceae*

**Genus:** *Indigofera indigo*

**Species:** *Indigofera arrecta*

**Vernacularnames:** *Ba-ba 2*: Hausa

*Natal indigo*: English

## **2.5.0 REVIEW OF SOME NATURAL PRODUCTS FROM PLANTS, TESTS AND THEIR USES.**

Natural products are those compounds derived from plants and animals. They can further be defined as the end-products of metabolism occurring in living organisms which can be products of primary metabolism and secondary metabolism [Akpuaka, 2009].

The primary metabolites are the sugars and their derivatives, the twenty essential amino acids, the common fatty acids and the simple carboxylic acids.

The secondary metabolites which are the products of secondary metabolism are derived from the primary metabolites. Their distribution is species-dependent. They are produced from the natural products of primary metabolism by specific, genetically controlled, enzymatically catalyzed series of reactions.

The plant natural products of secondary metabolism include alkaloids, flavonoids, saponins, glycosides, tannins, terpenes and steroids (Akpuaka, 2004).

Some plants are pharmacologically potent, some are toxic and lethal and some are used as flavours, perfumes, drugs, dyes etc due to the presence of one or more of these plants natural products. As such some of them may play a beneficial role to the plant and animals in general.

These natural products are present in one or all of the parts of a plant like the roots, stem bark, leaf, flower, fruit or seed.

### **2.5.1 ALKALOIDS**

Alkaloids (which means alkali-like) is the name given to all organic bases isolated from plants [Finar, 2005]. They can further be defined as a group of naturally occurring amine derivatives which have very powerful physiological effects on mammalian systems they include those basic substances which contain one or more Nitrogen atoms usually in combination as part of a cyclic system [Khan and Khan, 2006].

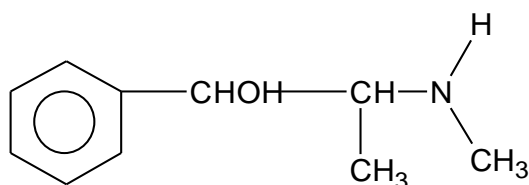
They are usually very poisonous but in small doses may have very useful physiological effects e.g. Quinine is an anti-malaria.

Even today, it is difficult to define an alkaloid but the term is generally limited to organic bases formed in plants. Not all authors do this, and so they specify those alkaloids obtained from plants as plant alkaloids or vegetable alkaloids. However, the basic properties complex structures, physiological action and plant origin are the main characters which define plant alkaloids.

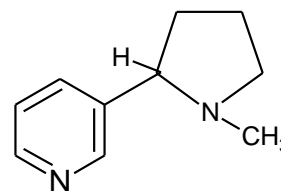
In effect, an alkaloid is a plant derived compound that is toxic or physiologically active, contains a nitrogen in a heterocyclic ring, is basic, has a complex structure and is of limited distribution in the plant kingdom. The Nitrogen in alkaloids generally makes the compound basic and the compound exists in the plant as a salt. Thus alkaloids are often extracted with water or mild acid and then recovered as crystalline material by treatment with a base. Alkaloids contain Nitrogen which are usually found in the rings although there are exceptions where the nitrogen

atoms are not included in the rings e.g. Ephedrine. They are usually bitter substances which can be attributed to their alkaline or basic pH.

Alkaloids are produced by a large variety of organisms including bacteria, fungi, plants and animals. Examples of alkaloids are the local anesthetic and stimulant cocaine, the stimulant caffeine, nicotine, the analgesic morphine, or the anti-malarial drug quinine and some of the alkaloids have a bitter taste [Arora, 1986].



Ephedrine



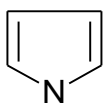
Nicotine

### Classification of alkaloids

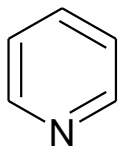
It is difficult to categorize alkaloids in precise terms. Thus the classification of alkaloids is still somewhat arbitrary owing to the difficulty of classifying them into distinct groups [Henry, 1995].

However some scientists usually classify them according to their biological source in which they occur. Hence, there are morphine or opium alkaloid from opium poppy (*Papaver somniferous*), tropane alkaloid (atropine) from *Atropa belladonna* and Ergot alkaloids from a fungus, *Claviceps purpureae*.

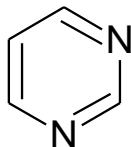
Others chose to classify them by the nature of the heterocyclic rings present in their molecular structure. These heterocyclic rings are pyrrole, pyridine, pyrrolidine, indole, quinoline, isoquinoline, purine etc.



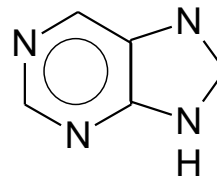
Pyrrole



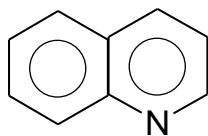
pyridine



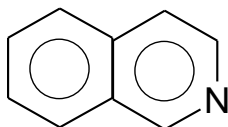
pyrimidine



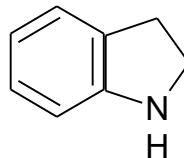
purine



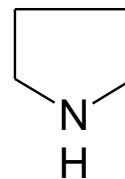
Quinoline



Isoquinoline



Indole



Pyrrolidene

Heterocyclic rings present in the alkaloid molecule [Morrison and Boyd, 2006]

### Properties of alkaloids

Alkaloids are usually colourless non-volatile solids, insoluble in water but soluble in chloroform, ether, etc. Some are liquids which are soluble in water e.g. Nicotine and conine. Few liquid alkaloids are coloured e.g. Berberine is yellow. Most alkaloids have bitter taste as in

*Vernonia* species (bitter leaf). Alkaloids are optically active (laevorotatory). They usually contain nitrogen atoms in the tertiary state. They also contain oxygen atoms. Alkaloids are soluble in hydrochloric acid.

Alkaloid form insoluble precipitates with solutions of many compounds such as:

Mayer's reagent [Potassium-Mercuric iodide] gives white precipitate

Draggendorf's reagent [Potassium bismuthic compound] gives red or orange precipitate

Marquis reagent (H<sub>2</sub>SO<sub>4</sub> and formalin mixtures) Wagner's reagent (iodine in Potassium iodide) gives chocolate or brown precipitate.

Many of these precipitates have definite crystalline shapes and colours and may be used in the identification of an alkaloid. Some of the reagents are used to detect alkaloids in Paper and Thin Layer Chromatography [Akpuaka, 2009].

### **Uses of alkaloids**

The usage, according to Raffaut, (1996), depends on the type of the alkaloid but generally, they can act as analgesics, stimulant anti-malaria local anesthetic or in diabetic therapy. For example, Quinine, an alkaloid, is used as an anti-malaria in killing malaria parasite in the blood of patient suffering with the disease. Cocaine is another alkaloid of great use to the doctor and dentist. It is used in the surgical and dental practice as an anesthesia for deadening the tissues to pain. Morphine and codeine are used as narcotic and analgesics while caffeine from coffee, tea, cocoa and cola is a central Nervous system stimulant [McGraw, 1997].

## Occurrence and distribution

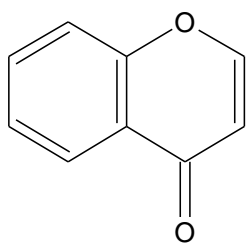
The Alkaloids are found most frequently in the higher seed-bearing plants and especially Dicotyledons. Occurrence in the lower seed bearers is rare.

Generally, they are usually found in the seeds, roots, leaves or bark of the plant and they are usually found as salts of various acids such as oxalic, citric, malic, tartaric and acetic acids.

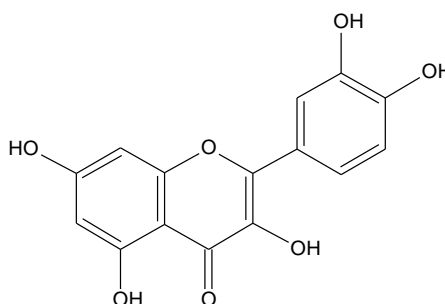
Alkaloids are regarded as end-products or by products of amino acid metabolism in plants.

### 2.5.2 FLAVONOIDS

The term flavonoids (or bioflavonoids) refers to a class of plants secondary metabolites which have the backbone molecular structure of flavones [2 -phenyl -4- chromone] or 2- phenyl-1-4-benzopyrone] (Wikipedia, 2008).



Chromone



Flavone

Molecular structure of the flavone backbone [2 phenyl-4- chromone] or 2-phenyl -1, 4-benzopyrone].

Flavonoids are compounds present in plants which are responsible for the colour of the plants. Flavonoids embrace the flavone, flavonols and all the compounds whose structures are based on flavones.

The flavones known as anthoxanthins are yellow pigments which occur as dust on flower, leaves, bark, root of the plant. They occur naturally on the free state or as glycosides (the aglycon is the anthoxathidin and the sugar is glucose etc) or associated with tannins. The flavonoids are the hydroxylated derivatives of flavones which may be partially alkylated.

The flavones show two absorption bands. Band I occurs at 330-350 and Band II at 250-270nm (Finar, 2005)]. Flavonol (3-hydroxyl flavone] or [3-hydroxy-phenyl chromone) with molecular formula  $C_{15}H_{10}O_3$  is also widely distributed among the plant kingdom. They occur usually in the form of glycosides.

Flavonols show two absorption bands. Band I at 350-390nm and Band II at 250-270nm. These taken in conjunction with their specific colour reactions make it possible to identify this group of compounds [Finar, 2005].

### **Sources and distribution of flavonoids.**

Flavonoids are widely distributed in plants fulfilling many functions including producing yellow or red or blue pigmentation on flowers and protection from attack by microbes and insects. The wide spread distribution of flavonoids, their variety and their relatively low toxicity compared to other active compounds (for instance alkaloids) mean that many animals including humans digest significant quantities in their diet. They can occur in the bark of a wide variety of plants and they can be bitter.

The important good dietary sources of flavonoids include all citrus fruits, berries, Onions Parsley, tea (especially white and green tea), red wine, sea buck and dark chocolate [with a cocoa content of 70% or greater] (Slumestand, 2007; Ewald et al, 1999 and Justesen, 2001).

Along the biosynthetic pathway of forming the flavones the Anthocyanins, the flavonols, proanthocyanidins (tannins) and a host of other polyphenolics are formed. Thus anthocyanins are one group of flavonoids [Finar, 2005].

### **Health benefits of flavonoids**

Flavonoids are most commonly known for their anti-oxidant activity. They show anti-allergic, anti-inflammatory, anti-microbial, anti-cancer and prevent cardiovascular diseases [Finar, 2005].

Flavonoids have been referred to as “nature’s biological response modifiers” because of strong experimental evidence of their inherent ability to modify the body’s reaction allergens, viruses and carcinogens. Consumers and food manufacturers have become interested in flavonoids for their medicinal properties especially the potential role in the prevention of cancers and cardiovascular diseases.

The beneficial effects of fruits, vegetables and tea or even red wine have been attributed to flavonoids compound rather than to known nutrients and vitamins. More recent researches have also indicated that flavonoids can also induce mechanisms that help tumour invasion [Stauth, 2007].

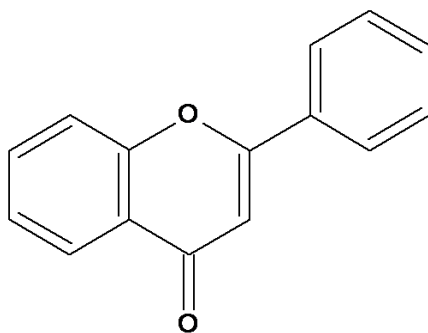
Flavonoids especially epicatechin, quercetin and zuteolin can inhibit the development of fluids that result in Diarrhoea, thus can serve as an anti-diarrhea.

Some flavonoids containing plants possess anti-spasmodic properties (e.g. Liquorices and parsley). There is therefore a possibility that the ascribed antispasmodic property to these plants may be due to flavonoids. Some flavonoids also decrease capillary fragility (e.g. Rutin). This explains the hypotensive property ascribed to some medicinal plants.

