PHARMACOGNOSTIC AND ANTIBACTERIAL STUDIES OF ACACIA SIEBERIANA VAR WOODII (FABACEAE) STEM BARK

BY

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DEPARTMENT OF PHARMACOGNOSY AND DRUG DEVELOPMENT

FACULTY OF PHARMACEUTICAL SCIENCES

AHMADU BELLO UNIVERSITY, ZARIA

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B. Sc. BOTANY (ABU, 2010)

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A THESIS SUBMITTED TO THE SCHOOL OF POSTGRADUATE STUDIES, AHMADU BELLO UNIVERSITY, ZARIA

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DEPARTMENT OF PHARMACOGNOSY AND DRUG DEVELOPMENT

FACULTY OF PHARMACEUTICAL SCIENCES

AHMADU BELLO UNIVERSITY

ZARIA

JANUARY, 2015

DECLARATION

I declare that the work in this thesis entitled **‘‘**Pharmacognostic and Antibacterial Studies of *Acacia sieberiana* Burtt Deavy var woodii (Fabaceae) Stem Bark’’ has been carried out by me in the Department of Pharmacognosy and Drug Development. The information derived from the literature has been duly acknowledge in the text and a list of references provided. No part of this thesis was previously presented for another degree or diploma at this or any institution.

Ibrahim, Hadiza Mohammed	_____	_____
Name of Student	Signature	Date

CERTIFICATION

This thesis entitled ‘‘**PHARMACOGNOSTIC AND ANTIBACTERIAL STUDIES OF ACACIA SIEBERIANA BURTT DEAVY VAR WOODII (FABACEAE) STEM BARK’’** by **HADIZA MOHAMMED IBRAHIM** meets the regulations governing the award of masters degree in Pharmacognosy of Ahmadu Bello University, Zaria and is approved for its contribution to knowledge and literary presentation.

Dr. G. Ibrahim	_____	_____
Chairman, Supervisory Committee	Signature	Date
Dr. U.A Katsayal	_____	_____
Member, Supervisory Committee	Signature	Date
Dr. G. Ibrahim	_____	_____
Head of Department	Signature	Date
Prof. A.Z Hassan	_____	_____
Dean, School of Postgraduate Studies	Signature	Date

DEDICATION

This research work is dedicated to all my siblings without whom my life will be meaningless. May Allah bless you all and keep the strong family tie together, Ameen.

ACKNOWLEDGEMENTS

Alhamdulillah, all praises due to Allah, the creator of all beings.

Kindness can never be replaced but it must be appreciated and passed on to others. In this regard, I will forever be grateful to my supervisors, Dr. G. Ibrahim and Dr. U. A . Katsayal for their unflinching support, contribution and insistence on excellence towards the success of this study.

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ABSTRACT

Acacia sieberiana var. Woodii (Fabaceae) is traditionally used as a remedy for stomach aches and ulcers among some localities in Northern Nigeria. The plant was investigated sequel to the reports of its ethnomedicine uses in the management of stomach ache and ulcers. Anatomical and physical constants studies were carried out using standard procedures. Phytochemical (preliminary studies, thin layer and column chromatography) were also carried out on the hexane and methanol extracts. Toxicity and antibacterial studies of the hexane and methanol extracts, and the isolated compound were investigated. The microscopical studies revealed the presence of cellulose cell wall, lignified and suberized cell walls, tannins and calcium crystals. Anatomical features identified in the stem bark consist of epidermis, phellogen, phelloderm, and cortex. The percentage moisture content and ash values were observed to be 9.0% and 10.5% respectively. Water extractive value was 1.2% while ethanol extractive value was 1.6%.The preliminary phytochemical studies as well as the TLC chromatogram of the hexane extract revealed the presence of steroids and triterpenes while the methanol extract contains tannins, flavonoids, alkaloids, steroids and triterpenes. Fraction AS1collected through column chromatography was suggested to be a hydrocarbon on the basis of its colourless and oily nature. The acute toxicity study carried out indicated that the hexane extract was slightly toxic while the methanol extract was moderately toxic. Antibacterial studies of the stem bark carried out showed zones of inhibition between 16 to 27 mm for the hexane and methanol extracts against *Helicobacter pylori, Escherichia coli, Salmonella typhi* and *Shigella dysenteriae.* The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were between the range of 2.5 and 10 mg/ml and 5.0 to

20 mg/ml respectively. Fraction AS1 exerted a dimension of zones of inhibition between 26 to 30 mm against all the bacterial strains except *S. typhi*. It was also able to exert MIC and MBC against *E. coli* and *H. pylori* at 12.5 and 25µg/ml respectively, while it had MIC and MBC at the lowest concentrations of 6.25 and 12.5 µg/ml against *S. dysenteriae*. Results of the present studies had shown that *A. sieberiana* and fraction AS1 collected from this plant have wide antibacterial property.

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ABBREVIATION, SYMBOLS AND GLOSSARIES

µg/ml: microgram per milliter

^{13}C NMR: carbon-13 nuclear magnetic resonance

^{1}H NMR: proton nuclear magnetic resonance

AS1: Compound Isolated from Hexane Extract of *A. sieberiana* Stem Bark

DMSO: Dimethyl sulphoxide

FAA: Formalin: Acetic acid: Methanol

HE: Hexane Extract of *Acacia sieberiana* Stem Bark

LD_{50} : Median Lethal Dose

MBC: Minimum Bactericidal Concentration

ME: Methanol Extract of *Acacia sieberiana* Stem Bark

GC-MS: Gas Chromatography, Mass spectroscopy

mg/kg: milligram per kilogram

mg/ml: milligram per milliliter

MIC: Minimum Inhibition Concentration

NMR: nuclear magnetic resonance

ppm: part per million

CHAPTER ONE

1.0 INTRODUCTION

1.1 Traditional Medicine

Traditional medicine can be said to be a comprehensive knowledge system that encompasses the utilization of substances, dosages and practices based on socio-cultural norms and religious beliefs as well as witnessed experiences and observations of a specific group (Tom *et al.,* 2008). The practice is used in various therapies by the indigenous population all over the world. It has been documented that about 80% of the people in developing countries rely on traditional medicines for their primary health care need, which can be attributed to increased poverty, ignorance as well as unavailability of modern health facilities (Omonike, 2010; Christiana *et al*., 2012).

1.2 Plants in Traditional Medicine

Plant based traditional remedies are the oldest forms of health care delivery known to mankind and it has been used in all cultures throughout history. Plants have been used as early as 5000-4000BC in different parts of the world for the treatment of various ailments (Nyananyo and Akada, 2011; Kunle *et al.,* 2012). A vast knowledge on how to use these plants against diseases may be expected to have accumulated in areas where the use of these plants is of great importance. The knowledge of these plants was developed gradually as it passed from generation to generation and has laid foundation of many health care systems all over the world (Azmatullah *et al.,* 2011; Kunle *et al.,* 2012).

During the past decade, there has been an increasing public interest and acceptance of natural therapies in both developed and developing countries, which could be attributed to the fact that medicinal plants reflect recognition of its validity based on many traditional claims regarding their medicinal values. They are also regarded to be relatively safer and better than synthetic drugs as they contain carbohydrate, protein, fats, vitamins and minerals believed to be naturally acceptable to the human body. They also produce diverse types of other bioactive molecules such as tannins, alkaloids, glycosides and terpenoids making them a rich source of different types of medicines (Azmatullah *et al.*, 2011; Christiana *et al.*, 2012; Kunle *et al.*, 2012).

1.3 Medicinal Plants as Antibacterial Agents

Bacterial infections including diarrhea, human intestinal diseases, urinary tract infections and gastroduodenal inflammations are the major causes of morbidity and mortality in developing countries. The use of plants for the treatment of these infectious diseases has been viewed as medical aids in most developing countries since plants have been established to produce bioactive components that are said to confer them with resistance against microbes as well as being responsible for their antimicrobial effects (Tayfun *et al.*, 2008; Doughari *et al.*, 2009; Mogahid *et al*., 2011).

Alternative antibiotics from plants have been revived for disease management due to the increased prevalence of multidrug resistance strains of bacterial isolates. This increase prevalence have been attributed to the indiscriminate uses of commercial antibiotics and this in turn has forced scientists to search for newer antimicrobial substances from various

medicinal plants. These plants appear to be the important approaches to the development of these antibiotics as metabolites such as phenolic compounds and essential oils which were reported to posses high antimicrobial properties which are of great importance to tackling microbial diseases (Satish *et al*., 2008; Doughari *et al.,* 2009; Oleyede *et al*., 2012; Deshpande, 2013).

1.4 Statement of Research Problem

There is need to establish pharmacognostic standards on the safety and efficacy of *A. sieberiana* stem bark as it is extensively utilized in traditional medicine for the treatment of various ailments including stomach pains and stomach ulcers with no scientific basis.

1.5 Justification

There is an increasing interest in the use of herbal medicines especially in developing countries where cost of living is high and there is less scientific research on the use of known medicinal plants including *A. sieberiana* in the treatment of diseases. Therefore, antibacterial studies of *A. sieberiana* stem bark will no doubt provide scientific basis to either support or debunk its uses in the treatment of stomach pain and ulcers associated with enteric bacteria.

1.6 Aim and Objective of the Study

The overall aim is to establish pharmacognostic standards and provide scientific basis for the use of the plant stem bark as antibacterial agents in ethno medicine.

The specific objectives are;

- To establish microscopical characters of the stem bark of *A. sieberiana*
- To determine the phytochemical constituents present in the stem bark of the plant.
- To establish the antibacterial activity of the plant stem bark as used in traditional medicine.

1.7 Hypothesis

Stem bark of *A. sieberiana* contains bioactive components with antibacterial properties against some enteric bacteria

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Botanical Description of Fabaceae

Fabaceae, formerly known as Leguminosae is the third largest family of Angiosperm after Orchidaceae and Asteraceae. It is reported to comprise of about 500 to 750 genera and 12,000 to 19,400 species divided into three subfamilies (Dejan Godevac *et al.,* 2008; Sayyah *et al.,* 2011; Karnal *et al.,* 2012; Wassila *et al.,* 2013). These subfamilies are Caesalpinaceae, Mimosaceae and Papilionaceae which thrive in both temperate and tropical climates. The largest genera are the *Astragalus* (Subfamily Papilionaceae) with more than 2,000 species, *Acacia* (Subfamily Mimosaceae) with more than 900 species and *Indigofera* (Subfamily Papilionaceae) with around 700 species (Robert *et al*., 2001; Dutta, 2005; Sayyah *et al.*, 2011).

The plant family comprises of mainly herbs, shrubs, trees, twiners and climbers. They vary in sizes from the smallest plants of the desert and arctic or alpine region to the tallest trees of the rain forest. They are characterised by large seeds and pods types of fruits. The pods possess parietal placentation with two or more ovules in the various species. Leaves are alternate, simple or compound, and sometimes reduced to tendrils. Flowers are bisexual with a superior carpels, arranged singly or in racemes, regular, zygomorphic or irregular and hypogynous or slightly perigynous (Muhammad *et al.,* 2001; Dutta, 2005; Martin *et al*., 2006).

