PHARMACOGNOSTIC AND MOLECULAR STUDIES ON THE LEAF OF *ALBIZIA CHEVALIERI* (HARMS) (MIMOSOIDEAE)

BY

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PHARMACOGNOSTIC AND MOLECULAR STUDIES ON THE LEAF OF *ALBIZIA CHEVALIERI* (HARMS) (MIMOSOIDEAE)

BY

Khadijah Isa IMAM, B.Sc Biology (UDUS) 2011
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SEPTEMBER, 2016

DECLARATION

I declare that the work in this dissertation entitled Pharmacognostic and Molecular Studies on the Leaf of *Albizia Chevalieri* (Harms) (Mimosoideae) has been carried out by me in the Department of Pharmacognosy and Drug Development. The information derived from the literature has been duly acknowledged in the text and list of references provided. No part of this dissertation was previously presented for another degree or diploma at this or any other institution.

……. ……..

Khadijah Isa IMAM Date

CERTIFICATION

This dissertation entitled PHARMACOGNOSTIC AND MOLECULAR STUDIES ON THE LEAF OF *ALBIZIA CHEVALIERI* (HARMS) (MIMOSOIDEAE) by Khadijah Isa IMAM, meets the regulations governing the award of the degree of Master of Science in Pharmacognosy of the Ahmadu Bello University, and is approved for its contribution to knowledge and literary presentation.

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DEDICATION

This work is dedicated to ALLAH (SWT) and my parent, Late ALH. Isa N. IMAM and HAJ. Ramatu IMAM, may Allah (SWT) reward and continue to bless you. (Ameen).

ACKNOWLEDGEMENT

All praises is due to Almighty Allah, the creator of the heavens and earth, the fountain of knowledge for His mercies, guidance and protection in my life, may His mercy continue to be upon the prophet Muhammed (SAW). My profound gratitude to my supervisors Prof. (Mrs.) N. Ilyas and Dr. A. Ahmed for their untiring support, commitment and encouragement throughout the course of undertaking this research work and also for their patience in monitoring the progress of the work in the laboratory and the paper work.

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ABSTRACT

Albiza chevalieri Harms (Mimosoideae) is a tree or shrub of 5-12 m tall and distributed in the dry savannah from Senegal, Niger and Nigeria. The leaf is known for its antioxidant activity, anti-dysentric and to treat diabetes mellitus. Pharmacognostic evaluation of the plant leaf was assessed based on macroscopy, microscopy, chemomicroscopy and physico-chemical parameters. The DNA sequence of chloroplast of the ribulose-1, 5-biphosphate carboxylase (rbcL) gene sequence profile of *A. chevalieri* were determined. The leaf is greenish in color and microscopy features showed hypostomatic distribution of numerous paracytic type of stomata, and lignified trichomes, prismatic crystals of calcium oxalate crystals and the epidermal cells of wavy or irregular in shape on both surface with straight anticlinal walls. Study of transverse section showed hypostomatic arrangement with palisade cells and fibres found at the vascular bundle. Chemomicroscopy showed the presence of tannins, starch, lignin, suberin, calcium oxalate crystals and absence of calcium carbonate. Quantitative leaf constants revealed the average of stomatal number (22.33), stomatal index (21.54), palisade ratio (17.25), vein islets number (5.00) and veinlet termination number (6.65). Physico-chemical parameters such as moisture content/loss on drying (9.11 %), total ash content (4.83 %), water soluble ash content (1.75 %), acid insoluble ash content (1.5 %) and ethanol had high extractive value of (21.00 %) compared to water which had extractive value of (15.62 %). The study showed that *A. chevalieri* (lcl|Query_108313) is phylogenetically related with Acacia and Albizia group on the basis of rbcL gene sequences and also distinguish between closely related genera or species. The specimen showed the highest level of sequence similarities with *Albizia amara*. The resulted rbcL gene (DNA) sequence profile of *A. chevalieri* will improve the identification process and delineate their medicinal use and appropriate selection along with pharmacognostic based methods. These findings may be useful towards establishing standards on identification, purity and quality of *A. chevalieri* leaf.

TABLE OF CONTENTS

CONTENT **PAGE**

LIST OF TABLES

TABLES **TITLE** **PAGES**

LIST OF FIGURES

FIGURES **TITLE** **PAGE**

LIST OF PLATES

PLATES **TITLE** **PAGE**

LIST OF APPENDICES

APPENDIX **TITLE** **PAGE**

ACRONYMS AND ABBREVIATIONS

%:	Percentage
Abs:	Absorbance
AFLP:	Amplified fragment length polymorphism;
ASAP:	Allele specific associated primers;
B.H. P:	British Herbal Pharmacopoeia
BLAST:	Basic Local Alignment Search Tool
CAPs:	Cleaved amplified polymorphic sequences.
CBOL:	The Consortium for the Barcode of Life
DMSO:	Dimethyl sulfoxide
DNA:	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
EDTA	Ethylenediaminetetraacetic acid
EST:	Expressed sequence tag markers;
F:	Forward primer
FAA:	Formalin Acetic acid Alcohol
Fig:	Figure
G.A.A:	Glacial Acetic Acid
g:	Gram
GenBank:	National Centre for biotechnology information
H_2SO_4:	Sulphuric acid
HCL:	Hydrochloric acid
HPTLC:	High performance thin layer chromatography;
kg:	Kilogram
matK:	Maturase K
µl:	Microlitre
mg:	Milligram
$MgCl_2$:	Magnesium chloride

ml:	Milliliter
mm:	Millimeter
MP:	Maximum Parsimony
ng:	Nanogram
NMR:	Nuclear magnetic resonance spectroscopy
No:	Number
PCR:	Polymerase chain reaction
R:	Reverse primer
RbcL:	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit.
ST:	Sequence tagged sites
TBE	Tris-Borate EDTA
TLC:	Thin Layer Chromatography
UV:	Ultraviolet Light
Vol:	Volume
W.H.O:	World Health Organization
w/w:	Weight per Weight

CHAPTER ONE

1.0 INTRODUCTION

1.1 Medicinal plant

Plants as medicinal agents have been used over thousands of years with records dating to Mesopotamia, (Newman *et al.,* 2000). Medical plants are of great importance to the health of individual and communities. These have been identified and used throughout human history. They are known to contain a variety of substances and the ability to synthesize a wide variety of these substance that are used to perform important biological functions and to defend against attack from predators such as insects, fungi and herbivorous mammals or living organisms. At least 12,000 such compounds have been isolated so far, a number estimated to be less than 10% of the total (Lai and Roy, 2004; Tapsell *et al.*, 2006).

Medicinal plants are used in the treatment of many kinds of ailments in traditional medicine. Most importantly is that they are taken by the majority of the population because they are inexpensive and available (Sofowora, 2008). In some Asian and African countries, 80% of the population depend on traditional medicine for primary health care. In many developed countries, 70% to 80% of the population has used some form of alternative or complementary medicine e.g. acupuncture (WHO, 2008). Ethnobotany, the study of traditional human uses of plants, is recognized as an effective way to discover future medicines. The use of and search for, drugs and dietary supplements derived from plants have accelerated in recent years. Pharmacologists, microbiologists, botanists, and natural-products chemists are combing the earth for phytochemicals and leads that could be developed for treatment of various diseases (Fabricant and Farnsworth, 2001).

