PHARMACOGNOSTIC AND ANTIBACTERIAL STUDIES OF THE LEAF EXTRACTS OF *SWARTZIA MADAGASCARIENSIS* DESV (FABACEAE)

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BY

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ZARIA, NIGERIA

NOVEMBER, 2015

DECLARATION

I declare that the work in this dissertation entitled "Pharmacognostic and Antibacterial Studies of the Leaf Extracts of *Swartzia madagascariensis* Desv (Fabaceae)" has been carried out by me in the Department of Pharmacognosy and Drug Development, Ahmadu Bello University, Zaria. The information derived from the literature has been duly acknowledged in the text and list of references provided. No part of this thesis was previously presented for another degree or diploma at this or any other institution.

Sani Muhammad HASSAN

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Name of Student	**Signature**	**Date**

CERTIFICATION

This dissertation entitled "PHARMACOGNOSTIC AND ANTIBACTERIAL STUDIES OF THE LEAF EXTRACTS OF *SWARTZIA MADAGASCARIENSIS* DESV (FABACEAE)" by Sani Muhammad, HASSAN meets the regulations governing the award of the degree of Master of Science in Pharmacognosy of the Ahmadu Bello University, Zaria, and is approved for its contribution to knowledge and literary presentation.

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DEDICATION

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ABSTRACT

This study evaluated the pharmacognostic standards and antibacterial properties of the leaf extracts of *Swartzia madagascariensis* Desv (Fabaceae*)*. This plant is widely distributed in Northern part of Nigeria; it has been exploited for both its medicinal and economic importance. It is used in the treatment of infections such as cutaneous wounds. Microscopic, chemo-microscopic, quantitative microscopic studies, qualitative and thin layer chromatographic studies, toxicity studies and antibacterial evaluation were carried out using standard methods. Microscopical studies revealed the presence of straight - walled anticlinal walls, polygonal epidermal cells, trichomes, anomocytic stomata on abaxial surface. The transverse section of the leaf through the lamina and midrib tissue was examined and revealed different anatomical features include: epidermal layers, palisade and spongy mesophyll cells, vascular bundles consisting of xylem and phloem vessels. Microscopy of the powder revealed fragments of starch grain, calcium oxalate crystals, lignified collenchyma, unicellular covering trichome and lignified fibre as a characteristic of the plant. Chemomicroscopical studies of the powdered leaves were found to have cellulose, lignin, calcium oxalate, tannins, and starch. The quantitative microscopic results were observed to be: stomatal number (201), stomatal index (9.5), palisade ratio (7.3), vein islets (29.5) and vein termination numbers (15.0). The physicochemical constants evaluated were moisture contents (6.7%), total ash value (6.0%), water soluble ash (4.5%), acid insoluble ash (1.0%), ethanol extractive value (15%) and water extractive value (24%). Phytochemical screening on the plant extracts revealed the presence of alkaloids, cardiac glycosides, saponins, flavonoids, tannins and anthracenes. Thin layer chromatographic analysis of the ethyl acetate extract (most active extract) confirms the presence of some chemical constituents in the leaf extracts namely flavonoids, steroids and triterpenes. Acute toxicity study

found the leaf extract to have an LD_{50} of 288.5mg/kg body weight (*i.p*) in mice. The antibacterial studies carried out on the leaf extracts showed zones of inhibition ranging from 17-18 mm, 24-27 mm and 21-22 mm for hexane, ethyl acetate and ethanol extracts respectively. These values were less than that of ciprofloxacin (32 – 37 mm) that was used as the standard antibacterial drug. Minimum Inhibitory Concentration (MIC) of the three extracts was determined at 0.5 scale Mc-farland's turbidity standard. The extracts at the concentrations of 20 mg/ml (hexane), 10 mg/ml (ethyl acetate) and 10 mg/ml (ethanol). They also gave Minimum Bactericidal Concentrations at 40 mg/ml each of hexane and ethanol extracts and 20 mg/ml for the ethyl acetate extract. It was observed that gram positive bacteria (*Staph. aureus* and *Strep. pyogenes*) were more sensitive to the extracts than the gram negative bacteria (*E. coli*). Results of the present studies had shown that *S. madagascariensis* is a promising medicinal plant with antibacterial property.

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ACRONYMS AND ABBREVIATIONS

ABUTH:	Ahmadu Bello University Teaching Hospital
B. P:	British Pharmacopoeia
Cm:	Centimeter
DMSO:	Dimethyl Sulphur Oxide
EUCAST:	European Union Committee for Antimicrobial Sensitivity Testing
Fig:	Figure
FAA:	Formalin Acetic acid Alcohol
g:	Gram
G.A.A:	Glacial Acetic Acid
HCL:	Hydrochloric acid
$H_2SO_{4:}$	Sulphuric acid
ILDIS:	International Legume Database and Information Services
LD_{50}:	Median Lethal Dose
ml:	Millimeter
MBC:	Minimum Bactericidal Concentration
MIC:	Minimum Inhibitory Concentration
NARICT:	National Research Institute for Chemical Technology

NILEST:	National Institute for Leather Science and Technology
TLC:	Thin Layer Chromatography
UV:	Ultraviolet Light
USEPA:	United State Environmental Protection Agency
Vol.:	Volume
w/w:	Weight per Weight
W.H.O:	World Health Organization
Z.I:	Zones of Inhibition
+:	Positive
%:	Percentage
- :	Negative

CHAPTER ONE

1.0 INTRODUCTION

1.1 Traditional Medicine

Traditional medicine is the sum total of the knowledge, skills and practices based on the theories, beliefs and experiences indigenous to different cultures, whether explicable or not, used in the maintenance of health, as well as in the prevention, diagnosis, improvement or treatment of physical and mental illnesses. This has been used for thousands of years with great contributions made by practitioners to human health, particularly as primary health care providers at the community level (WHO, 2000).

With these descriptions, various forms of medicines and therapies such as herbal medicine, massage, homeopathy, mud bath, music therapy, wax bath, reflexology, dance therapy, hydrotherapy, mind and spirit therapies, self-exercise therapies, radiation and vibration, osteopathy, chiropractice, aromatherapy, preventive medicine, radiant heat therapy, therapeutic fasting and dieting, spinal manipulation, psychotherapy, etc. are elements of traditional medicine. It does show that a large country of the size of Nigeria, with diverse cultures and traditions, should be rich in traditional medicine and should have eminent and respected traditional healers to take care of the teeming population (Adeshina, 2008).

It is important to stress the relevance of traditional medicine to the majority of Nigerian and the world. Most Nigerian, especially those living in rural communities don't have access to orthodox medicine and it is estimated that about 80% percent of the populace still prefer to solve their health problems consulting traditional healers. Where access to orthodox medicine exists, the rising cost of imported medications and other commodities used for medicine has posed big problems. Beside, many rural communities have great faith in

traditional medicine, particularly in the explicable aspect as they believe that it is the wisdom of their fore-fathers which also recognize their socio-cultural and religious background which orthodox medicine seems to neglect. Traditional medicine is the oldest, most tried and tested form of medicine and is as old as man himself (WHO, 2003).

Traditional medicine covers a wide variety of therapies and practices which vary from country to country and region to region. In some countries, it is referred to as "alternative" or "complementary" medicine (CAM) (WHO, 2000).

Plants, especially used in traditional medicine can provide biologically active molecules and lead structures for the development of modified derivatives with enhanced activity and /or reduced toxicity. The small fractions of flowering plants that have so far been investigated have yielded about 120 therapeutic agents of known structure from about 90 species of plants. Some of the useful plant drugs include: vinblastine, vincristine, taxol, podophyllotoxin, camptothecin, digitoxigenin, gitoxigenin, digoxigenin, tubocurarine, morphine, codeine, aspirin, atropine, pilocarpine, capsaicine, allicin, curcumin, artemesinin and ephedrine. In some cases, the crude extract of medicinal plants may be used as medicaments. On the other hand, the isolation and identification of the active principles and elucidation of the mechanism of action of a drug is of paramount importance. Hence, work in both mixture of traditional medicine and single active compounds are very important (Kumar *et al.,* 1997).

Advances in organic chemistry added a new dimension to the description and quality control of these drugs, and the discipline has since expanded to include discovery of novel chemical therapeutic agents from the natural world (Betz *et al.,* 2011).

The standardization of a crude drug is an integral part of establishing its correct identity. Before any crude drug is included in herbal pharmacopoeia, Pharmacognostic as well as other standard parameters must be established (Bako *et al.,* 2002). Therapeutic efficacies of medicinal plants depend upon the quality and quantity of chemical constituents. It has been established that chemical constituents of a plant species vary with regard to climate and seasons (Bapodara *et al.,* 2011).

The accurate identification of an herb is a pre-requisite for quality control and conservation procedures. The classification of plants used in the traditional system is based on some quality or property of the plant, while in other instances; the plants are classified on the basis of their therapeutic value. It may also take into account their odour, texture of leaves, reaction when touched and the sensation felt on contact (Bako *et al.,* 2002).

1.2 Standardization of Crude Drugs

Standardization of herbal formulations is essential in order to assess the quality of drugs, based on the concentration of their active principles, physical, chemical, phyto-chemical, standardization, and *In-vitro*, *In-vivo* parameters. The quality assessment of herbal formulations is of paramount importance in order to justify their acceptability in modern system of medicine (Satheesh *et al.,* 2011). One of the major problems faced by the herbal industry is the unavailability of rigid quality control profiles for herbal materials and their formulations (WHO, 1998).

The WHO has developed a series of technical guidelines and documents relating to the safety and quality assurance of medicinal plants and herbal materials. WHO had published the ‘Quality Control Methods for Medicinal Plant Materials, a collection of recommended test procedures for assessing the identity, purity, and content of medicinal plant materials to

assist national laboratories engaged in drug quality control (WHO, 1998). In 2003, WHO published the 'Guidelines on good agricultural and collection practices (GACP) for medicinal plants' and in a new guideline 'WHO guidelines for assessing quality of herbal medicines with reference to contaminants and residues' was formulated (WHO, 2003).

Quality assurance of botanicals and herbal preparations is a prerequisite of clinical trials. The certification for this is based on parameters such as identification, water content and chemical assay of active ingredients, inorganic impurities (toxic metals), microbial limits, mycotoxins, pesticides and others. For herbal preparations, in addition to these tests, disintegration, dissolution, hardness/friability and uniformity of dosage unit should also be presented (WHO, 2003).

1.3 Plants as Antibacterial Agents

Plant materials are of wide use in traditional systems of medicine, and in several communities of the developing world, they are the only resources available for the treatment of different infections. In some Asian and African countries, 80% of the population depends on traditional medicine for primary healthcare and more than 100 countries have regulations for herbal medicines (WHO, 2000).

Infectious diseases are one of the leading causes of morbidity and mortality worldwide, especially in developing countries (WHO, 1998). Therefore, the use of medicinal plants play a vital role in covering the basic health needs in developing countries and these plants may offer a new source of antibacterial agents with significant activity against infective bacterial strains (WHO, 2003).

Bacteria, in general, possess the genetic ability to acquire and transmit resistance to therapeutic agents. Following the massive use of antibiotics in human therapy, bacteria

have developed several resistance mechanisms including the efflux of antibiotics. Several mechanisms have been proposed, such as target site modification, expression of the efflux pumps, and metabolic inactivation, which contribute to the drug resistance in multiple drug resistant bacteria (Gowsiya *et al.,* 2014).

The evolution of resistant strains is a natural phenomenon that happens when microorganisms are exposed to antibacterial drugs, and resistant traits can be exchanged between certain types of bacteria. The misuse of antibacterial medicines accelerates this natural phenomenon. Poor infection control practices encourage the spread of antibacterial resistance (ABR). Infections caused by resistant bacteria often fail to respond to the standard treatment, resulting in prolonged illness and greater risk of death. The death rate for patients with serious infections treated in hospitals is about twice that in patients with infections caused by non-resistant bacteria (WHO, 2014).

Alternative antibacterial drugs from plants have been revived for disease management due to the increased prevalence of multidrug resistance strains of bacterial isolates. This increases prevalence have been attributed to the indiscriminate uses of commercial antibiotics and this in turn has forced scientist to search for new antibacterial substances from various medicinal plants. These plants appears to be the important approaches to the development of these antibiotics as metabolites such as phenolic compounds and essentials oils have been reported to posses high antibacterial properties which are of great importance to tackling bacterial diseases (Gowsiya *et al.,* 2014).

1.4 Phytochemistry

Phytochemistry essentially deals with the enormous different types of organic substances that are not only elaborated but also accumulated by plants. The detailed phytochemical study of an unknown plant may be accomplished right from elucidation of chemical structures of pure constituents to the elaborated study of their biological characteristics (Evans, 2009).

The biosynthesis and breakdown of proteins, fats, nucleic acids and carbohydrates, which are essential to all living organisms, is known as primary metabolism with the compounds involved in the pathways known as "primary metabolites" (Dewick *et. al* 2008). The mechanism by which an organism biosynthesizes compounds called 'secondary metabolites' (natural products) is often found to be unique to an organism or is an expression of the individuality of a species and is referred to as "secondary metabolism"(Evans, 2009). Secondary metabolites are generally not essential for the growth, development or reproduction of an organism and are produced either as a result of the organism adapting to its surrounding environment or are produced to act as a possible defense mechanism against predators to assist in the survival of the organism (Colegate and Molyneux, 2006). The biosynthesis of secondary metabolites is derived from the fundamental processes of photosynthesis, glycolysis and the Krebs cycle to afford biosynthetic intermediates which, ultimately results in the formation of secondary metabolites also known as natural products (Dewick *et. al*, 2008). It can be seen that although the number of building blocks are limited, the formation of novel secondary metabolites is infinite. The most important building blocks employed in the biosynthesis of secondary metabolites are those derived from the intermediates: Acetyl coenzyme A

(acetyl-CoA), shikimic acid, mevalonic acid and 1-deoxyxylulose-5-phosphate. They are involved in countless biosynthetic pathways, involving numerous different mechanisms and reactions (alkylation, decarboxylation, aldol, Claisen and Schiff base formation (Dewick *et. al*, 2008). It is hypothesized that secondary metabolism utilizes amino acids and the acetate and shikimate pathways to produce "shunt metabolites" (intermediates) that have adopted an alternate biosynthetic route, leading to the biosynthesis of secondary metabolites. Modifications in the biosynthetic pathways may be due to natural causes (e.g. viruses or environmental changes) or un-natural causes (chemical or radiation) in an effort to adapt or provide longevity for the organism (Sarker *et.al*, 2006).