2.1.1 Economic and medicinal importance of Fabaceae

Fabaceae is second only to Poaceae in terms of their agricultural and economic importance. They are used as pulses, vegetables, as natural fertilizers, source of timber, gums, and tannins. Legumes are important to agronomy due to their symbiotic capacity for biological nitrogen fixation. Their roots possess tubercles containing bacteria which are responsible for their nitrogen fixing bacteria properties (Robert *et al*., 2001; Dutta, 2005; James *et al*., 2006).

Medicinally, plants belonging to Fabaceae such as *Entada phaseoloides, Butea manosperma,* and *Bauhinia forticata* have been reported in scientific literature for their antidiabetic and antinociceptive activities (Richa, 2010; Tanzila *et al.,* 2012). Several species of the family including *Alysicarpus* species, *Crotalaria* species, *Cassia sieberiana* and *Lephrosa* species are been utilized traditionally for treatment of skin eruptions, rheumatic pains, and in treatment of syphilis, gastritis, cough, fever, ringworm, leprosy, epilepsy, dysentery, mouth ulcers, as vermicide and contraceptive (Obidah *et al*., 2009; Rahmatullah *et al.,* 2010; Gupta *et al*., 2013). Other plants such as *Medicago sativa, Glycyrihiza glabra, Indigo tinctoria, Albizzia lebbek,* and *Cassia fistula* among others are been utilize in treatment of haemorrhage, diuretic, bronchitis, hypertension, diabetes, typhoid and malarial fever, dysentery, expectorant, skin diseases, inflammation, asthma, ulcers, cramp and colic antitussive, stomach pains and ulcers, as blood tonic, antifertility, laxatives, in treatments of fevers, arthritis, and cardiac conditions (Fennel *et al.,* 2004; Alesiani *et al.,* 2007; Lalchhandama, 2011; Seyedeh *et al*., 2011; Stella *et al*., 2011; Annie and Muthulingam, 2012; Meghendra and Ashwani, 2013; Shaheen *et al*., 2013). *Parkia*

species have been reported to possess antioxidant, antimicrobial, gastroprotective and hypertensive effects. Generally, species of fabaceae are known to yield resins, balsams and dyes with few possessing astringent, narcotic, emetic, purgative, tonic and restorative properties (Meghendra and Ashwani, 2013; Vivianne *et al*., 2013).

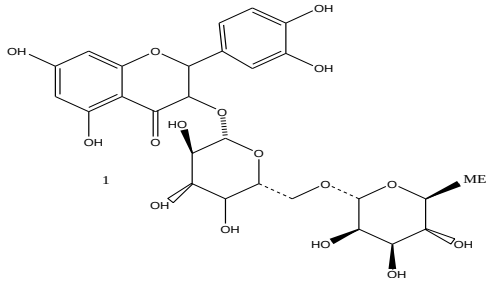
2.1.2 Reported phytochemical constituents of Fabaceae

Legumes are particularly rich in flavonoids when compared to other families. About 28% of all flavonoids and 95% of isoflavonoid aglycone structures known to the plant kingdom are produced by the legumes. Chemotaxonomically, the three subfamilies are united by the absence of 5-hydroxyl group with 50% of the flavonoids and 66% of the isoflavonoid structures showing this characteristic (Hengnauer and Renpe, 1993; Constantino and David, 2006). Some flavonoids isolated from Fabaceae are genistein, and prunetin from *Butea pendula* stem bark, quercetin-3-O-rutinoside and quercetin from *Bauhinia monandra*, while isoquercetin, avicularin, apigenin-7-O-L-D-glucoside, cassiaocidentalin B, isoorientin were reported from the aerial part of *Mimosa pudica* (Dejan Godevac *et al.*, 2008). Others include liquintin, isoliquintin, liquitigenin, isoliquintegenin, rhamnoliquinin, and 2-methylisoflavones, pongaflavonol, pongaglabol, karanjin, pongapin, pongachin, ponganone III, pongamone A-E, ovalichromene- B, 50-methoxypongapin, karanjachromene and luteolin from the roots of *Pongamia pinnata* (Harleen *et al*., 2011). Purpurin, pongamol, karanjin, lanceolatin-B were also reported from *Tephrosia purpurea* seeds. O-glycoside, rutin **(1)** and C-glycosides vitexin **(2)** and vitexin 2’’-0-α-rhamnopyranosides **(3)** were isolated from the aerial parts of *Onobrychis montana* (Harleen *et al*., 2011). Also, phytochemical studies of *Flemingia macrophylla* revealed the

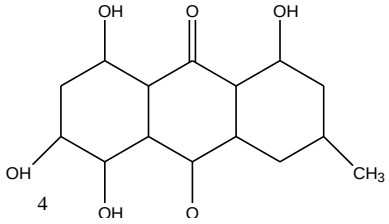
presence of flavonoids, flavonones, flemiflavanones A, B, C and D, flemichin A, B, C and D, narigenin, homoflemingin, procyanidin and α-amyrin. While *Dalbergia* species contains butin **(4)**, cajanin **(5)**, alpinetin **(6)** and biochanin A **(7)** among others (Kavita *et al.,* 2012; Sanjib *et al*., 2013).

Astragalus species have been reported to contain saponins, phenolic and polysaccharides, imidazoline alkaloids and selenium derivatives such as astragaloside VIII, and nicotiflorin, (Wassila *et al.,* 2013). *Bauhinia purpurea* has been reported to contain a bauhiniastatins, pentacyclic triterpenoids, fatty acids esters, and barbigerone. Carotenoids, coumarins, tannins, sugars, polyphenols, alkaloids, amino acids, triterpenoids and saponins have been reported in *Indigofera tinctoria* (Annie and Muthulingam, 2012; Gupta *et al*., 2013).

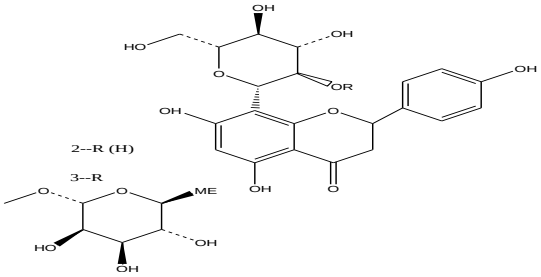
Major saponin triterpenoids, glycyrrhizic acids have been obtained from *Glycyrrhiza* species (Taro *et al*., 2002; Lalchhandama, 2011; Annie and Muthulingam, 2012; Gupta *et al*., 2013). *Cassia reingera* is also known to be a rich source of anthraquinones such as 1,5,6,8-tetrahydroxy-3-methyl-anthraquinone **(8)** , and 8-0-α-L-glucosides. While *Lotus garanii* was found to contain Garceine, isophytol, hexadecanoic acids, cholesterol, oleanolic acids, betulinic acids, gurosides and garthiol (Muhammad *et al*., 2001; Lalitha and Mukthar, 2005; Vivianne *et al.,* 2013).



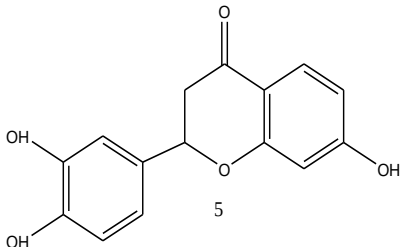
Rutin **(1)**



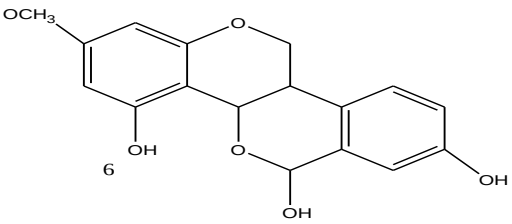
1,5,6,8-tetrahhydroxy-3-methyl-anthraquinone **(4)**



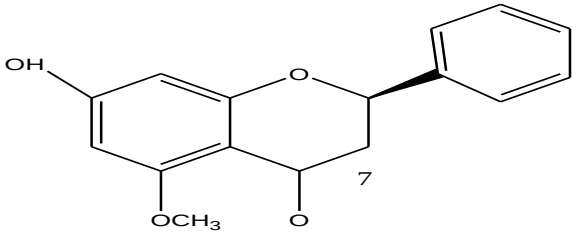
Vitexin **(2)** and Vitexin-2'-0-α-rhamnopyranoside **(3)**



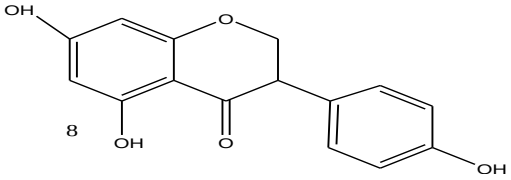
Butin **(5)**



Cajanin **(6)**



Alpinetin **(7)**



Biochanin A **(8)**

Figure I: Some Phytochemicals reported from Members of the Family Fabaceae

2.2 Botanical Description of Genus *Acacia*

The second largest and economically richest genus of the Fabaceae and the largest of the Mimosoideae subfamily is the *Acacia* comprising more than 13, 000 species occurring in all habitats. They are indigenous to tropical and subtropical savannah and widespread in Australia, Africa, Asia and the America. About 700-800 of the species occur in Australia, 129 in Africa, and a few in Asia with 200 species distributed in the rest of the world. The genus consists of three subgenera including *Acacia, Aculeiferum* and *Phyllodineae* (Duarte and Wolf, 2005; Mokoboki *et al.*, 2005; Saurabhi *et al*., 2012; Sakinah *et al*., 2012).

Leaves of *Acacia* are compound and pinnate, with leaflets suppressed. The petioles becomes the tendrils which serve as the leaves in some species, while in other species, modified leaf-like photosynthetic stem called the cladodes are present. The flowers possess very small petals with long stamens that are arranged in dense globular or cylindrical clusters. They range in colour from yellow, white, red, purple, or cream. Stipules may be spinescent or non spinescent with exine outline broken or continous (Singh, 2004; James *et al*., 2006).

2.2.1 Economic and medicinal importance of genus *Acacia*

Plants from the genus *Acacia* have been reported to be of great importance in industries, rural development and conservation. In agriculture, they act as nodulation inducers as most of their roots have been found in symbiotic association with rhizobium in the soil. Gums of *Acacia* are utilized extensively in pharmaceuticals, cosmetics and confectionery industries.

These gums are used as binders in solid oral dosages, formulations in pastilles and lozenges (James *et al*., 2006; Shittu *et al*., 2010).

The genus *Acacia* has medicinal uses in the treatment of diarrhea, urinary tract infections, throat, gastritis, headaches, skin, stomach and tooth problems (Duarte and Wolf, 2005). Pharmacological studies revealed that some members have antitumor, cytotoxic, antimutagenic, antimicrobial antiameobic, anti-inflammatory, hypotensive, gastrointestinal disorders and antiparasitic activity (Jesus *et al.,* 2007; Gaara *et al*., 2008; Napar *et al*., 2012; Saurabhi *et al*., 2012). Traditionally, different parts or whole plant of the *Acacia* species are utilized extensively for treatment of various ailments. Several species such as *A. nilotica, A. leucophloea,* and *A. albida* are utilized for the treatment of cold, congestion, hemorrhage, small pox, syphilitic and oral ulcers, tuberculosis, diarrhea, pneumonia, sexual disorders, tumors of ear and eyes, dysentery, sterility, skin diseases, toothaches, vomiting, arthritis, diabetic, vaginal douche, malaria, gastrointestinal disorders, constipation, and convulsion (Mokoboki *et al.,* 2005; Gupta *et al*, 2010; Lalitha *et al.,* 2012; Lawal *et al*., 2012; Saurabhi *et al*., 2012; Saba *et al.,* 2012; Saad *et al.,* 2013).