### Important flavonoids

The important flavonoids include:

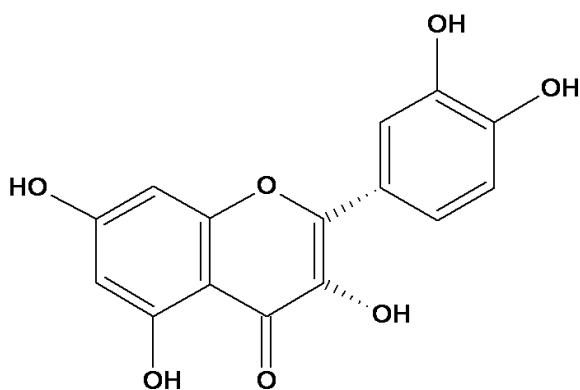
- i. **Quercetin**, a flavonol: Studies have shown that quercetin is the most active of the flavonoids and many of the medicinal plants owe much of their activity to their high quercetin content. Quercetin have demonstrated significant anti-inflammatory activity, exerts potent antioxidant activity and also help to prevent some types of cancer. It also helps to protect against heart attack and strokes [Harbonne, 1998 and Williams, 2000].



Quercetin

## ii. Epicatechin

Epicatechin improves blood flow and thus seems good for cardiac health. Cocoa, the major ingredient of dark chocolate contains relatively high amounts of Epicatechin and has been found to have nearly twice antioxidant content of red wine up to three times that of green tea in-vitro tests [Wikipedia, 2008].



Epicatechin (EC)

### 2.5.3 SAPONINS

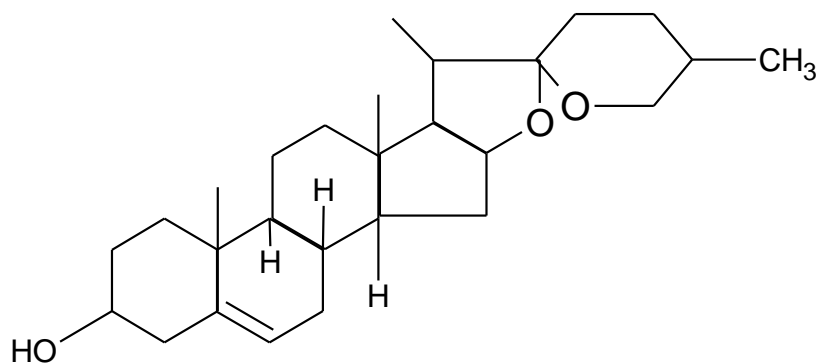
Saponins are group of steroidal glycosides which foam in water [like soap solution]. A characteristic property of the saponins is the haemolysis caused by an intravenous injection of their aqueous solutions into animals. Finar, (2005) stated that these solutions are comparatively harmless when taken orally. Saponins also form molecular complexes with various 3 $\beta$ -hydroxy steroids and this is particularly characteristics of digitonin.

## Occurrence

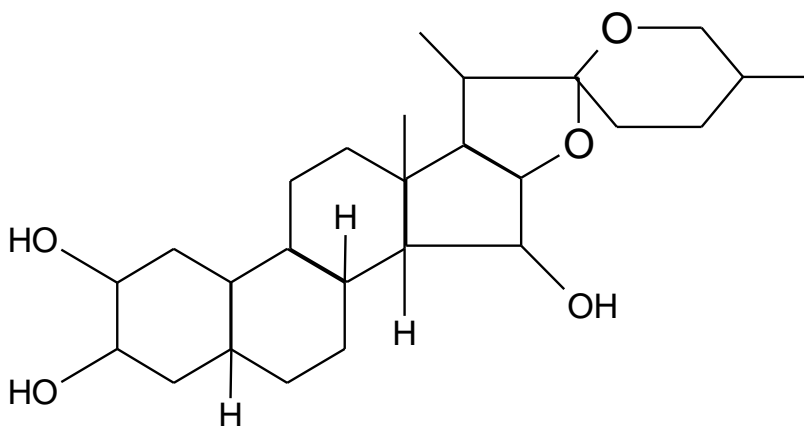
The saponins occur in many plants and are often associated with the cardiotonic Glycosides e.g. Digitonin has been isolated from various species of *Digitalis* and also from *Vernonia* species (Bitter leaf) [Akpuaka, 2004].

Sapogenins are the aglycons of saponins and are characterized by the presence of a spiroketal side chain. Example of a sapogenin is Diosgenin obtained from the root of *Trillium erectum*. Diosgenin is the starting material for the partial synthesis of progesterone.

Diosgenin



Digitogenin



Saponins can serve as anti-carcinogenic, anti-cholesterol and anti-inflammatory compounds

#### **2.5.4 GLYCOSIDES**

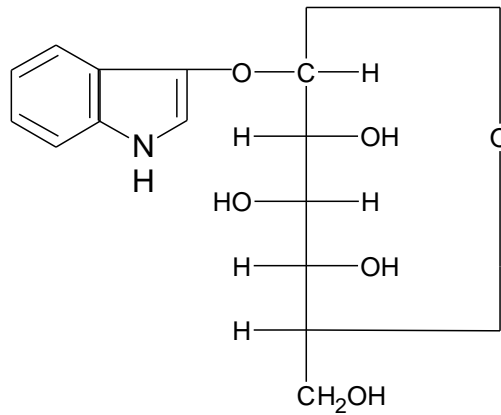
Glycoside is a generic name given to sugars that react in their ring forms with a molecule of an alcohol to form the Acetal derivative. The non-sugar part of a glycoside is known as the aglycon and in many glycosides that occur naturally, the aglycon is often a phenolic compound. In other words, Glycosides are ether-like combinations of sugars with other organic structure (non-sugar aglycon or genin).

Many glycosides particularly those containing phenolic group as the non-sugar part occur in most parts of a plant e.g. anthocyanins which are water soluble pigments that occur in the aqueous cell saps and are responsible for the large variety of colours in flowers from red to violet to blue (Finar, 2005).

#### **Properties**

The simple, glycosides are colourless, soluble in water and are optically active. They do not reduce Fehling's solution. On hydrolysis with inorganic acids, glycosides give a sugar and a hydroxylic compound, the aglycon which may be an alcohol or a phenol.

Most glycosides are hydrolyzed by emulsion therefore they are  $\beta$ -glycosides. In the natural state, each glycoside is usually associated with an enzyme which occurs in different cells of the plant. Maceration of the plant thus produces hydrolyses of the glycoside by bringing the enzyme in contact with the glycoside. Glucose has been found to be the most common sugar component e.g. Indican.



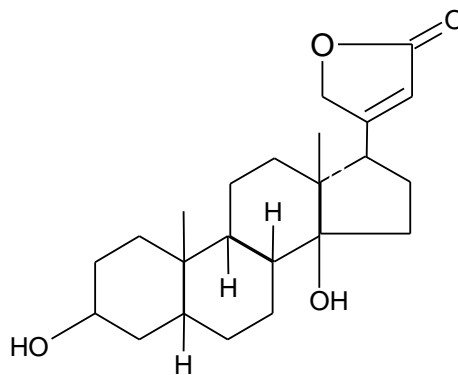
Indican which occurs in the leaves of Indigo plant and wood plant [Finar, 2005].

### Cardiac glycosides

These are plant steroids that occur as glycosides and they have the property of stimulating heart muscle. They are referred to as cardiac-active glycosides or cardiotonic glycosides.

These glycosides when hydrolyzed give one or more sugars and an aglycon e.g. Digitoxigenin

Thus, these glycosides can serve as heart stimulant and diuretics [Sharpa, 1998].



Digitoxigenin

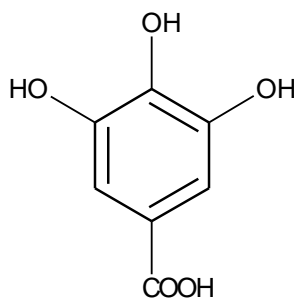
## Cyanogenic glycosides

They are phytotoxins that occur in plant substances that give off prussic acid or hydrogen cyanide [HCN] upon hydrolysis either by enzymatic or non-enzymatic hydrolysis.

The ease of detection of HCN with picrate paper is a reliable test for cyanogenic glycosides (Harbonne, 1998).

### 2.5.5 TANNINS

The tannins are colourless non-crystalline substances which form colloidal solutions in water. These solutions have astringent bitter taste, thus tannins act as a barrier to herbivores. Tannins are polyphenolics and they precipitate proteins from solutions. They form a bluish-black colour with ferric salts; a property which is used in the manufacture of ink. Tannins also precipitate many alkaloids from their solution. Tannins can be hydrolyzed by boiling with dilute acid to yield a phenolic compound, usually a derivative of gallic acid or sugar.



Gallic acid

Tannins are widely distributed in plants and many are glycosides [Finar, 2005]. The name tannin is derived from their ability to tan leather and is not based on a class of compounds with a common basic structure. There are two groups of Tannins, the hydrolysable tannins, which are

esters of gallic acid and also glycosides of these esters. The other type are the condensed tannins which are polymers derived from various flavonoids.

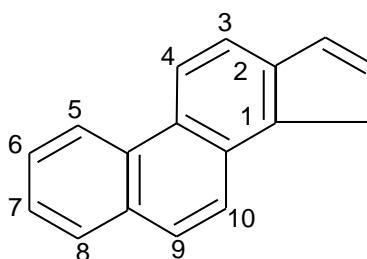
Tannins possess toxic and therapeutic functions [Sharpa, 1998]. It is anti-inflammatory thus can control gastritis, oesophagitis and irritating bowel disorders. Its anti-microbial powers heal burns and stops bleeding [[http://www.en.wikipedia.org/wiktannin.](http://www.en.wikipedia.org/wiktannin), 2007].

The presence of tannins ensures inhibition of microorganisms by coagulating their protoplasm. The non-specific activity of tannins has been ascribed to their ability to complex to metal ions, scavenge and reduce active oxygen species and form tight complexes with a wide array of proteins and polysaccharides [Marja and Anu, 1999].

### 2.5.6 STEROIDS

Steroids form a group of structural related compounds which are widely distributed in animals and plants. Included in the steroids class are the sterols e.g. vitamin D, the bile acids, a number of sex hormones, the adrenal cortex hormones, certain sapogenins, etc. The structure of the steroids are based on the 1,2-cyclopentenophenanthrene skeleton.

1,2-cyclopentenophenanthrene



Sterols occur in plants and animals as oils and fats. They are crystalline compounds and contain an alcoholic group. They occur free or as Esters of the higher fatty acids and are isolated from the unsaponifiable portion of oils and fats.

The sterols that are obtained from animal sources are often referred to as zoosterol e.g. Cholesterol and those obtained from plant sources are called the phytosterols e.g. ergosterol and stigmasterol. A third group are those obtained from yeasts and fungi; these are mycosterols.

Due to their physiological actions, they are used in producing drugs. Phytosterol can reduce the risk of coronary heart disease, cancer, and high blood pressure [Sharpa, 1998].

#### **2.5.7. TERPENOIDS**

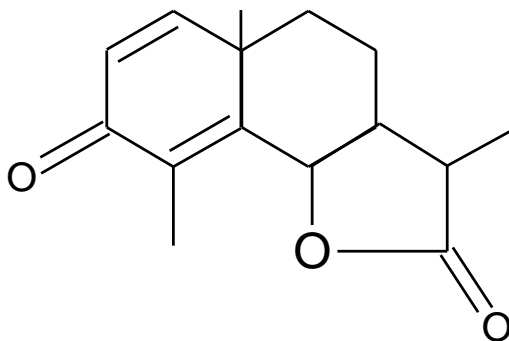
They are a group of compounds, the majority of which occur in the plant kingdom and they are the chief constituents of essential oils. The essential oils are the volatile oils obtained from the sap and tissues of certain plants and trees and the odour of many crushed plants is due to the presence of these volatile compounds.

The essential oils are sweet smelling liquid extract which are insoluble in water and used in perfumery, flavours and medicine from the earliest times.

They are obtained from flowers leaves, fruits seeds, bark and roots of plants.

This group of compounds was originally classified as the terpenes and although this name is still used, there is a tendency to use the more general name terpenoids. This is due to the fact that since the suffix 'ene' signifies unsaturated hydrocarbons, the name terpene is inappropriate to include compounds such as alcohols, aldehydes or ketones. The term terpene is restricted to the hydrocarbon  $C_{10}H_{16}$ , that is, the monoterpenoids.