Herbal remedies are very common in Germany, herbal medications are dispensed by apothecaries (e.g., Apotheke). Herbal remedies are seen by some as a treatment to be

preferred to pure phytochemicals which have been industrially produced (James and Duke 2000). Furthermore, adulteration, inappropriate formulation, or lack of understanding of plant and drug interactions have led to adverse reactions that are sometimes life threatening or lethal (Elvin-Lewis, 2001). Herbal medicines without established efficacy may unknowingly be used to replace medicines that do have corroborated efficacy (Ernst, 2007).

1.2 Authenticity and Standardization

In herbal medicines, plant material is used either in fragment/powdered single or also as multicomponent mixture making identification difficult. Misidentified collections could lead to introduction of unsuitable or unwanted plant species in medicinal preparations. In order to avoid this problem proper identification of plant species of these powders or fragment is essential (Sanjay and Rajendra, 2015).

Authentication are implemented in the whole process from the collection of the raw material to the finished products. In addition, they are used to eliminate adulterants and fraudulent behaviour of unscrupulous individuals. However, there is no single authentication method that can be applied to every medicinal plant. The chain of custody are formed by combining various technologies and practices that are needed: microscopic and macroscopic analyses, identification via the phytochemical profiling, identification via DNA sequences of marker genes (Kalpana *et al.,* 2004; Florian and Micheal, 2014).

According to WHO guidelines authenticity, purity and safety are important aspects of standardization and in evaluation of traditional medicines, the first step is authentication (WHO 2011; Shinde *et al.,* 2007). The adulteration of herbal materials usually occur as a result of materials not having readily distinguishable morphological features, materials sharing similar common names and the substitution of economically valuable materials with inexpensive herbs (Ming *et al.,* 2011). Proper authentication process is necessary to prevent

the adulteration of target plant with other plant materials. For the standardization of botanical preparations most of the regulatory authorities and pharmacopeias suggest macroscopic, microscopic and chemical evaluation. As macroscopic identity of botanical materials is based on parameters like shape, size, color, texture, surface characteristics, fracture characteristics, odor, taste and such organoleptic properties that are compared to a standard references. Microscopy involves comparative microscopic examination of broken or powdered crude botanical materials. Chemical profiling establishes a characteristics chemical pattern for a plant material. Chromatography tools like Thin Layer Chromatography (TLC), High performance thin layer chromatography (HPTLC) and High-performance liquid chromatography (HPLC) are routinely used for qualitative determination of small amounts of impurities (Bharkatiya, 2005). Macroscopic and microscopic examinations and chemical analysis can be used as rapid and inexpensive method for plant identification and detection of contaminants (Techen *et al.,* 2004). However these methods have limitations because the composition and relative amount of chemicals in a particular species of plant varies with growing condition-harvesting period, post harvesting period and storage conditions and processing the drug went through before being sold on the market and thus no profile is identical (Sharma *et al.,* 2008) and are also influenced by both genetic and environmental factors (Mukherjee *et al.,* 2010). Chemical markers are further sensible to severe errors, since they need to be specific for the species, stable during storage and modification processes and should represent the therapeutically relevant compound (Srivastava and Mishra, 2009).

Authentication of botanicals which are medicinally valuable is an important issue globally because of unavailability/underutilization of appropriate tools for standardization. Since DNA is more stable and does not vary seasonally with age of the plant. DNA based fingerprinting techniques have greater role in the authentication of botanicals which are medicinally important. Genome-based methods have been developed for the identification of medicinal

plants starting in the early 1990's as the genetic composition is unique for each species irrespective of plant part used and is not affected by age, physiological condition as well as environmental factors (Shaw *et al.*, 2002; Techen *et al.*, 2004; Sucher and Carles, 2008; Hao *et al.*, 2009., Pravin *et al.*, 2012;).

Determination of various component, properties plays a significant role for standardization of the indigenous crude drugs (Hina *et al.*, 2011). Whether they are being taken as dietary supplements by the general public or being evaluated in a clinical study, the authenticity and standardization of botanical products is a matter of paramount concern (Smillie and Khan, 2010). In order to protect consumers and promote development of the herb medicine, reliable authentication of plant materials is critically important (Hao *et al*., 2010).

1.3 Pharmacognostic Evaluation of Medicinal Plant

Pharmacognosy is the study of medicines derived from natural sources, mainly from plants. It basically deals with standardization, authentication and study of natural drugs. Most of the research in pharmacognosy has been done in identifying controversial species of medicinal plants, and authentication of commonly used traditional medicinal plants. The importance of pharmacognosy has been widely felt in recent times. Unlike taxonomic identification, pharmacognostic study includes parameters which help in identifying adulteration in dry powdered form also. It lays down standardization parameters which helps and prevents adulterations and ensures reproducible quality of herbal products, its safety and efficacy (Sumitra, 2014).

Herbal raw material shows a number of problems when quality and authentication aspects are considered. This is because of nature of herbal parts, ingredients and different phytochemicals present in plants (WHO, 2011). To ensure quality of herbal medicines, proper control of starting raw material is very important.

1.4 DNA Fingerprinting of Medicinal Plant

DNA fingerprints are a bar-code like patterns generated by amplification of chromosomal DNA of an individual which can distinguish the uniqueness of one individual from another (*DNA fingerprinting,* 2005). Also called DNA typing, genetic fingerprinting and DNA profiling. (*DNA fingerprinting,* 2015). DNA fingerprinting in plants can be applied to a number of applications and uses (James *et al.,* 2001), DNA based marker analysis has been proven as an important tool in herbal drug standardization. DNA is the basic component of all living cells, the characteristics, traits and morphological features of plants are determined by the specific arrangement of DNA base pair sequences in their cell. DNA in cell is made of nucleotides i.e., adenine, guanine, thymine and cytosine and pentose sugar joined by phosphate bonds. These regulate the production of specific metabolites like enzymes and proteins (Pravin *et al.,* 2012). DNA fingerprinting is based on the identity of an organism at molecular level i.e., genetic characteristics. The basic technique of DNA Fingerprinting of species was discovered by Great Britain geneticist Alec J. Jeffrey in 1984.

DNA profiling is primarily used in botanicals for protection of biodiversity, identifying markers for traits, identification of gene diversity and variation etc. (*DNA fingerprinting in plants,* 2006). They are also used in molecular biology and biotechnology experiments where they are used to identify a particular sequence of DNA. As the DNA sequences are very highly specific, they can be identified with the help of the known molecular markers, which can find out a particular sequence of DNA from a group of unknown (Kurane *et al.,* 2009). It mimics the basic process used to copy DNA in a cell during chromosomal replication. Normally the length of the "sequence read" can vary from about 50 to more than 1,000 bases. Plastid rbcL (ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit) is the most commonly sequenced gene for identification and phylogenetic studies of plants (Schuettpelz

et al., 2006), in which the success rate of PCR and sequencing is higher compared with the other selected plant characterization genes such as matK (maturase K) (*CBOL Plant Working Group,* 2009; Parveen *et al.,* 2012). The DNA fingerprinting is so specific technique and widely used for authentication of plant species of medicinal importance (Himesh *et al.,* 2015). This is especially useful in case of those that are frequently substituted or adulterated with other species or varieties that are morphologically and/or phytochemically indistinguishable (Srivastava and Misha, 2009). DNA based tools for authentication of medicinal plants is an evolving new pharmacognostic measure aimed at quality control and quality assurance in medicinal plant research as well as in clinical usage (Kumar *et al.,* 2001).