2.2.2 Reported Phytochemical Constituents of *Acacia* species

Flavonoids such as 3,4-dihydrobenzoic acids, 7,8.3’,4’-tetrahydroxy-4-methoxyflavan-3-ol, 7,8,3’- trihydroxy-3,4’-dimethoxyflavone, and 7,3’,4’-trihydroxyflavone have been identified from the heartwood of genus *Acacia*. Others include stigmasterol, β-sitosterol, β-sitosterol-3-O-β-D-glucoside, stigamasterol-3-O-β-D-glucoside, lupenone, taraxerone, apigenin, luteolin, quercetin, gallic acid **(9)**, erythrodiol, salicylic acids, gallic acid methyl

ester **(10)**, gallocatechin-3-gallate, naringenin **(11)**, catechol, (+)-catechin **(12)**, 2-O-β-Arabinofuranosyl, flavan-3-ol-gallate and nilobamate **(13)** were isolated from *A. cochliacantha*, *A. confusa*. and *A. nilotica* among other species (Chalk *et al*., 1968; Muhaisen *et al.*, 2002; Jyh-horng *et al*., 2005; Bala, 2006; Jesus *et al*., 2007; Jyh-horng *et al*., 2008; Valentine *et al*., 2012; Sakinah *et al*., 2012).

Pentacyclic terpenoids and triterpenoids such as (2OX) 3-oxolupane-30al, (2OS) 3-oxolupane-30al, 30-hydroxylup-20(29)-en-3-one, 30-hydroxylup-20(29)-en-3β-ol, methyl-2-hydroxy-4-hydroxy-3,6-dimethybenzoate, β-sitosterol, β-O-glucoside and linoleic acids were isolated from stem bark of *A. mellifera* (Mutai *et al*., 2007).

Several other secondary metabolites such as hydrolysable and condensed tannins, terpenes, cyanogenic glycosides and gums with the occurrence of typtamine alkaloids limited to the genus *Acacia* among other genera of the plant family (Constantino and David, 2006; Jesus *et al*., 2007).

2.3 Botanical description of *Acacia sieberiana*

Acacia sieberiana is a perennial savannah tree commonly called white thorn, umbrella thorn, paper bark thorn, or flat-topped thorn in English. In Africa, it has different local names such as *Papierbasdoring* in Afrikaans, *Daneji* in Fulani, *Farar Kaya* in Hausa, *Mgunga* in Swahili, *Aluki* in Yoruba, and *Umkhaya* in Zulu language (Orwa *et al*., 2009; Christiana *et al*., 2012). The plant grows in the savannah, woodland and sometimes along river banks or low grounds, occurring in the entire Sahel and other semi-arid regions in

Africa. It is documented as a tree indigenous to Africa and occurs in many West African countries including Nigeria, Ghana, Cameroun, Niger and Benin (Burkill, 1995; Orwa *et al.*, 2009).

The plant is a tree 3-25 m tall, bole straight to 6 m long by 1 m diameter with a rather rounded crown and trunk 6 m high. The bark is rough and yellowish with grey-brown scales. The branches bear short to massive 10 cm long straight spines (Orwa *et al*., 2009).

Kingdom: Plantae

Phyllum: Magnoliophyta

Class: Magnoliopsida

Order: Fabales

Family: Fabaceae

Genus: *Acacia*

Species: *sieberiana*

Variety:*Woodii*

This classification of *Acacia sieberiana* is according to the International Legume Database Information Service (ILDIS, 2013).

Leaves are usually sparsely hairy, bunched in to small clusters. The leaves also possess pairs of pinnae that are densely crowded and overlap with a common stalk 5 to 10 cm long which ends in a minute spine. The colour of the flowers ranges from cream, to white or yellow with a whorl of tiny bracts near the apex. Fruits are shiny brown in colour, straight

or slightly falcate with more or less parallel margins. Indehiscent, and release about 12 seeds which are about 1 cm long, hard, flat and embedded in a yellowish-greenish pulp (Burkill, 1995; Orwa *et al.*, 2009).

2.3.1 Economic and medicinal importance of *Acacia sieberiana*

Gums from *A. sieberiana* are edible and used as chewing gum, in making ink, for cosmetics, and is included in turbans and head-cloths in Senegal. It is also used as an astringent and as emulsifier. The flowers are good bee forage as they are used as home for hives while the pods, leaves and shoots are used as forage for live stocks. The bark and pod are also utilized in tanning while the wood is used as firewood and charcoal. The wood is used in making furniture, tool handles and mortars as they are termite resistant. The forked branches are used in hut-building and form handles for the large bent hoe (Burkill, 1995; Orwa *et al*., 2009).

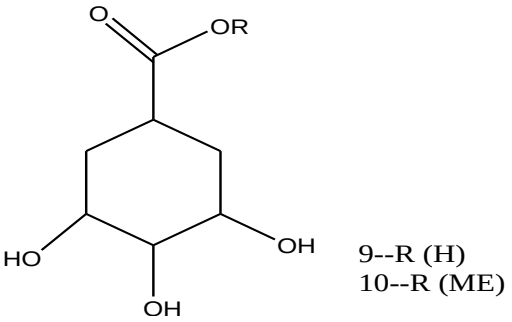
The antimicrobial activities of *A. sieberiana* leaf and bark extracts have been reported on *Mycobacterium aurum*, *Bacillus subtilis*, *Staphyllococcus aureus*, *Escherichia coli*, *Kiebsella pneumonia*, and *Staphyllococcus epidermis* (Rabe and Van Staden, 1997; Eldeen *et al*., 2005; Eldeen and Van Staden, 2007). Traditionally, *A. sieberiana* is utilized by different communities for the treatment of various ailment including inflammation, tiredness, joint pains, bilharzia, fever, enemas and taeniasis. The bark and root extract both rich in tannins are used in treating schistosomiasis, fever, stomach ache, jaundice, opthalmia, cough, sexual impotence, erectile dysfunction, hemorrhoids, syphilis, uterine problems and to improve lactation after child birth (Christiana *et al*., 2012). The leaves are

taken orally for the treatment of urinary tract disorders, tapeworm, headache, bilharzia, kidney problem, rheumatism, circulatory system disorders and as a vermifuge. While the pods are utilized as an emollient (Burkill, 1995; Orwa *et al.*, 2009).

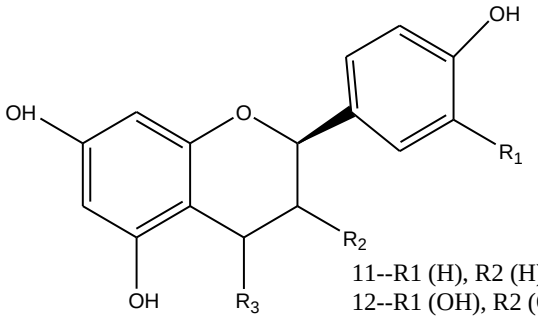
2.3.2 Chemistry of *Acacia sieberiana*

Gums of *A. sieberiana* were reported to consist of complex carbohydrate polymers of arabinose, galactose, and traces of protein. Preliminary phytochemical screening of the plant was reported to show the presence of saponins, tannins, cardiac glycosides, flavonoids and anthraquinones (Burkill, 1995; Anisa, 2010; Mahdi *et al*., 2013). Cyanogenic glycosides, Acacipetalin (14) have been reported present in the *A. sieberiana* with Dihydro-acacipetalin isolated from the leaves of the plant (Butterfield *et al.,* 1975; Siegler *et al*., 1975). HPLC profiles of the leaves of the plant also revealed tentatively the presence of two flavonols and a flavone (Anisa, 2010). Glucosides, 2-β-D-Glucopyranosyloxy-2-methylpropanol and (2R)-2-(β-D-Glucopyranosyloxy)-3-hydroxy-3-methylbutanetrile which correlated with proacacipetalin by oxymercuration and (2S)-2-[(6-O-α-L-Arabinopyranosyl-β-D-Glucopyranosyl)oxy]-3-methylbut-3-enenitrile were isolated from the pods of *A. sieberiana* (Nartey *et al.*, 1981; Brimer *et al*., 1981; Brimer *et al.*, 1982).

The seeds of *A. sieberiana* were reported to contain 4% concentration of fixed oils with a composition of 44% oleic acids and 31% palmitic acids. The bark was also reported to contain about 3.8% of condensed tannins, 4.9% and 5.1% catechin (Shittu *et al*., 2010; Mahdi *et al*., 2013).



9--R (H)
10--R (ME)



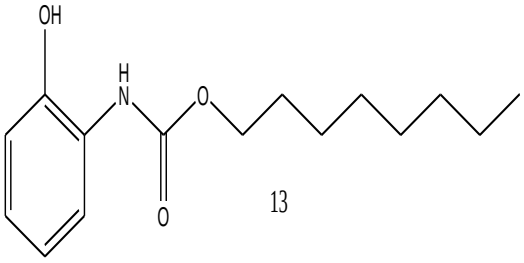
11--R1 (H), R2 (H), R3 (OH)
12--R1 (OH), R2 (OH), R3 (H)

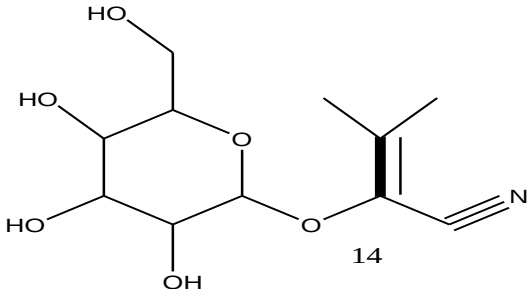
Gallic acid **(9)**

Gallic acid methyl ester **(10)**

Naringenin **(11)**

Catechin **(12)**





Nilocarbamate **(13)**

Acacipetalin **(14)**

Figure II: Some Phytochemicals reported from Members of the genus *Acacia*

2.4 Enteric Bacteria

Enteric bacteria or enterobacteria are the largest families of gram-negative that are, peritrichously flagellated or non motile, facultative anaerobic, straight rods with simple nutritional requirements. They are all capable of degrading sugar by means of Embden-meyerhof pathways and cleave pyruvic acids to yield formic acid in formic acid fermentation. *Escherichia* species, *Salmonella* species and *Shigella* species are among the groups that carry out mixed fermentation to produce lactate, acetate, succinate, formate and ethanol (Prescott *et al*., 2005).