Most natural terpenoid hydrocarbons have the molecular formula.  $[C_5H_8]_n$  which is called the isoprene unit and the value of n is used as a basis for classification e.g.  $C_{15}H_{24}$  is sesquiterpenoid. Santonin a drug that is widely used in medicine as an ant-helminthic (it has the power to expel worms) is a sesquiterpenoid lactone  $[C_{15}H_{18}O_3]$  [Finar, 2005].



Santonin

#### **2.6.0 FACTORS WHICH CAN AFFECT THE LEVEL OR THE COMPOSITION OF THE ACTIVE INGREDIENTS IN MEDICINAL PLANTS**

The composition of the active principles in a medicinal plant has been shown through natural product chemistry research to vary to a large extent with the phonological age of the plant, geographical location, climatic condition, soil conditions/nutrients physiological state of the plant, time of the day of harvesting, genetic constitution and the method of extraction or even the solvent for extraction [Morah, 2007]. These factors and many others are possible sources of variation for the chemical compositions, toxicity and bioactivity of the plant extracts. For example the yield of Morphine from poppy at 9am is about four times the yield about 9pm in the same plant [Morah, 2007].

In the case of age, certain principles may be absent at the beginning of a plant's life but may later start accumulating with age. The seed of *Vinca rosea* (Periwinkle) for example does not contain the active principle; it appears during germination, spreads, only to disappear three weeks after germination. It reappears in the eight week old plant and reaches its maximum level during flowering. Again the young leaves and matured leaves of the same plant are known to contain different levels of the active principles. For example *Ocimum gratissium* is a febrifuge, stomach laxative and a remedy for diabetes mellitus, constipation and rheumatism. The essential oil contains 32-65% Thymol and serves as a mosquito repellent. The young leaves have been reported to contain much higher level of thymol than the matured leaves [Sofowora, 1970].

The production of these natural products may be characteristic of all organs in a plant including the seeds or may be localized in certain organs of the plant. For instance, the seeds of Tobacco plant are devoid of alkaloids but the leaves contain nicotine.

Generally active principles from plants of closely related genera are similar in structure while those from remotely related genera usually differ markedly

### **2.7.0 SOME MICROORGANISMS AND THEIR EFFECTS ON THE HUMAN BODY**

Most of the pathogenic micro-organisms used for the microbial test of plant extract include the following: *Staphylococcus aureus*, *Streptococcus pyrogenes*, *Streptococcus pneumonia*, *Corynebacterium ulcerans*, *Klebsiella pneumonia*, *Salmonella typhi*, *Escherichia coli*, *Candida albicans*, *Candida tropicalis*, *Candida krusei*, *Candida stellatoidea*.

### **2.7.1 STAPHYLOCOCCI**

Staphylococci are gram negative spherical cells, usually in grapelike irregular clusters. They grow readily on many types of media and are active metabolically, fermenting carbohydrates and producing pigments that vary from white to deep yellow. The genus staphylococcus has at least 40 species. The three frequently encountered species of clinical importance are *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Staphylococcus saprophyticus*. *Staphylococcus aureus* is coagulase-positive, which differentiates it from the other species. *Staphylococcus aureus* is a major pathogen for humans. Almost every person will have some type of *Staphylococcus aureus* infection during lifetime, ranging from food poisoning or minor skin infections to severe life-threatening infections. Staphylococci are non-motile and do not form spores. Under the influence of drugs like penicillin, staphylococci are lysed. Staphylococci are early cultured on most bacteriological media under aerobic or microaerophilic conditions. Staphylococci can produce disease both through their ability to multiply and spread widely in tissues and through their production of many extracellular substances. Some of these substances are enzymes. However, it is difficult to eradicate pathogen staphylococci from infected persons, because the organisms rapidly develop resistance to many antimicrobial drugs and the drugs cannot act in the central necrotic part of a suppurative lesion. It is difficult to eradicate the *S. aureus* carrier state (Bronner et al., 2004).

### **2.7.2 STREPTOCOCCI**

The streptococci are gram-negative spherical bacteria that characteristically form pairs or chains during growth. They are widely distributed in nature. Some are members of the normal human flora; others are associated with important human diseases attributable in part to infection by streptococci, in part to sensitization to them. Streptococci elaborate a variety of extracellular

substances and enzymes. The streptococci are a large and heterogeneous group of bacteria and no one system suffices to classify them. Yet, understanding the classification is key to understanding their medical importance. Examples of streptococci are *streptococci pyogenes*, *streptococciagalactiae*, *streptococcidysgalactiae*, *streptococci anginosus*, *streptococci pyogenes*, *streptococci pneumococci* are susceptible to penicillin G and most are susceptible to erythromycin. Some are resistant to tetracycline. Streptococci pneumococci are sensitive to many antimicrobial drugs, early treatment usually result in rapid recovery and antibody response seems to play a much diminished role (Musher, 2010).

### **2.7.3 CANDIDA**

Several species of the yeast genus *Candida* are capable of causing candidiasis. They are members of the normal flora of the skin, mucous membranes and gastrointestinal tract. *Candida* species colonizes the mucosal membranes and gastrointestinal tract. *Candida* species colonizes the mucosal surface of all humans during or soon after birth and the risk of endogenous infection is ever present. Candidiasis is the most common system mycosis, and the most common agents are *Candida albicans*, *Candida tropicalis*, *Candida krusei*, *Candida stellatoidea*, *Candida glabrata*, *Candida guilliermindii*, etc. The risk factors associated with superficial candidiasis include AIDS, diabetes, young or old age birth control pills and trauma (burns, maceration of the skin). It is often difficult to establish an early diagnosis of systemic candidiasis, the clinical signs are not definitive and cultures are often negative. Furthermore thrush and other mucocutaneous forms of candidiasis are usually treated with topical nystatin or oral ketoconazole or fluconazole (calderone, 2002).

#### **2.7.4 ENTEROBACTERIACEAE**

The enterobacteriaceae are a large, heterogeneous group of gram-negative rods whose natural habitat is the intestinal tract of humans and animals. The family include many genera such as: *Escherichia shigelle*, *Salmonella enterobacter*, *Klebsiella serratia*, *Proteus*, and others. Some enteric organisms e.g. *Escherichia coli*, are part of the normal flora and incidentally cause disease, while others, the Salmonellae and Shigellae are regularly pathogenic for humans. The enterobacteriaceae are facultative anaerobes, ferment a wide range of carbohydrates, possess a complex antigen structure and produce a variety of toxins and other virulence factors.

#### **2.7.5 KLEBSIELLA**

*Klebsiella pnemoniae* is present in the respiratory tract and faeces of about 5% of normal individuals. It causes a small proportion (about 1%) of bacterial pneumonias. *Klebsiella pneumonia* can produce extensive haemorrhagic necrotizing consolidation of the lungs. *Klebsiella* sp. Rank among the top ten bacterial pathogens responsible for hospital-acquired infections.

#### **2.7.6 ESCHERICHIA COLI**

*Escherichia coli* is the most common cause of urinary tract infection and accounts for approximately 90% of first urinary tract infections in young women. It causes diarrhea and it is common worldwide. These *E.coli* are classified by the characteristics of their virulence properties and each group causes disease by a different mechanism.

### **2.7.7 SALMONELLAE**

Salmonella are often pathogens for humans or animals when acquired by the oral route. They are transmitted from animals and animal products to human, where they cause enteritis, systemic infections and enteric fever. Examples of other forms of salmonellae are as follows: Salmonellatyphi, Salmonella cholerasuis, Salmonella paratyphi A, Salmonella paratyphi B. Salmonellae vary in length and grow on simple media. The most important is salmonella typhi which reaches the small intestine after ingestion of salmonellae, from which they enter the lymphatics and then the blood stream. It can be treated with antibiotics.

## CHAPTER THREE

### 3.0 MATERIALS AND METHODS

#### 3.1 Materials

##### 3.1.1 Solvents

Solvents used were of general purpose grade and were distilled before used, they are:

N-hexane, Ethyl acetate, Methanol and Chloroform.

##### 3.1.2 Equipment

The equipment used include the following: Soxhlet extractor, Top loading balance, Rotary evaporator, Syringe (2ml and 5ml), Petri dishes, UV lamp (254-326nm), Oven, Vacuum pump, GC-MS, NMR (500mhz).

##### 3.1.3 REAGENT

95% Ethanol, 10% Ammonium solution, 10% Hydrochloric acid, 20% sodium hydroxide solution, 1% Ferric chloride solution, 10% sodium nitrate, Fehlings solution, 10% H<sub>2</sub>SO<sub>4</sub>, Iodine solution, Wagner's reagent, Dragendoffs reagent, Molisch reagent and Meyers reagent

### 3.1.4 MICROBIAL MEDIA, TEST ORGANISMS AND EQUIPMENT FOR

#### ANTIMICOBIAL TEST

##### a) Microbiological media

- I. Nutrient broth
- II. Nutrient agar and
- III. Sabouroud Dextrose agar

##### b) Test Organisms(Clinical cultures)

*Staphylococcus aureus*

*Streptococcus feacalis*

*Streptococcus pyogene*

*Corynebacterium ulcerans*

*Escherichia coli*

*Salmonella typhi*

*Proteus vulgaris*

*Candida albicans*

*Candida tropicalis*

### **3.1.5 THE IDENTIFICATION AND PREPARATION OF PLANT MATERIAL.**

The plant, *Indigofera arrecta* stem, were collected from Makurdi, Benue State in the month of March, 2013. They were properly identified at the Herbarium, Department of Biological Sciences, Ahmadu Bello University, Zaria and a sample specimen with Herbarium Number 631 was depicted there.

The stem bark were dried for five weeks in readiness for experiment. The dried stem were pounded using wooden mortar and pestle into coarse powder.

#### **Solvents/Reagents**

Solvent used were of general purpose grade and were distilled before use. Also, Pre-coated silica gel plates used were those of Merck silicagel 60F<sub>254</sub>

### **3.1.5 EXTRACTION PROCEDURE FOR CRUDE EXTRACTS**

#### **Extraction of Plant Material.**

Dried powder (800g) was macerated in methanol until complete extraction. The solvent was removed *in-vacuo* to yield methanol extracts of 47.60g. The methanol extract was partitioned into n-hexane (23.20g), ethyl acetate (8.7g) and chloroform extracts

### **3.2 PRELIMINARY PHYTOCHEMICAL SCREENING**

A portion of each of the three extracts was subjected to preliminary phytochemical screening using standard methods (Trans and Evans 2007).

### **3.2.1 TEST FOR STEROIDS/TERPENES**

#### **Liebermann-Buchard test**

1ml of anhydrous acetic acid was added to 1ml chloroform and cooled to 0 °C then one drop of concentrated sulphuric acid was added to the cooled mixture followed by the extract. The solution was observed for any blue, green, red or orange color that changes with time (Silva *et al.*, 1998).

#### **Salkowski test**

A little quantity of the extract was dissolved in 1ml chloroform and to it 1ml of concentrated sulfuric acid was added down the test tube to form two phases. Formation of red or yellow coloration was taken as an indication for the presence of sterols. (Silva *et al.*, 1998).

### **3.2.2 TEST FOR FLAVONOIDS**

#### **Shinoda test**

To an alcoholic solution of the extract three pieces of Magnesium chips were added followed by a few drops of concentrated hydrochloric acid. Appearance of an orange, pink or red to purple color indicates the presence of flavonoids. (Sofowora,1993).

#### **Sulphuric acid test:**

The sample was dissolved in concentrated sulfuric acid and the color change was observed (Silva *et al.*, 1998).

### **Ferric Chloride test**

Extract was boiled with water and filtered. To 2ml of the filtrate, two drops of freshly prepared ferric chloride solution was added; green, blue or violet colorations indicate the presence of phenolic hydroxyl group. (Finar,2005)

### **Sodium Hydroxide test**

2mls of the extract was dissolve in 10% aqueous sodium hydroxide solution and filtered to give yellow color, a change in color from yellow to colorless on addition of dilute HCl indicate the presence of flavonoids.(Finar,2005).

### **3.2.3 TEST FOR ALKALOIDS**

0.5g of the extract was stirred with 5ml of 1% aqueous hydrochloric acid on a water bath and filtered. Three ml of the filtrate was divided into three. To the first portion few drops of freshly prepared Dragendoff reagent was added and observed for formation of orange to brownish precipitate. To the second portion 1 drop of Mayer reagent was added and observed for formation of white to yellowish or cream color precipitate. To the third portion 1 drop of Wagner reagent was added to give a brown or reddish or reddish- brown precipitate (Silva *et al.*, 1998).