1.5 Antibacterial Activity

Bacterial infections are observed to be a significant cause of mortality and morbidity in spite of advancement in synthetic medicine and new antifungal agents (WHO, 2014). The use of plants for the treatment of these infectious diseases have 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 (Mogahid *et al.*, 2011). Alternative antibacterial drugs from plants have been revived for disease management due to the increased prevalence of multidrug resistance strains of bacterial isolates. This increases prevalence have been attributed to the indiscriminate uses of commercial antibiotics and this in turn has forced scientist to search for new antibacterial substances from various medicinal plants (Deshpande, 2013). Herbal drugs are now considered as an alternative in such situations (Sofowora, 2008). It is important to explore effective treatments of microbes. Researchers are therefore taking much attention in folk medicine in search of better drugs against microbial infections (Nester, 2004). A number of plants contain compound that have

antimicrobial property (Khan *et al.*, 2011). Compounds such as emetine, berberine and qunine which are derived from plants are very effective for the infectious microbes. Plants extracts from more than 157 plant families have been described which have potential antimicrobial properties (Gowsiya *et al.,* 2014).

1.6 Statement of Research Problems

Bacterial resistance has been reported to be a difficult problem for hospital immunocompromised patients. The use of antibacterial agents on patients has resulted in the adaptation of mutants' organisms, which can be very resistance to the most potent drug, thus scaling up health care cost and for this reason, the masses cannot afford it, the result is increase in disease severity and death from certain infections (Nester *et al.*, 2004). Hence, this has remained the major challenge in bacterial therapy worldwide.

1.7 Justification

In various countries across Tropical Africa, *S. madagascariensis* has been used without proper standardization, despite its use by some populace as herbal remedy for various diseased conditions such as dysentery and venereal diseases (Tom *et al.,* 2008; Mark *et al.,* 2002). It becomes extremely important to make an effort towards setting standards of the plant as crude drug and also to establish scientific evidence of its traditional use as antibacterial agent.

1.8 Hypothesis

Swartzia madagascariensis contains some phytochemicals with antibacterial activity.

1.9 Aim and Objectives

The overall aim of the study is to provide some pharmacognostic standards and scientific evidence for the use of *S. madagascariensis* leaves in ethnomedicine as antibacterial agent.

1.9.1 Specific objectives

i. To evaluate some pharmacognostic characters of *S. madagascariensis* with a view to providing parameters for its correct identification.
ii. To investigate the antibacterial activity of the leaf extracts of the plant
iii. To develop thin layer chromatographic profile of the most active extract of the plant
iv. To evaluate the safety margin of the plant.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Description of Family Fabaceae

Fabaceae, also called Leguminosae or bean and pea family, is the third largest family of angiosperms after Orchidaceae (orchids) and Asteraceae (daisies, sunflowers), and second only to Poaceae (grasses) in terms of agricultural and economic importance. Legumes includes a large number of domesticated species harvested as crops for human and animal consumption as well as the oils, fiber, fuel, fertilizers, timber, medicinal, chemicals, and horticultural varieties. The family also includes several species studied as genetic and genomic model systems (e.g pea- *Pisum sativum*, barrel medic- *Medicago truncatula*, and trefoil- *Lotus corniculatus*) (Lewis *et al.*, 2005).

Legumes vary in habit from annual and perennial herbs to shrubs, trees, vines/lianas, and even a few aquatics. Ranging in size from some of the smallest plants of deserts and arctic/alpine regions to the tallest of rain forest trees. Legumes are a conspicuous, and often dominant, component of most of the vegetation types distributed throughout temperate and tropical regions of the world (Rundel, 1989).

Legumes are particularly diverse in tropical forests and temperate shrub lands with a seasonally dry or arid climate. This preference for semi-arid to arid habitats is related to a nitrogen demanding metabolism. While many species have the ability to colonize barren and marginal lands because of their capacity to "fix" atmospheric nitrogen via a symbiotic association with root-nodulating bacteria, this is just one of several ways in which legumes

obtain high levels of nitrogen to meet the demands of their metabolism (McKey, 1994; Sprent, 2001) .

Over the past 30 years, the study of legume classification and biology has benefitted from major advances in our understanding of the morphology, evolution and systematics, and ecology of the family (Polhill, 1994; Lewis *et al.*, 2005).

2.2 Morphological Characteristics of Fabaceae

Fabaceae is characterized by leaves that are simple to compound (pinnate, rarely palmate, or bipinnate), unifoliate, trifoliate, sometimes phyllodic (many species of *Acacia*), or reduced to a tendril (as in *Lathyrus*), spirally arranged, with stipules present that are sometimes large and leaf-like (*Pisum*) or developed into spines (*Prosopis and Robinia*) (Lewis *et al.*, 2005).

Flowers are usually regular or irregular, bisexual, with a single superior carpel (hypogynous to perigynous), pentamerous, arranged singly or in racemes, spikes, or heads. The principal unifying feature of the family is the fruit, the legume (Polhill, 1994). With a few exceptions, legumes are typically one-chambered pods (one locule), with parietal placentation along the adaxial suture, ovules 2 to many, in two alternating rows on a single placenta, typically dry and dehiscent along one or both sutures (legume), occasionally constricted into 1-seeded sections (loments) or indehiscent (samara, drupe, achene) (Sprent, 2001).

2.3 Taxonomy of Fabaceae

Taxonomically, *Fabaceae* has been traditionally divided into three subfamilies, the *Caesalpinioideae, Mimosoideae, and Papilionoideae* (although sometimes these have been ranked as separate families, as in *Caesalpiniaceae, Mimosaceae, and Papilionaceae),* and considered most closely related to the *Connaraceae* and *Sapindaceae* on the basis of anatomy, morphology, and biogeographic distributions (Polhill and Raven, 1981).

The recognition of three subfamilies is based on characteristics particularly of the flower, including size, symmetry, aestivation of petals, sepals (united or free), stamen number and heteromorphy, pollen (single or polyads), but also presence of a pleurogram, embryo radicle shape, leaf complexity, and presence of root nodules (Lewis *et al.,* 2005). Differences in these characteristics led to the view that the *Mimosoideae* and *Papilionoideae* are unique and distinct lineages in the family which arose independently within a paraphyletic "basal" *caesalpinioid* assemblage. The *Dimorphandra* group of tribe *Caesalpinieae* and *papilionoid* tribe *Swartzieae* were considered likely transitional groups between them, respectively (Polhill, 1994).

The last formal classification by Polhill (1994), published prior to the advent of family-wide molecular phylogenetic studies, recognized 39 tribes and some 670 genera. The recent update of the tribal and generic classification of the family, having the benefit from more than 10 years of intensive molecular phylogenetic studies, recognizes 36 tribes, 727 genera and 19,327 species (Lewis *et al.,* 2005). The family contains at least four genera of 500 or more species (*Acacia, Astragalus, Crotalaria,* and *Indigofera*) and at least 40 genera with

100 spp. or more. At the other extreme, nearly 500 genera are small, either being monospecific or containing up to 10 species (Lewis *et al.,* 2005).

As Lewis *et al.* (2005) point out, while there has been some disagreement as to whether *Fabaceae* should be treated as one family (composed of three subfamilies) or three, there is a growing body of evidence from morphology and molecules to support the legumes being one monophyletic family. This view has been reinforced not only by the degree of interrelatedness of taxonomic groups within the legumes compared to that between legumes and its relatives, but also by recent molecular phylogenetic studies (Kajita *et al.,* 2001; Wojciechowski, 2003; Wojciechowski *et al.,* 2004) showing strong support for a monophyletic family that is more closely related to *Polygalaceae, Surianaceae, and Quillajaceae,* which together form the order *Fabales* (Herendeen *et al.,* 2003).

2.4 Chemotaxonomy of Fabaceae

The concept that plants can be classified on the basis of their chemical constituent is not new. Compared to morphological characters, chemical constituents are often more precisely definable and can be of more fundamental significance for classification purpose. It involves the use of scientific methods for the investigations of the potential chemical characters for the study of the problems of plant taxonomy and plant phylogeny. Medicinal plants are thus classified on the basis of the chemical they contain and it incorporates the principles and procedures involved in the use of chemical evidence for classification purposes.

The characters employed in chemical taxonomy are those of intermediate distribution in the plant kingdom. The study of these constituent in relation to taxonomy include the

investigation of the pattern of compounds occurring in the plants and preferentially in all various individuals parts of the plant such as bark, root leaves, wood, cuticle and seeds. Such integrated investigations are necessary in other to obtain a convincing evidence for the relationship of the plants. Although there are many not yet understood irregularities in the distribution of plant constituents and the overall information available at present does not yet suffice for a complete appreciation of their real contribution to a total look of a taxon, they are as valid as morphological characters for taxonomic work (Hegnauer, 1992). 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 posession of 5-deoxyflavonones and C-glycosylflavonoids (Constantino and David, 2006).

Some of these 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* (Harleen *et al.*, 2011).

2.5 Distribution and Habitat of Fabaceae

The *Fabaceae* is the most common family found in tropical rainforests and in dry forests in the Americas and Africa. The *Fabaceae* have an essentially worldwide distribution, being found everywhere except Antarctica and the high arctic. The trees are often found in tropical regions, while the herbaceous plants and shrubs are predominant in extra tropical regions. The *leguminosae* have a wide variety of growth forms including trees, shrubs or herbaceous plants or even vines or lianas. The herbaceous plants can be annuals, biannual

or perennials, without basal or terminal leaf aggregations. They are upright plants, epiphytes or vines. The latter support themselves by means of shoots that twist around a support or through cauline or foliar tendrils. Plants can be heliophytes, mesophytes or xerophytes (Angiosperm Phylogeny Group, 2003).

2.6 Description of the species: *Swartzia madagascariensis*

It is a small deciduous tree, 3-4 m in height. The stem and branches often much twisted and contorted, bark is dark grey, rough and fissured, common throughout wooded savanna to Nigeria and into Mozambique, Tanzania, Congo and Malawi. The leaves are compound, imparipinnate with a common stalk of 7.5-10 cm long with short hairs. It has 5-11 alternate to sub-opposite leaflets, and the leaflets are elliptic to obovate. Fruits are cylindrical with dark brown to black colour, up to 30 cm, indehiscent. Flowers in lax 2-10 flowered sprays, with one large petal and a mass of orange yellow stamens, sweetly scented. In its native range, *S. madagascariensis* (Plate 1) flowers from September to November (Curtis and Mannheimer, 2005). The tree is valuable and well distributed throughout tropical West Africa.

S. madagascariensis is a semi deciduous shrub or small tree up to 15 m tall, multi-stemmed or with a single bole up to 60 cm in diameter. The bark surface is deeply furrowed and ridged, flaking off in irregular pieces, grey to black (Amri, 2011). It is called Snake bean in English, ‘Bayama’ or ‘Gama Fada’ in Hausa, ‘Yawolawogi’ in Nupe, ‘Hil Igwom’ in Tiv. *S. madagascariensis* (Desv.) is synonymous to *S. marginata* Benth and *S. sapini* Wild (Hostettmann *et al.*, 2000).

Plate 1. *S. madagascariensis* in its Natural Habitat (http://www.medicinalplants**innigeria.com).**

2.6.1 Other Species From Genus Swartzia

Some species of the genus *Swartzia* are *S. acreana, S. acuminata, S. acutifolia, S. amazonica, S. anomala, S. apetala, S. apiculata, S. aptera, S. argentea, S. aymardii, S. bannia, S. bombycina, S. benthamiana, S. buntingii, S. cabrerae, S. calva, S. canescens, S. caudate, S. cardiosperma, S. davisii, S. dipetala, S. eriocarpa, S. fasciata, S. glabrata, S. gracilia, S. guttata, S. hostmannii, S. ingifolia, S. jenmanii, S. leptopetala, S. lucida, S. macrophylla and S. polyphylla* (ILDIS, 2012).

2.7 Chemical Constituents Reported from *S. madagascariensis*

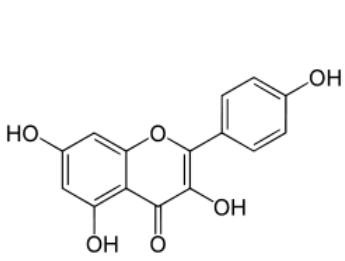
Preliminary Phytochemical screening of the *S. madagascariensis* leaves indicates the presence of chemical substances such as flavonoids, triterpenes, steroids, tannins, alkaloids, saponins, and glycosides. Literatures reports the isolation of some compounds from the plant, including flavonoids such as quercetin isolated from the stem bark of *S. madagascariensis.* Chromatographic fractionation led to the isolation of main active components as methyl paraben and lupeol (Adeyemi *et al.,* 2010).

The Phytochemical investigation of the plant led to the Isolation of highly glycosylated flavonoids such as kaempferol **(1)** and quercetin **(2)** isolated from the pod methanolic extract of *S. madagascariensis* (Stevenson *et al.,* 2010). Insects antifedant activity of quercetin isolated from the stem bark of *S. madagascariensis* have been reported including: Methyl paraben **(3)** and lupeol **(4)** from the chloroform extract by spectroscopic means of both the extract and the isolated constituent against the red flour beetle, *Tribolium casteneum* (Adeyemi *et al.,* 2010). Phytoalexins have been reported from *Swartzia* Species which is defined as a "low molecular weight antimicrobial compounds that are both synthesized by and accumulate in plants after exposure to micro-organisms". As inducible

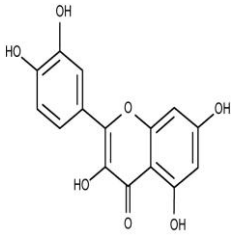
metabolites, phytoalexins are distinct from the secondary products which accumulate constitutively in plants and which have also been implicated in disease resistance (Gregor, 1995). Phytochemical investigation of the dried fruits of *S. madagascariensis* (Desv) afforded five triterpenoids saponins. They were shown to be glucuronides of oleanolic acid **(5)** and of gypsogenin **(6)** by chemical and spectral means. One of the isolated compounds, oleanolic acid was responsible for the high molluscicidal activity against the schistosomiasis- transmitting snails, *Biomphalaria glabrata* and *Bulinus globusus* (Wolfender *et al.,* 1998). The seed pod of *S. madagascariensis* contains two acidic saponins, swartziasaponin A and B and swartziagenin, a mixture of oleanolic and O-acetyloleanolic acid **(7)** (Jewers *et al.,* 2003).