1.5 Statement of Research Problem

Herbal medicines are promising choice over modern synthetic drugs. They may show minimum/no side effects and are considered to be safe. Herbal medicine are mostly marketed as multicomponent mixtures which are usually difficult to identify through physical means. Since the use of plants as therapeutic agents is paramount in virtually all systems of traditional medicine. It is necessary to have the correct knowledge/develop mechanisms for such crude drug which is a very important aspect in preparation, safety and efficiency of the herbal product (Nivedithadevi *et al.,* 2012). The plant *Albizia chevalieri* has been reported in management of diabetes mellitus, treatment of cancer, and remedy for dysentery, cough in traditional medicine with numerous uses. This plant are difficult to be differentiated from similar species in the same genera/family on the basis of morphological characters

1.6 Justification of Study

In recent times the use and demand of herbal preparations has been growing in Western countries where medicinal plants have gained popularity and attention among physicians and patients. The plant *Albizia chevalieri* preparation have reports of usage in traditional

medicine for centuries without any obvious negative effects and research on its phytochemical constitiuent, antioxidant and hematotoxicity on the leaf extracts (Yusuf *et al.,* 2007; Aliyu *et al.,* 2009). This recent work investigate the pharmacognostic features and DNA fingerprint of the leaf.

1.7 Aim and Objectives of Study

1.7.1 Aim

The aim of this research work is to provide some scientific parameters for identification and quality control of crude drug of *Albizia chevalieri* leaf

1.7.2 Objectives

1. To evaluate the pharmacognostic features of *A. chevalieri* leaf.
2. To provide profile of DNA fingerprint of *A. chevalieri*.

CHAPTER TWO

2.0 Literature Review

2.1 Description of the Plant *Albiza chevalieri* Harms (Mimosoideae).

The plant *Albizia chevalieri* is known in Hausa as *Katsari* and Zarma as *Nkolo.* It is a tree or shrub of 5-12 meter tall, often branching low down, trunk up to 30 cm diameter, rounded crown and open. The bark is corky, pale gray, scaly deeply cracked rectangular and thick enough, revealing brown areas when they stand. The edge is red to dark brown. The branch is also beige, white lenticels, gray pubescence. The sheet is alternate, bipinnate, dark green above and gray pubescent beneath. Petiole is crater with a gland before the first pair of pinnae and smaller at the base pairs 1 or 2 last pairs of pinnae. In venation only the main rib is visible near the upper edge of the blade and parallel. The infructescence is booklet terminal located at the base of the leaves and spherical clusters composed of 1-2 bright yellow, peduncle up to 6 cm long, 1-2.5 cm in diameter. Flower has greenish calyx, corolla purple, white stamens long tube whose base is not more than the corolla. Fruit is flat oblong pod more or less pubescent becoming glabrous, membranous, pale brown at maturity, containing 7-10 seed (Alhassane, 2013). The family is concentrated in the arid tropics with about 16,000 species in nearly all of the world's habitats. Northeast Tropical Africa: Chad. West-Central Tropical Africa: Cameroon. West Tropical Africa: Burkina Faso; Cote D'Ivoire; Ghana; Guinea; Mali; Niger; Nigeria; Senegal (Mark *et al.*, 2015).

Plate I. Tree of *Albizia chevalieri* in its natural habitat

2.1.1 Habitat

Albiza chevalieri is distributed in the dry savannah from Senegal, Niger and Northern Nigeria.

2.2 Ethnomedicinal and Ethnobotanical value

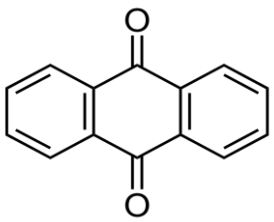
Alibizia chevalieri leaf is used in Borno-North eastern Nigeria as purgative, dysentery, diarrhoea, taenicide and also remedy for coughs. A decoction of leaves is used in Northern Nigeria as remedy for dysentery (Le Houèrou, 2009). The leaf extract of *A. chevalieri* is used either as cold water decoction or dried, ground and sieved leaf mixed with pap, for the management of diabetes mellitus by traditional medical practitioners in some parts of Niger Republic and Sokoto, Nigeria (Saidu *et al.*, 2007). The bark is use as vermifuge, purgative, for cough and tanning of hides (Alhassane, 2013). There are also reports on the local use of the leaves extract for cancer treatment in Zaria city, Kaduna state and also reported to have anti-oxidant activity, a significant hypoglycemic effect (Aliyu *et al*., 2009; Yusuf *et al.,* 2007).

Other *Albizia* species like *Albizia lebbeck*, is an astringent, also used by some cultures to treat boils, cough, to treat the eye, flu, gingivitis, lung problems, pectoral problems, abdominal tumors and is used as a tonic. Its sweet-smelling gum or resin is used in cosmetics in some African countries. The bark is poisonous but is used medicinally by the Zulu of South Africa who also sometimes make a love charm from the plant. *Albizia procera* is prepared as an infusion (hot or cold) from the bark and roots to treat skin diseases such as scabies, inflamed eyes, bronchitis. Seeds of *Albizia amara* are regarded as astringent, and used in the treatment of piles, diarrhea and gonorrhoea, leprosy, leucoderma, erysipelas and abscesses (Yadava and Reddy 2001). Tree parts of *A. julibrissin* are used in China as medicine, Bark extract is applied to bruises, ulcers, abscesses, boils, hemorrhoids and fractures, and has displayed cytotoxic activity (Higuchi *et al.,* 1992). *Albizia schimperiana* has antihistaminic property (Babu *et al.,* 2009). *Albizia gummifera* revealed very promising anti-trypanosomal activity

(Rukunga and Waterman, 1996). The lipophilic extracts of *A. zygia* showed high antimalarial activity (Muna and Hartmut 2012). Lipophilic extracts of *Albizia gummifera* revealed very promising anti-trypanosomal activity (Steinrut *et al.,* 2011). *Albizia saman* and *Albizia inundata* was found to have good antiplasmodial and anti-candida activity (Gupta *et al.,* 2006). *Albizia odoratissima* is used in the treatment of leprosy, ulcers and cough. *Albizia mollis* is well known for its sedative and sleeping pill properties (Zou *et al.,* 2000).

2.3 Phytochemistry of the genus *Albizia*

Phytochemical investigation of different species belonging to genus *Albizia* showed different classes of secondary metabolites. The preliminary phytochemical screening of methanol leaf and bark extracts of *Albizia chevalieri* revealed the presence of saponins, triterpenes, flavonoids, tannins, and alkaloids (Aliyu *et al.,* 2009; Le Houèrou, 2009). Anthraquinone glycosides **1** and saponin **2** were isolated from *A. lebbeck* leaf and bark (Ganguli and Bhatt, 1993; Jangwan *et al.,* 2010), and from *A. subdimidiata* root (Abdel-kader *et al.,* 2001). New Macrocyclic spermine alkaloids (budmunchiamines) **3** isolated from *A. inopinata* leaf (De Assis *et al.,* 1999). Spermine alkaloids **4** isolated from *A. adinocephala* stem bark (Ovenden *et al.,* 2002).



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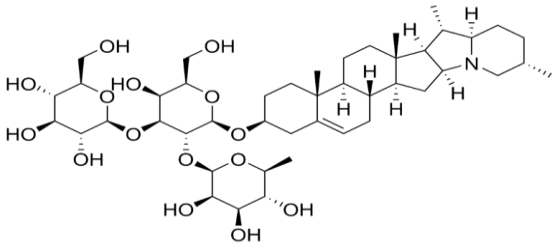

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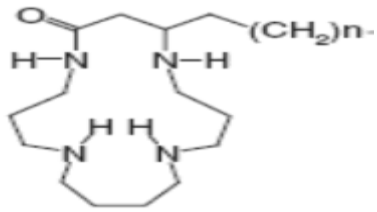

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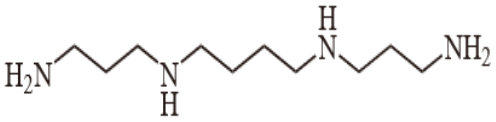

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2.4 Pharmacological Properties of the Plants from the Genus *Albizia*

Plants from the genus of *Albizia* possess the following pharmacological properties.