### **3.2.4 TEST FOR TANNINS**

A small quantity of the extract was boiled with water and filtered. Two drops of ferric chloride was added to the filtrate, formation of a blue-black, or green precipitate was taken as evidence for the presence of tannins (Trease and Evans, 1996).

### **3.2.5 TEST FOR ANTHRAQUINONES**

#### **Free anthraquinones**

The extract was shaken with 10 ml of benzene, the content was filtered, and 5ml of 10% ammonia solution was added to the filtrate, the mixture was shaken. Presence of a pink, red or violet color in the ammoniacal layer (Lower phase) indicates the presence of free anthraquinone.

#### **Combined anthraquinones**

The extract was boiled with 10 ml of aqueous sulphuric acid and filtered hot. The filtrate was shaken with 5ml benzene, the benzene layer was separated and half its own volume, 10%  $\text{NH}_4\text{OH}$  was added. A pink, red or violet colouration in the ammonia phase (lower phase) indicates the presence of combined anthraquinone or anthraquinone derivative.(Finar,2005).

### **3.2.6 TEST FOR SAPONINS**

About 0.5g of the extract was shaken with water in a test tube. Frothing which persisted for 15 minutes indicates the presence of saponins. (Silva *et al.*, 1998)

### **3.2.7 TEST FOR GLYCOSIDE (FeCl<sub>3</sub> TEST)**

To about 0.5g of the extract/fraction, 5mL of conc.  $\text{H}_2\text{SO}_4$  was added and boiled for 15 min. This was then cooled and neutralized with 20% KOH. The solution was divided into two portions. Three drops of ferric chloride solution was added to one of the portions, and a green to black precipitate indicated phenolic aglycone as a result of hydrolysis of glycoside.

### 3.3.0 ANTIMICROBIAL SCREENING

#### The test organism

Stock culture of microbes used for this study were clinical isolates of the following: *Staphylococcus aureus*, *Streptococcus pyogenes*, *Streptococcus faecalis*, *Corynebacterium ulcerans*, *Escherichia coli*, *Salmonella typhi*, *Proteus vulgaris*, *Candida albicans*, and *Candida tropicalis*. They were obtained. All the isolates were checked for purity and maintained in a slant of nutrient agar.

- a. **Equipment:** Incubator, agar punches, swab sticks, pipettes, Petri dishes, straight wire, wire loop, meter rule, a pair of calipers
- b. **Reagents and Media:** Nutrient Agar, distilled water, Sabouraud dextrose Agar, and half strength solvent

#### 3.3.1 Preparation of bacterial test organism

Stock culture of bacterial species such as *Staphylococcus aureus*, *Streptococcus pyogenes*, *Streptococcus faecalis*, *Corynebacterium ulcerans*, *Escherichia coli*, *Salmonella typhi* and *Proteus vulgaris*, were collected.

Each organism was sub-cultured into a fresh nutrient agar medium and incubated for 18-24 hours, that is overnight at 37°C so as to obtain cultures of the test organism for use.

Using a sterile wire-loop, a loopful of each organism was emulsified in 5ml of normal saline. This enhances a smooth suspension of the organism under test for use.

### **3.3.2 Preparation of fungal test organisms**

Stock cultures of *Candida albicans* and *Candida tropicalis* were collected from the Department of medical microbiology Ahmadu Bello University teaching Hospital Shika, Zaria. Each organism was sub-cultured into a fresh Sabouraud Dextrose Agar SDA plates. They were incubated for 4 days. The *Candida* culture grew well within 3 days. Three universal bottles each containing few glass beads were sterilized in the autoclave, and 5ml sterile distilled water was pipetted into each, and scrapings were made from the young fungal culture to give a light spore suspension after moderate shaking and breaking up with the glass any clump remaining were allowed to settle at the bottom of the bottles before use.

### **3.3.3 The stock dilution of the plant extracts**

These were made using 50% acetone and sterile distilled water, Two (2ml) of each solvent were added to each of the plant extracts to form a stock solution ready for use.

### **3.3.4 Preparation of the Nutrient Agar**

20g of the Nutrient Agar medium was weighed and dispensed in 500ml of diluted water. This was allowed to soak for 10 minutes followed by gentle shaking to mix. The pH was adjusted to 7.4 with pH meter, the mixture was boiled to dissolve completely; with shaking and poured into separate 250ml screw capped bottles and sterilized by autoclaving for 15 minutes at 121°C. This was allowed to cool to 45°C before pouring into sterile Petri dishes. The media was allowed to gel after which the plates were packed and dried overnight (18-24 hrs) at 37°C. This ensures non-contamination of the plates.

### **3.3.5 Preparation of the Sabouraud dextrose agar media**

16.25g of the Sabouraud Dextrose Agar (SDA) powder was weighed and dispensed into 250ml of distilled water bottle at 60°C. This was stirred thoroughly . The pH was adjusted to 6.0 with pH meter. It was sterilized by autoclaving for 20 minutes at 115°C, allowed to cool to 45°C and mixed very well before pouring into sterile Petri dishes. The media was allowed to gel after which the plates were packed and dried overnight at 37°C, to test for sterility, and any contamination.

### **3.3.6 The Punched Agar Diffusion Method [Bryant, 1972].**

#### **Procedure:**

The prepared plates were allowed to dry by keeping them half-open and face downward in incubator at 37°C for 30 minutes, this reduced the moisture content of the media. With the aid of sterile, 6mm diameter agar puncher, four wells or holes in diameter were made on each plate. These were sufficiently spaced to prevent any inhibition zone overlapping later on. Respective wells were carefully labeled accordingly using grease pencil to match with the extracts. The cut off agar pieces were removed with sterile inoculating loop.

#### **Culture media**

The culture media used were Mueller Hinton agar (MHA) and Mueller Hinton broth (MHB). All the media were prepared according to manufacturer's instruction.

### **3.3.7 Preparation of inoculums of test organisms**

The McFarland turbidity standard scale 1 was used to standardise the organisms. The scale was prepared by adding 9.9ml of 1% sulphuric acid to 0.1ml of 1% barium Chloride

(BaCl<sub>2</sub>). The volumes of the prepared stock solution were mixed according to the McFarland nephelometer standard.

Suspensions of the organisms were made in sterile distilled water and compared with the McFarland turbidity standard, until the opacity matched with the scale number 1, which corresponds to  $3.6 \times 10^6$  bacterial densities.

### **3.3.8 Sensitivity test of the extract using agar diffusion method**

The agar diffusion method (Nostro, 2000) was used. The antimicrobial activities of the n-hexane, ethyl acetate and methanol extract of the stem bark of *Indigofera arrecta* were determined using stock concentration of 100mg/ml. Five appropriately labelled wells were punched into each agar plate. Extract of known concentration (0.2ml) was placed in each well and then allowed to diffuse into the agar. An extra plate was streaked with the inocula isolate and ciprofloxacin standard (10µ/disc) was placed in it. The plates were incubated at 37°C for 24hours. While for the fungi, Sabraud dextrose broth was used and the incubation period was 48hours. The antimicrobial activities were expressed as diameters of inhibition zones produced by the plant extracts.

### **3.3.9 Determination of minimum inhibitory concentration using tube dilution method**

The minimum inhibitory concentrations of the extracts were determined using the tube dilution method as outlined by the National Committee For Clinical Laboratory Standards (NCCLS). Dilutions (3.25-60mg/ml,) of concentration of extract that exhibited sensitivity against the test organisms were prepared in test tubes containing Mueller Hinton broth (MHB) (Table 5). The organisms were inoculated into each tube containing the diluted extracts. The tubes were

incubated at 37°C for 24 hours for bacteria and 5-7 days for fungi growth. The lowest concentration in the series showing no visible growth of the test organisms was considered to be the minimum inhibitory concentration (MIC).

### **3.3.10 Minimum bactericidal concentration (mbc)**

The minimum bactericidal concentration of the extract was determined as outlined by the NCCLS on the nutrient agar plates. Minimum bactericidal concentrations were determined by assaying the test tube content of the MIC determinations. A loopful of the content of each tube was inoculated known concentration of extracts active in the microbes by streaking on a solidified nutrient agar plate and then incubated at 37°C for 24 and 48 hours for bacteria and fungi respectively after which it was observed for microbial growth. The lowest concentration of the subculture with no growth was considered as minimum bactericidal concentration.

## **3.4 CHROMATOGRAPHIC PURIFICATION OF EXTRACTS**

### **3.4.1 Thin Layer Chromatography (TLC)**

Thin layer chromatography was carried out on TLC aluminum sheet silica gel 60F<sub>254</sub> pre-coated with layer thickness of 0.2mm.

**Technique:** One way ascending

**Spotting and development:** Spots were applied manually using capillary tube; plates were dried using air blower and developed at room temperature using a Shandon chromatotank.

**Detection of Spots:** Spots on TLC plates were visualized under UV light (254 and 366 nm) and spraying with 10% sulphuric acid, followed by heating at 110°C for 5-10 min.

### 3.4.2 Column Chromatography

The following column conditions were employed in running the column chromatography.

- (a) *Technique* - Gradient elution.
- (b) *Column* - A glass column of dimensions (75 by 3.5cm) was used.
- (c) *Stationary phase* - Silica gel, 60 – 120 mesh size.
- (d) *Column packing* - Wet slurry method.
- (e) *Sample loading* - The sample was by the dry loading method (Cannell, 1998); the sample was dissolved in minimum amount of suitable organic solvent, mixed with a small quantity of silica gel, dried, triturated and then loaded on top of the previously packed column.

### 3.4.3 Solvents system/Elution:

Various solvent systems comprising hexane/ethyl acetate mixtures were used in eluting the column by gradient elution.

### 3.4.4 Gel Filtration Chromatography

**Stationary phase:** Sephadex<sup>TM</sup> LH-20 (Sigma).

**Packing method:** Sephadex<sup>TM</sup> LH-20 was suspended in methanol and allowed to saturate for 24 hours prior to use. It was then introduced into a glass column and allowed to settle tightly.

**Sample application:** The sample was dissolved in a minimum amount of eluting solvent and applied as a thin layer on top of the column.

**Eluting Solvent:** Methanol.

### **3.4.5 Thin layer chromatography of the n-hexane Extract**

The n-Hexane extract of *Indigofera arrecta* was subjected to thin layer chromatography using pre-coated aluminum TLC plate and Hexane: Ethyl acetate (1:1) as solvent system to determine the profile of this extract. Two prominent spots were observed on visualization using UV light.

### **3.3.6 Column Chromatography of n-hexane fraction**

N-Hexane extract(7g) was chromatographed over silica gel packed column of dimension 75 by 3.5cm, the column was eluted continuously using neat n-Hexane, and then n-Hexane: Ethyl acetate mixtures as solvent systems. Eighty four fractions, 100ml each were collected. The fractions were pooled together based on their TLC profile to give four major fractions(F<sub>1</sub>,F<sub>2</sub> F<sub>5</sub>and P<sub>1</sub>) and the column was finally washed with methanol. Fraction 1 which showed one major spot and some minor impurities were washed with methanol,pure white crystal which showed a single spot when spotted was collected and labeled as compound EB.Compound EB was subjected to spectroscopic analysis to elucidate its chemical structure.

## CHAPTER FOUR

### 4.0 EXTRACTION OF PLANT MATERIAL

**Table 4: % yield of the extract of the stem of *Indigofera arrecta***

<b>Solvent</b>	<b>Mass of Extracts (g)</b>	<b>Percentage yield (%)</b>
<b>n-hexane</b>	23.20	2.90
<b>Ethyl acetate</b>	8.70	1.08
<b>Methanol</b>	47.60	5.95

#### 4.1.0 PHYTOCHEMICAL SCREENING OF EXTRACTS

**Table 4.1: Phytochemical screening results of methanol, ethyl acetate and n-hexane extracts of the stem bark of *Indigofera arrecta***

Phytochemical	Methanol extract	Ethyl acetate extract	n-hexane extract
Anthraquinones	-	-	-
Cardiac glycosides	+	+	+
Saponins	+	-	-
Steroids and terpenes	+	+	+
Flavonoids	+	+	-
Tannins	+	-	+
Alkaloids	-	-	-
	-	-	-

Key: + = Present, - = Absent.