2.7.1 Reported Chemical Constituents from other Species of Swartzia

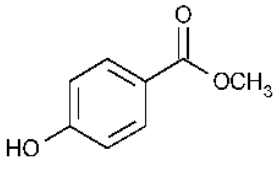
The antifungal activity of the hexane extract of *Swartzia apetala* afforded these constituents: Stilbene **(8)**, Pterocarpan **(9)**, maackiain **(10)**, stigmasterol **(11)**, pinocembrin **(12)**, campesterol **(13)**, sitosterol **(14)** and homopterocarpin **(15)** were first reported from *swartzia* genus by adopting a bioactivity-guided fractionation. Identification of the isolated compounds was achieved using physical, chemical and spectroscopic data (Marcelo *et al.,* 2009). Report on Bioassay-guided fractionation of the ethanolic extract of *S. prophylla* heartwood resulted in the isolation and characterization of naringenin **(16)** and biochanin A. Chemical constituent like isoflavone **(17)** isolated from the heartwood of *S. prophylla* through bioassay-guided fractionation where it shows promising results in the inhibition of protein kinase C (Dubois and Sneden, 1995).



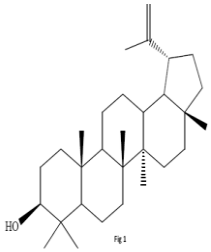
(1) Kaempferol



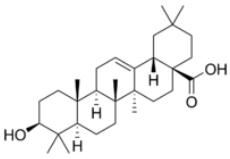
(2) Quercetin



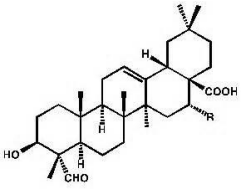
(3) Methyl paraben



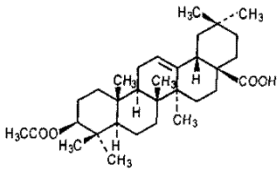
(4) Lupeol



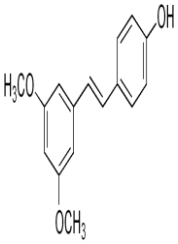
(5) Oleanolic acid



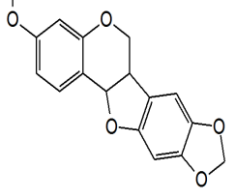
(6)Gypsogenin



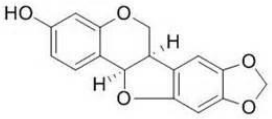
(7)Acetyloleanolic acid



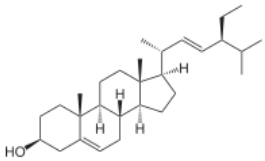
(8)Stilbene



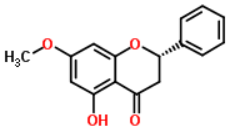
(9) Pterocarpan



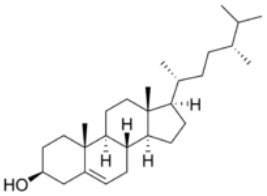
(10) Maackiain



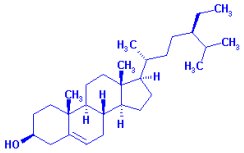
(11) Stigmasterol



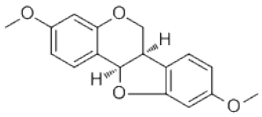
(12) Pinocembrin



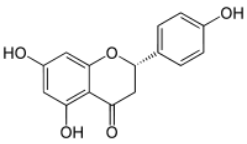
(13) Campesterol



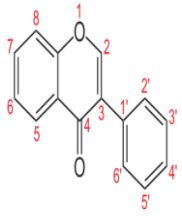
(14) Sitosterol



(15) Homopterocarpan



(16) Naringenin



(17) Isoflavone

Fig. 2.1 Chemical Structures of Some Compounds Isolated From *Swartzia* Species (Marcelo *et al.*, 2009).

2.8 Biological Activities of *Swartzia* Species

Various biological activities have been reported for various *Swartzia* species. Biological activities ranging from antimicrobial, antileukemic, antifungal, anti-inflammatory and chemopreventive properties have been associated with plant members of the genus *Swartzia*. Scientific investigations involving a number of various Swartzia species including *S. madagascariensis* proved numerous activities such as antifungal and antimalarial (Ouattara *et al*., 2006). Also, molluscicidal activity of *S. madagascariensis* fruits was reported to be active against the schistosomiasis- transmitting snails (Wolfender *et al*., 1998). Aqueous and methanolic extracts of *S. madagascariensis* fruit pulp exhibited highest trypanocidal activity (Atawodi *et al*., 2003).

Other biological investigations have been reported on molluscicidal activity of the pods of *S. madagascariensis* (Hostettmann *et al.,* 2000). Studies were carried out on the isolated compounds such as quercetin, gypsogenin, pterocarpan and methyl paraben from *S. madagascariensis* extract which had shown various pharmacological activities like antifungal, antifeedant, anti-inflammatory and molluscicidal activity (Adeyemi *et al.,* 2010), potential cancer chemopreventive and cytotoxic agents from *S. prophylla* which was found to be an isoflavone, biochanin A and naringenin (Sithisarn, 2012). Investigations reported that *S. prophylla* showed antimicrobial, antifungal, cytotoxic, antimutagenic and antiproliferative properties (Sithisarn, 2012). Antifungal agents of hexane extract isolated from *S. apetala* were found promising as antimycoses (Marcelo *et al.,* 2009).

Acute anti-inflammatory activity was evaluated using carrageenan-induced rat paw oedema test with the extract *of S. leptopetala (*Jimenez *et al.,* 2001*).* Different toxicity was carried out in various species of *Swartzia* these includes: Toxicity study (brine shrimp lethality)

test of fruit extract of *S. leptopetala* was carried out according to McLaughlin and Rogers (1998) method, using microsomes of the tested animals which were found effective.

2.9 Ethnomedicinal and Economic Importance of *S. madagascariensis*

Medicinal uses of *S. madagascariensis* plant include the use of the leaves as a cure for scabies and cutaneous infections, the bark for tooth and mouth troubles and bark resin as astringent for severe diarrhoea and dysentery. The roots are used to induce abortion, counteract venomous stings and bites, kill or expel intestinal worms and to treat leprosy. It has also been found useful in the treatment of fever (Coates, 2002).

The powdered fruits, seeds and sometimes roots are widely used as poison to stupefy fish and make them easy to catch (Neuwinger, 2004).

Trunk-bark, root-bark is used for the management of diseases such as: genital stimulants/depressants, malnutrition, debility, pregnancy, antiaborifacients, yaws etc. the leaves appear to have some effect on bilharzias snails. A decoction of the fruits has been used to induce vomiting to remove poison from the stomach, and to treat bilharzias, leprosy and ear- ache (Burkill, 1995).

Economically, the wood of *S. madagascariensis* is used for poles and posts for houses, fences and was found to be good for furniture, ship building, agricultural implements and turnery. The wood is excellent slow- burning firewood, and it is so popular for charcoal making that it is called ‘charcoal tree’ in parts of coastal Tanzania and Mozambique (Kirkbride and Wiersema, 1997).

The foliage of *S. madagascariensis* is a nutritious fodder for farm animals and Burkina faso has an active market for this which is in high demand by sheep farmers for fodder (Burkill, 1995).

2.10 Habitat and Distribution of *S. madagascariensis*

S. madagascariensis is widely distributed in semi- arid tropical, from Senegal and Gambia eastward to the Central African Republic, and south of the rainforest belt from DR Congo to Tanzania and southward to the Caprivi Strip in Namibia, Northern Botswana and Mozambique. It is occasionally cultivated in South Africa (Kirkbride and Wiersema, 1997). Inspite of its scientific name, it does not occur in Madagascar (Amri, 2011).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Materials, Chemicals, Equipments, Solvents, Reagents/Solutions

3.1.1 List of Reagents and Solvents

Acetic acid

Aluminium chloride

Anisaldehyde (Sigma-Aldrich, St. Lous, MO, USA)

Antimony trichloride

Chloral hydrate

Chloroform (Sigma-Aldrich, St. Lous, MO, USA)

Dragendorff's reagent

Ethanol (Park Scientific Ltd, Northampton, UK)

Ethyl acetate (Qualikems)

Fast green

$FeCl_3$ (Sigma-Aldrich, St. Lous, MO, USA)

Formalin

Glycerol.

Hexane (JHD, AR; Lobal Chem, India),

Hydrochloric acid

Libermann-Burchard's reagent

Methylene blue

Paraffin wax (BDH Laboratory Chemicals Division, POOLE. England)

Phloroglucinol,

Safranin

Sodium Hypochlorite

Sudan Red Solution

Tetraoxosulphate (iv) acid (Sigma-Aldrich, St. Lous, MO, USA)

TLC silica gel 60 F_{254} pre-coated plates (Merk-Germany)

Xylene (Sigma-Aldrich, St. Lous, MO, USA)

$ZnCl_2$ Solution

3.1.2 List of Equipments

Ash less filter paper

Camera Lucida

Compound microscope (Fisher Scientific, UK)

Dessicator

Disposable syringes 1ml, 5ml, and 10ml.

Glass Slides and Cover slips

Laboratory glass wares (Funnel, Conical flask, Beakers, Measuring cylinder)

Mechanical shaker (Stuart Scientific Flask Shaker, Great Britain)

Metallic cages and feeding bottles for mice

Microtome (C 740527, Cambridge Instrument Company Ltd, London and Cambridge, England).

Photographic camera

Plant press (local made)

Slide dryer (Hospital and Lab. Supply Ltd, London, UK)

Stage Micrometer and Ocular Lens (Graticules Ltd, Ton bridge, Kent. England)

TLC tanks (Uni kit® TLC Chromatank® , Shandon Southern Germany)

UV lamp

Water bath (HHS, Mc Donald Scientific International,)

3.2 Collection, Identification and Preparation of *S. madagascariensis*

The plant was collected at Kufena village, Zaria, Kaduna State, Nigeria. It was identified by the taxonomist (Malam M. Muhammad) at the herbarium section of the Department of Biological Sciences, Ahmadu Bello University, Zaria Nigeria. The plant was dried at room temperature, powdered and sieved with 30 – 40 mesh size and stored in a closed container for further use.

3.3 Extraction of the Leaves of *S. madagascariensis*

The plant material (500g) was extracted with 2 liters of Hexane using soxhlet apparatus and the extract was filtered and marc was pressed to dryness and then further extracted with 2 Liters of ethyl acetate and finally with ethanol and the extracts were then filtered and dried over water bath and the percentage yield was calculated using the formula: Percentage Yield of extract $(\%w/w) = \frac{\text{Weight of total extract}}{\text{weight of powdered material}} \times 100$

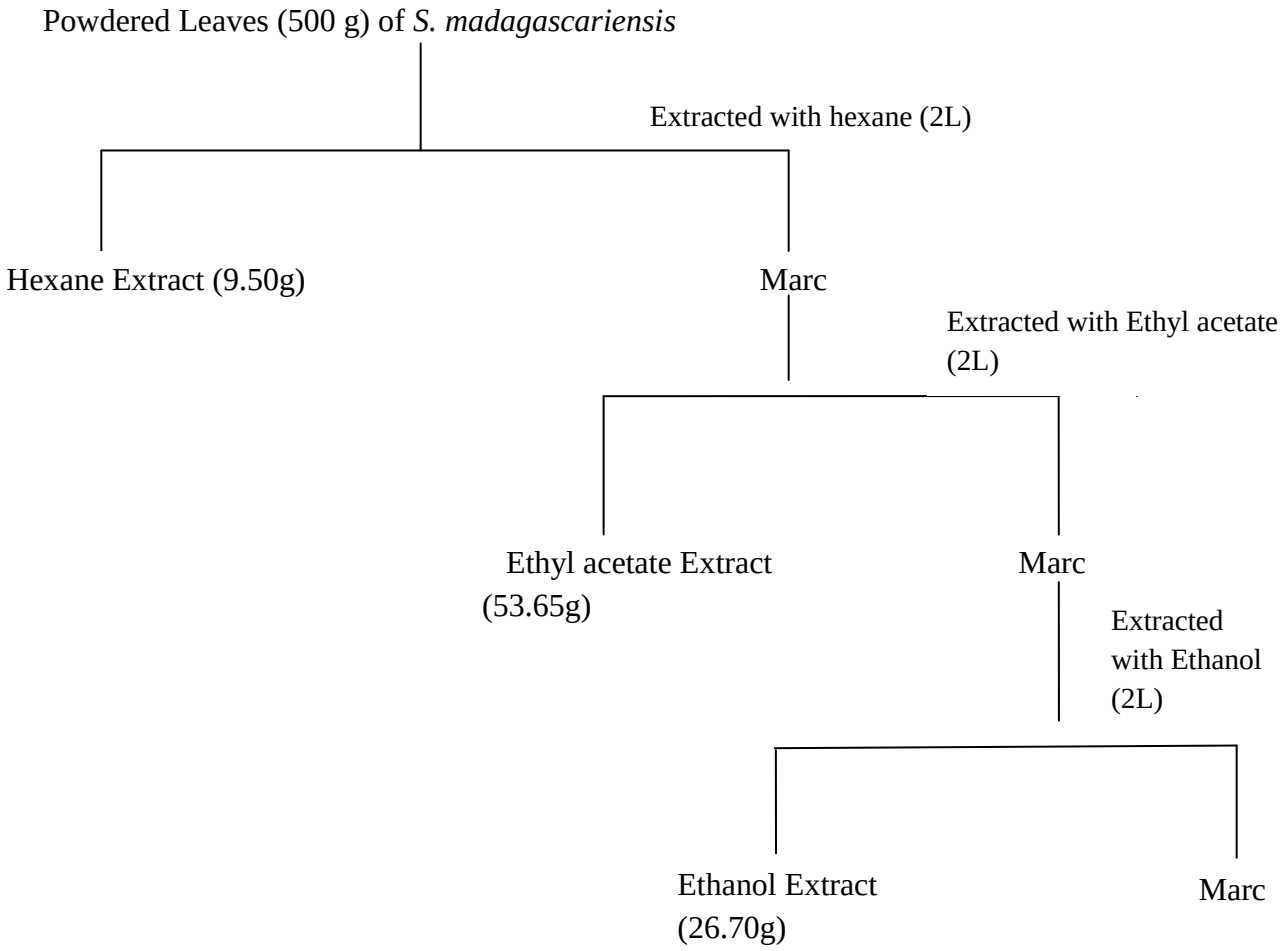


Fig. 3.1: Flowchart of Extraction Profile of *S. madagascariensis* Leaves (Bapodara *et al.*, 2011)

3.4 Some Pharmacognostic Characters of *S. madagascariensis*

Experimental Design

Detailed pharmacognostic studies of leaves of *S. madagascariensis* were carried out by examining the microscopical and chemomicroscopical characteristics of the leaves in order to establish some Pharmacopoeial Standards.