2.4.1 Antioxidant Properties

There are many reports on the antioxidant property for *Albizia* species. *A. julibrissin* foliage produced quercetin derivative, hyperoside (quercetin-3-O-galactoside)**5** and quercitrin (quercetin-3-O-rhamnoside)**6** that showed excellent antioxidant activity (Lau *et al.,* 2007). The albibrissinoside B **7** was found to be a radical scavenger on the 1, 1- diphenyl-2-picrylhydrazyl (DPPH) radical (Jung *et al.,* 2004). Khatoon *et al.,* (2013) studied the antioxidant activity of *A. procera* leaves through DPPH, reducing power and total antioxidant capacity. Their leaf extract exhibited an IC_{50} value of about 90% among that of DPPH radicals. The aqueous ethanol extract of *A. anthelmintica* showed its significance for both analgesic and antioxidant activities.

An isolation from plant produced quercetin-3-O-β-Dglucopyranoside **8**, kaempferol-3-O-β-D-glucopyranoside **9**, kaempferol-3-O-(6β-O-galloyl-β-D-glucopyranoside) **10** and quercetin-3-O-(6β-Ogalloyl-β-D-glucopyranoside) **11** exhibited potent antioxidant scavenging activity towards diphenyl-picrylhydrazine (DPPH) of *A. myriophylla* (Steinrut *et al.,* 2011) showed the highest antioxidant activity on DPPH radical assay (EC_{50} value

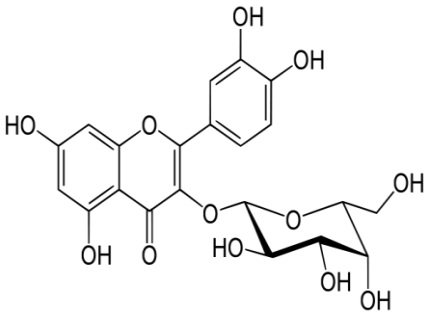
14.45%), lipid peroxidation assay (IC_{50} value 0.70%). Aurantiamide acetate **12** was the most active compound isolated from the leaf of *A. adianthifolia* through antioxidant activity (DPPH) and trolox equivalent antioxidant capacity (TEAC) assays were used to detect the antioxidant activity EC_{50} values 9.51 µg/ml and 78.81 µg/ml, respectively. The bark extracts of *A. lebbeck* possess free radical scavenging activity against 1, 1-di diphenyl-2-picrylhydrazyl radical (DPPH) and reducing power assays. Their results on DPPH free radical scavenging at 1000 µg/ml indicated maximum antioxidant activity of 91.82% and 90.08% respectively. Ethanolic extract of *A. procera* showed strong scavenging activity against free radicals compared to various standards. These in-vitro assays indicate that these plant extracts are a better source of natural antioxidant, which might be helpful in preventing the progress of various oxidative stresses. Aliyu *et al.,* (2009) studied the antioxidant activity of *A. chevalieri* leaves through DPPH, by exhibiting an IC_{50} value of about 94.7 % against the standard ascorbic acid (94.81 %). *A. amara* leaves extracts showed highest antioxidant activity, which were studied by three different methods, 2,2-diphenyl-1-picrylhydrazyl radical assay (IC_{50} value 164 %), nitric oxide free radical scavenging assay (IC_{50} value 205 %) and reducing power assay (EC_{50} value 0.087 µg/ml), when compared to standard samples.

2.4.2 Anticancer Properties

Three triterpenoid saponins from *A. julibrissin* bark, served as anti-tumorals by the induction of apoptosis in certain cell types (human acute leukemia junket T-cells) and butanol extract from the bark of *A. julibrissin* (Zheng *et al.,* 2006). A cytotoxic compound, Echinocystic acid 3, 16-O-bisglycosides **13** from the bark of *A. procera*. In contrast to other cytotoxic echinocystic acid glycosides with N-acetyl glucosamine unit, the new glycosides were found inactive when assayed by MTT method for their cytotoxicities against the HEPG2, A549, HT29 and MCF7 cell lines (Miyase *et al.,* 2010). Three oleanane-type triterpene saponins

named grandibracteosides A−C **14** from *A. grandibracteata* showed significant inhibitory activity against KB and MCF7 tumor cell lines in vitro (Sabrina *et al.,* 2005). Saponins from *A. procera*, characterized as 3-O-[β-D-xylopyranosyl-(1→2)-α-L-arabinopyranosyl-(1→6)-2-acetamido-2-deoxy-β glucopyranosyl] echinocystic acid **15** exhibited cytotoxicity against HEPG2 cell line with IC_{50} 9.13 μg/ml (Melek *et al.,* 2007). Three oleanane type triterpene saponins named albizosides A-C **16** from *A. chinensis*. These compounds showed cytotoxic activity against a small panel of human tumor cell lines as well as hemolytic activity against rabbit erythrocytes (Rui *et al.,* 2009).

An oleanane-type saponin named coriariosides A **17**, along with known saponin was isolated from the roots of *A. coriaria*. These compounds when tested for cytotoxicity against two colorectal human cancer cells, showed excellent activities viz. HCT 116 (IC_{50} 4.2 μM) and HT- 29 (IC_{50} 6.7 μM) cell lines (Not *et al.,* 2009). *A. harveyi* showed a significant cytotoxic activity on the RT-4 cell line (percentage survival 23 %) at 10 μg/ml. It showed a weak cytotoxic activity on the HT-29 cell line. Two diastereomeric saponins named julibrosides J1-2 **18** which show cytotoxic activity from *A. julibrissin*. Triterpenoidal saponin with a xylopyranosyl moiety from *A. julibrissin* showed marked inhibitory action against Bel-7402 cancer cell line at 10 micro/ml (Zou *et al.,* 2006). Two active cytotoxic saponins viz. Albiziatrioside A and B from *A. subdimidiata* showed significant effects against A2780 cell line (Lau *et al.,* 2007). Cytotoxic oleanane-type triterpenoid saponins named gummiferaosides **19** from *Albizia gummifera* showing cytotoxicity against the A2780 human ovarian cancer cell line with IC_{50} values of 0.8, 1.5 and 0.6 μg/ml respectively.