#### 4.2.0ANTIMICROBIAL ACTIVITY SCREENING

**Table 4.2:Resultof Sensitivity test of the microorganisms to varying concentrations of the extracts**

Test organism	ME	EA	N-hex	Ciprofloxacin 5mg/ml
<i>S.aureus</i>	S	S	S	S
<i>S.feaecalis</i>	R	R	R	S
<i>S.pyogenes</i>	S	S	S	S
<i>E.coli</i>	S	S	S	S
<i>S.typhi</i>	R	R	R	S
<i>C.ulcerans</i>	S	S	S	S
<i>P. vulgaris</i>	S	S	S	S
<i>C.albicans</i>	S	S	S	R
<i>C.tropicalis</i>	R	R	S	S

ME = Methanol, EA = Ethyl acetate, N-hex = Normal hexane,R=Resistance,S=Sensitive

**Table 4.3:Antimicrobial Screening result of the extracts showing zone of inhibition.**

Test organism	Zone of inhibition (mm)				
	ME(mg/ml)	EA(mg/ml)	N-hex(mg/ml)	Ciprofloxacin 5mg/disc	Fluconazole 5 mg/disc
<i>S. aureus</i>	22	20	25	37	<b>0</b>
<i>S.faealis</i>	25	18	28	40	<b>0</b>
<i>S.pyogenes</i>	0	0	0	40	0
<i>E.coli</i>	27	17	27	35	0
<i>S.typhi</i>	0	0	0	42	0
<i>C.ulcerans</i>	20	16	26	34	0
<i>P. vulgaris</i>	20	21	40	47	0
<i>C.albicans</i>	21	16	21	0	37
<i>C.tropicalis</i>	0	0	20	0	33

ME = Methanol, EA = Ethyl acetate, N-hex = Normal hexane.

**Table 4.4: Minimum inhibitory concentration (MIC)**

<b>Test organisms</b>	<b>ME(mg/ml)</b>	<b>EA (mg/ml)</b>	<b>N-hex(mg/ml)</b>
<i>S.aureus</i>	15	15	15
<i>S.pyogenes</i>	30	15	15
<i>S.feacalis</i>	15	15	15
<i>E.coli</i>	15	15	7.5
<i>C.ulcerans</i>	15	30	7.5
<i>S.typhi</i>	30	60	15
<i>P. vulgaris</i>	30	30	30
<i>C.albicans</i>	30	60	60

Table 4.5: Minimum bactericidal concentration (MBC)

Test organisms	ME(mg/ml)	EA (mg/ml)					N-hex(mg/ml)				
		60	30	15	7.5	3.25	60	30	15	7.5	3.25
<i>S.aureus</i>	60										30
<i>S.pyogenes</i>	60										60
<i>S.feacalis</i>	30										15
<i>E.coli</i>	30										30
<i>C.ulcerans</i>	60										30
<i>S.typhi</i>	60										60
<i>P. vulgaris</i>	30										60
<i>C.albicans</i>	60										60

#### 4.4.0 RESULT OF CHROMATOGRAPHIC SEPARATION

##### Thin layer chromatography of the n-hexane extract

The n-hexane extract of *Indigofera arrecta* was subjected to thin layer chromatography using pre coated silica aluminum TLC plates and Hexane: Ethyl acetate (7:3) as solvent system to determine the profile of this extract. Three prominent spots were observed after visualization under VU light (Table 7.0).

Table 4.5: TLC of n-hexane extracts using hexane: ethyl acetate (7:3)

Spot	R <sub>f</sub> Value	Color in 10% H <sub>2</sub> SO <sub>4</sub>
1	0.85	Orange
2	0.67	Yellow
3	0.55	purple

##### 4.5.0 Column Chromatography of n-hexane fraction

The n-hexane extract(7g) was chromatographed over silica gel packed column of dimension 75cm by 3.5cm and the column was eluted continuously using neat n-Hexane, and then n-Hexane: Ethyl acetate mixtures as solvent systems (Table 6.0).Eighty four of 100ml each were collected. The concentrated fractions were pooled together based on their TLC profiles to give four major fractions and the column was finally washed with methanol to give the fifth major mixture.

**Table 4.6: Fractions from column chromatography of n-hexane extract.**

Fraction	Eluting solvent	Number of major spots
1	Hexane:ethyl acetate 05:95%	3
2	Hexane:ethyl acetate 10:90%	2
3	Hexane:ethyl acetate 20:80%	2
4	Hexane:ethyl acetate 30:70%	1
5	Methanol	4

#### 4.6.0 Isolation of EB

The fourth fraction which showed one major spot and some minor spots were subject to washing using absolute n-hexane to remove some non-polar dirt or impurities. The fraction was further subjected to preparatory TLC using four plates of precoated aluminium sheets, the zones that contained this spot were all scratched from the plates and were pooled together and re-extracted from methanol, labeled as compound EB (59.0mg). EB was isolated as a white crystalline solid. It was also subjected to spectroscopic analysis to elucidate its chemical structure.

#### 4.7.0 TLC analysis of EB

TLC analysis of EB using Hexane:ethyl acetate (3:7) gave a single homogenous spot ( $R_f$  0.53) while the TLC analysis with chloroform:ethyl (1:1) also gave a single homogenous spot ( $R_f$  0.84). Spraying with 10%  $H_2SO_4$  and heating gave an orange colored spot Table 7.0.

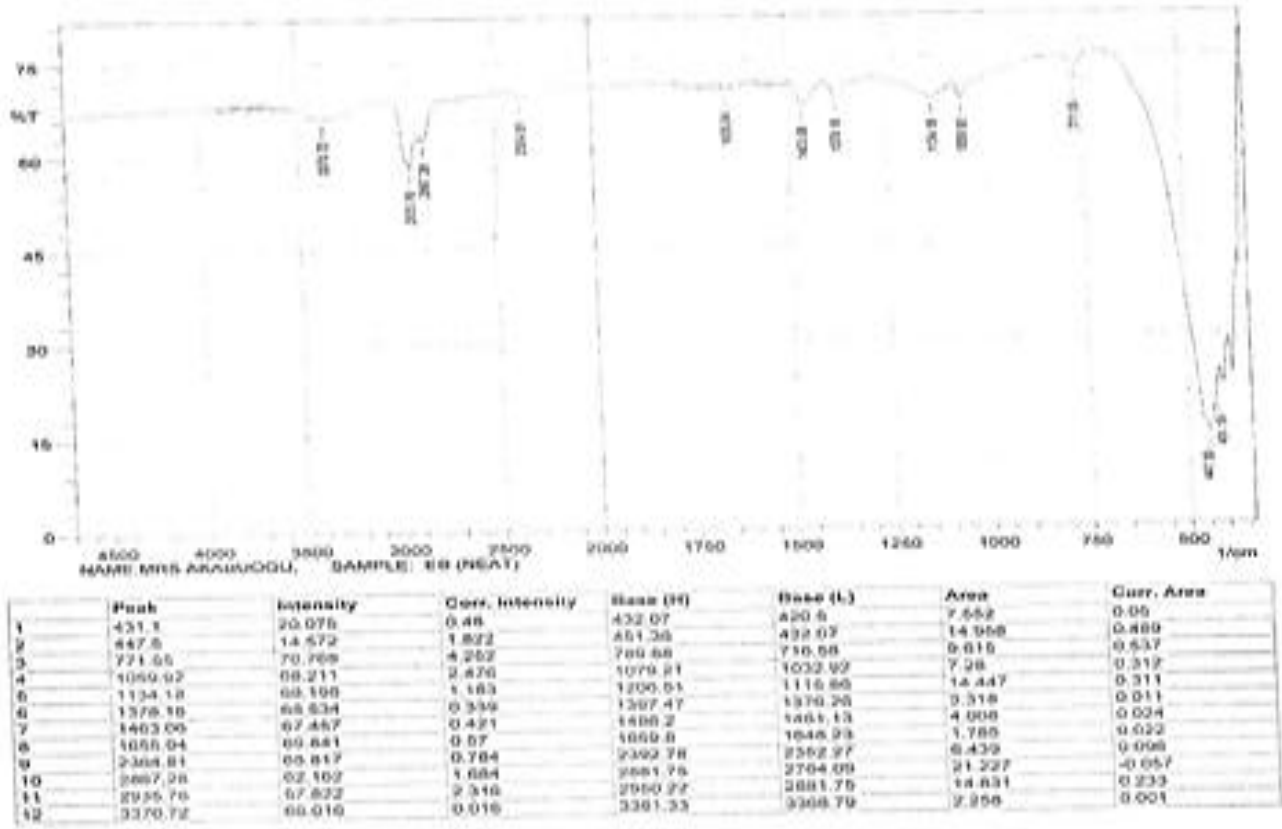
Table 4.7: TLC analysis of EB using different solvent systems

Solvent system	Number of spot(s)	Color of spot on heating	R <sub>f</sub> value
Hexane: Ethyl acetate (7:3)	1	Pink	0.53
Chloroform: Ethyl acetate (1:1)	1	Pink	0.84

#### **4.8.0 Melting point Result**

The melting point range of EB was found to be 141-145<sup>0</sup>C

Figure 2



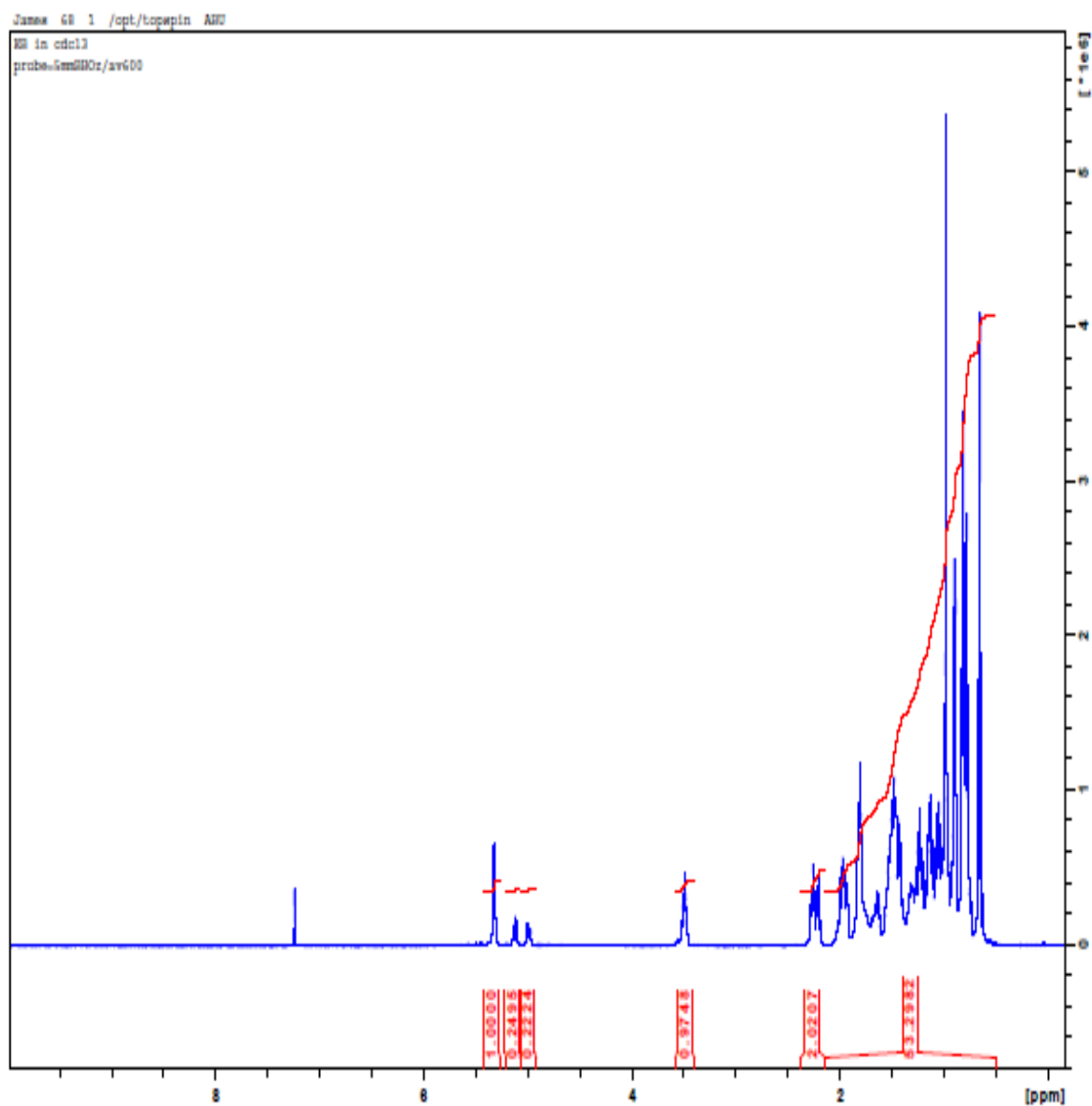
Comment:  
 NAME: MRS AKABUOGU, SAMPLE: EB (NEAT)