3.4.1 Microscopical Examination of Leaves of *S. madagascariensis*

3.4.1.1 Anatomical Section/Surface Preparations

Anatomical section of the leaf sample was examined under the microscope and features were described by using the terms according to Dutta, (2003) and Ahlam and Bouran (2011).

Methods

Fixation: the fresh Leaves of *S. madagascariensis* was picked off directly from the plant and dipped immediately into the fixative, Formal acetic acid (70% Ethanol, 40% formalin and glacial acetic acid) at the ratio 90:5:5 and was allowed to stand for 24 hours.

Dehydration: the sample above was transferred in to 30%, 50%, 70%, 95% and 100% ethanol. This was carried out in each graded alcohol for 2 hours each.

Clearing: the sample was transferred in to Ethanol: Chloroform (75:25), Ethanol: Chloroform (50:50), Chloroform: Ethanol (75:25), and 100% Chloroform. This was also carried out after every 2 hours each.

Infiltration and Embedding: chips of paraffin wax were added slowly in to the leaf sample and this was left to stand for 24 hours before transferring in to the oven at 60^{o}C. After melting, the paraffin containing the sample was transferred in to the embedding box and allowed to solidify. This was then trimmed and mounted on the microtome to get the transverse or cross section of the leaf. The transverse section was then transferred on to slides.

Staining: the transverse section of sample was dewaxed in xylene, by changing twice for 5 minutes each. This was then hydrated in 95%, 70%, 50%, and 30% ethanol for 2 minutes each. The transverse section was then transferred in to safranin and left to stand for 30 minutes before washing with water. It was then transferred in to 0.5% HCl in 70% ethanol shortly before dipping in to fast green for 2 minutes and washed with water. The transverse section was then further dehydrated in 30%, 50%, 70%, 95% and 100% ethanol for 2 minutes each and cleared in xylene for another 2 minutes. Gum (Balsam) was sprayed along the transverse section and cover slip was placed (Ahlam and Bouran 2011). This was then observed under the microscope and appropriate images were taken with photographic camera and documented.

3.4.1.2 Microscopic Examination of Powdered S. madagascariensis Leaves

Small amounts of ground powdered *S. madagascariensis* were used for the microscopy. The sample was cleared in chloral hydrate solution in test tubes by boiling in water bath and mounted using dilute glycerol and viewed with the microscope (Evans, 2009).

3.4.2 Quantitative Microscopy of the Leaf of *S. madagascariensis*

3.4.2.1 Calibration of Eyepiece Micrometer

Calibration of the ocular micrometer was performed as described in the WHO manual (2011). The ocular micrometer was placed in the eyepiece of the microscope. The stage micrometer was placed on the microscope stage and focused on the scale divisions. The eyepiece was turned to place the scales in a parallel position. The first point at which the two scales superimpose was noted. Another point where two division lines were exactly superimposed was noted as far long along the scale. The number of divisions on the ocular micrometer and the corresponding length on the stage micrometer scale were counted in

order to determine the length that is equivalent to one division on the ocular micrometer scale that is the calibration factor.

3.4.2.2 Determination of Dimensions

Fragment of the leaf of *S. madagascariensis* was used for the microscopy. The sample was cleared in chloral hydrate solution in test tube in boiling water bath, mounted on microscope glass slide using dilute glycerol and viewed with the microscope (Evans, 2009).

The stomatal size, stomatal number, stomatal index, vein islets number, veinlet termination number and palisade ratio were studied as described in Brain and Turner (1975) and Evans (2009).

The stomatal size was determined using methods described in WHO manual (2011). The leaf specimen was placed on the microscope stage and stomata were focused. The ocular micrometer scale was superimposed and the length and width of the stomata was recorded. The number of scale divisions was multiplied by the calibration factor (2.5µm) to obtain the actual dimension in micrometers of the stomata. Measurement was done by using x40 (objective) and x10 eyepiece lenses.

3.4.3 Setting up the Camera Lucida for Quantitative Leaf Microscopy

Camera lucida was set up in such a way that: the light from the object passes directly to the observer's eye through an opening in the left – hand prism. At the same time, light from the drawing paper and the pencil were reflected by the right – hand prism, so that the pencil appears superimposed on the object, which was also traced. The illumination of both the object and the paper was suitably adjusted and the paper was tilted at the correct angle to avoid distortion, as the position of the drawing board to which the paper was pinned was

correctly determined. Thereafter, the stage micrometer was placed on the microscope stage and its divisions were traced (Brain and Turner, 1975).

3.4.4 Chemomicroscopical Studies of Leaves of *S. madagascariensis*

These were carried out on the powdered leaves of the plant. Small amount of the finely ground powdered leaves of the plant was cleared in a test-tube containing 70% chloral hydrate solution. They were boiled on a water-bath for about thirty minutes to remove obscuring materials. The cleared sample was mounted on a microscope slide, using dilute glycerol. Using various detecting reagents, the presence of some cell inclusions and cell wall materials were detected in accordance with Brain and Turner (1975); Evans (2009) as follows:

3.4.4.1 Cell wall Materials

(i) Test for Cellulose: About 2 drops of iodinated zinc chloride were added to the cleared sample on a slide, and this was allowed to stand for few minutes. One drop of sulphuric acid was added, cover- slipped and observed under the microscope for blue colour which indicated the presence of cellulose in the cell walls of epidermal cells.

(ii) Test for Suberins: Two drops of Sudan red was added to the cleared sample on a slide, cover slip was applied and this was gently heated over hot water bath for 2 minutes. The slide was then observed under the microscope for red coloration which indicates the presence of suberin in the cell wall.

(iii) Test for Lignins: Two drops of phloroglucinol was added to the cleared sample and allowed to stand until almost dry. A drop of sulphuric acid was added and cover slip applied and this was

observed under the microscope which showed the appearance of red coloration on the anatomical section indicating the presence of lignin.

3.4.4.2 Cell Contents/Cell inclusions

(i) Test for Calcium carbonates: The appearance of effervescences on addition of concentrated hydrochloric acid to the powdered sample on the slide showed the presence of calcium carbonate.

(ii) Test for Calcium oxalates: To the cleared sample, cover slip was applied and this was observed under the microscope. Two drops of hydrochloric acid was then added and also observed under the microscope and dissolution of shining crystals on the anatomical sections of the leaves indicated the presence of calcium oxalates.

(iii) Test for Tannins: A single drop of ferric chloride was added to the cleared sample and cover slip was applied and this was observed under the microscope. The appearance of greenish black coloration on some cells of the anatomical sections of the leaves indicated the presence of tannins.

(iv) Test for Starch: Two drops of N/50 iodine solution were added to the cleared sample and cover slip was applied and this was observed under the microscope and the appearance of blue-black coloration in some parenchyma cells of the leaf powder indicated the presence of starch.

3.4.5 Determination of Some Leaf Constants

(i) Determination of Stomatal Number

Fragment of the leaf from the middle of the lamina was cleared with chloral hydrate solution and viewed with the microscope, and the number of stomata was counted in 10

field of view. The average stomatal number per mm^2 of leaf epidermis was calculated and result recorded.

(ii) Determination of Stomatal Index

Number of stomata and epidermal cells were counted in 10 fields of view and the stomatal index was calculated as percentage.

$$\text{Stomatal Index} = \frac{S \times 100}{S + E}$$

Where S = stomatal number

E = Number of epidermal cells

(iii) Determination of Veinlet Termination Number

The veinlet termination number was determined by counting the number of vein terminations per mm^2 of leaf surface midway between midrib and margin.

(iv) Determination of Vein-islets Number

The vein-islets number was determined by counting the number of vein-islets per mm^2 of the leaf surface midway between midrib and margin.

(v) Determination of Palisade Ratio

The average number of palisade cell beneath each epidermal cell was obtained by counting the number of palisade cells lying beneath a group of four contagious epidermal cells; the figure obtained was divided by four.

3.5.0 Determination of Physicochemical Constants of the Powdered Leaves of *S. madagascariensis*

3.5.1 Moisture Content (loss on drying).

Loss on drying method was used in which powdered sample (3 g) in a crucible was heated in an oven at 105^0C until a constant weight was obtained and noted. The loss in weight was determined by subtracting the weight of the dish and the sample after heating from the weight of dish and content before heating and the percentage was calculated. Three determinations were conducted and the average of these was taken as the moisture content of the drugs (WHO, 2011).

3.5.2 Total Ash

Ground air dried powdered leaves (2 g) was weighed in a platinum crucible. It was ignited by gradually increasing the heat, until it became white. It was cooled in a dessicator and weighed. The contents of the total ash per dried-powdered were calculated and result was recorded (WHO, 2011).

3.5.3 Acid Insoluble Ash

To the crucible containing the total ash, 25ml of dilute hydrochloric acid was added and covered with a watch glass and boil gently for 5 minutes. About 5ml of hot water was used to rinse the cover glass into the crucible. The insoluble matter was collected on an ash less filter paper and washed with hot water until the filtrate was neutral. This was then transferred back to the crucible and dried on a hot plate and ignited to a constant weight. The residue was allowed to cool in a dessicator for 30 minutes. It was then weighed without delay. The contents of the acid insoluble ash per air dried powdered were calculated and recorded (WHO, 2011).

3.5.4 Water Soluble Ash

To the crucible containing the total ash, 25ml of water was added and boiled for 5 minutes. The insoluble matter was collected in a sintered glass crucible. It was then washed with hot water and ignited in a crucible for 15 minutes at 105^{o}C. The weight of the residue was subtracted from the weight of the total ash. The content of water soluble ash per air dried powdered sample was calculated and recorded (WHO, 2011).

3.6.0 Extractive Values

Determinations of water and ethanol soluble extractives were determined by using some common methods as follows:

3.6.1 Water Soluble Extractives Value

Powdered leaves of the plant (5g) was macerated in 100 ml of water in a closed flask for 24 hours with frequent shaking for the first 6 hours by using mechanical shaker and was allowed to stand for 18 hours. It was then filtered rapidly and 20 ml of filtrate was evaporated to dryness in a tarred, flat bottomed, shallow dish and dry at 60^{o}C to constant weight (WHO, 2011).

3.6.2 Ethanol Soluble Extractives Value

Powdered leaves of the plant (5g) was macerated in 100 ml of ethanol in a closed flask for 24 hours with frequent shaking for the first 6 hours by using mechanical shaker and was allowed to stand for 18 hours. It was then filtered rapidly and 20 ml of filtrate was evaporated to dryness in a tarred, flat bottomed, shallow dish and dry at 60^{o}C to constant weight (WHO, 2011).

3.7.0 Phytochemical Screening of the Leaf Extracts of *S. madagascariensis*

The leaf extracts (hexane, ethyl acetate and ethanol) were subjected to phytochemical screening in order to identify the phytochemical constituents of the plant using the method described by (Evans, 2009, Musa, 2005, Sofowora 2008).

3.7.1Tests for carbohydrates

(a) Molish's (General) Test for Carbohydrates: To 1 ml of the filterate, 1 ml of Molish's reagent was added in a test tube, followed by 1 ml of concentrated sulphuric acid down the test tube. A reddish coloured at the interfacial ring indicates the presence of carbohydrate (Evans, 2009).

(b) Barford's Test for Monosacchrides: To 1 ml of the filtrate, 1ml of Barfoed reagent was added and the mixture heated on water bath for 5 minutes. A red precipitate indicates the presence of monosaccharide (Evans, 2009).

(c)Fehling's Test; To 2 ml of the filtrate 5 ml equal mixture of Fehling solutions A and B were added and the mixture boiled for 5 minutes. Brick red coloured precipitates indicate the presence of reducing sugar (Evans, 2009).

(d) Selivanoff's Test for Ketose Sugars: To 1 ml of the filtrate, some crystals of resorcinol were added, followed by an equal volume of concentrated HCl and the mixture was placed on water bath for 5 minutes. A rose colour indicates the presence of ketose sugar (Evans, 2009).

3.7.2 Test for alkaloids: The crude (hexane, ethyl acetate and ethanol) extracts of the plant were dissolved in 2 N HCl. The mixtures were filtered and the filtrates were divided into 4 equal portions. One portion was treated with few drops of Dragendorff's reagent; one

portion was treated with equal amount of Mayer's reagent, the other portion was treated with an equal amount of Wagner's reagent and to the fourth portion of the filtrate 1 ml of picric acid solution was added drop by drop. Appearance of orange, cream, reddish brown and yellow precipitates respectively indicated the presence of alkaloids (Salehi-Surmaghi *et al.*, 1992).

3.7.3 Tests for Tannins:

(i) Ferric chloride test: Two grams of the extracts (hexane, ethyl acetate and ethanol) were dissolved in 10 ml of water each and filtered. Two drops of ferric chloride solution were added to the filtrate. Appearance of blue or green precipitate indicates the presence of phenolic nucleus (Musa, 2005).

(ii) Lead sub-acetate test: To 2 g of the extracts (hexane, ethyl acetate and ethanol), two drops of lead sub-acetate solution were added and appearance of whitish-yellow precipitate indicated the presence of tannins (Musa, 2005).

3.7.4 Test for Flavonoids

(i) Shinoda test: About 0.5 g of the extracts (hexane, ethyl acetate and ethanol) was dissolved in 2 ml of 50% methanol. 0.2g of magnesium fillings and 3 drops of concentrated hydrochloric acid were added and the pink coloration indicated the presence of flavonoids (Evans, 2009).

(b) Sodium hydroxide Test: To the second portion of the filtrate 2 ml of 10% sodium hydroxide solution was added. A yellow solution indicates the presence of flavonoid which on addition of dilute HCl becomes colorless (Evans, 2009).

3.7.5 Tests for Saponins

(i) Frothing test: About 0.5 g of the extracts (hexane, ethyl acetate and ethanol) was dissolved in 10 ml of water and shaken vigorously in a test tube for 30 seconds and allowed to stand for 30 minutes the occurrence of frothing column or honey comb-like of at least 1 cm in height and persisting for at least an hour indicates the presence of saponins (Sofowora, 2008).