Diarrheal diseases are significant causes of morbidity and mortality in most developing countries. This disease can either be non-inflammatory caused by the production of toxins by the bacteria, *Escherichia coli* or it can inflammatory characterised by presence of fever and blood in stool caused by *Salmonella typhi* and *Shigella dysentriae*. *E. coli* is present in human intestines and has been established to cause gastrointestinal ailments, nausea, vomiting, coleocystitis, septiceamia and induces acute and chronic urinary tract infections (Mahady, 2005; Sharif *et al.,* 2009; Mogahid *et al*., 2011). While *Salmonella typhi* is the only bacteria presently associated with typhoid fever, a disease estimated to have caused 21.6 million illnesses and 216, 500 deaths globally in the 2000, affecting all ages (Bhan *et al*., 2005).

Another enteric bacterium that is of major health concern worldwide is *Helicobacter pylori*. This bacterium is a spiral, gram-negative, micoraerophilic, motile, curved rod organism that inhabits the gastric mucosa of the stomach. It is reported to have the ability of establishing infection in the human stomach for decades (Ronita *et al*., 2009; Nicoline

and Roland, 2013). The bacteria is said to be related with a number of gastro-duodenal pathologies such as chronic gastritis, peptic ulcers, duodenal ulcers, non-ulcer dyspepsia, mucosal associated gastric cancer and gastric mucosa-associated lymphoid tissue lymphoma. The prevalence of these bacteria is indicated to be approximately 50% of adults in developed countries and 90% in developing countries. Presently, *H. pylori* are reported in almost all patients with duodenal ulcers and 80% of patients with gastric ulcers (Anwar *et al*., 2006; Tayfun *et al*., 2008; Nicoline and Roland, 2013).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Materials, Solvents and Reagents

3.1.1 Materials

These include paraffin wax, microtome, embedding box, oven, microscope**,** slides and cover slip**,** bunsen burner**,** camera, silica gel plate (Merck 60 F_{254}) Aluminium coated, Magnesium fillings, capillary tubes, TLC tank, silica gel (60-120 mesh), glass column, beakers, funnel, stirring rod, test tubes, Mueller Hinton agar, petric dishes, cork borer.

3.1.2 Solvents

These include methanol, Chloroform, Hexane, Ethyl acetate, Ethanol (Sigma Aldrich) and Distilled water.

3.1.3 Reagents

These include hydrochloric acid (HCl), Acetic acids, Phloroglucinol, Zinc chloride, Iodine solution, Sudan red, Potassium hydroxide, Sulphuric acid (H_2SO_4), Ferric chloride, formalin, Sodium hypochlorite**,** Glycerol, P-anisaldehyde/ Sulphuric acid solution, Mayers reagent, Wagner reagent, Dragendrof reagent, Sodium hydroxide, Benzene, Acetic anhydride, Molisch reagent, Fehling solutions A and B, Ammonia, Dimethyl sulphoxide, Barium sulphate.

3.2 Collection and Identification of *A. sieberiana*

The plant was collected from Basawa area of Sabon Gari Local Government Area of Kaduna state, Nigeria in May, 2013. It was identified and authenticated by Malam U. S Gallah at the Herbarium Unit of the Department of Biological Sciences, Ahmadu Bello University, Zaria and was assigned a voucher number: 900248.

3.3 Preparation of *A. sieberiana*

Stem bark of *A. sieberiana* was air dried under shade and pulverized using mortar and pestle. The powdered plant material was then transferred into an air tight container for proper storage before use.



Leaves and flowers of *A. sieberiana*



Pods of *A. sieberiana*



Thorns and stem bark of *A. sieberiana*



Aerial part of *A. sieberiana*

Plate I: *Acacia sieberiana* var woodii in its Natural Habitat

3.4 Determination of Botanical Features and Physical Constants of *A. sieberiana* Stem Bark

3.4.1 Microscopical studies of the stem bark

Fresh sample of the stem bark was fixed in 5ml of 40 % formalin, 90 ml of 70 % methanol, and 5ml of glacial acetic acid (FAA) immediately after collection from the field and was allowed to stand for 24 hours. This was followed by dehydration of the stem bark in graded methanol of 30, 50, 70, and 100% for 2 hours each. Clearing was then carried out in methanol (100%) at the initial stage and Chloroform added gradiently from 25-100% and kept overnight.

The sample was then collected from the chloroform and chips of paraffin wax were added gradually until gel was formed. This was left to stand in an open space for some few hours. Impregnation of the sample with more paraffin wax was further carried out after transferring the sample in vial and was left to stand for 24 hours. The vial was placed in an oven at $60^{O}C$ before transferring the content into an embedding box and allowed to solidify.

The solidified embedded sample was then trimmed using razor and placed on the microtome to get the transverse section (TS) of the sample. The section was dewaxed in xylene twice at 5 minutes each and hydrated in graded methanol 100, 95, 70, 50 and 30% each for 2minutes. The sample was then transferred into safranin for 30 minutes before washing with water. It was then transferred into 0.5% HCl in 70% methanol briefly before dipping into fast green for 2 minutes and rinsed with water. Dehydration in graded methanol of 30, 50, 70, 95 and 100% was then carried out for 2 minutes each before

clearing with xylene for another 2 minutes. Distyrene plasticizer xylene (DPX) was used as the mountant (Gattuso *et al*., 2008). The anatomical sections were observed under the microscope and appropriate photographs were taken.

3.4.2 Chemo microscopical studies of the stem bark

The powdered sample of the stem bark of the plant was placed in a clean beaker. Small quantity of sodium hypochlorite was added to the sample in the beaker and placed in hot water bath for 30 minutes. The sample was thoroughly washed with fresh water and used for the chemo microscopical examination. The mountant used was glycerol and the reactions were observed under a light microscope following standard procedures (WHO, 2011).

a. Cellulose cell walls: Iodinated zinc chloride (2 drops) was added to the cleared sample on a slide, and this was allowed to stand for few minutes. Sulphuric acid (1 drop) was added and cover slip was applied before observation.
b. Suberized cell walls: Sudan red (2 drops) was added to the cleared sample on a slide, cover slip was applied and this was gently heated over hot plate for few seconds.
c. Lignified cell walls: Few drops of phloroglucinol was added to the cleared sample and allowed to stand until almost dry. Sulphuric acid (1 drop) was added and cover slip applied.
d. Calcium carbonate and calcium oxalate: To the cleared sample, few drops of acetic acid were added, cover slip applied and this was observed under the microscope. Few drops of dilute hydrochloric acid were added.

e. Tannin: A single drop of ferric chloride solution was added to the cleared sample and cover slip was applied and this was observed under the microscope.

f. Inulin: A drop each of 1-napthol and sulphuric acid was added to the cleared sample and cover slip was applied. This was then observed under the microscope.

g. Hydroxyanthraquinones: A drop of potassium hydroxide was added to the cleared sample and cover slip was applied.

3.4.3 Physical constants determination of the stem bark

The experiment was carried out using standard procedures (WHO, 2011).

3.4.3.1 Moisture content: The powdered stem bark (2g) was weighed into three crucibles and the original weight was recorded before placing the crucibles in an oven at 105^{o}C for 1 hour. This was removed and weighed before placing them back in to the oven for 30 minutes and reweighed. This was repeated until a constant weight was recorded. The percentage loss on drying was then calculated using the formular as;

Moisture Content (%) = $\frac{Weight\ of\ water\ lost}{initial\ weight\ of\ powdered\ plant} \times 100$

3.4.3.2 Ash values: The crucibles containing the powdered material used for determining the moisture content was placed over open flame until ashes were achieved. The crucibles were then transferred into dessicator and allowed to cool before weighing. Hydrochloric acid (25ml) was added to one of the crucible and distilled water (25ml) was added to the other crucible. The crucibles were placed over hot water bath for 5 minutes. The filtrate was collected in a beaker using ashless filter paper and the filter paper was placed in an

oven for some few minutes. The filter paper was then removed and placed in a crucible and allowed to dry until the ashless paper was completely burned. The crucible were then removed and placed in a dessicator before weighing. The percentage total ash, acid insoluble ash and water insoluble ash were calculated respectively using the following;

Total ash value (%): $\frac{Weight\ of\ residual\ ash}{initial\ weight\ of\ powdered\ plant} \times 100$

Water soluble ash (%): $\frac{initial\ weight\ of\ ash - weight\ of\ residual\ ash}{initial\ weight\ of\ powdered\ plant} \times 100$

Acid-Insoluble ash value (%): $\frac{Weight\ of\ residual\ ash - \ weight\ of\ crucible}{initial\ weight\ of\ powdered\ plant} \times 100$

3.4.3.3 Extractive values: The powdered stem bark (5g) was weighed in six different conical flasks. 100 ml of ethanol was added to three of the conical flasks, and 100ml of distilled water was added to the other three flasks. The flasks were then attached to the shaker and allowed for 6 hours. These were removed from the shaker and allowed to stand for another 18 hours. Filtrates from the conical flasks were collected in a beaker using filter paper and 25 ml each of the extracts was collected and transferred to six different evaporating dishes until dryness to constant weight. The different extracts were weighed and the percentage extractive values were calculated using the formular as;

Extractive Values (%) = $\frac{\text{Weight of yield extract}}{\text{initial weight of powdered plant}} \times 100$

3.5 Determination of Phytochemical Constituents of *A. sieberiana* Stem Bark

3.5.1 Extraction of the stem bark

The powdered stem bark (1.2kg) was weighed and transferred into an extraction thimble. The thimble was placed in the Soxhlet apparatus and 2 litres of hexane added and the plant material was extracted at 60^{o}C. This was left to extract for 12 hours before recovering of the solvent. The extract collected was transferred in to a crucible and placed over hot water bath to evaporation and the extract (HE) was obtained. Aqueous Methanol (70%) was added to the Soxhlet apparatus for methanol extraction. This was also left to stand for 12 hours and the aqueous methanol extract was collected in a crucible and placed over hot water bath for evaporation and the extract (ME) obtained.