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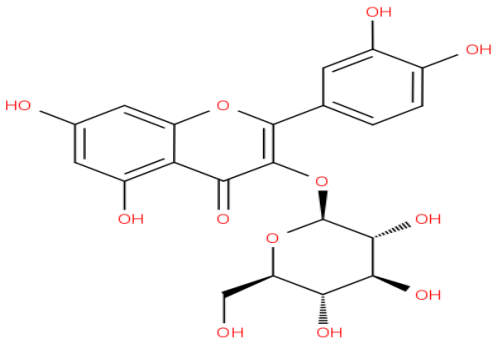

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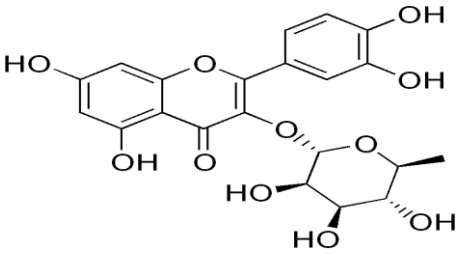

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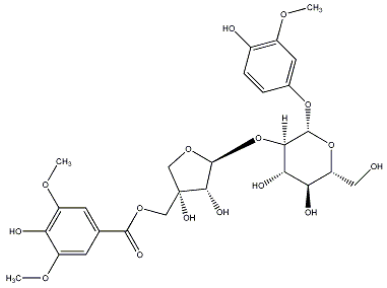

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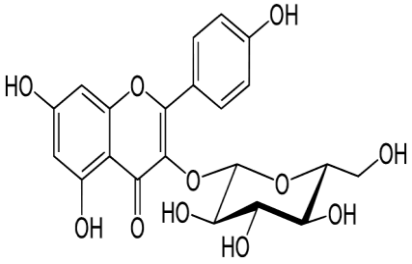

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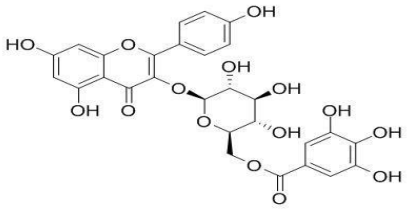

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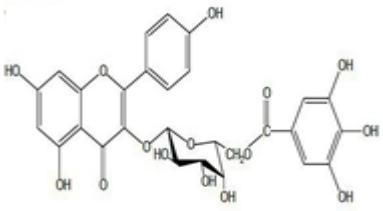

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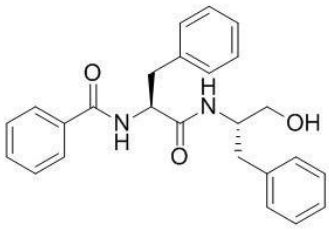

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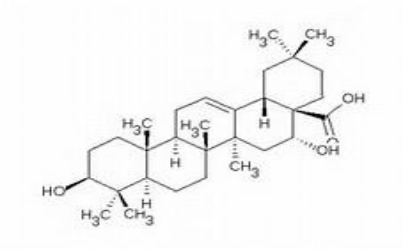

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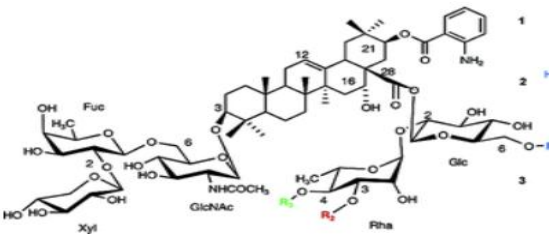

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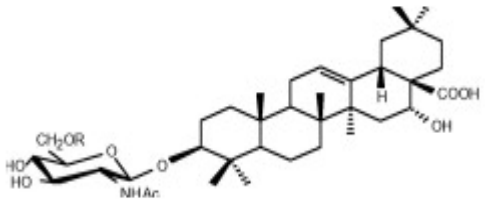

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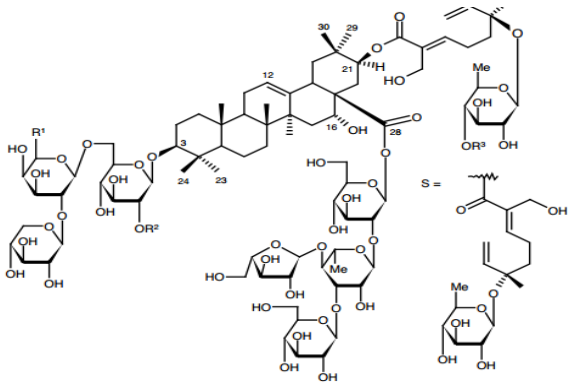


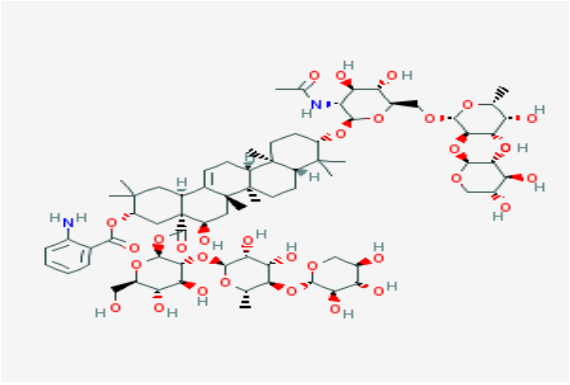
Albizoside A: R^1 = M, R^2 = H, R^3 = S

Albizoside B: R^1 = Me, R^2 = GLc, R^3 = S

Albizoside C: R^1 = H, R^2 = GLc, R^3 = H

Glc: β-D-glucopyranosyl

16



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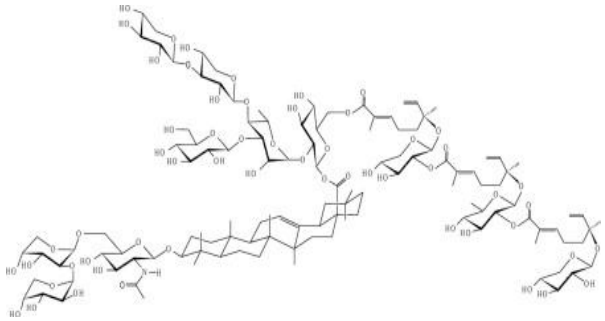

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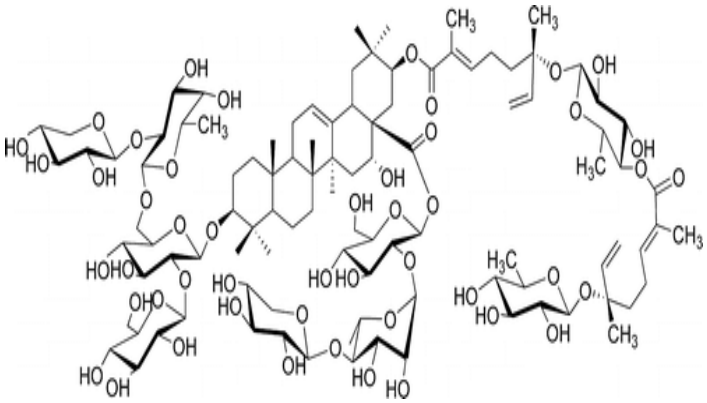

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2.4.3 Antidiabetic Properties

Two flavonol glycosides, quercitrin and isoquercitrin from the flowers of *A. julibrissin* showed diabetic activity (Kang *et al.,* 2000).

2.4.4 Anti-inflammatory Properties

A novel flavonol glycoside of *A. procera* stem showed moderate anti-inflammatory action on albino rats by using non-immunological carrageen an induced hind paw edema method. *A. lebbeck* seed ethanolic extract showed highest anti-inflammatory activity was observed at 200 mg/kg dose. The aqueous ethanol extract of *A. anthelmintica* showed moderate anti-inflammatory activity.

2.4.5 Antibacterial Properties

The bark of *Albizia lebbeck* and its extract showed antimicrobial activity. Novel macrocyclic alkaloids isolated from *A. amara.* They were also found to have antiplatelets aggregation and bactericidal activity (Yadava and Tripathi, 2000). A new biologically active flavonol glycoside from the seeds of *A. julibrissin* was fairly active against gram positive and gram negative bacteria. The extracts of *A. ferruginea* were also reported to have significant anti-microbial activity on selected microorganisms.

2.4.6 Hepatoprotective effect

A. procera, *A. lebbeck*, *A. inopinata* and *A. amara*, seem to exhibit potent hepatoprotective activity along with various pharmacological activities such as CNS activity, cardiotonic activity, lipid-lowering activity, antioxidant activity, hypoglycemic activity etc. (Mar *et al.*, 1991).