(ii) Liebermann-Burchard's test: About 1 ml of acetic anhydride was added to 0.5 g of the extracts (hexane, ethyl acetate and ethanol). Two drops of sulphuric acid were then added gently by the side of the test tube to the solution above and at the junction of the two liquids a formation of reddish brown or violet brown ring, the upper layer bluish green or violet indicates the presence of sterols and triterpenes (Evans, 2009).

(iii) Salkowski's test: About 2 ml of chloroform and two drops of sulphuric acid were carefully added to about 0.5 g of the extracts (hexane, ethyl acetate and ethanol) from the side of the test tube to form a lower layer. A reddish brown coloration at the interface indicated the presence of steroidal ring (Sofowora, 2008).

3.7.6 Test for Cardiac glycosides

(i) Kella-killiani's test: About 0.5 g of the extracts (hexane, ethyl acetate and ethanol) was dissolved in glacial acetic acid containing ferric chloride and 1drop of sulphuric acid was added to the solution, the appearance of reddish-brown coloration indicated the presence of cardiac glycoside or cardinolide (Sofowora, 2008).

(b) Kedde's Test: To the second portion of the first filtrate 1ml of 2% solution of 3, 5-dinitrobenzoic acid in 95% alcohol was added. The solution was made alkaline by the

addition of 5% Na0H; the presence of purple- blue colour indicates the presence of cardinolides (Evans, 2009).

3.7.7 Test for Anthracenes

(i) Bontrager's test: About 0.5 g of the extracts (hexane, ethyl acetate and ethanol) was added to 10 ml of benzene and shaken. This was then filtered and 5 ml of 10% ammonia solution was added to the filtrate; stirred and the reaction was observed**.** The presence of pink or cherish red color indicated the presence of anthracenes (Evans, 2009).

(ii) Modified Bontrager's Test: The powdered (0.5g) drug was boiled in 5 ml 10% HCl for 5 minutes on a water bath. The mixture was filtered while hot and then allowed to cool to room temperature. The filtrate was extracted with 5 ml benzene in a separating funnel. The upper benzene layer was pipette-off into a test tube and shaken with half its volume of 10% ammonium hydroxide. If the lower layer becomes rose pink or cherry red, it indicates the presence of anthraquinone derivatives in its free or combined form (Evans, 2009).

3.8.0 Determination of Acute Toxicity (LD_{50}) of *S. madagascariensis* Leaf Extract.

The LD_{50} was determined using the method of (Lorke, 1983). The study was conducted in two phases using a total of 13 mice. In the first phase, nine mice were grouped into three groups of three mice each. The groups received the ethyl acetate extract at 10, 100 and 1000 $mgkg^{-1}$ *i.p.* The second phase involved four groups of one mouse each which were administered with 140, 225, 370 and 600 mg/kg body weight of the ethyl acetate extract respectively. The LD_{50} was calculated as the geometric mean of the minimal lethal dose that caused death and the maximal non-lethal dose that did not cause death.

$$LD_{50} = \sqrt{\boldsymbol{minimum\ lethal\ dose} \times \boldsymbol{maximum\ non-lethal\ dose}}$$

3.9.0 Antibacterial Activity of the Leaf Extracts of *S. madagascariensis*:

3.9.1 Collection of Clinical Isolates

Four (4) clinical isolates were collected from Department of Medical Microbiology Ahmadu Bello University Teaching Hospital (ABUTH, Shika) the experiment was carried out using agar well diffusion method as described by Azoro (2002) and Mobasher *et al.* (2005) in the Microbiology units of National Institute of Leather Science and Technology (NILEST) Zaria, Kaduna State, Nigeria. The clinical isolates used were obtained from human wounds and include:

1. *Corynebacterium ulcerans*
2. *Escherichia coli*
3. *Staphylococcus aureus*
4. *Streptococcus pyogenes*

3.9.2 Preparation of Stock Solution

Hexane, Ethyl acetate and ethanol leaf extracts (0.4g) each of *S. madagascariensis* leaves were weighed and dissolved separately in 10 ml of DMSO to obtain a concentration of 40 mg/ml as an initial concentration. From the stock solution, two-fold serial dilution are made to obtain 20 mg/ml, 10 mg/ml, 5 mg/ml and 2.5 mg/ml concentration of each extracts. Standard antibiotic - Ciprofloxacin was used as the positive control drug for antibacterial agents (Azoro, 2002).

3.9.3 Preparation of Culture Media

Culture media for antibacterial was prepared using the Muller Hinton agar and was sterilized at 121^0C for 15 minutes, poured into sterilized petri dishes; it was then allowed to cool and solidified. Diffusion method was used to screen the extracts; the sterilized medium was seeded with 0.1 ml of the standard inoculums of the test microbes and was spread

evenly over the surface of the medium with a sterile swab. A well was cut at the centre of each inoculated medium using a standard cork – borer of 6 mm in diameters; 0.1 ml of the extract solution of the concentration of 40 mg/ml was introduced into each well on the inoculated medium.

The incubation of the medium was made at 37^0C for 24 hours, after which the plates were observed for the zone of inhibition of growth which was measured with a transparent ruler and the result recorded in millimeters (Azoro, 2002 and Mobasher *et al.*, 2005).

3.9.4 Determination of Minimum Inhibition Concentration (MIC)

The minimum inhibitory concentrations of the different extracts were carried out using the broth dilution method (EUCAST, 2000).

Mueller Hinton broth was prepared, 10 ml was dispensed into test tubes and sterilized at 121^0 C for 15 minutes and the broth was allowed to cool. The turbidity was made possible by preparing the Mc – farland's turbidity scale number 0.5. The test microbes were inoculated into the test tubes by dissolving 10 ml of the prepared normal saline and then incubated at 37^0 C for 6 hours. Dilution of the test microbes was done in the normal saline until the turbidity marched with that of the Mc – farland standard by visual comparison and the concentration was determined at that point. Two – fold serial dilution of the extract in the sterile broth was prepared and concentrations were obtained as 40, 20, 10, 5 and 2.5 mg/ml.

The test microbes (0.1 ml) in the normal saline were inoculated at different concentrations, incubated at 37^0 C for 24 hours after which the test tubes are observed for turbidity (growth). The lowest concentration of the extract in the broth that will not allow turbidity indicates minimum inhibitory concentration (EUCAST, 2000).

3.9.5 Determination of Minimum Bactericidal Concentration (MBC)

Minimum bactericidal concentrations of the different extracts were carried out to determine whether the test microbes were killed or their growth was inhibited. The contents of the MIC in the serial dilution was then sub – cultured onto the prepared medium and incubated at 37^0 C for 24 hours, after which each plate of the medium was observed for colony growth. The minimum bactericidal concentration was determined as the plates with lowest concentration of the extract without colony growth (EUCAST, 2000).

3.10 Thin Layer Chromatographic Profile of the Most Active Extract of *S. madagascariensis*

The crude extract of ethyl acetate was subjected to thin layer chromatography and the Retention factor (R_f) value of each spot was determined. The solvent system and adsorbent silica gel G used were as follows; (Evans, 2002).

Adsorbent: Precoated silica gel G plate (Merck), thickness 0.25mm activated at 105^0C for 1hr.

Solvent systems used:

Hexane: Ethyl acetate 9:1, 4:1, 7:3, 3:2, 1:1

Hexane: Ethyl acetate: Acetic acid 3:6:2, 3:6:3, 3:7:1

Chloroform: Methanol 9:1, 4:1, 7:3

Ethyl acetate: Methanol 9:1

Ethyl acetate: Formic acid 9:1

Chloroform: Ethyl acetate 1:1

Chloroform: Ethyl acetate: Methanol 15: 8:4

Chloroform: Methanol: Water 5:4:1

Hexane: Ethyl acetate: Methanol 6: 3: 1

Butanol: Acetic acid: Water 8:1:1, 10:1:1

Detection techniques

(a) General reagents

(i) Daylight

(ii) Iodine vapour

(ii) Ultra Violet (UV) at 254 and 366nm

(iv) p-anisaldehyde/H_2SO_4 heated at 105^0 C for detection of phenols, sugars, steroids and terpenes.

(b) Specific reagents

(i) Aluminium chloride for flavonoids

(ii) Ferric chloride for (phenolic compounds).

(iii) Dragendorff reagent for (alkaloids).

(iv) Borntragers for Anthraquinones

3.11 Statistical Analysis of Data

Data collected were statistically analysed using student t-test at ($P<0.05$) to compare the zones of inhibition displayed with the standard drug against each clinical isolates.

CHAPTER FOUR

4.0 RESULTS

4.1 Plant Collection, Identification and Preparation

The leaves of *S. madagascariensis* were collected from Kufena village, Zaria Local Government Area of Kaduna State, Nigeria in the month of September, 2014, it was identical to a herbarium sample with voucher number 7231 deposited in Department of Biological Sciences, Ahmadu Bello University, Zaria.

4.2 Extraction of the Leaves of *S. madagascariensis:*

The yield of extraction from 500g powdered leaves was 9.50 g for hexane, 53.65 g for ethyl acetate and 26.70 g for ethanol extracts and the percentage yield for hexane, ethyl acetate and ethanol is reported in (Table 4.1).

Table 4.1: Mass and Percentage Yield of the Leaf Extracts of *S. madagascariensis*

S/N	Extract	Mass (g)	Percentage Yield (%w/w)
1.	Hexane	9.50	1.90
2.	Ethyl acetate	53.65	10.73
3.	Ethanol	26.70	5.34

4.3 Pharmacognostic Evaluation of the Leaves of *S. madagascariensis*

4.3.1 Microscopical Studies of the Leaf of *S. madagascariensis*

Microscopical examination of the leaves of *S. madagascariensis* revealed the presence of some important diagnostic characters on both upper and lower epidermal layers these includes: Polygonal epidermal cells (29.3 – 30.25 – 31.2) with straight - walled anticlinal walls, anomocytic or ranunculaceous (irregular-celled) types of stomata (28.3 – 29.30 – 30.2) as summarized in Table 4.2; Plate II and III.

Table 4.2 Microscopic Features of the Epidermises of the Leaf of *S. madagascariensis*

Characters	Observations
Upper Epidermis	
Epidermal cells	Polygonal in shape (24.5µm)
Anticlinal walls	Straight - walled
Stomata	Absent
Trichomes	Present
Lower Epidermis	
Epidermal cells	Irregular in shape (30.2µm)
Anticlinal walls	Straight - walled
Stomata	Anomocytic (29.3µm)
Trichome	Present

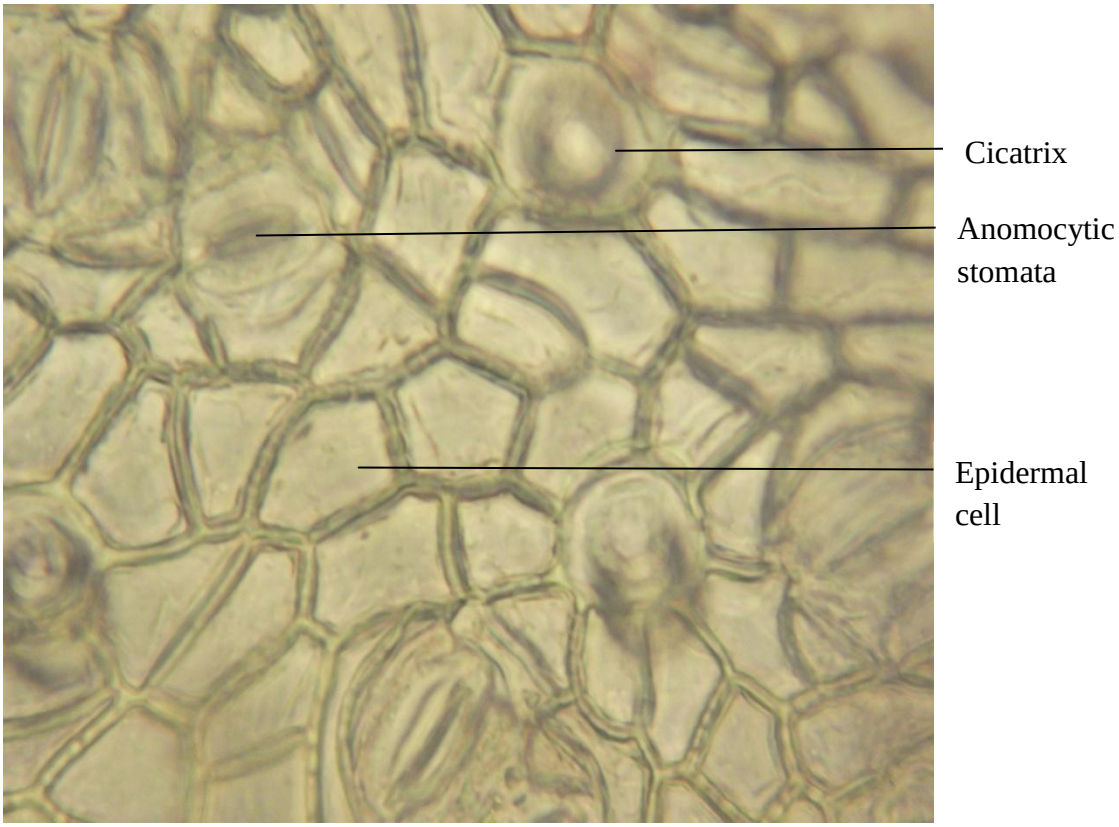


Plate II: Photomicrograph of the Lower Epidermal Layer of *S. madagascariensis* Leaf showing Anomocytic stomata; x100 (Fujifilm, 16mega pixel).