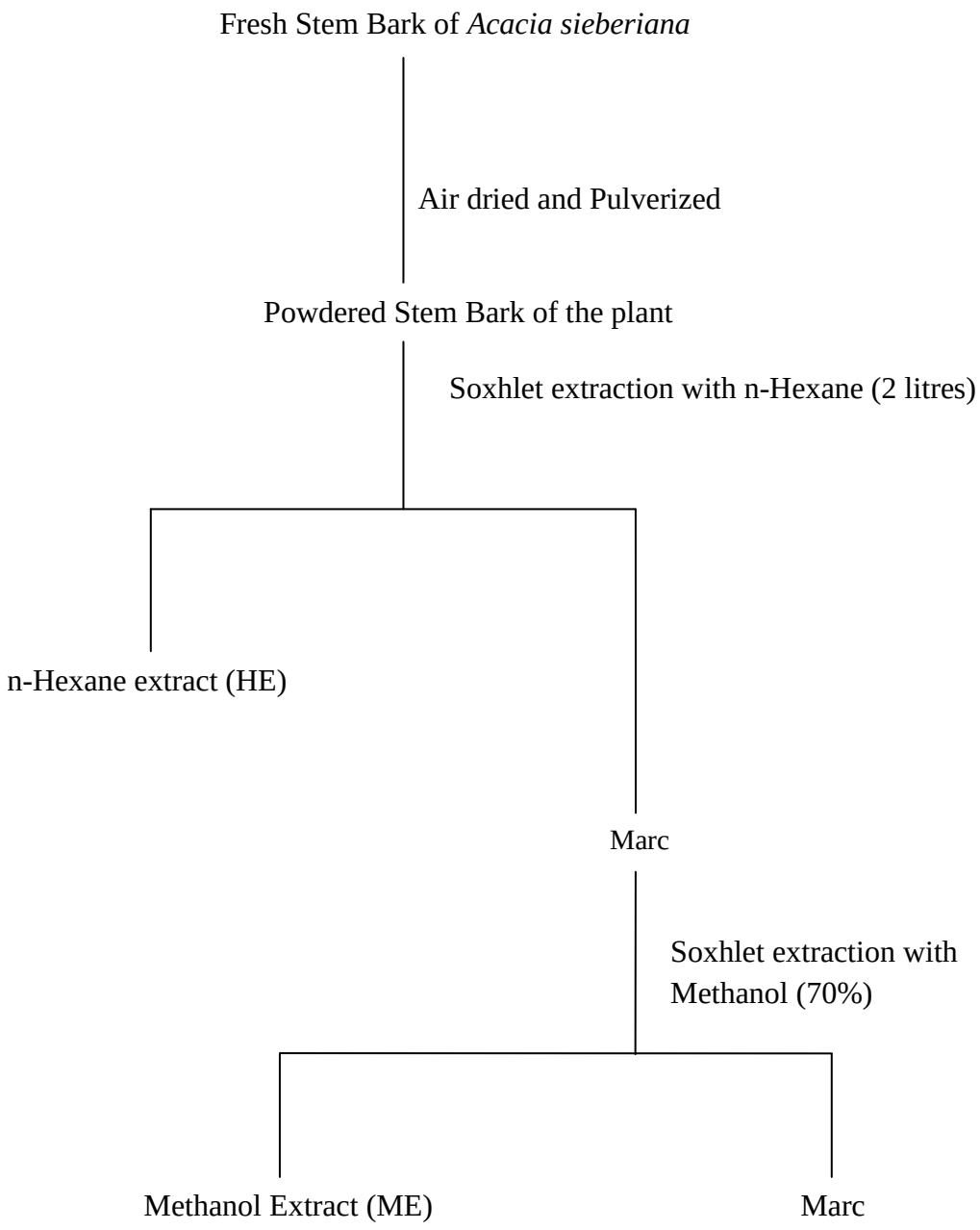


Figure III: Schematic Chart for the Extraction of *A. sieberiana* stem bark.

3.5.2 Preliminary phytochemical studies of methanol and hexane extracts of the stem bark

3.5.2.1 Test for phenolic compounds

a. Ferric chloride Test: The extracts (0.5 g) were dissolved in 10 ml of water each and filtered. Few drops of ferric chloride were added to the filtrate and the colour reaction was observed (Sofowora, 2008).

3.5.2.2 Test for tannins

a. Lead sub-acetate test: Lead sub-acetate solution (3 drops) was added to the extract solution and reaction was observed (Evans, 2009).
b. Gold-beater skin test: The powdered stem bark (2 g) was placed in 10ml of 50 % alcohol and filtered. The gold-beater skin was soaked in 2 % HCl and rinsed with water and transferred to the extract for 5 minutes. The skin was then removed, washed with water and placed in solution of ferrous sulphate and observed (Evans, 2009).

3.5.2.3 Test for saponins, steroids/ triterpenes

a. Frothing test: The extract (2 g) was dissolved in 10 ml of water and shaken vigorously for 30 seconds and allowed to stand for 30 minutes before observing (Sofowora, 2008).
b. Salkowski test: Chloroform (2 ml) and few drops of sulphuric acid were added to 2 g of the methanol and hexane extract and the reaction was observed (Sofowora, 2008).

c. Lieberman-Burchard test: Acetic anhydride (1 ml) was added to 1ml of the extract. Few drops of sulphuric acid were then added to the solution above and the reaction was observed (Sofowora, 2008).

3.5.2.4 Test for flavonoids

a. Shinoda test: The extract (0.5 g) was dissolved in 2 ml of 50 % methanol. Few drops of magnesium fillings and 3 drops of hydrochloric acid were added and the reaction observed (Evans, 2009).
b. Sodium hydroxide test: Few drops of sodium hydroxide were added to 5 ml of the extract and the reaction was observed and recorded (Evans, 2009).

3.5.2.5 Test for cardenolides

a. Keller-killiani test: The extract (2 ml) was dissolved in glacial acetic acid containing ferric chloride and 1 ml of sulphuric acid was added to the solution. The reaction was observed (Sofowora, 2008).

3.5.2.6 Test for alkaloids

a. Mayer's test: Potassium Mercuric Iodide was added to the extract solution and reaction was observed (Evans, 2009).
b. Wagner's test: Iodine in potassium Iodide was added to the extract solution and reaction was observed (Evans, 2009).
c. Dragendrof's test: Solution of potassium Bismuth Iodide was added to the extract solution and reaction was observed (Evans, 2009).

3.5.2.7 Test for cyanogenic glycoside

Picric acid test: Picrate paper was prepared by using strips of filtered paper were soaked in aqueous solution of 0.05 M picric acid previously neutralized with sodium bicarbonate and

filtered. The impregnated paper was left to dry at room temperature. The test was carried out by placing fresh sample of the stem bark in test tube of 1.5 ml distilled water and 6 drops of chloroform. The test tube was stoppered with a cork containing the strips of picrate-impregnated paper hanging down the stopper and incubated at room temperature and observed at the interval of 2 hours, 24 hours and 48 hours (Francisco and Maria, 2000).

3.5.3 Thin layer chromatographic studies (TLC) of hexane extract from *A. sieberiana*

The TLC were obtained by spotting the hexane extract on the TLC plates and developed in Hexane-Ethyl acetate (9:1, 8:2, 7:3 and 6:4). The plates were sprayed with p-anisaldehyde/ sulphuric acid solution as the detecting reagent followed by heating at 110^{o}C for 5-10 minutes. Specific sprays/ reagents namely Dragendroff and Liebermann-Burchard were used as detecting reagents. Photographs of the chromatograms were taken accordingly (Evans, 2009; WHO, 2011).

3.5.4 Column chromatography of hexane extract from *A. sieberiana*

Silica gel (100 g) was carefully packed using wet slurry method to about 30 cm high in a heavy walled glass tube leaving 40cm head space. The extract (2 g) preabsorbed in a small quantity of silica gel was added on to the packed adsorbent using 100 % hexane and allowed to stabilize for 9 hours before elution. Hexane (100 %) was used as the initial eluent to collect fraction 1-5. Hexane-Ethylacetate (95:5) was used to collect fraction 5-19. Fraction 20-34 was collected using Hexane-Ethylacetate (90:10). The solvent systems

Hexane-Ethylacetate (85:15 and 70:30) were used to collect fraction 35-84 and 85-11 respectively. Fraction 111-120 was collected using Hexane-Ethylacetate (60:40) while fraction 121-138 was collected using Hexane-Ethylacetate (50:50). The column was washed with 100% hexane to collect fraction 139-148. Fractions of 20ml each were collected, allowed to concentrate at room temperature and monitored using TLC with solvent systems Hexane-Ethylacetate 7:3 and 8:2 respectively using p-anisaldehyde/sulphuric acids as detecting reagent (WHO, 2011). Fractions that showed similar R_f values from the TLC chromatogram were pooled together and weighed.

3.6 Determination of Margin of Safety of *A. sieberiana* Stem Bark Extracts

3.6.1 Acute toxicity study (LD_{50})

Acute toxicity for the methanol and hexane extracts were carried out using 26 mice of both sexes with 13 mice for each of the extract to determine the median lethal dose (LD_{50}).

The stock solution of both extracts were prepared by dissolving 1g of the extract each in 10ml of distilled water with 3 drops of tween 80 to enhance solubility of the hexane extract. Serial dilution of 100 mg/ml and 1000 mg/ml of the extracts were used in the first phase. In the second phase, serial dilutions from the stock solutions were obtained at 200, 400, 800 and 1600 mg/ml for both extracts.

In the initial phase, mice were divided into 3 groups of 3 mice each and were treated with 10, 100 and 1000 mg/kg of the extracts per body weight intraperitoneally (i.p). They were observed for 24 hours for any signs of toxicity, including death.

In the final phase, 3 mice were divided into 3 groups of one mouse each and treated with 200, 400, 800 and 1600 mg/kg for the methanol and hexane extract. The LD_{50} was calculated from the results of the final phase as square root of the product of the lowest lethal dose and the highest non-lethal dose (Lorke, 1983).

Median Lethal Dose (LD_{50}) = $\sqrt{\text{minimum dose of death} \times \text{maximum dose of survival}}$

3.7 Determination of Antibacterial Studies of *A. sieberiana* Stem Bark against Some Enteric Bacteria

3.7.1 Bacterial isolates

Four bacterial isolates namely *Helicobacter pylori, Salmonella typhi, Shigella dysentriae,* and *Escherichia coli* were obtained during the month of August, 2013 from the Department of Microbiology, Ahmadu Bello University Teaching Hospital, Zaria, Kaduna State, Nigeria. The experiment was carried out using agar well diffusion method at the Microbiology Unit of the National Institute of Leather Science and Technology (NILEST) Zaria, Kaduna State, Nigeria.

3.7.2 Preparation of stock solution

Hexane (HE) and methanol (ME) extracts of the stem bark were weighed (200 mg each) and dissolved in 10ml of 0.5 Moles Dimethylsulfoxide (DMSO) to obtain a concentration of 20 mg/ml. From the stock solution, serial dilution to obtain 10 mg/ml, 5.0 mg/ml, 2.5 mg/ml and 1.25 mg/ml concentrations were made of each extract. Standard antibiotic, Sparfloxacin was used as the positive control drug.

While for the fraction AS1, 500 µg was weighed and dissolved in 10 ml of DMSO to obtain a concentration of 50 µg/ml. From this stock solution, serial dilution was carried out to obtain 25 µg/ml, 12.5 µg/ml, 6.25 µg/ml and 3.125 µg/ml. Standard antibiotics, Ciprofloxacin was used as the positive control drug.

3.7.3 Preparation of media for bacterial growth

Mueller Hinton agar was used as the growth medium for the bacteria strains. The agar (20 g) was prepared by mixing in distilled water and autoclaving for 15 minutes at 121^{oc}. The sterilized media was then transferred into petric dishes and allowed to solidify. The media was then sealed with 0.1 ml of the standard inoculum of the test microbes and spread evenly over the surface of the media with a sterile swab. A diameter of 6 mm well was made at the centre of each medium using a standard cork borer initially sterilized with acetone (Azoro, 2002; Mobasher *et al*., 2005).

3.7.4 Mc-Farland 0.5 Barium sulphate turbidity standard

This was pepared by dissolving 0.5 ml of 0.04 M barium sulphate in 99.5ml, 0.3 N sulphuric acids using normal saline (Azoro, 2002; Mobasher *et al*., 2005).