Date/Time: 1/1/2001 3:07:23 AM  
 Application:

User: Administrator

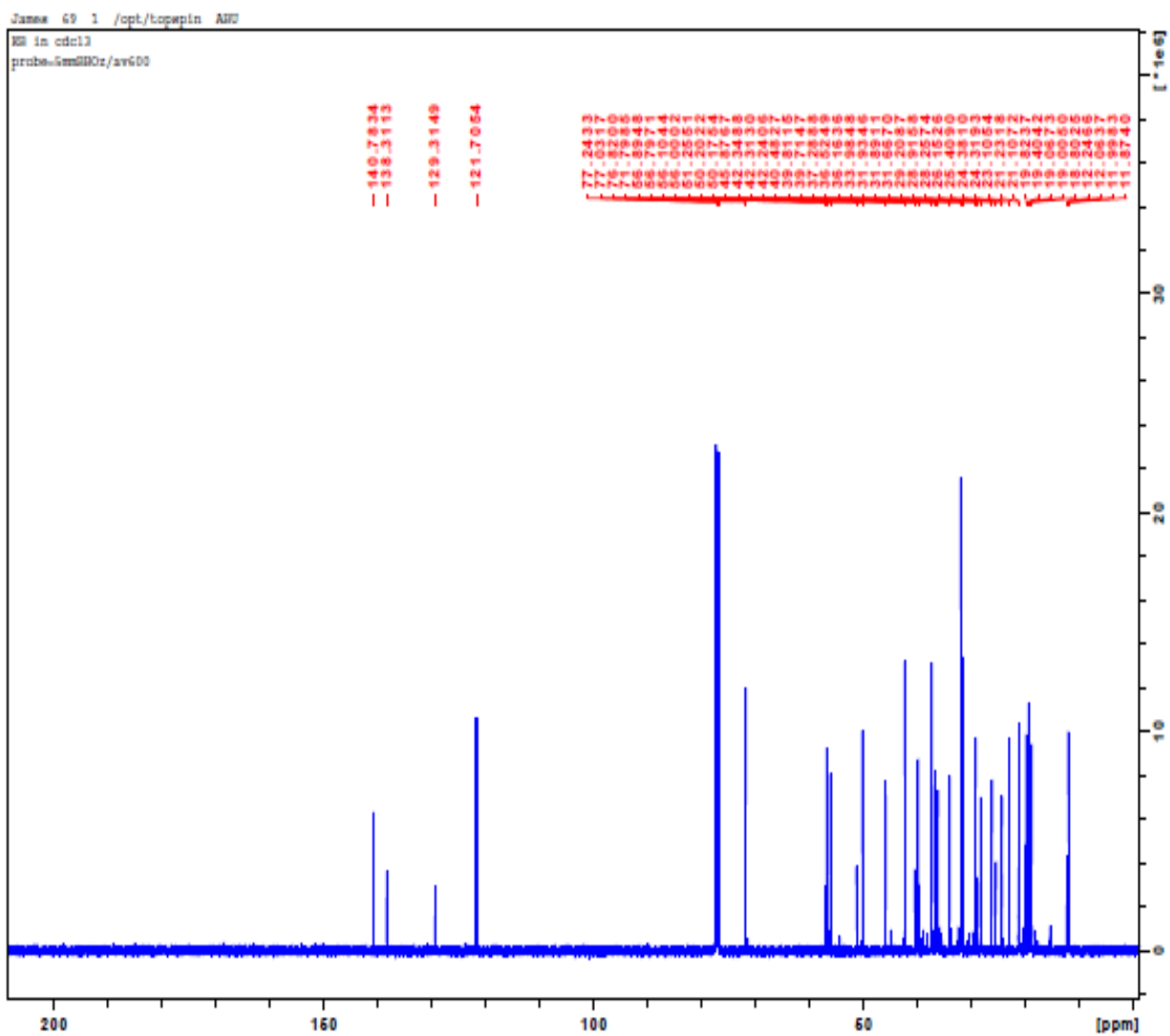
FTIR OF COMPOUND EB

**FIGURE: 3**



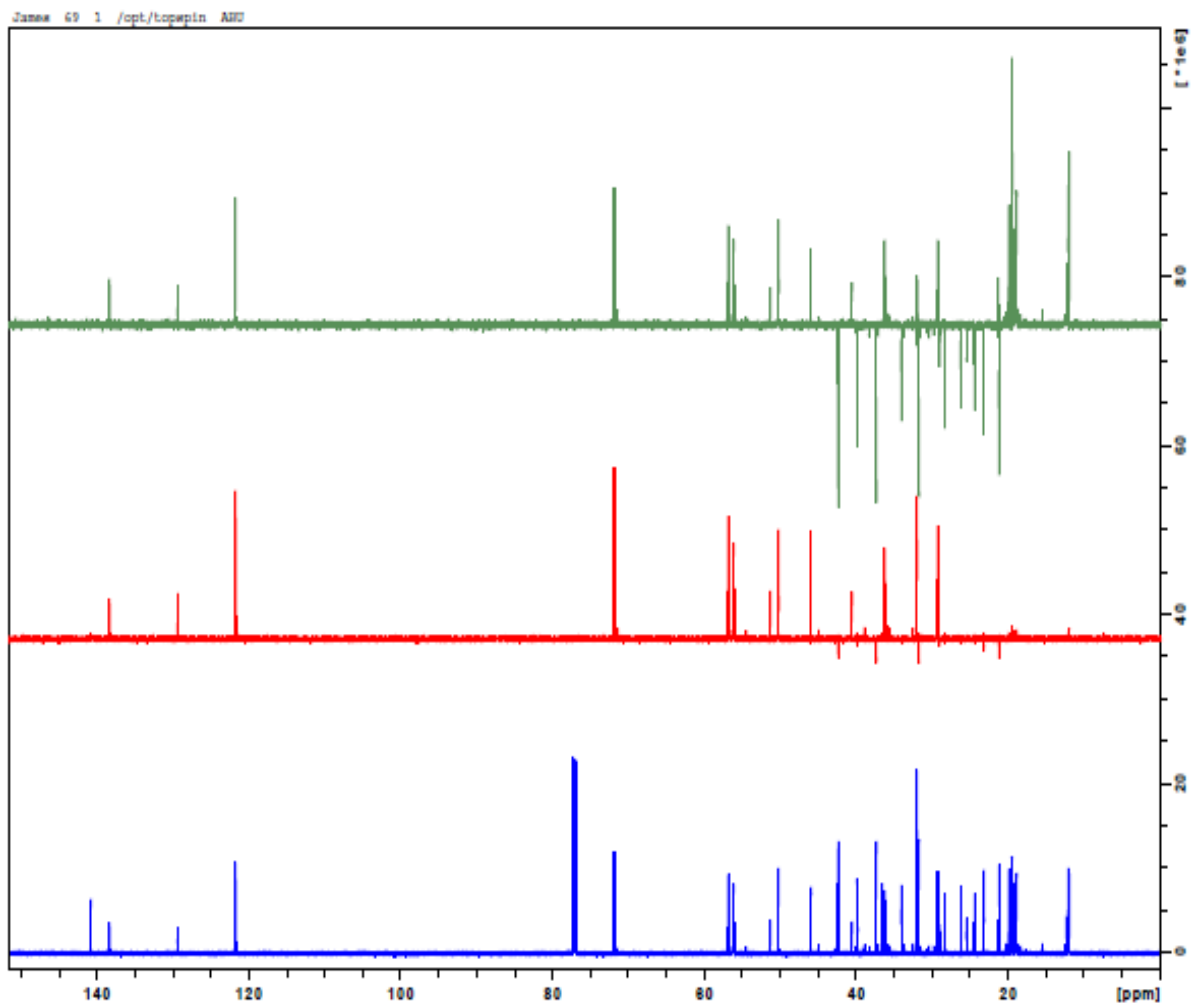
$^1\text{H}$  NMR

Figure 4



**<sup>13</sup>C NMR SPECTRUM OF COMPOUND EB**

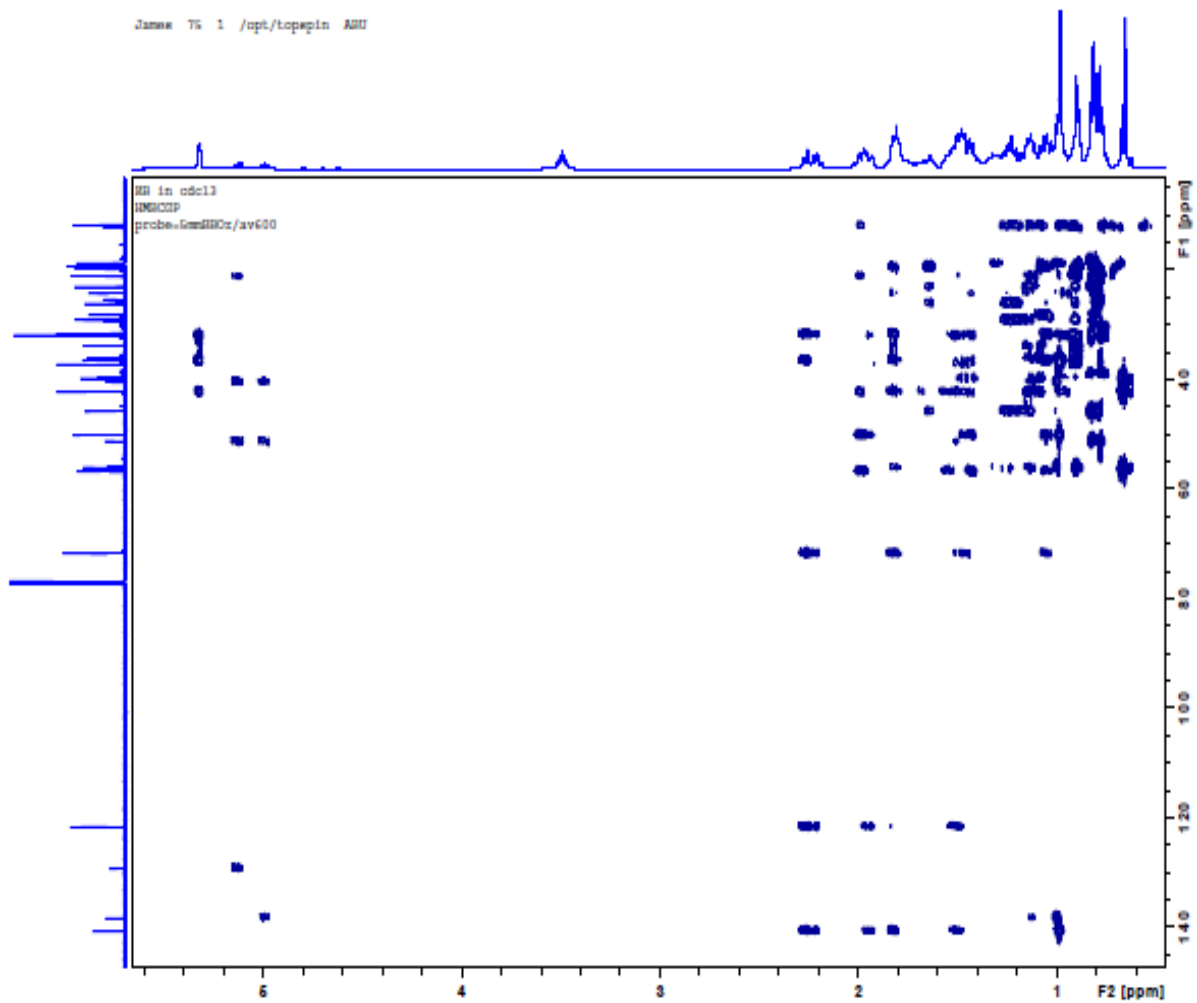
**Figure 5**



**DISTORTIONLESS ENHANCEMENT BY POLARISATION TRANSFER (DEPT)**

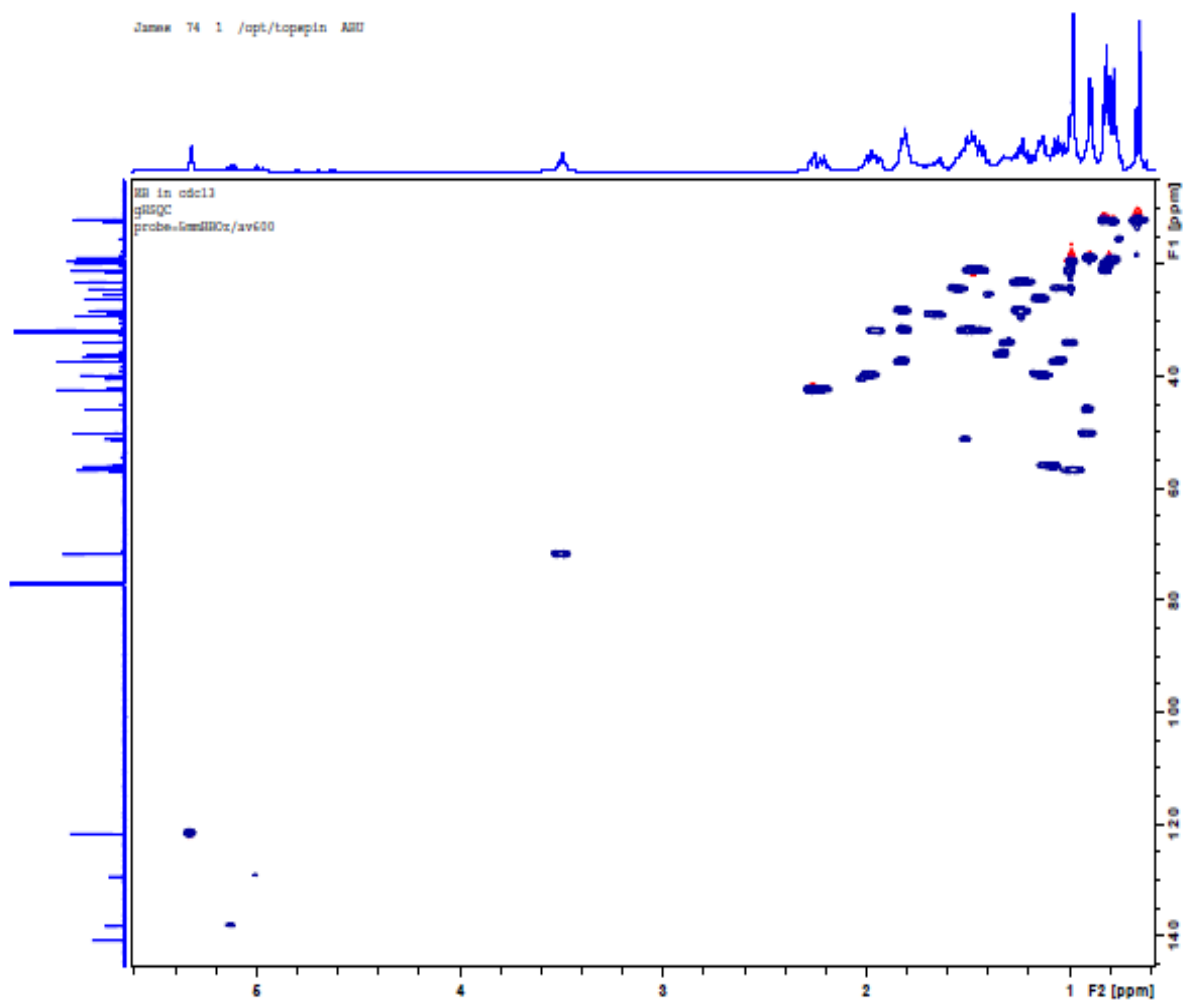
**SPECTRUM OF COMPOUND EB**

Figure 6



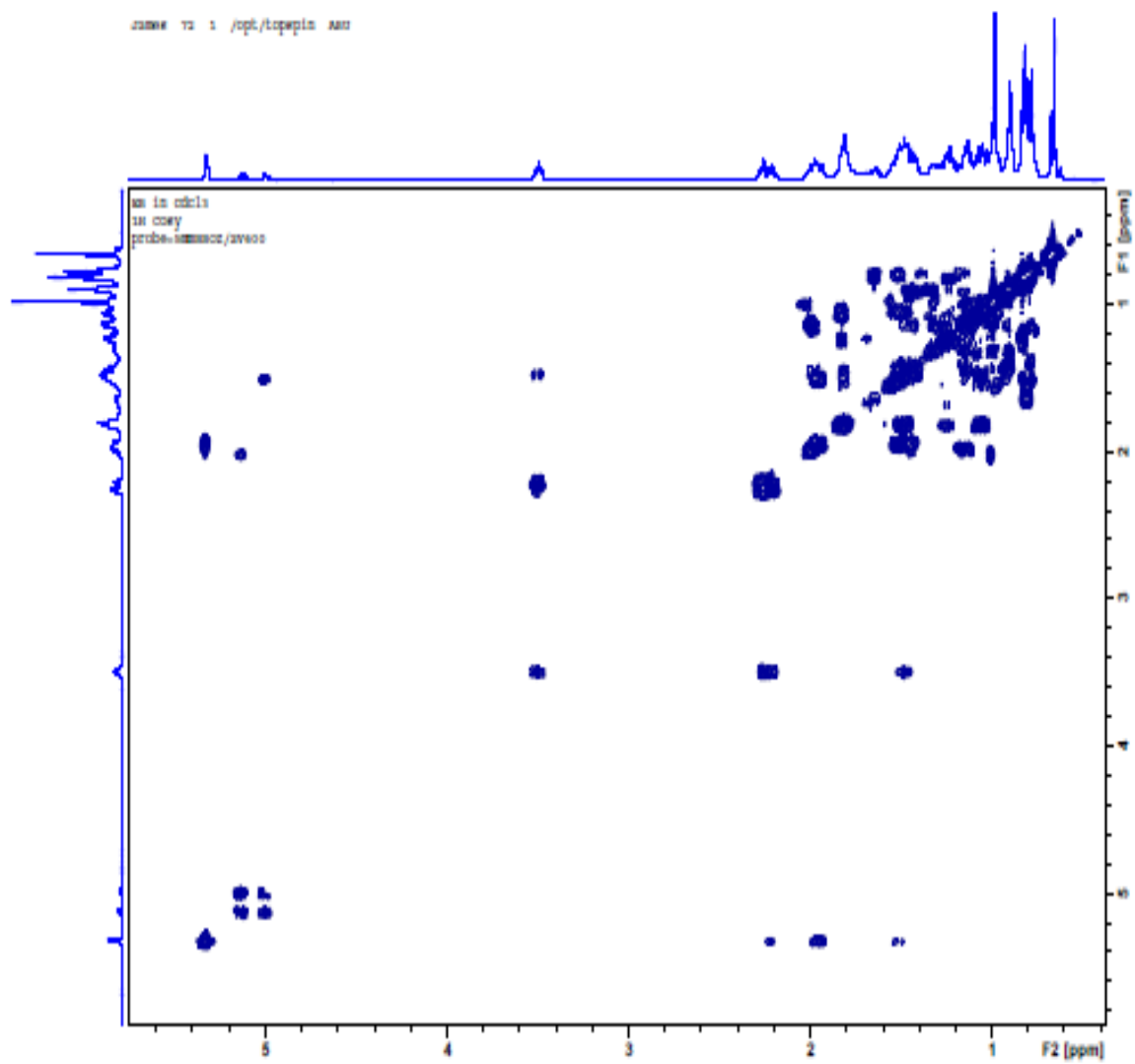
HMBC

Figure 7



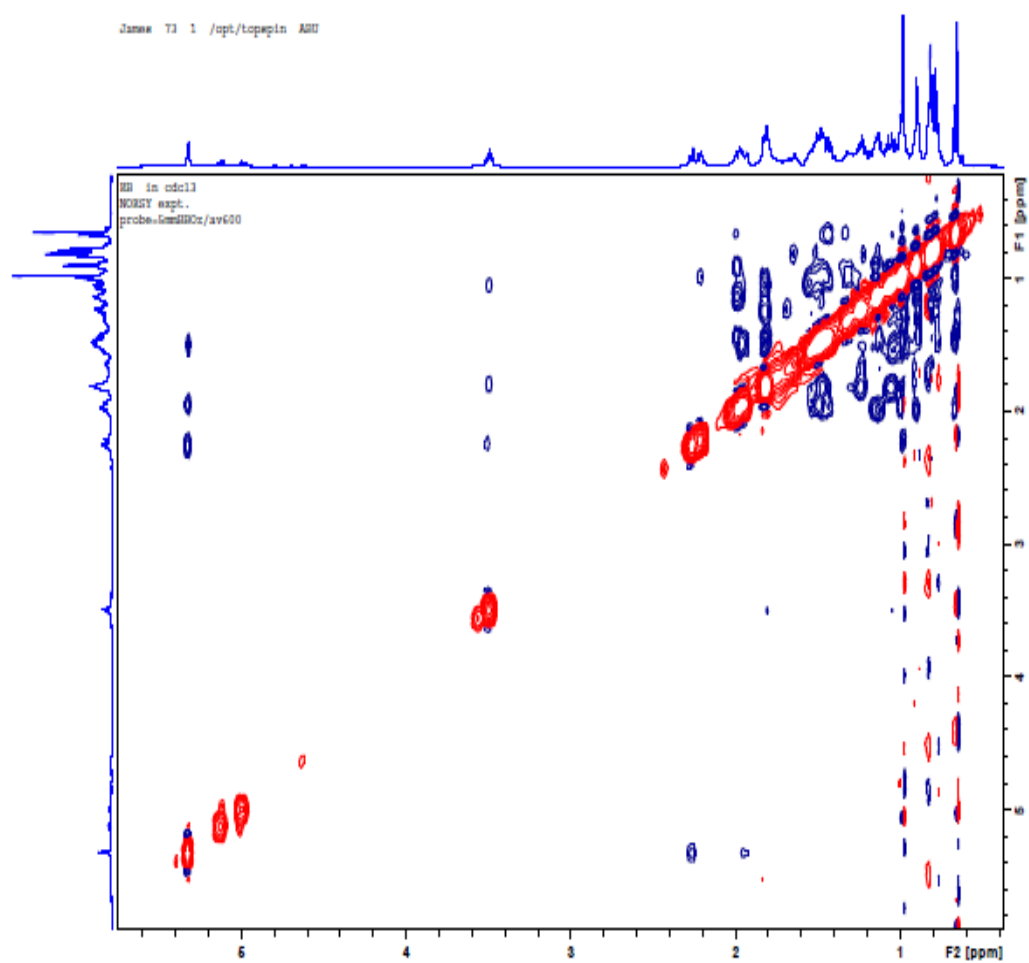
HSQC

Figure 8



COSY

**FIGURE 9**



**NOESY**

**Table 4.8: <sup>13</sup>C NMR assignment of Sample EB**

<b>Carbon</b>	<b>δ(ppm)</b>	<b>CHn</b>
1	37.28	CH <sub>2</sub>
2	31.66	CH <sub>2</sub>
3	71.79	CH
4	42.31	CH <sub>2</sub>
5	140.78	C
6	121.71	CH
7	31.89	CH <sub>2</sub>
8	31.93	CH
9	51.25	CH
10	36.52	C
11	21.10	CH <sub>2</sub>
12	39.71	CH <sub>2</sub>
13	42.31	C
14	56.89	CH
15	24.38	CH <sub>2</sub>
16	28.91	CH <sub>2</sub>
17	56.00	CH
18	12.06	CH <sub>3</sub>
19	21.23	CH <sub>3</sub>
20	40.48	CH
21	21.23	CH <sub>3</sub>
22	138.31	CH <sub>2</sub>
23	129.31	CH <sub>2</sub>
24	50.17	C
25	31.66	CH
26	21.23	CH <sub>3</sub>
27	19.40	CH <sub>3</sub>
28	25.40	CH
29	12.24	CH <sub>3</sub>