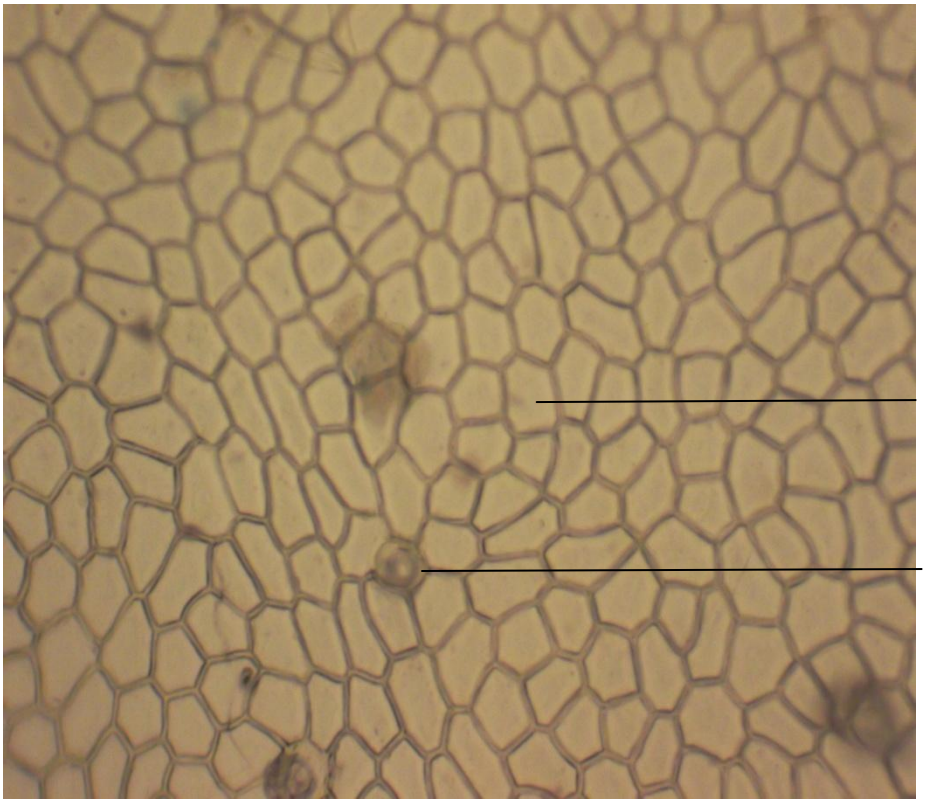


Plate III: Photomicrograph of Upper Epidermal Layer of *S. madagascariensis* Leaf showing Polygonal Epidermal cells; x100 (Fujifilm, 16mega pixel).

The transection of the leaf revealed that the leaf is isobilateral; this is a distinguishing characteristic of the leaf from most dicot leaves which is of diagnostic importance to the plant. It exhibits both upper and lower epidermis. The continuity of both epidermises is interrupted by the presence of raised stomata with ledges of wall material on the upper and lower sides and trichomes. Below the upper epidermis are found a layer or two of palisade tissues and also above the lower epidermis. These tissues are separated from each other by spongy mesophyll. The vascular bundles of the vein are embedded in the mesophyll (Plate IV).

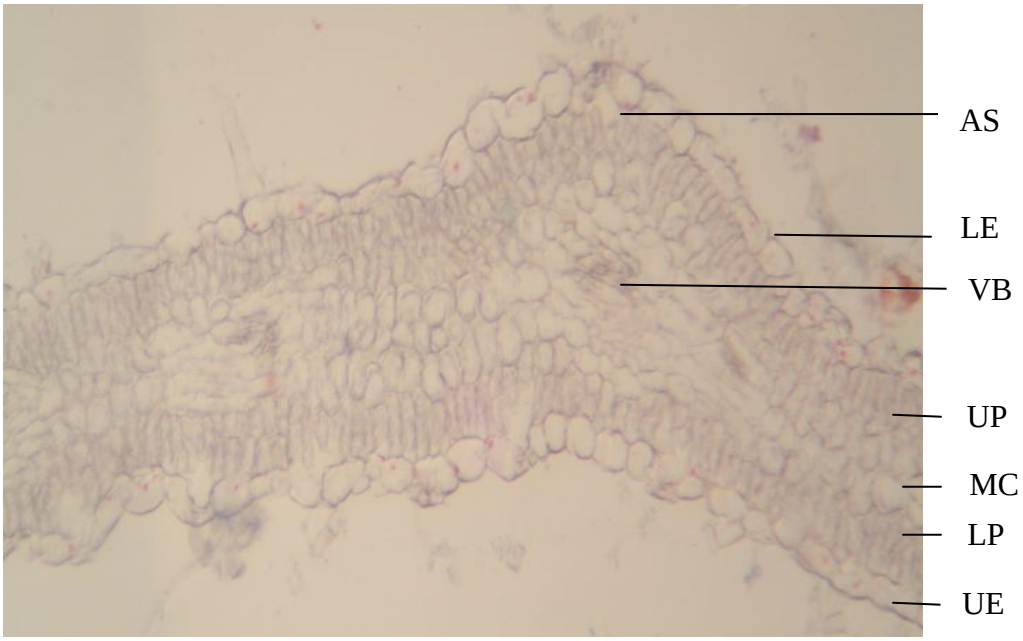


Plate IV: Photomicrograph of the Leaf Transection of *S. madagascariensis* stained with Saffranin and Fast green solution. ×100: AS- Air Space; UE- Upper epidermis; VB- Vascular bundle; UP- Upper palisade cells; MC- Mesophyll cells; LP- Lower palisade cells; LE- Lower epidermis.

4.3.2 Microscopy of Powdered Leaves of *S. madagascariensis*

Fragments of starch grains, calcium oxalate crystals, lignified collenchymas, fragment of uniseriate, unicellular covering trichome with pointed apex and bulbous base were found in abundance and Fragment of a lignified fibre which is slender in shape (Plate V - IX).

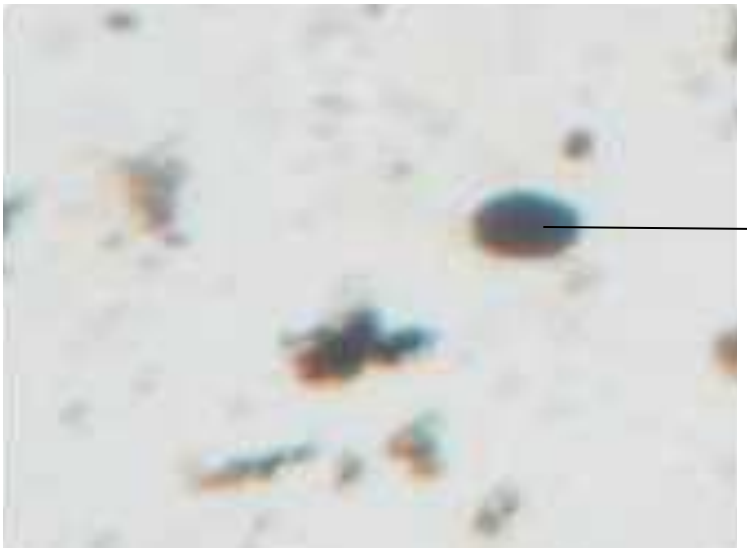


Plate V: Photomicrograph of fragments of starch grains (×100).

Plate VI: Photomicrograph of fragments of calcium oxalate crystals (×100).

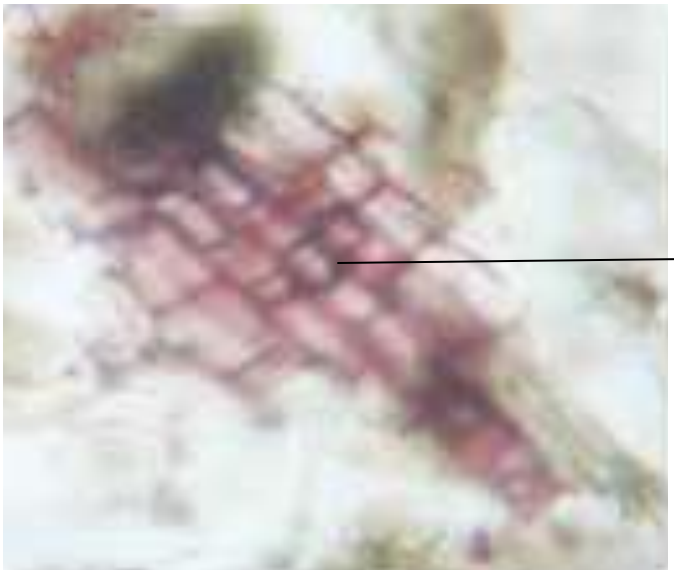


Plate VII: Photomicrograph showing lignified collenchyma (×100).

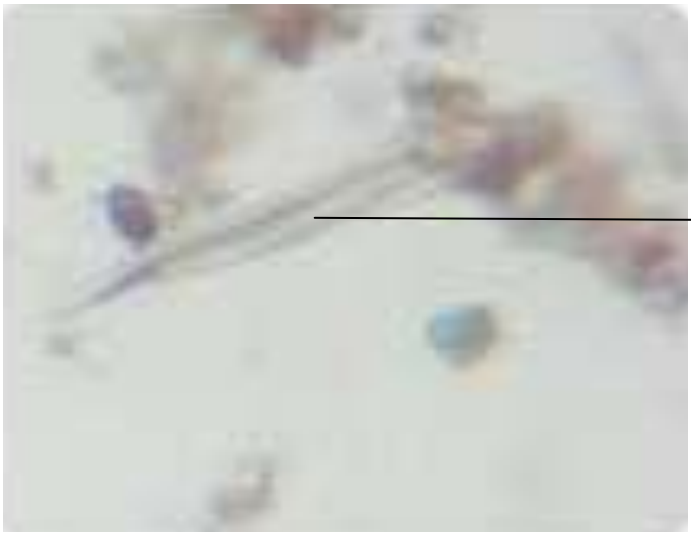


Plate VIII: Photomicrograph of fragments of covering trichome (×100).

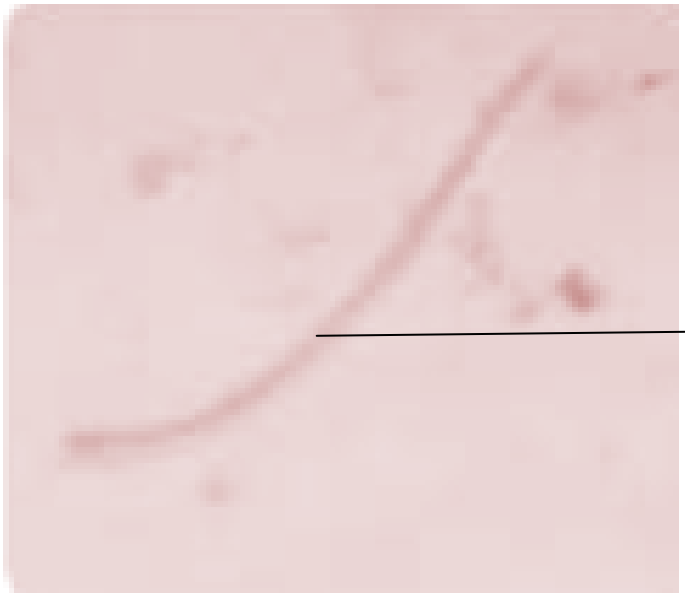


Plate IX: Photomicrograph of fragments of lignified fibre (×100).

4.3.3 Chemomicroscopical Studies

Chemomicroscopical examination of the powdered leaves of *S. madagascariensis* revealed the presence of cellulose cell wall, lignified cell wall, tannins, starch, and calcium oxalate as described below and summarized on table 4.3.

a. Test for Starch

Blue-black coloration observed on some starch grains in parenchyma cells of transverse section in the leaf powder, this indicate the presence of starch.

b. Test for Lignin

Red coloration was observed on the vascular tissue (Xylem and Phloem) of the powdered drugs on the addition of Phloroglucinol, which indicate the presence of Lignin.

c. Test for Cellulose

Blue coloration was observed on the epidermal cell of powder drugs when treated with Chlor-zinc iodine, this indicated the presence of cellulose.

d. Test for Tannins

Greenish black coloration in some parenchyma cells was observed when treated with Ferric chloride, this indicate the presence of tannins.

e. Test for Calcium oxalate

Brightly color was observed on the addition of 80% H_2SO_4acid to the cleared sample drugs and the appearance of bright color indicates the presence of calcium oxalate.

f. Test for Calcium carbonate

Calcium carbonate was dissolved upon addition of acid without effervescence observed, this indicate the absence of calcium carbonate.

TABLE 4.3: Chemomicroscopical Features of *S. madagascariensis* Powdered Leaves

Constituents	Detecting reagents	Observation	Inference
Starch	N/50 iodine	Blue-black colour on some grains within the chloroplast.	Starch present
Lignin	Phloroglucinol	Red-pink colour on the walls of some lignified collenchymas cell.	Lignin present
Tannins	5% $Fecl_3$	Greenish-black colour in some parenchyma cells.	Tannins present
Suberin	Sudan red	No Orange colour on walls of epidermal cells.	Suberin absent
Calcium oxalate	80% H_2SO_4	Dissolution of some brightly coloured crystals in some collenchyma cells.	Crystals of calcium oxalate present
Calcium carbonate	80% H_2SO_4	No effervescence in some dissolving crystals in the collenchyma cells.	$CaCO_3$ absent
Cellulose	Chlor – zinc iodine	Blue colour on the walls of epidermal cells.	Cellulose present

4.3.4 Quantitative Microscopy on the Leaves of *S. madagascariensis*

On the average, stomatal number (201), and index (9.5), palisade ratio (7.3), vein islets (29.5) and vein termination numbers (15.0) were determined and recorded (Table 4.4).

Table 4.4: Quantitative Microscopical Values for the Leaf of *S. madagascariensis*

Evaluative Parameter	**Values***
Stomatal number	170.8 – 201 - 231.2
Stomatal index	8.1 – 9.5 - 10.9
Palisade ratio	6.2 – 7.3 - 8.4
Veinlet termination number	12.7 – 15.0 - 17.3
Vein islet number	25.1 – 29.5 - 33.9

***** Mean Value of 5 counts

4.3.5 Determination of Physicochemical Constants of Powdered Leaves of *S. madagascariensis*

The result of average moisture contents using loss on drying method was calculated to be 6.7% and the percentage yield of total ash, acid insoluble and water soluble matter were recorded in percentage values as 6.0%, 1.0% and 4.5% respectively. The extractives obtained were 15% and 24% for alcohol and water solvents respectively (Table 4.5).

Table 4.5: Physicochemical Constants of *S. madagascariensis* Leaf Powder

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Parameters	**Values (%w/w) ± SEM**
Moisture content	6.7 ± 0.06
Total ash value	6.0 ± 0.06
Acid Insoluble ash	1.0 ± 0.04
Water Soluble ash	4.5 ± 0.06
Ethanol Extractives	15 ± 0.04
Water Extractives	24 ± 0.04

Average values of three determinations.