3.7.5 Determination of zones of inhibition for fraction AS1, hexane and methanol extracts from *A. Sieberiana*

Solution of 0.5ml of the 20 mg/ml of each extract was introduced in to each of the wells made on the medium. While in the media for fraction AS1, 50 µg/ml of the fraction was introduced. All the media were then inoculated at 37^{o}C for 24 hours and the zone of

inhibition were measured using a transparent ruler and the results were recorded in millimeter (Azoro, 2002; Mobasher *et al*., 2005).

3.7.6 Determination of minimum inhibitory concentration of fraction AS1, hexane and methanol extracts from *A. sieberiana*

The agar (10 ml each) was dispensed in to sterile test tubes and the test microbes were inoculated in the medium for 6 hours at 37^{o}C. This was closed in normal saline until the turbidity was observed to march that of Mc-farland standard by visual comparison which was at 1.5×10^{8}cfu/ml. Two fold of serial dilution in the sterile froth were then prepared to obtain concentrations of 20, 10, 5.0, 2.5 and 1.25 mg/ml of the microbe in normal saline for each of the extracts. While a fold was prepared as above to obtain concentrations of 50, 25, 12.5, 6.25 and 3.125 µg/ml of the microbe in normal saline. Each plate were incubated at 37^{o}C for 24 hours after which the froth was observed for growth turbidity. The lowest concentration of the extract which had no turbidity was recorded as the Minimum Inhibition Concentration (Azoro, 2002; Mobasher *et al*., 2005).

3.7.7 Determination of minimum bactericidal concentration of fraction AS1, hexane and methanol extracts from *A. sieberiana*

The content of the Minimum Inhibition Concentration was sub cultured in to a freshly prepared agar medium and incubated at 37^{o}C for 24 hours. The growth of the colony was then observed to determine the Minimum Bactericidal Concentration (Azoro, 2002; Mobasher *et al.,* 2005).

CHAPTER FOUR

4.0 RESULTS

4.1 Determination of Botanical Features and Physical Constants of *A. sieberiana* Stem Bark

4.1.1 Microscopical studies of the stem bark

Microscopical examination of the stem bark of *A. sieberiana* showed the presence of epidermis, phellogen (cork-cambium), phelloderm (secondary cortex), cortex, and phleom (Plate II).

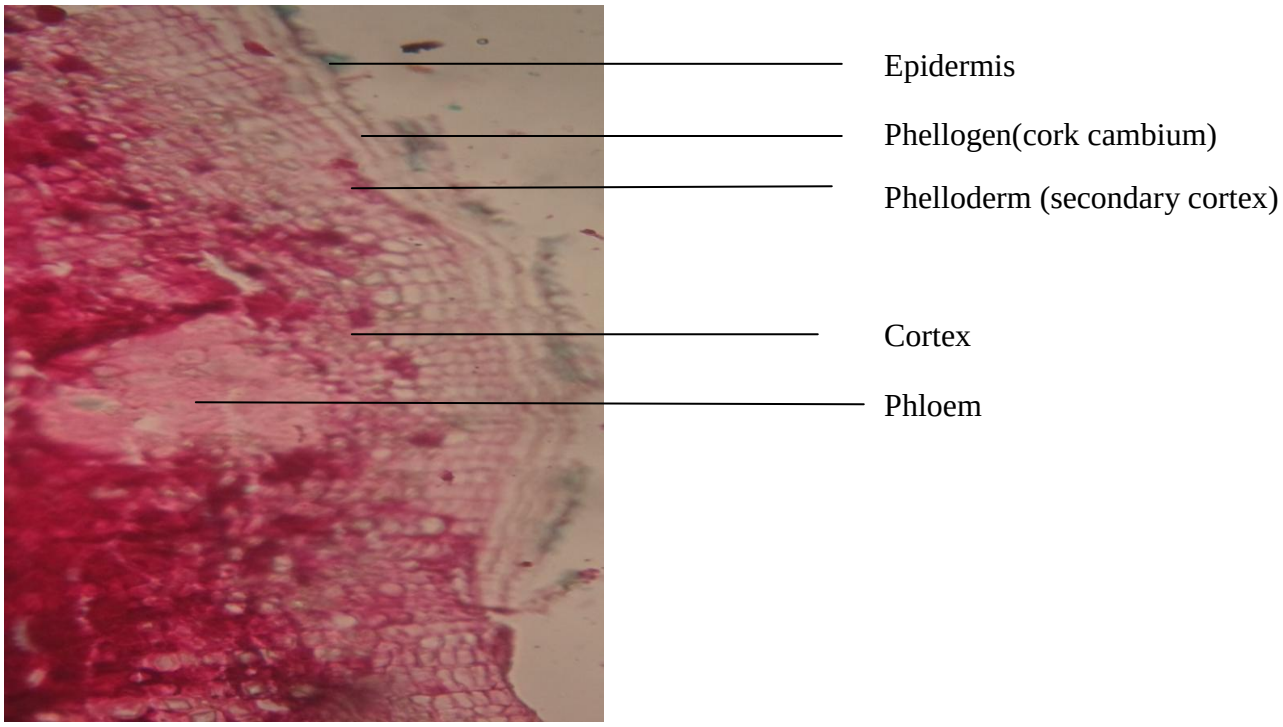


Plate II: Anatomical Features of *A. sieberiana* Stem Bark, X400

4.1.2 Chemo microscopical studies of the stem bark

The different cell wall materials and cell inclusions determined were as follows:

a. Cellulose cell walls: Blue to blue-violet colour was observed which indicated the presence of cellulose in the cell wall.
b. Suberized cell walls: Cherry pink colour was observed which indicated the presence of suberin in the cork cells.
c. Lignified cell walls: Orange red colour was observed which indicated the presence of lignin in xylem elements.
d. Calcium oxalate and calcium carbonate: Prismatic crystals were observed along some fibres which indicated the presence of calcium oxalate which dissolved gradually on addition of hydrochloric acid.
e. Tannin: Greenish-black colour was observed which indicated the presence of tannins on the cell walls.
f. Inulin: No crystals or colour change was observed which indicated the absence of inulin.
g. Hydroanthraquinones: Brown colour was observed which indicated the absence of hydroanthraquinones.

4.1.3 Physical constants determination of the stem bark

The various physical constants determined for the stem bark of *A. sieberiana* were as follows:

4.1.3.1 Moisture content

The mean obtained for the three crucibles was 0.18 g. The percentage yield from the loss on drying was calculated to be 9.0 % as in Table 4.1.

4.1.3.2 Ash values

The percentage total ash, water soluble ash and acid insoluble ash values were 10.5, 6.0 and 4.5 % respectively as in Table 4.1.

4.1.3.3 Extractive values

The mean of the ethanol extractive value was calculated to be 0.08 g while that of the water extractive value 0.06 g. The percentage yield when calculated for the ethanol extract was 1.6 % while the percentage yield of the water extract was 1.2 % as in Table 4.1.

TABLE 4.1: Physical Constants of *A. sieberiana* Stem Bark

Constants	Mean Percentage Yield (%)
Moisture content	9.0 ± SEM
Total ash	10.5 ± SEM
Acid-insoluble ash	4.5 ± SEM
Water-soluble ash	6.0 ± SEM
Alcohol extractives	1.6 ± SEM
Water extractives	1.2 ± SEM

Number of replicates = 3

4.2 Screening of Phytochemical Constituents of *A. sieberiana* Stem Bark

4.2.1 Extraction of the stem bark

Hexane extract (HE) was obtained as 0.42% (5.09g) while the methanol extract (ME) was 5.83% (70g) from 1200g of the powdered stem bark of *A. sieberiana*.

4.2.2 Preliminary phytochemical studies of the stem bark

Preliminary phytochemical studies of *A. sieberiana* stem bark revealed the presence of steroids and triterpenes in the hexane extract.

The methanol extracts gave positive results for the presence of tannins, flavonoids, saponins, alkaloids, steroids and triterpenes.

Cardiac glycosides, anthraquinones and cyanogenic glycoside were observed absent as in Table 4.2.

TABLE 4.2: Preliminary Phytochemical Studies of *A. Sieberiana* Stem Bark

Constituents/Test	Observation	Inference	
		HE	ME
Test for Phenolic nucleus			
Ferric chloride	Blue-black colour	Absent	Present
Test for Tannins			
Lead sub-acetate	Brown precipitate	Absent	Present
Gold beater skin	Black colour	No data	Present
Test for Saponins, Steroids and Triterpenes			
Frothing test	Persistent honey-comb	Absent	Present
Liebermann-Burchard	froth	Present	Present
Salkowski test	Reddish-brown colour	Present	Present
	Reddish-brown at interphase		
Test for Flavonoids			
Shinoda	Orange/ Pink colour	Absent	Present
Sodium Hydroxide	Yellow colour	Absent	Present
Test for Cardenolides			
Keller-kiliani	Purple ring at interphase	Absent	Present
Kedde	Dirty brown precipitate	Absent	Absent
Test for Alkaloids			
Dragendroff	Red rose precipitate	Absent	Present
Wagners	Creamy precipitate	Absent	Present
Mayers	Brown creamy precipitate	Absent	Present

Keywords: Hexane extracts (HE), Methanol extract (ME).

4.2.3 Thin-layer chromatographic (TLC) studies of hexane extract from *A. sieberiana*

The TLC of the *A. sieberiana* stem bark hexane extract was observed to give separation using Hexane: Ethyl acetate (7:3) as shown in plates III and IV.

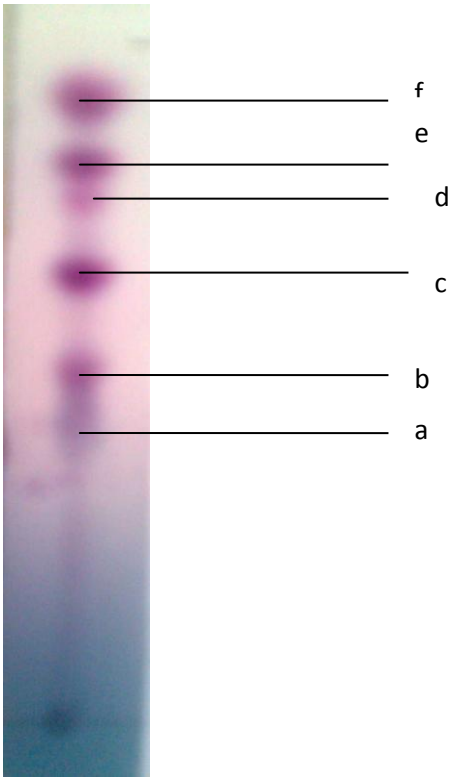


Plate III: TLC Chromatogram of Hexane extract from *A. sieberiana* stem bark developed in Hexane: Ethyl acetate (7:3), sprayed with P-anisaldehyde/H_2SO_4 solution, heated at 110^oC for 5-10 minutes and observed under day light with R_f Values of 0.54 (a), 0.62 (b), 0.66 (c), 0.72 (d), 0.74 (e), and 0.86 (f) respectively.