CHAPTER THREE

3.0 Materials and Methods

3.1 Materials,Equipment, Solvents, Reagents/Solutions

3.1.1 Reagents and Solvents

Acetic acid.

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

Applied BioSystems®3130 Genetic analyser (Thermo Scientific)

Big Dye terminator cycle sequencing kit (Thermo Scientific. cat. No. 4336935).

Chloral hydrate.

DNeasy plant mini kit (Qiagen, Cat No 69106).

dNTPs (Thermo Scientific).

EDTA solution.

Ethanol (JHD, AR; Lobal Chem, India).

Ferric chloride.

Formalin.

Glycerol.

Hi-Di™ Formamide buffer (Applied Biosystems™ Cat No. 4311320).

Hydrochloric acid.

Invitrogen 1kb plus ladder (GEHealthcare).

Methylene blue.

$MgCl_2$.

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

PCR buffer.

PCR Purification Kit (QIAquick, cat. No.28106).

Phloroglucinol.

Primer (rbcLaF & rbcLaR).

Safranin.

Sodium Hypochlorite.

Taq polymerase (Thermo Scientific).

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

3.1.2 Equipments

Ash less filter paper.

Autoclave.

Bead-mill (Retsch®MM 300).

Centrifuges.

Compound microscope (Fisher Scientific, UK).

Desiccator.

Freezer,

gel Electrophoresis.

Genogrinder (SPEX inc 2000).

Glass Slides and Cover slips.

Homogenizer (Ultra-Turrax™).

KERN EW Electronic Balanced.

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

Magnetic heater/stirrer.

Mechanical shaker (Stuart Storage containers, Scientific Flask Shaker, Great Britain).

Microfuge tubes.

Micropipettes.

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

Microwave oven.

NanoDrop®2000 Spectrophotometer (Thermo Scientific Inc.).

Applied Biosystems GeneAmp® PCR System 9700 (Thermo Scientific Inc).

Photographic camera.

QIAcube & Qiagen (Cat No 69106).

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

Thermal cycler.

Thermometers.

UV-transparent trays.

Vortex mixer.

Water bath (HHS, Mc Donald Scientific International).

Whatman 21 filter paper.

3.2 Pharmacognostic Studies of *A. chevalieri* Leaf

3.2.1 Collection and Identification of *A. chevalieri*.

The leaf of *A. chevalieri* were collected from Kufena village, Zaria Local Government Area of Kaduna state, Nigeria. The plant was identified at the Herbarium unit, Biological Sciences Department, Ahmadu Bello University, Zaria. As compared with a voucher specimen number (900247) available in the herbarium.

3.2.2 Processing of Plant material

The plant material were air –dried thoroughly under shade (at room temperature), grinded into powdered and stored in air-tight container for further analysis.

3.2.3 Macroscopical Examination of the Leaf of *A. chevalieri*

The general feature of the fresh leaf of the plant was studied. The size (length and width) of the lamina were measured with a ruler. The shape, composition, venation, type of the margin, apex and base of the lamina was observed and noted. Organoleptic character (odor, taste. color and texture) of both fresh and powered leaf sample was also determined using standard methods (Evans, 2009).

3.2.4 Microscopical Examination of Leaf of *A. chevalieri*

3.2.4.1 Anatomical Section/Surface Preparations

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

Fixation: the fresh leaf of *A. chevalieri* 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.

Temporary slide preparation. Fresh sample of the leaves were detached into petri dish, and hypochlorite solution was added enough to cover the surface and left for leaf complete bleached. Microscopy was accrued out on the upper and lower leaf surfaces; and transection of the leaf across the midrib. This was then observed under the microscope and appropriate images were taken and documented.

Permanent slide preparation: Dehydration of the fresh leaf 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 (25:75), 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 prepared sample was then transferred on to slides.

Staining: The sections 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. It 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 section and cover slip was placed (Ahlam and Bouran 2011).

3.2.4.2 Qualitative Microscopy of Leaf of A. chevalieri

The stomata number, stomata index, veinlet termination number and palisade ratio of fresh leaf were studied as described by Evans (2009). Measurement of dimension (length and width) of the various diagnostic microscope characters of the plant. It was carried out by using a binocular microscope with the aid of graticles (Kokate, 2008).

3.2.4.3 Chemomicroscopical Studies of leaf of A. chevalieri

This was carried out on the powdered leaf of the plant. Small amount of the finely ground powdered leaf of the plant was cleared in a test-tube containing 70% chloral hydrate solution.

It was boiled on a water-bath for 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 (Evans 2009).

(i) Test for Cellulose: Two drops of iodinated zinc chloride were added to the cleared sample on a slide, and this was allowed to stand for 2 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.

***(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 hydrochloric 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.

***(iv)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.

***(v) 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 leaf indicated the presence of calcium oxalates.

(vi)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 leaf indicated the presence of tannins.

(vii)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.

3.3 Physicochemical Parameters of the Powdered Leaf of *A. chevalieri*

Plant powdered material of *A. chevalieri* leaf was be subjected for determination of physicochemical parameters such as moisture content, Ash-values, extractive values determination procedure described per WHO 2011.

3.3.1 Determination of Moisture Content.

3 g of powdered leaf of *A. chevalieri* was accurately weighed in moisture disc. For estimation of loss in drying, it will be dried at 105^0c for 3 hours in an oven, cooled in a desiccator for 30 minutes and weighed without delay. The loss of weight was calculated as the content in mg per g of air-dried material.

$$\% \text{ Moisture content} = \frac{Initial\ Weight\ of\ Powder - Final\ Weight\ of\ Powder}{Initial\ Weight\ of\ Powder} X\ 100$$

3.3.2 Determination of Total ash.

2 g of powdered leaf of *A. chevalieri* was accurately weighed and place in tarred crucible. The material was ignited (350^0c for 1 hour) gradually until was white, indicating the absence of carbon. Cooled in a desiccator and weighed. Total ash content was calculated in mg per g of air dried material.

$$\% \text{ Total Ash Value} = \frac{Weight\ of\ Residual\ Ash}{Original\ Weight\ of\ Powder} X\ 100$$

3.3.2.1 Determination of Acid-insoluble ash.

25 ml of hydrochloric acid was added to the crucible containing total ash, covered with a watch glass and boiled gently for 5 minute. 5 ml of hot water and the liquid were added to

crucible. The insoluble matter was collected on an ash less filter-paper (Whatmann 21) and washed with hot water until the filtrate is neutral. The filter-paper containing the insoluble matter was transferred to the original crucible. Ignited gradually to constant weight. Residue was allowed to cool in a desiccators for 30 minutes and weigh a gain. Ash-insoluble ash content was then calculated as mg per g of air dried material.

$$\% \text{ Acid insoluble Ash} = \frac{\text{Weight of Residual Ash}}{\text{Original Weight of Powder}} X\ 100$$

3.3.2.2 Determination of water-soluble Ash.

25 ml of water was added to the crucible containing total ash, covered with a watch glass and boiled gently for 5 minutes. Insoluble matter was collected on ash less filter-paper. It was washed with hot water and ignited in a crucible for 5 minutes at a temperature not exceeding 45^0c in a muffle furnace. Residue was allowed to cool in a suitable desiccator for 30 minutes, and then weighed without delay. The weight of the residue was subtracted in mg from the weight of total ash.

$$\% \text{ Water Soluble Ash} = \frac{\text{Weight of Total Ash} - \text{Weight of Residual Ash}}{\text{Original Weight of total ash}} X\ 100$$

3.3.3 Extractive Values

Determinations of water and ethanol soluble extractives were determined by using a common method as follows:

3.3.3.1 Water Extractives values

Exactly 4 g of air-dried leaf powder was weighed into a 250 ml glass stoppered conical flask and 100 ml of water was added to macerate the powder for 6 hours with frequent shaking by using mechanical shaker and was allowed to stand for 18 hours. It was then filtered rapidly and 25 ml of filtrate was transferred into a previously dried and weighed evaporating dish and evaporated to dryness on a hot water bath. This was further dried in the oven at 105^0C for 6 hours, cooled in a desiccator for 30 minutes and then weighed without delay. The percentage water extractive value was calculated using the following formula:

$$\%\ Water\ Extractive\ Value = \frac{Weight\ of\ Extract\ in\ 25ml\ X\ 4}{Original\ Weight\ of\ Powder}\ X\ 100$$

3.3.3.2 Alcohol Extractives value

The procedure above (subsection 3.3.3.1) was repeated with ethanol in place of water and the percentage ethanol extractive value was calculated using the following formula:

$$\%\ Ethanol\ Extractive\ Value = \frac{Weight\ of\ Extract\ in\ 25ml\ X\ 4}{Original\ Weight\ of\ Powder}\ X\ 100$$

3.4 DNA Fingerprint profile of *A. chevalieri*

The basic methodology of DNA profiling in plants involve first the extraction of DNA from plant cells, quantification and quality assessment of extracted DNA, PCR amplification and the further step are of DNA sequence. The important steps involved in DNA fingerprinting are shown in Fig I.

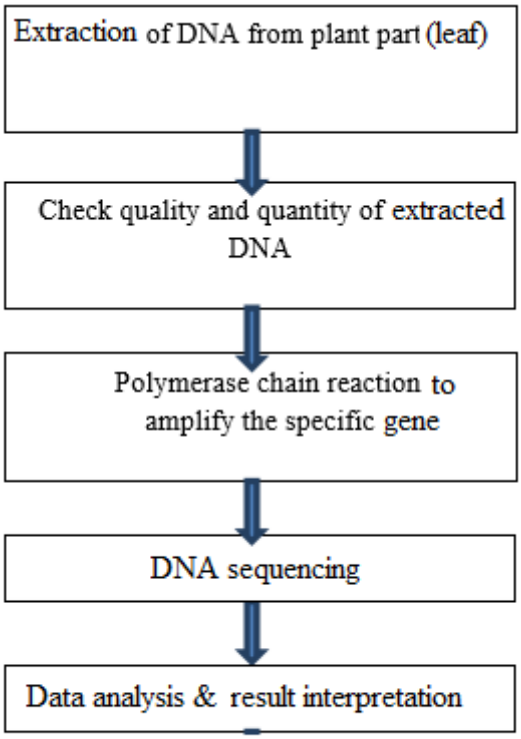


Figure 1: Flow Chart of DNA Profiling

3.4.1 DNA Extraction of *A. chevalieri*

DNA extraction from fresh leaf of *Albizia chevalieri* was carried out according to QIAGEN Genomic DNA Handbook (2001) procedure. Two hundred milligram (200 mg) each of lyophilized plant tissues were grinded using genogrinder and transferred into well labelled microcentrifuge tubes. Genomic DNA extraction was carried out using DNeasy plant mini kit (Qiagen) and an automated DNA extraction instrument (QIAcube, Qiagen) were used for

DNA isolation. The protocol was according to manufacturer manual. 400 µl of Buffer AP1 and 4 µl of RNase, a stock solution (100 mg/ml) were added to each tube containing the powdered tissue. The mixture was incubated for 10 min at 65°C and mixed 2–3 times during incubation by inverting tube. 130 µl of buffer AP2 was added to the lysate, mixed, and incubated for 5 min on ice. The lysate were centrifuged for 5 min at full speed and the supernatant was transferred to QIAshredder spin column sitting in a 2 ml collection tube and centrifuged for 2 min at maximum speed. The flow-through was transferred into labelled microcentrifuge tubes. 1.5 volumes of buffer AP3/E was added to the cleared lysate and mixed by pipetting. 650 µl of the mixture from above was pipetted to DNeasy mini spin column sitting in a 2 ml collection tube and centrifuged for 1 min at 6000 x g. The flow-through was then discarded and the spin columns placed back into the collection tubes. This step was repeated for the remaining solution. The DNeasy column were placed in new 2 ml collection tubes and 500 µl of buffer AW was added to the DNeasy column and centrifuged for 1 min at 6000 x g. The flow-through was discarded and 500 µl of buffer AW was added to the DNeasy column and centrifuged for 2 min at maximum speed to dry the membrane. The DNeasy column was transferred to 1.5 ml microcentrifuge tube and 200 µl of preheated (65°C) buffer AE was directly added onto the DNeasy membrane. It was Incubated for 5 min at room temperature and then centrifuged for 1 min at 6000 x g to elute DNA.

DNA quality and concentration were checked by running 5 µl diluted genomic DNA sample on 1 % agarose gel and 2 µl of genomic DNA on NanoDrop®2000 spectrophotometer. Isolated plant genomic DNA was stored at -80oC until use.

3.4.2 Polymearase Chain Reaction (PCR) Amplification

A set of primer, rbcLaF (5′ATGTCACCACAAACAGAGACTA3′) and rbcLaR (5′ GAAACGGTCTCTCCAACGCAT3′) was used in this study for the amplification of rbcL

gene (Kress and Erickson, 2007). PCR reaction were carried out in a total volume of 25 µl containing 100 ng of genomic DNA, 2.5 µl of 10× PCR buffer, 1 µl of 50 mM of $MgCl_2$, 2 µl of 2.5 mM dNTPs, 0.1 µl Taq polymerase, 1 µl DMSO, 1 µl each of forward and reverse primer and 11.3 µl of H_2O. Touch-down PCR was used for amplification as follows: initial denaturation step of 5 mins at 94°C, followed by 9 cycles each consisting of a denaturation step of 20 sec at 94°C, annealing step of 30 sec at 65˚c, and an extension step of 72°C for 45 sec, this is followed by another 30 cycles each consisting of a denaturation step of 20 sec at 94°C, annealing step of 30 sec at 55˚C, and an extension step of 72°C for 45sec. Amplification reactions was performed in a Applied Biosystems GeneAmp® PCR System 9700.

3.4.3 Agarose Gel Electrophoresis

To ascertain the extraction of genomic DNA and the amplified PCR product for the presence or absence of the band with their sizes. The eluent and PCR products resulting from the primer pair were subjected to agarose gel electrophoresis using 1kb plus ladder (Invitrogen) prepared as stated below:

One percent (1 %) gel was prepared by adding 0.3 g agarose to 30 ml of 1X TBE (Tris-Borate electrophoresis buffer) in a 250 ml beaker flask. The solution was heated in a microwave oven to dissolve the agarose for 50 seconds until agarose is dissolved. 5 µl of ethidium bromide (5 mg/ml, Ethidium bromide stains DNA by intercalating between the bases of DNA) was added to the dissolved agarose solution and mixed. Two dams are put into the slots on each side of the gel plate and fit tight. Melted agarose are then poured onto the gel plate in the agarose gel electrophoresis box and casting comb (well-maker) are then placed nearest to the black electrode (cathode) as the DNA migrates towards the red electrode (anode). The gel is allowed to cool at room temperature. The casting comb was carefully

removed after the gel had completely solidified. 250 ml of TBE (electrophoresis buffer) was then poured into the reservoir until the buffer covers the agarose gel. 8.5 µl of 1 kb plus ladder (0.5 µl), water (5 µl) and loading dye (3 µl) were mixed and added to the first well. 5 µl of sample plus 3 µl of the loading dye (50 mM EDTA, 0.2 % SDS, 50 % glycerol, 0.05 % w/v bromophenol blue) were mixed and carefully put into the other wells using pipette. The sample was electrophorese at 100 V for 30 minutes. At the completion of the electrophoresis, the gel was removed from the buffer (TBE) and the gel viewed under a trans-illuminator UV light of wavelength 302 nm. The band pattern of the DNA fragment was then photographed with a Polaroid camera and documented using an electrophoresis gel documentation system.