4.4.0 Phytochemical Screening of Leaves of *S. madagascariensis*

Phytochemical screening of the extracts indicated the presence of alkaloids, saponins, cardiac glycosides, phenolic compounds, triterpenes and anthracenes. The results of phytochemical screening of hexane, ethyl acetate and ethanol extracts are described below and summarized in table 4.6.

a. Test for alkaloids

Appearance of orange, cream, reddish brown and yellow precipitates indicated the presence of alkaloids.

b. Test for tannins

Blue or green and whitish- yellow precipitates indicated the presence of tannins.

c. Test for flavonoid

Formation of pink or yellow solution indicated the presence of flavonoids.

d. Test for saponins

Frothing Test: appearance of frothing column or honey comb- like that persisted for almost an hour indicated the presence of saponins.

Liberman burchard's Test: reddish brown or violet brown ring at the junction and a bluish green or violet at the upper layer indicated the presence of sterols and triterpenes.

Salkowski Test: reddish brown coloration at the interface indicated the presence of steroidal ring.

e. Test for cardiac glycosides

Kella- killiani's Test: formation of reddish brown colour upon addition of a drop of sulphuric acid indicated the presence of cardiac glycosides.

Kedde's Test: appearance of alkalinity of the solution upon the addition of 5% NaOH indicated the presence of cardinolides.

f. Test for anthracenes

Borntrager's Test: formation of pink or cherish red colour indicated the presence of anthracenes.

Modified Borntrager's Test: rose pink or cherry colour at the lower layer indicated the presence of anthraquinone derivatives.

g. Test for carbohydrates

Molish's Test: reddish colour at the interfacial ring indicated the presence of carbohydrates.

Barford's Test: reddish colour precipitates observed indicated the presence of monosaccharides.

Fehling's Test: brick red coloured precipitates indicated the presence of reducing sugar.

Seliwanoff's Test: appearance of rose colour solution indicated the presence of ketose sugar.

Table 4.6: Phytochemical Screening of the Leaf Extracts of *S. madagascariensis*

Constituents/Test	Observation	Inference		
		Hexane	**Ethyl acetate**	**Ethanol**
Alkaloids				
Dragendorff 's	Orange precipitate	Absent	Absent	Present
Mayer's	Whitish precipitate	Absent	Absent	Present
Wagner's	Brown precipitate	Absent	Absent	present
Picric acid	Yellow precipitate	Absent	Present	Present
Tannins				
Ferric chloride	Greenish appearance	Absent	Absent	Present
Lead sub acetate	Whitish precipitate	Absent	Absent	Present
Flavonoids				
Shinoda	Pink/Red coloration	Absent	Present	Present
Sodium hydroxide	Yellow precipitate	Absent	Present	Present
Saponins				
Frothing	Frothing or honey comb-like	Absent	Present	Present
Salkowski's	Red precipitate	Present	Present	Present
Libermann burchard's	Purple to violet ring	Present	Present	Present
Cardiac glycosides				
Keller Kiliani's	Appearance of reddish brown color	Absent	Present	Present
Kedde's Test	Purple- blue colour	Absent	Present	Present
Anthracenes				
Bontrager's	Pink red to violet Color	Absent	Absent	Present

Modified Borntragers	Rose pink colour at the lower layer	Absent	Present	Present
Carbohydrate				
Molish's	Reddish colour at the interfacial ring	Present	Present	Present
Barford's	Red colour precipitate	Present	Present	Present
Fehling's	Brick red coloured precipitate	Present	Present	Present
Seliwanoff's	Rose colour solution	Present	Present	Present

4.5.0 Determination of Median Lethal Dose (LD_{50}) of Ethyl acetate Extract of *S. madagascariensis*

The median lethal dose (LD_{50}) of ethyl acetate leaf extract of the plant was determined intraperitonially *(i.p)* in mice and was found to be 288.5mg/kg body weight (Table 4.7).

Table 4.7: Median Lethal Dose (LD_{50}) of Ethyl acetate Extract from *S. madagascariensis*

DOSE (mg/kg)	RESULTS*
First Phase	
10	0/3
100	0/3
1000	3/3
Second Phase	
140	0/1
225	0/1
370	1/1
600	1/1

* $LD_{50}= \sqrt{(370 \times 225)} = 288.5 mg/kg\ i.p$

4.6.0 Evaluation of Antibacterial Activities of the Leaf Extracts of *S. madagascariensis*

4.6.1. Zones of Inhibitions (mm)

The hexane, ethyl acetate and ethanol extracts showed antibacterial activities on the test organisms used, zones of inhibition were significant at ($P < 0.05$) and ranged from 17- 18 mm, 24- 27 mm and 21-22 mm for the hexane, ethyl acetate and ethanol respectively and lower than that of the control (Ciprofloxacin) which had 32-37 mm. The diameter of zones of inhibition of the extracts against test organisms (mm) was highest for ethyl acetate extract which had activity against all the test organisms, the ethyl acetate extract exhibited strong activity against gram positive bacteria, *S. aureus* at a diameter zone of 27 mm and *S. pyogenes* with diameter zone of inhibition of 25 mm; while it had no activity against *C.ulcerans*, and for the gram negative bacteria was recorded as *E. coli* 24 mm.

The ethanol extract also had an effect on the bacterial strains with diameter zones of inhibition of 22 mm for *S. aureus*, 21 mm for *S. pyogenes*, while it is in- active on *C. ulcerans* and 22 mm for *E. coli*. The hexane extract had minimal effect on the test organisms compared to ethyl acetate and ethanol extracts, with diameter zones of inhibition of 18mm for *S. aureus,* 18 mm for *S. pyogenes*, no effect against *C. ulcerans* and 17 mm for *E. coli* (Tables 4.8).

Table 4.8: Zones of Inhibition of Hexane, Ethyl acetate and Ethanol extracts (mm)

Test organisms	Hexane	Ethyl acetate	Ethanol	Ciprofloxacin
Corynebacterium ulcerans	0	0	0	32
Escherichia coli	17*	24*	22*	35
Staphylococcus aureus	18*	27*	22*	37
Streptococcus pyogenes	18*	25*	21*	35

*Significant at $P < 0.05$

4.6.2 Minimum Inhibition Concentration (MIC)

The minimum inhibition concentration (MIC) was 10 mg/ml for the ethyl acetate extract against all pathogens except for *S. aureus* which was observed at 5 mg/ml, MIC of the ethanol extract was observed at 10 mg/ml against all pathogens. Whereas, MIC of the hexane extract was at 20 mg/ml. Ethyl acetate extract had exhibited better activity at MIC of 5 mg/ml compared to ethanol extract with MIC of 10 mg/ml and hexane extract with MIC of 20 mg/ml (Table 4.9).

Table 4.9: Minimum Inhibitory Concentration of Hexane, Ethylacetate and Ethanol Extracts of *S. madagascariensis*

	Hexane (mg/ml)					**Ethyl acetate (mg/ml)**					**Ethanol (mg/ml)**				
Organisms	**40**	**20**	**10**	**5**	**2.5**	**40**	**20**	**10**	**5**	**2.5**	**40**	**20**	**10**	**5**	**2.5**
C. ulcerans	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
E. coli	-	*	+	++	+++	-	-	*	+	++	-	-	*	+	++
Staph. aureus	-	*	+	++	+++	-	-	-	*	+	-	-	*	+	++
S. pyogenes	-	*	+	++	+++	-	-	*	+	++	-	-	*	+	++

KEY: - = Clear (No growth), *** = MIC**, + =Turbid (Light growth), ++ = (Moderate turbid), +++ = (High turbidity), 0 =In-active.

4.6.3 Minimum Bactericidal Concentration (MBC)

Ethyl acetate had a better activity against all isolates at MBC of 20 mg/ml followed by ethanol that was active at MBC of 40 mg/ml against the bacterial strains. Hexane extract was general at MBC of 40 mg/ml against the three isolates (*E. coli, Staph. aureus* and *S. pyogenes*) (Table 4.10).

Table 4.10: Minimum Bactericidal Concentration of Hexane, Ethyl acetate and Ethanol Extracts of *S. madagascariensis*

	Hexane (mg/ml)		Ethyl acetate (mg/ml)		Ethanol (mg/ml)	
Organisms	**40**	**20**	**40**	**20**	**40**	**20**
C. ulcerans	0	0	0	0	0	0
E. coli	*	+	-	*	*	+
Staph. aureus	*	+	-	*	*	+
S. pyogenes	*	+	-	*	*	+

KEY: - = Clear **(**No growth**),** * = (MBC), + = Scanty colonies growth (Light growth), 0 = In-active.

4.7.0 Thin Layer Chromatographic Profile of The Most Active Extract of the Leaves of *S. madagascariensis*

The Thin Layer Chromatographic studies of ethyl acetate leaf extract of *S. madagascariensis* leaves was carried out to obtain a suitable solvent system for the separation of the extracts. The solvent systems, hexane: ethyl acetate: methanol (6:3:1) showed a good and clear chromatogram separation (Plate X- XII).

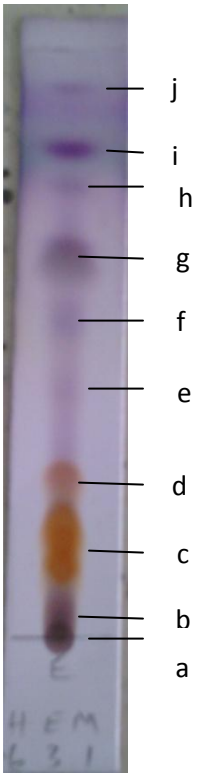

Plate X: TLC Profile of ethyl acetate leaf extract of *S. madagascariensis* Developed in Hexane: Ethyl acetate: Methanol (6:3:1) and sprayed with p-anisaldehyde in sulphuric acid reagent. R_f values for the respective spots are: a (0.10), b (0.12), c (0.15), d (0.20), e (0.30), f (0.55), g (0.69), h (0.76), i (0.81), j (0.94).

Plate XI: Ethyl acetate Extract of *S. madagascariensis* developed in a Hexane: Ethyl acetate: Methanol (6:3:1) and sprayed with Liebermann Burchard's reagent. R_f values for the respective spots are: a (0.10), b (0.13), c (0.20), d (0.27), e (0.38), f (0.55), g (0.69).

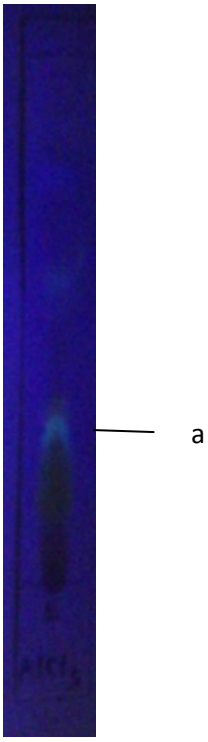

Plate XII: Ethyl acetate Extract of *S. madagascariensis* Developed in Hexane: Ethyl acetate: Methanol (6:3:1) and sprayed with Aluminium Chloride Reagent and viewed under UV Lamp. R_f value for the fluorescence spot is: a (0.20).

CHAPTER FIVE

5.0 DISCUSSION

Microscopically, the leaf was observed to be hypostomatic with anomocytic type of stomata on the abaxial surface only. The epidermal cells were observed to have straight anticlinal walls and the epidermal cells at the abaxial surface appear to be larger in size than the cells at the adaxial layer. The occurrence of the above mentioned characteristic features was observed among members of the Fabaceae (Sathis *et al.,* 2011).

Transverse section of the leaf of *S. madagascariensis* across the midrib portion had shown that the leaves are isobilateral. This is a good distinguishing and diagnostic anatomical feature for the leaf because most dicotyledonous leaves are known to be dorsiventral (Dutta, 2003).They palisade tissues occurred on both epidermises of the leaf. The vascular bundle tissues (xylem and phloem tissues) are of the conjoint type (Plate IV). These characteristics are observed among members of the Fabaceae (Sathis *et al.*, 2011).

The main diagnostic features found in the powdered leave include fragment of unicellular covering trichome and lignified fibre. These features observed are in accordance with those described by Sathis *et al.*, 2011 and Borate *et al.,* 2004 for the Fabaceae family.

Anatomical features of the internal structures of plant drugs provides an important diagnostic features for the identification of both entire and powdered crude drugs and detection of adulterants in plant materials (Ghani, 1990). Microscopical evaluation of crude drugs is aimed at identification of right variety and search for adulterant in plant materials (WHO, 1996).

Quantitative microscopy was used to study microscopic characters not easily characterized by general microscopy. Stomatal size, stomatal number, stomatal index, palisade ratio and vein islets number and veinlet termination numbers have all been investigated and reported for the first time from the leaves of the plant. Stomatal number for lower epidermis was found to be (170.8 – 201 - 231.2). Unfortunately, the limits of the numbers are wide and have been shown to vary quite widely according to the environmental conditions in which the plant was grown (Brain and Tuner, 1975). Evans, (2009) stated that early investigation by Timmerman indicated that stomatal numbers are useless in distinguishing between closely allied species.

The stomatal index of lower surface was found to be (8.1 – 9.5 - 10.9). The stomatal index is a more useful value and supportive evidence which, taken together with other factors, can make a positive identification possible and it is less subjected to variations with external conditions (Brain and Turner, 1975).

The vein islets number (25.1 – 29.5 - 33.9) and veinlet termination number (12.7 – 15.0 - 17.3) of the plant were of diagnostic importance. The vein islet and termination number may appear to vary according to the preliminary treatment the leaf has received (Evans, 2009).

The palisade ratio (6.2 – 7.3 - 8.4) observed is important. Palisade ratio can be determined even on quite fine powders unlike the veinlet termination and vein islet numbers which require fresh and large portions of leaves and are preferably determined on a particular part of a leaf and for these reasons; it is an important parameter that is used primarily for evaluating intact leaves rather than powder (WHO, 2011).

Chemomicroscopical studies of the powdered leaves of *S. madagascariensis* were found to have cellulose cell, lignin, calcium oxalate, tannins, starch, while mucilage was found to be absent (Table 4.5). These findings were consistent with that of sathis *et al.* (2011) for the family fabaceae. The Physicochemical constants were determined from the leaf powder of the plant, the moisture content (6.7%), Total ash value (6.0%), Acid insoluble ash (1.0%), Water soluble ash (4.5%), Alcohol (Ethanol) extractives value (15%) and Water extractives value (24%). These values are useful as the criteria to judge the identity and purity of crude drug (WH0, 1996). It also indicates presence of various impurities like carbonate, oxalate and silicate in plant materials (Kaneria and Chanda, 2011).