**TABLE 4.9: COMPARISON OF <sup>13</sup>C NMR DATA OF EB WITH LITERATURE DATA**

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<b>POSITION</b>	<b><sup>13</sup>C(ppm)</b>	<b><sup>13</sup>C LIT(Isah et al 2012)</b>
C <sub>1</sub>	37.28	37.26
C <sub>2</sub>	31.66	31.68
C <sub>3</sub>	71.79	71.82
C <sub>4</sub>	42.31	42.32
C <sub>5</sub>	140.78	140.76
C <sub>6</sub>	121.71	121.72
C <sub>7</sub>	31.89	31.90
C <sub>8</sub>	31.93	31.90
C <sub>9</sub>	51.25	51.24
C <sub>10</sub>	36.52	36.52
C <sub>11</sub>	21.10	21.09
C <sub>12</sub>	39.71	39.69
C <sub>13</sub>	42.31	42.32
C <sub>14</sub>	56.89	56.87
C <sub>15</sub>	24.38	24.37

<b>C<sub>16</sub></b>	<b>28.91</b>	<b>28.92</b>
<b>C<sub>17</sub></b>	<b>56.00</b>	<b>55.96</b>
<b>C<sub>18</sub></b>	<b>12.06</b>	<b>1205</b>
<b>C<sub>19</sub></b>	<b>21.23</b>	<b>21.22</b>
<b>C<sub>20</sub></b>	<b>40.48</b>	<b>40.49</b>
<b>C<sub>21</sub></b>	<b>21.23</b>	<b>21.22</b>
<b>C<sub>22</sub></b>	<b>138.31</b>	<b>138.30</b>
<b>C<sub>23</sub></b>	<b>129.31</b>	<b>129.28</b>
<b>C<sub>24</sub></b>	<b>50.17</b>	<b>50.17</b>
<b>C<sub>25</sub></b>	<b>31.66</b>	<b>31.68</b>
<b>C<sub>26</sub></b>	<b>21.23</b>	<b>21.22</b>
<b>C<sub>27</sub></b>	<b>19.40</b>	<b>19.40</b>
<b>C<sub>28</sub></b>	<b>25.40</b>	<b>25.41</b>
<b>C<sub>29</sub></b>	<b>12.24</b>	<b>12.25</b>

#### **4.9.0 Result of antimicrobial activity of compound EB**

Table 4.9.1: Sensitivity test of compound EB

Test organism	Mean Zone of inhibition(mm)
<i>S.aureus</i>	28
<i>S.feacalis</i>	27
<i>C.ulcerans</i>	34
<i>E.coli</i>	0
<i>S.typhi</i>	0
<i>P.vulgaris</i>	35
<i>C.albicans</i>	34

## CHAPTER FIVE

### 5.0 Discussion

#### 5.1 Extraction

The percentage yields of the extracts are shown in Table 1. Methanol gave the highest yield while ethylacetate which gave the least yield.

#### 5.2.0 Phytochemical Screening

The phytochemical screening of the extracts of *Indigofera arrecta* indicated the presence of cardiac glycosides, Saponins, Steroids, triterpenes, flavonoids and tannins, while anthraquinones and alkaloids were absent as shown in Table 4.1

Methanolic and ethyl acetate extracts showed the presence of saponins and cardiac glycosides while only methanolic extract showed the presence of tannins.

The plant extracts demonstrated the presence of good number of vital secondary metabolites that are precursor for drug synthesis due to their activities, especially flavonoids which are good scavengers of free radicals as a result of their high reactivity due to the hydroxyl group in them (Korkina and Afana, 1997) and saponins which are known to possess both antimicrobial and anti-inflammatory activities (Mathias et al., 2007).

#### 5.3.0 Antimicrobial Screening

The three crude extracts were tested against eight pathogens; *Staphylococcus aureus*, *Streptococcus faecalis*, *Streptococcus pyogenes*, *Corynebacterium ulcerans*, *Escherichia coli*, *Salmonella typhi* and *Proteus vulgaris*.

The results obtained are shown on Table 4.2. It indicated that *Salmonella typhi* were resistant to all the extracts and the remaining microorganisms were sensitive to all the extracts except *E.coli* which is resistance to ethyl acetate and n-Hexane fractions. The n-Hexane have shown to be the most active of all, followed by the methanol extract and then ethyl acetate extract.

Table 4.3 showed the zone of inhibition of the extracts against the test organisms. The n-hexane being the most active of all showed most activity on the test organisms, followed by methanol, while the ethyl acetate extract showed the least. The zones of inhibition of a known drug used as standard (Ciprofloxacin and Fluconazole) was also shown in the Table 4.4

The result of the Minimum Inhibitory Concentration (MIC) of n-hexane extract is shown in Table 4.5. It revealed that n-hexane extract could inhibit the growths of six out of the eight tested microorganism with *Corynebacterium ulcerans* being the most sensitive one with MIC of 7.5mg/ml while *Staphylococcus aureus*, *Streptococcus pyogenes* and *Streptococcus faecalis* having the least MIC of 15mg/ml.

The result of the Minimum Bactericidal Concentration (MBC) and Minimum Fungicidal Concentration (MFC) determinations of the n-hexane, ethyl acetate and methanolic extracts are shown in Table 6. The MBC for n-hexane extract was 60mg/ml for all the test organisms, the MBC for n-hexane showed that, for *Staphylococcus aureus*, *Streptococcus pyogenes* and *Corynebacterium ulcerans* was 30mg/ml and 15mg/ml for *Streptococcus faecalis*. The MBC of the methanolic extract was 60mg/ml for *Streptococcus pyogenes*, *Staphylococcus aureus*, *Corynebacterium ulcerans*, and *Proteus vulgaris*. For all the extracts, the highest MBC value was 60mg/ml while the lowest was 15mg/ml.

Result of TLC of the n-hexane fraction is seen in Table 4.6. Hexane and ethyl acetate in the ratio of 7:3 gave the best profile with three prominent spots. After developing the plate, the colours and  $R_f$  of the spots were; 0.55 for the purple spot, 0.67 for the yellow spot and 0.85 for the orange spot.

#### **5.4.0 Physical and Chemical Properties of EB**

EB was a white crystalline solid with melting point range of 140-144<sup>0</sup>C, which showed positive to steroids and triterpenes test of Salkowski and Liebermann-Buchard and was obtained from the chromatographic separation of n-Hexane extract.

#### **5.4.1 Spectral Analysis**

The structure of EB was analysed using FTIR Fig 2, <sup>1</sup>H NMR Fig 3, <sup>13</sup>C NMR Fig 4, Distortionless Enhancement polarization Transfer (DEPT) Fig 5, (HMBC) Fig 6, Heteronuclear Single Quantum Correlation (HSQC) Fig 7, (COSY) Fig 8, nuclear overhauser Enhancement spectroscopy (NOESY) Fig 9 and Compound EB Figure 10.

#### **5.4.2 FTIR**

The IR signal absorption band observed at 3370.72cm<sup>-1</sup> is characteristic of OH stretching. The absorption at 2935.76cm<sup>-1</sup> is typical of cyclic olefinic -HC=CH- Stretching, while the absorption at 1655.94cm<sup>-1</sup> is typical of C=C absorption

### 5.4.3 <sup>1</sup>H NMR

There are forty eight signals identified from the <sup>1</sup>H NMR of compound EB. The signals range from 0.6 to 5.4. Signals from 0.6 to 0.1.45 may be due to the saturated hydrogen atoms, the signals from 1.5 to 2.35 may be due to allylic hydrogen atom, signals at 3.5 is due to hydrogen attached to oxygen, while the signals at 5.2 and 5.4 were due to olefinic protons

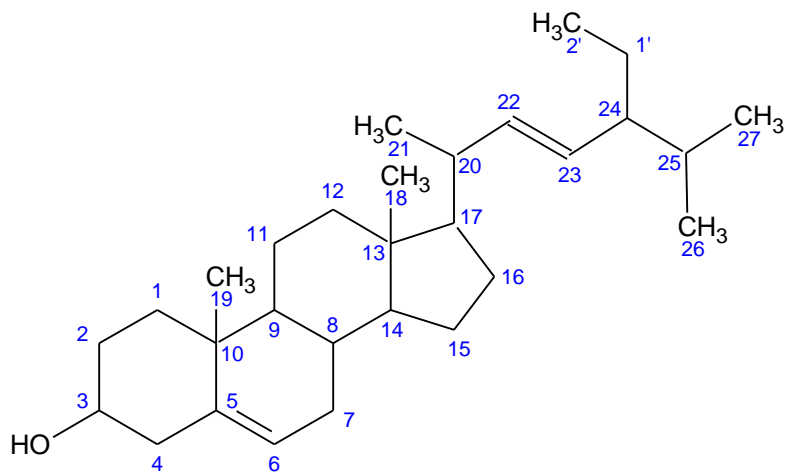
### 5.4.4 <sup>13</sup>C NMR

The <sup>13</sup>C NMR spectrum shows twenty nine signals, ranging from 11.9 to 140.8. The signals at 11.5 ppm to 58.5 ppm are due to overlapping of methyl methylene and methane carbon atoms. The signal at 72 ppm was due to methine carbon, the olefinic carbons appeared at 128 ppm, 138 ppm and 140.78 ppm respectively.

### 5.4.5 DEPT

The DEPT experiment also revealed the presence of carbon atoms to be twenty nine in the compound just like the <sup>13</sup>C NMR, but this experiment distinguished the carbon atoms into C, CH, CH<sub>2</sub> and CH<sub>3</sub>. From the DEPT it was observed that there was three quaternary carbon atoms at 138.3, 37.3 and 42.3 respectively. Nine methine carbon atoms (CH) were observed namely the 71.8, 129.8, 31.9, 51.2, 56.9, 56.8, 39.7, 36.5 and 121.7 peaks. The carbon atoms identified to be CH<sub>2</sub> i.e methylene carbons peaked at 38.8, 29.7, 45.8, 31.7, 23.1, 42.2, 26.1, 29.2, 33.9 and 36 ppm respectively. The remaining six carbon atoms were all methyl carbons (-CH<sub>3</sub>) at 11.9, 19.8, 18.8, 21.2, 21.1 and 12.3 respectively. Therefore, the DEPT experiment revealed the presence six CH<sub>3</sub>, eleven CH<sub>2</sub>, nine CH and three quaternary carbons

Based on spectral comparison with available data base the observed peaks from the spectral data were in complete agreement with the structure of Stigmasterol which is a tetracyclic compound.



**EB;3 $\beta$ ,22E-Sigmasta-5,22-dien-3-ol(C<sub>29</sub>H<sub>48</sub>O,412.7g/mol)**

## CHAPTER SIX

### 6.0 Summary, conclusion and Recommendation

#### 6.1.0 Summary

The whole plant of *Indigofera arrecta* were collected from Makurdi, Benue state. Having been properly identified at the Herbarium unit of Department of Biological science, A.B.U Zaria. The stem was subjected to air drying, pulverization and extraction. The crude extracts obtained were further subjected to preliminary phytochemical screening as well as antimicrobial activity tests. n-hexane fraction being the most active of all, was subjected to chromatographic separation and a compound named EB was obtained, which after undergoing some spectroscopic analysis was considered tentatively to be Stigmasterol.

#### 6.2.0 Conclusion

*Indigofera arrecta* is a plant used in traditional medicine for the treatment of several diseases such as ulcer, sore treatments, epilepsy and as a chief source of blue dyes, etc. Preliminary phytochemical screening revealed the presence of some secondary metabolites which may be responsible for the observed biological activity seen against the tested microorganisms. So, the claim made by the local practitioners was verified to be true.

#### 6.3.0 Recommendation

Based on the work carried out on the stem bark of *Indigofera arrecta*, the following recommendation were made;

- I. Further work should be carried out on the remaining parts of the plant, root and the leaves to also justified the general use of the plant as a medicinal plant,
- II. More compounds could be isolated from the plant's parts using solvents of different polarity with the intention of revealing all the possible compounds with antimicrobial activity and
- III. Other biological activity or pharmacological activity tests should be carried out on the plant to explore the efficacy of the plant as medicinal plant.

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