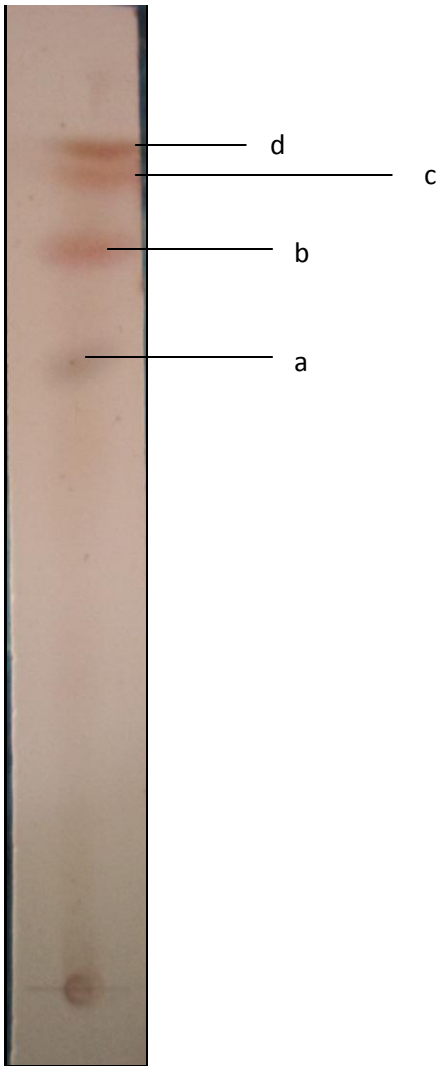


Plate IV: TLC Chromatogram of Hexane Extracts of *A. sieberiana* stem bark developed in Hexane-Ethyl acetate (7:3), sprayed with Liebermann-burchard, heated at 110^{o}C for 5-10minutes and observed under daylight with R_f values of 0.68(a), 0.74 (b), 0.86 (c), 0.89 (d).

TABLE 4.3: Thin Layer Chromatogram of Hexane Extract from *A. sieberiana* using Specific Spray Reagent

Constituents	Observation	Inference
General test for steroids and triterpenes Liebermann-Burchard Reagent	Reddish and violet colour	Four spots indicating presence of steroids and triterpenes
Specific test for alkaloids Dragendroff reagent	No colour change	Alkaloids absent
Specific test for cardiac glycoside 2,4-Dinitrobenzoic acid + KOH	No colour change	Cardiac glycosides absent

Keynotes: Potassium hydroxide (KOH)

4.2.4 Column chromatography of hexane extract from *A. sieberiana*

Fractionations of the hexane extract stem bark of *A. sieberiana* by column chromatography produced 148 fractions and with the aid of TLC using the solvent systems Hexane-Ethylacetate (7:3 and 8:2), similar fractions were identified and pooled together as one fraction and assigned code as presented in Table 4.4. The TLC plate for fraction AS1 is presented in plate 4.5.

TABLE 4.4: Fractions Collected from Column Chromatography of Hexane Extract from *A. sieberiana*

Fractions	Solvent System	Codes	Yield weight(mg)
5-16	95:5 (Hexane-Ethyl acetate)	AS1	60
17-19	95:5 (Hexane-Ethyl acetate)	AS2	54
20-26	90:10 (Hexane-Ethyl acetate)	AS3	67
27-34	90:10 (Hexane-Ethyl acetate)	AS4	32
35-84	85:15 (Hexane-Ethyl acetate)	AS5	89
85-110	70:30 (Hexane-Ethyl acetate)	AS6	56
111-120	60:40 (Hexane-Ethyl acetate)	AS7	20
121-138	50:50 (Hexane-Ethyl acetate)	AS8	59
139-148	100% (Hexane)	AS9	84



Plate V: TLC Chromatogram of Fraction 5-16 coded AS1 from Hexane Extracts of *A. sieberiana* stem bark developed in Hexane-Ethyl acetate (7:3), sprayed with anisaldehyde/H_2SO_4 solution, heated at 110^oC for 5-10minutes and observed under day light with R_f Values of 0.87.

4.3 Margin of Safety of *A. sieberiana* Stem Bark Extracts

4.3.1 Acute toxicity study (LD_{50}) of the stem bark extracts

These revealed that the hexane extract of *A. sieberiana* stem bark at 10, 100, 200, 400, and 800 mg/ml exerted no signs of distress or death in the animals after 24 hour monitoring. But at 1000 and 1600 mg/ml, stretching of limbs in the animals within 20 minutes of extract administration as well as death were recorded (Table 4.7).

Death was recorded in all the groups of animals that were administered methanol extract except at 10mg/ml as presented in Table 4.6.

The LD_{50} values of the hexane and methanol extracts were calculated as 894.42 and 31.62 respectively.

TABLE 4.5: Median Lethal Dosage (LD_{50}) for Hexane and Methanol Extracts of *A. sieberiana* against Mice

Phase	Dose of extract (mg/kg)	Hexane extract	Methanol extract
I	10	0/3	0/3
	100	0/3	2/3
	1000	2/3	3/3
II	200	0/1	1/1
	400	0/1	1/1
	800	0/1	1/1
	1600	1/1	1/1

Keywords: 0/1(no death in group with one mouse), 1/1(all death in group with one mouse), 0/3(no death in group with three mice), 2/3(two death in group with three mice), 3/3(all died in group with three mice).

4.4 Determination of Antibacterial Studies of *A. sieberiana* Stem Bark Extracts against Some Enteric Bacteria

Sensitivity test showed that all the four bacterial strains were susceptible to the methanol and hexane extracts. They were also susceptible to compound AS1 except *S. typhi* which showed resistance against the compound.

4.4.1 Zones of inhibition of fraction AS1, hexane and methanol extracts from *A. sieberiana* against some enteric bacteria

Methanol and hexane extracts of *A. sieberiana* stem bark at a concentration of 20mg/ml each showed zones of inhibition between 16 and 27 mm for all the four bacterial strains. Both extracts were observed to have higher zones of inhibition against *H. pylori* at 27 mm for methanol extract and 22 mm for the hexane extract. This was followed by *E. coli* at 23 mm for methanol extract and 19 mm for the hexane extract. Zones of inhibition for *S. dysenteriae* and *S. typhi* were observed at 20 and 21 mm respectively for the methanol extract and at 16 mm for hexane extract. Sparfloxacin showed a wider zone of inhibition between 35 and 39 mm for all the four bacterial strains which were more than both the methanol and hexane extracts as in Table 4.6.

Fraction AS1at 50 µg/ml showed higher zones of inhibition at 30 and 27 mm against *S. dysenteriae* and *E. coli* respectively. The fraction exhibited no effects on *S. typhi* but had zones of inhibition at 26 mm against *H. pylori*. This is in contrast to the results obtained from ciprofloxacin which showed zones of inhibition at 42 mm against *S. typhi* and had no effects on *H. pylori* as in Table 4.6.

TABLE 4.6: Zones of Inhibition of Hexane and Methanol Extracts and Fraction AS1 from *A. sieberiana* Stem Bark against the Four Bacteria Strains (mm)

Test organism	Zones of Inhibition (mm)				
	Methanol Extract (20 mg/ml)	Hexane Extract (20 mg/ml)	Fraction AS1 (50 µg/ml)	Ciprofloxacin (50 µg/ml)	Sparfloxacin (20 mg/ml)
Helicobacter pylori	27	22	26	0	37
Escherichia coli	23	19	27	39	35
Shigella dysenteriae	20	16	30	45	39
Salmonella typhi	21	16	0	42	37

4.4.2 Minimum inhibitory concentration of fraction as1, hexane and methanol extract from *A. sieberiana* against some enteric bacteria

The Minimum Inhibition Concentration (MIC) for *H. pylori* was observed at 2.5 and 5.0 mg/ml for the methanol and hexane extracts respectively while that of *E. coli, S. dysenteriae* and *S. typhi* were observed at 5.0 and 10 mg/ml respectively for the methanol and hexane extracts respectively as in Table 4.7.

The MIC for *E. coli* and *H. pylori* was recorded 12.5 µg/ml for fraction AS1, while that of *S. dysenteriae* was observed at 6.25 µg/ml as in Table 4.7.

TABLE 4.7: Minimum Inhibitory Concentration of Fraction AS1, Methanol/Hexane Extracts from *A. sieberiana* Stem Bark against the Four Bacteria Strains (mg/ml)

Test organisms	Minimum Inhibitory Concentration (MIC)														
	Methanol extract (mg/ml)					Hexane extract 9mg/ml)					Fraction AS1 (µg/ml)				
	20	10	5.0	2.5	1.25	20	10	5.0	2.5	1.25	50	25	12.5	6.25	3.125
Helicobacter pylori	__	__	__	__*	+	__	__	__*	+	++	__	__	__*	+	++
Escherichia coli	__	__	__*	+	++	__	__*	+	++	+++	__	__	__*	+	++
Shigella dysenteriae	__	__	__*	+	++	__	__*	+	++	+++	__	__	__	__*	+
Salmonella typhi	__	__	__*	+	++	__	__*	+	++	+++	No data				

Key words: No Turbidity (__), MIC(__*), Light Turbidity (+), Moderate Turbidity (++).

4.4.4 Minimum bactericidal concentration of fraction as1, hexane and methanol extract from *A. sieberiana* against some enteric bacteria

Results of this study shows that the methanol and hexane extract had MBC for *S. dysenteriae* and *S. typhi* at 20 mg/ml. The MBC for *E. coli* was observed at 10 and 20 mg/ml for methanol and hexane extracts respectively. While the methanol and hexane extracts showed MBC at 5.0 and 10 mg/ml respectively against *H. pylori* as in Table 4.8.

The MBC for fraction AS1 as shown in the table above for all the four bacterial strains showed that the fraction had MBC effects against *H. pylori* and *E. coli* at 25 and at 12.5 µg/ml for *S. dysenteriae* as in Table 4.8.

TABLE 4.8: Minimum Bactericidal Concentration of Fraction AS1, Methanol/Hexane Extracts from *A. sieberiana* Stem Bark against the Four Bacteria Strains (mg/ml)

Test organisms	Minimum Bactericidal Concentration (MIC)														
	Methanol extract (mg/ml)					Hexane extract (mg/ml)					Fraction AS1 (µg/ml)				
	20	10	5.0	2.5	1.25	20	10	5.0	2.5	1.25	50	25	12.5	6.25	3.125
Helicobacter pylori	__	__	__*	+	++	__	__*	+	+	++	__	__*	+	+ +	++
Escherichia coli	__	__*	+	++	++	__*	+	++	++	+++	__	__*	+	++	++
Shigella dysenteriae	__*	+	++	+++	++	__*	+	++	++	+++	__	__	__*	+	++
Salmonella typhi	__*	+	++	+++	++	__*	+	++	++	+++	No data				

Key words: No Turbidity (__), MBC(__*), Light Turbidity (+), Moderate Turbidity (++).