Quantitative evaluation is an important parameter in setting standard of crude drugs and the physical constant parameters could be useful in detecting any adulterant in the drug (Musa, 2005). Moisture content (6.7%) is not high which indicated less chances of microbial degradation of the drug during storage. The general requirement of moisture content in crude drug is that, it should not be more than 14% (B. H. P, 1990) and the value obtained in this research work was within the accepted range. Moisture is considered an adulterant because of its added weight as well as the fact that excess moisture is conducive to the promotion of mold and bacterial growth and low moisture content in a crude drug suggest better stability against degradation of product (WHO, 1996). Total ash value (6.0%) represents both the physiological and non-physiological ash from the plant. The non-physiological ash is an indication of inorganic residues after the plant drug is incinerated. The acid insoluble ash values (1.0%) obtained in this study indicated that the plant was in good physiological condition and it contained little extraneous matter such as sand, silica

and soil. The total ash value is used as criteria to judge the identity and purity of drugs (WHO, 1996).

Estimation of extractive values determines the amount of the active constituents in a given amount of plant material when extracted with a particular solvent. The extractions of any crude drug with a particular solvent yield a solution containing different phytoconstituents. The compositions of these phytoconstituents depend upon the nature of the drug and the solvent used. It also gives an indication whether the crude drug is exhausted or not (Tatiya *et al.,* 2012).This study indicated that the water had high extractive value of (24% w/w) compared to ethanol which had extractive value of (15% w/w).

Preliminary phytochemical screening gives an idea about the qualitative nature of active phytochemical constituents present in plant extracts, which will helps the future investigators regarding the selection of the particular extract for further investigation or isolating the active principle (Mishra *et al.*, 2010).

Phytochemical analysis of the leaf extracts had revealed the presence of some secondary metabolites namely alkaloids, tannins, flavonoids, cardiac glycosides, saponins (triterpenes and steroids) and anthracenes; this result is in agreement with the finding of Neuwinger, (2004) who detected the presence of phytochemicals namely flavonoids, tannins, saponins, cardiac glycosides and alkaloids in the plant.

The hexane, ethyl acetate and ethanolic extracts of *S. madagascariensis* had inhibited the growth of the test organisms (Table 4.10). Antibacterial activity of other plants has been reported by (Arshad *et al.,* 2010; Kamba and Hassan 2010; Korche *et al.,* 2011). The ability of plant extract to inhibit the growth of the pathogenic organisms may be as a result of the bioactive compounds such as saponin, alkaloids, and phenolic compounds in their leaves (Osuagwu *et al.,* 2007) and some of these were observed in this particular plant species.

Research findings indicated that the antibacterial properties of plants are conferred in them by the presence of secondary metabolites (Edeoga and Eriata, 2005; Enaba *et al.,* 2007; Bishnu *et al.,* 2009; Dahanukar *et al.,* 2000).

Phenolic compounds are essentially representing varieties of natural antioxidants, which are used as nutraceuticals and also in control of human pathogens (Puupponen-Pimiä *et al*., 2008). Triterpenoidal saponins extracted from aqueous and ethanolic extracts of *Allophylus cobbe* and *Allophylus serratus* are found to have potential antibacterial activity against *B. subtilis* (Chavan and Gaiwad, 2013). Saponins are reported to be sensitive against six strains of *E. coli* compared to standard drugs streptomycin (Michal *et al.,* 2012). Active crude flavonoids separated from *Mimba pudica* (Fabaceae*)* have been evaluated and found sensitive against gram positive *Staph. aureus* and gram negative *P. aeruginosa* bacteria with zones of inhibition of 15 - 22 mm (Doss *et al.,* 2011).

The intraperitoneal median lethal dose of *S. madagascariensis* ethyl acetate extract in mice was found to be 288.50 $mgkg^{-1}$. This suggests that the plant extract is moderately toxic when administered intraperitonially (Loomis and Hayes, 1996). Acute toxicity is a useful index for assessing the safety margin of a substance even though differing results are obtained on repetition or when determinations are carried out in different laboratories hence, should not be regarded as a biological constant (Lorke, 1983).

Antibacterial activity was expressed as diameter of zones of inhibition (Table 4.8). A zone of observable inhibition of growth of each organism served as a criterion for declaring an extract sensitive and was indicated by a clear zone around the well. The extent of antibacterial activity of the extracts based on the diameter zones of inhibition has been described as low (12-18 mm), moderate (19-22 mm) and strong activity (23-38 mm) by Ahmad *et al.,* (1999). The diameter of zones of inhibition of extract against test organisms

(in mm) was highest for ethyl acetate extract with zone of 27 mm exhibited against gram positive *S. aureus* compared to standard drug with the highest zone of inhibition of 37 mm. The least zone of inhibition exhibited by ethyl acetate extract was 24 mm against gram negative *E. coli* which also had a strong activity. While highest zone of inhibition in ethanol extract was observed at 22 mm against gram positive *S. aureus* and gram negative *E. coli* and the least was observed at 21 mm against gram positive *S. pyogenes.* Also, the highest zone of inhibition in hexane extract was observed at 18 mm against gram positive *S. aureus* and *S. pyogenes* and the least zone of inhibition was observed at 17 mm against gram negative *E. coli* which had a weak activity. The extracts (hexane, ethyl acetate and ethanol) had no activity against gram positive *Corynebacterium ulcerans* in this study, observed only against the standard drug (Ciprofloxacin) at 32 mm.

Minimum inhibitory concentration (MIC = 10 mg/ml) of ethyl acetate extract was observed and higher against gram positive *S. pyogenes* and gram negative *E. coli* and it is in lined with the studies carried out by Pateh, (2006) in the antimicrobial studies of the leaves of *Stylochiton loncifolius*. Whereas, (MIC = 5 mg/ml) of ethyl acetate extract against *Staph. aureus.* The minimum inhibitory concentrations of ethanol extract against *Staph. aureus, S. pyogenes* and *E. coli* was observed at 10 mg/ml. While the minimum inhibitory concentrations (MIC = 20 mg/ml) of hexane extract was observed against *S. aureus, S. pyogenes* and *E. coli.* The above MIC was also observed in the antibacterial screening of *Crotalaria capensis* (Fabaceae) ranges from 10 - 30 mg/ml as reported by Dzoyem *et al.*, (2014).

Minimum bactericidal concentration (MBC = 20 mg/ml) of ethyl acetate extract was observed with a higher activity against gram positive *S. aureus, S. pyogenes* and gram negative *E. coli.* Ethanol and hexane extracts were observed at (MBC = 40 mg/ml) against

gram positive *Staph. aureus, S. pyogenes* and gram negative *E. coli* (Table 4.10) . These are in consistent with the finding of Bhawna and Bharti, (2010) in the antimicrobial studies of *Acacia catechu* (Fabaceae) where it exerted the activity at MBC between 20- 40 mg/ml.

In all the antibacterial screening of the extracts against bacteria, it was observed that gram positive bacteria are the most susceptible to the extracts compared to the gram negative bacteria. The gram positive cell wall is simple while that of gram negative is complex consisting of lipoproteins outer membrane and lipopolysaccharides (Nester *et al.,* 2004). The outer membrane of gram negative cell envelopes does block the penetration of large molecules and hence the relative resistance of gram negative bacteria to some antimicrobial drugs (Nester *et al.,* 2004).

Antibiotic resistance also appears as a result of changes in genes or the acquisition of genes that allow the pathogen to evade the action of antimicrobial drugs. Resistance mechanisms include structural changes in or around the target molecule that inhibit the drug's ability to bind to it; reduced permeability of the cell membrane to the drug and actively pumping the drug out of the cell after it has entered; and production of enzymes that inactivate the antibiotic after it has been taken up by the cell this phenomenon mostly occur in gram negative bacteria (Ken *et al.,* 2012).

Thin layer chromatographic technique which has been used for the analysis of natural and synthetic substances was used in this study to separate extract of ethyl acetate of *S. madagascariensis* into its various components using various solvent systems which were composed of mixtures of varying chemical polarity. The plates developed were viewed under UV light in order to detect the presence of fluorescent compounds and visualized with different detecting reagents.

The ethyl acetate crude extract developed in hexane – ethyl acetate - methanol (6:3:1) produced colored spots with anisaldehyde in sulphuric acid. Spots of various colors; violet, blue, grey and green revealed. These colors obtained are indicative of the presence of classes of terpenoids (Evans, 2009). Phenols, terpenes, sugars and steroids turn violet, blue, red, grey or green colors with p-anisaldehyde. Liebermann- Burchard reagent yielded orange-brown, orange and violet colored spots which imply the presence of triterpenoids/ steroids (Gibbs, 2006).

Spots indicating the presence of flavonoids were also detected in the crude ethyl acetate extract. This was observed as a yellow colored fluorescence with R_f value of 0.20 obtained with UV after spraying plate with aluminium chloride which was used for detection of flavonoids. This is in agreement with Talukdar *et al.* (2010), the variations in R_f values provide an important understanding of the polarity of the various phytoconstituents of the extract and also help in the selection of appropriate solvent system for the separation of the constituents into their pure form by column chromatography.

CHAPTER SIX

6.0 SUMMARY, CONCLUSION AND RECOMMENDATION

6.1 SUMMARY

Microscopic features of the leaf revealed the presence of some important diagnostic features namely: polygonal epidermal cells, straight-walled anticlinal wall, prominent and unicellular covering trichomes, anomocytic stomata on lower epidermal layer (Table 4.2 Plate II and Plate III).

The transverse section of the leaf through the lamina and midrib tissue was examined and revealed different anatomical features include: epidermal layers, palisade and spongy mesophyll cells, vascular bundles which enclosed xylem and phloem vessels (Plate IV).

The physicochemical constants in (%w/w) of the plant are moisture content (6.7%), total ash (6.0%), acid insoluble ash value (1.0%), water soluble ash value (4.5%), water extractives value (24%) and ethanol extractives value (15%).

Phytochemicals investigation revealed the presence of chemical constituents including: alkaloids, flavonoids, saponins, triterpenes, steroids and cardiac glycosides.

The median lethal dose (LD_{50}) of the ethyl acetate extract was found to be 288.50mg/kg and considered moderately toxic.

The hexane, ethyl acetate and ethanol extracts showed zones of inhibition between 17 mm to 27 mm against the four clinical pathogenic isolates used with an MIC of 5 mg/ml to 20 mg/ml against *E. coli, Staph. aureus, and S. pyogenes* while *C. ulcerans* was resistant to all the extracts. MBC of the extracts ranges from 20- 40 mg/ml against all sensitive isolates.

6.2 CONCLUSION

The present studies had established:

(i) Microscopic features namely anomocytic stomata (29.30µm), chemomicroscopic and physical constants (moisture content (6.7%), ash value (6.0%), acid insoluble ash (1.0%), water soluble ash (4.5%), alcohol extractives (15%) and water extractives (24%) of the leaves of *S. madagascariensis* as its important diagnostic features.

(ii) The ethyl acetate leaf extract of *S. madagascariensis* had the most antibacterial activity that may explain and support its traditional use in the treatment of infectious diseases such as cutaneous wound and venereal diseases.

(iii) The thin layer chromatographic profile of the ethyl acetate leaf extract of the plant had shown that it contained chiefly flavonoids, triterpenes and steroids among other secondary metabolites which can be said to be responsible for its antibacterial activity.

6.3 RECOMMENDATION

It is recommended that more research should be carried out to establish pharmacognostic profile of the whole plant for inclusion into the Nigerian/African Pharmacopoeia which will assist in standardization of the plant as a tool for quality control.

Further work needs to be done to determine, identify, purify and quantify the antibacterial compound within this plant and also to determine its full spectrum of efficacy.

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APPENDIX:

Appendix I: Details of the Calculation Procedure of the Physical Constants Determination

Moisture Content

(i) Weight of drug = 3 g

Constant weight of empty crucible = 38.30 g

Weight of crucible + drug = 41.30 g

Constant weight of crucible + drug after drying = 41. 10 g

Weight of moisture = 41.30 – 41.10 = 0.2 g

Thus; Moisture = $\frac{0.2}{3} \times 100$ = 6.7 %

(ii) Ash Value

Weight of drug = 2 g

Constant weight of empty crucible = 21.60 g

Weight of crucible + drug = 21.6 + 2 = 23.60 g

Constant weight of crucible + residual ash = 23.48 g

Thus: Weight of Ash = 23.60 – 23.48 = 0.12 g

Thus; Total ash value = $= \frac{0.12}{2} \times 100$ = 6.0 %

(iii) Acid – insoluble ash value

Initial weight of drug = 2 g

Constant weight of crucible = 21.60 g

Weight of crucible + drug = 23.60 g

Weight of crucible + residual ash = 23.58 g

Weight of acid - insoluble ash = 23.60 – 23.58 = 0.02 g

% Acid insoluble ash = $= \frac{0.02}{2} \times 100$ = 1.0 %

(iv) Water soluble ash

Weight of drug = 2 g

Constant weight of crucible = 21.62 g

Weight of crucible + drug = 23.62 g

Weight of crucible + residual ash = 23.53 g

Weight of water soluble ash = 23.62 – 23.53 = 0.09

% of water soluble ash $= \frac{0.09}{2} \times 100$ = 4.5 %

(v) Ethanol soluble extractive value

Weight of drug = 5 g

Constant weight of empty crucible = 64.85 g

Constant weight of crucible + 20ml residue = 65 g

Thus; weight of residue from 20ml filtrate = 65 – 64.85 = 0.15 g

Weight of residue in 100ml = 0.15 × 5 = 0.75 g

Thus; % of Ethanol soluble extractive value$= \frac{0.75}{5} \times 100$ = 15 %

(vi) Water soluble extractive value

Weight of drug = 5 g

Constant weight of empty crucible = 123.03 g

Constant weight of crucible + 20ml residue = 123.27 g

Thus; weight of residue from 20ml filtrate = 123.27 – 123.03 = 0.24 g

Weight of residue in 100ml = 0.24 × 5 = 1.2 g

Thus; % of Water soluble extractive value$= \frac{1.2}{5} \times 100$ = 24 %

II: Median Lethal Dose (LD_{50})

$LD_{50} = \sqrt{minimum lethal\ dose \times maximum\ non - lethal\ dose}$

$= \sqrt{370 \times 225}$

$= \sqrt{83250}$

= 288.50 mg/kg.