CHAPTER FIVE

5.0 DISCUSSION

Microscopically, the stem bark of the plant was observed to possess a layer of epidermis as in line with all dicot stem bark. The presence of phellogen and phelloderm in the external cortical region is in line with the reports on the stem organisation of Fabaceae family (Duarte and Wolf, 2005; Stella and Duarte, 2011). Chemomicroscopically, the stem bark was observed to contain cellulose cell wall along with lignified and suberized cell walls. Calcium crystals along with tannins were also observed. This observation was in line with some studies of the plant related genus that have been reported to possess calcium oxalate and tannins as in *A. leucophloea* and *A. podalyrrifolia* (Duarte and Wolf, 2005; Gupta *et al.,* 2010; Gupta *et al.,* 2012). The percentage loss on drying recorded for *A. sieberiana* stem bark at 9.0% is slightly higher than that of *A. pennata* which was reported at 7.0%, and also higher than that of *A. leucophloea* which were reported at 3.4% (Gupta *et al*., 2010; Reena *et al*., 2013). This value can assist in the differentiation of the plant from its closely related species when dried under similar condition.

Ethanol can be said to be a better solvent for extraction of the plant stem bark as the percentage extractive value was recorded at 1.6% higher than water extractive value at 1.2%. This result was similar to the percentage extractive values of *A. leucophloea, A. pennata* and *A. nilotica* where alcohol was reported to give a higher percentage extractive yield than aqueous and chloroform (Gupta *et al*., 2010; Reena *et al*., 2013). The percentage ash value at 10.5% was observed to be relatively higher than that of *A. pennata,* and *A. leucophloea* (Gupta *et al*., 2010; Singh *et al.,* 2013).

The preliminary phytochemical studies of the methanol extract showed the presence of flavonoids which is in line with previous studies on Fabaceae where it was reported that legumes are particularly rich in flavonoids when compared to other plant families (Hengnauer and Renpe, 1993). The presence of alkaloid, tannins, steroids and triterpenes in the stem bark was also similar to previous reports on the genus *Acacia* (Jesus *et al*., 2007; Shittu *et al*., 2010). Anthraquinones, cyanogenic glycosides and cardiac glycosides was observed absent in the present study but were reported present in the stem barks of closely related species (Kubmarawa *et al*., 2007; Chaudhary *et al.,* 2009; Anjaneyulu *et al.,* 2010; Okpanachi *et al*., 2012; Suman *et al.,* 2011; Deshpande, 2013). The fraction AS1 is a colourless and oily substance that was observed to be highly soluble in hexane. It was based on these characteristic that it was suggested to be a hydrocarbon (Ali *et al.*,2011; Ali and Jabbar, 2011; Milena *et al*., 2011).

Hydrocarbons are biologically stable and common in plant species. They are biosynthesized from decarboxylation of long chain fatty acids (Lanzon *et al.,* 1994). Gas Chromatography-Mass Spectroscopy and Gas Liquid Chromatography of some plants namely *Cestrum nocturnum, Amomum subulatum, Leea indica, Inula viscosa, Anthocleista species, Capsicum annum* and *Actinolema macrolema* were reported to contain hydrocarbons in their various parts (Nicolino *et al*., 2002; Mubo *et al*., 2007; Sharif *et al*., 2009; Aneta *et al.,* 2011; Supriya *et al*., 2012). Hydrocarbons such as heptadecane, pentadecane and tetracosane have been reported in several species of the Fabaceae family including *Glycrrhiza glabra, Samanea saman, Cassia* and *Senna* species (Bisby et al., 1994; Vijayalakshmi and Abhilasha, 2013). Recent report on GC-MS of the leaves of *A.*

*nilotic*a of the same genus with *A. sieberiana* indicated the presence several hydrocarbons (Sheema *et al*., 2014).

According to Matsumura (1975) and Corbett *et al*., (1984) toxicity category, the methanol extract with LD_{50} 31.62mg/kg is highly toxic while the hexane extract with LD_{50} 894.43mg/kg is slightly toxic to the experimental mice.

Methanol and hexane extracts of *A. sieberiana* stem bark was observed to possess antibacterial activity against the four bacterial isolates that were used during the present study. The wider zones of inhibition (35-39 mm) exhibited by sparfloxacin indicated that it has higher antibacterial activity as compared to the plant extracts. Although, both extracts showed appreciable zones of inhibition (16-27 mm), the methanol extract exhibited a wider dimension (21-27mm) as compared to the hexane extract (16-22 mm). The results from the MIC and MBC also revealed that the methanol extract (2.5 and 5.0 mg/ml) had double the inhibitory strength of the hexane extract (10 and 5.0 mg/ml). Conclusively, from the overall studies, methanol extract was observed to have more antibacterial activity at various concentrations than the hexane extract. This can be supported by the claims that methanol is a better solvent for the extraction and isolation of phytochemicals with antimicrobial activities (Bhawna and Bharti, 2010; Poovendron *et al*., 2011). Medicinal efficacy of any plant may not be due to the presence of only one main constituent, but due to the combined action of other chemical constituents such as tannins, flavonoids, terpenoids, saponins, alkaloids and other secondary metabolites (Poovendron *et al.*, 2011). The preliminary phytochemical studies of the methanol extract of the stem bark of *A.*

sieberiana revealed the presence of all the secondary metabolites mentioned above with the presence of steroids and triterpenes in the hexane extract. Therefore, the efficacy of the methanol extracts over the hexane extract can be attributed to the presence of saponins along with other secondary metabolites. Phenolic compounds that were observed in the methanol extract may also be responsible for their antimicrobial activities as previous reports had shown that phenolic compounds exhibit antimicrobial activities against disease causing microorganisms (Ismail and Abdulsamad, 2010; Nyananyo and Akada, 2011; Hussein, 2012). Plants containing tannins have also been reported to be utilise traditionally in the treatment of diarrhea and dysentry while saponins were also reported to have the natural tendency to ward off microbes (Adaramola *et al*., 2012). From these reports, the methanol extract of *A. sieberiana* stem bark can be said to be more active against the four enteric bacteria used in the present study possibly due to the presence of saponins, tannins and flavonoids.

A wider dimension for the zones of inhibition (39 to 45 mm) was recorded for ciprofloxacin which was higher than that of the fraction AS1 (26 to 30 mm). From this observation, ciprofloxacin can be said to exhibit a higher antibacterial activity against *E. coli, S. dysenteriae* and *S. typhi* than fraction AS1. Interestingly, ciprofloxacin exerted no effects on *H. pylori* while the fraction AS1 exhibited a dimension at 26 mm against the bacterial strain, although it exerted no effect on *S. typhi*. Hydrocarbons such as 34-octahexacantanol, heptatriacont-8-ol, tricosan-17-ene-5-ol, hexaeicosn-16-ol and nonacosan-23-ene-3-ol from *Psidium guajava* were reported to show zones of inhibition (6.8 to 11 mm) against *Shigella species, S. aureus, E.coli* and *Salmonella typhimurium*

(Mehta *et al.,* 2012). Subsequently, the MIC and MBC of the fraction AS1 against *E. coli* and *H. pylori* was observed at 12.5 and 2 5µg/ml respectively. While that of *S. dysenteriae* was observed at 6.25 µg/ml for MIC and at 12.5 µg/ml for MBC. Therefore, fraction AS1 can be said to have exhibited antibacterial activity against *S. dysenteriae* as compared to *E. coli* and *H. pylori*. This finding can be supported from the report that leaves of *Saraca indica* and *Enterolobium cyclocarpum* which have been reported to contain certain percentage of hydrocarbons exhibited MIC at 18.6 and 16.9µg/ml against *S. aureus* and *P. aeruginosa* respectively (Hawas *et al.,* 2012). The antibacterial activity of the fraction AS1can be said to be attributed to previous reports which suggested that antimicrobial activities of hydrocarbons is determined by the ability of the compound to penetrate the cell walls of bacteria as they possess hydrophobic molecules (Lay-Jing *et al*., 2012).

CHAPTER SIX

6.0 SUMMARY, CONCLUSION AND RECOMMENDATION

6.1 Summary

The microscopical study of the stem bark was observed to posses epidermis, phellogen, phelloderm and cortex similar to the plants belonging to the family Fabaceae. While the chemo microscopy studies revealed the presence of lignified and suberized cell wall along with tannins and calcium oxalate.

The preliminary phytochemical studies as well as the TLC chromatogram of hexane extract showed the presence steroids and triterpenes. The methanol extract was observed to contain tannins, flavonoids and alkaloid with the absence of cyanogenic glycosides and anthraquinones in the plant stem bark. A fraction (5-16) coded AS1 collected from column chromatography of the hexane extract was suggested to be a hydrocarbon based on its characteristic nature.

The methanol extract with LD_{50} value of 31.62 was highly toxic while the hexane extract with LD_{50} value of 894.42 was slightly toxic from the acute toxicity study.

The hexane and methanol extracts showed zones of inhibition (16 to 27 mm) against the four bacterial strains used with an MIC at 5.0 and 10 mg/ml against *E. coli*, *S. dysenteriae* and *S. typhi* for hexane and methanol extracts respectively while the MIC for *H. pylori* was at 2.5 and 5.0 mg/ml respectively. A similar result was observed for MBC with the methanol extract exhibiting high antibacterial activity at a lower dose than the hexane

extract. The proposed hydrocarbon was also observed to exhibit antibacterial activity against all the four bacterial strains used except *S. typhi*.

6.2 Conclusion

The phytochemical studies revealed that hexane stem bark extract of *A. sieberiana* may contain hydrocarbons among other secondary metabolites which can be said to be responsible for its antibacterial activity against *E. coli, S. dysenteriae* and *H. pylori*.

This study has scientifically justified the traditional uses of *A. sieberiana* stem bark as an antibacterial agent associated with stomach aches and ulcers related to *E. coli, S. dysenteriae, S. typhi* and *H. pylori*.

6.3 Recommendation

Although *A. sieberiana* stem bark is found to have antibacterial activity against some enteric bacteria associated with stomach ache and ulcer, precaution on the use of stem bark extracts as a traditional remedy should be taken as it was found to be highly toxic on the animals tested.

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APPENDICES

APPENDIX A (I-VIII): Composition of Some Reagents/ Sprays

I: p-anisaldehyde/H_2SO_4 (0.5ml of Anisaldehyde + 10ml glacial acetic acid + 85ml methanol + 4.5ml H_2SO_4)

II: Ferric chloride (5g of $FeCl_3$ + 100ml of H_2O)

III: Wagners Reagent (3g of potassium iodide + 2g of iodine + 100ml of H_2O)

IV: Mayers Reagent (1.36g of mercuric chloride dissolved in 60ml of H_2O + 5g of potassium iodide dissolved in 20ml of H_2O)

V: Dragendroff Reagent (50g of tartaric acid + 4.25g of bismuth oxynitrate + 100ml potassium iodide + 200ml of H_2O)

VI: Chloral hydrate (250g of chloral hydrate + 100ml of H_2O)

VII: Liebermann-Burchard (5ml of chloroform + 1ml of acetic anhydride + 1ml of H_2SO_4)

VIII: Phloroglucinol (1g of phloroglucinol dissolved in 100ml of methanol).