3.4.4 DNA Sequencing of *A. chevalieri*

The amplified product was first purified using manufacturer's protocol (QIAquick PCR Purification Kit). Sequence was determined directly using the dye-terminator sequence method adopted variant of Sanger sequence (Smith *et al.,* 1986) with a DNA sequencer (Applied Biosystems® 3130 xl Genetic Analyser) and a Big Dye terminator cycle sequencing kit, according to manufacturer's instructions. Unincorporated dye terminators were purified and precipitated using ethanol EDTA solution. The purified amplified product was diluted in water (2 µl of PCR x 6 µl H_2O) and mix with 2 µl of the Dye ready reaction termination and then run in thermal cycler. The pellets were then re-dissolved in Hi-Di™ formamide buffer then run on for 6 hrs.

3.4.5 Sequence Alignment and Data Analysis

BLAST searches were applied to the produced sequence using the available online databases. Sequences of *rbcL* that matched closely with the query sequences retrieved from DDBJ/EMBL/GenBank database. The sequences were aligned using CLUSTAL X (version 1.81) (Thompson *et al.,* 1997). Phylogenetic analyses were conducted in MEGA4 (Tamura et

al., 2007). Phylogenetic trees were constructed using maximum Likelihood (Tamura and Nei, 1993), and Neighbor Joining (Saitou and Nei, 1987) methods.

3.5 Statistic Analysis

Statistical analysis of the results obtained in each experiment were carried out by the use of the Ms. Excel 2007 statistical software and expressed as mean ± standard errors of the mean (SEM) for all values.

CHAPTER FOUR

4.0 Results

4.1 Pharmacognostic Studies of *A. chevalieri* Leaf

4.1.1 Macroscopical Examination of the Leaf of *Albizia chevalieri*

Albizia chevalieri leaf are compound paripinnate, glabrous, and elliptical in shape with entire margin. Apex of leaf is obtuse and petiolate base with equal. Reticulate venation with midrib/vein depressed as shown in Table 4.1. Leaf is greenish in color with characteristics taste and odourless. Mature leaves are 0.7 - 0.9 x 0.3 - 0.5 cm with smooth texture as per Table 4.2.

Plate II: Leaf of *Albizia chevalieri* showing arrangement and size

Table 4.1: Macromorphological Characters of *A. chevalieri* leaf

CHARACTERS	OBSERVATIONS
Surface appearance	Glabrous
Shape	Elliptic
Margin	Entire
Lamina	Compound paripinnate
Apex	Obtuse
Base	Petiolate equal
Venation	Reticulate

Table 4.2: Organoleptic Characters of *A. chevalieri* leaf

CHARACTERS	OBSERVATIONS
Colour	Green
Size(cm)	0.7 - 0.9 x 0.3 - 0.5 cm (mature leaves)
Odour	Odorless
Taste	Characteristics

Texture	Smooth
Fracture	Soft

4.1.2 Microscopical Examination of the Leaf of *Albizia chevalieri*

Microscopical examination of the leaf of *A. chevalieri* revealed the presence of some important diagnostic characters such as wavy or irregular epidermal cells with anticlinal walls on both adaxial (upper) and abaxial (lower) epidermal layers. The presence of paracytic stomata or rubiaceous (irreular-celled) types with two subsidiary cells with their long axes parallel to the pore of stomata (2.31 ± 0.10 x 1.19 ± 0.08 µm) present in only abaxial (lower) epidermal layers. Lignified hair trichomes were 26.11 ± 2.12 x 1.00 ± 0.00 µm in diameter present on both adaxial (upper) and abaxial (lower) epidermal layers. The calcium oxalate crystals found are the prisms type (1.32 ± 0.13 x 4.1 ± 0.71 µm) along the veins as shown in Table 4.3 and plate III - VI

The transverse section of the leaf through the midrib tissue was examined and revealed different anatomical features namely adaxial (upper) and abaxial (lower) epidermis, mesophyll cells and vascular bundle with fibers.

Table 4.3: Microscopical Characters of *A. chevalieri* leaf

PARAMETERS	RESULTS	OBSERVATIONS
Epidermal cells	Shape	Wavy or irregular
Stomata	Type	Paracytic type
	Position	Abaxial (lower) epidermis
	Frequency	Numerous
	Size	2.31 ± 0.10 x 1.19 ± 0.08 µm
Trichomes	Type	Lignified trichomes
	Position	Adaxial (upper) and abaxial (lower) epidermis
	Frequency	few
	Size	26.11 ± 2.12 x 1.00 ± 0.00 µm
Calcium oxalate crystals	Type	Prism crystals
	Position	Along the veins
	Frequency	Numerous
	Size	1.12 ± 0.13 x 0.99 ± 0.10 µm

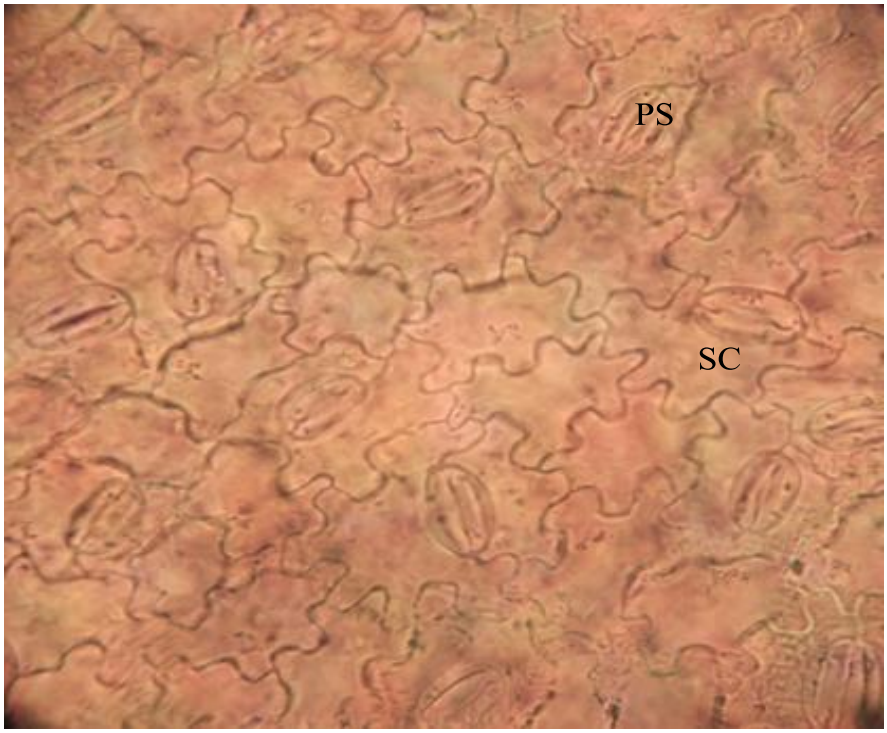


Plate III: Lower Epidermal Layer of *A. chevalieri* Leaf (x400); PS- paracytic stomata, SC- subsidiary cell

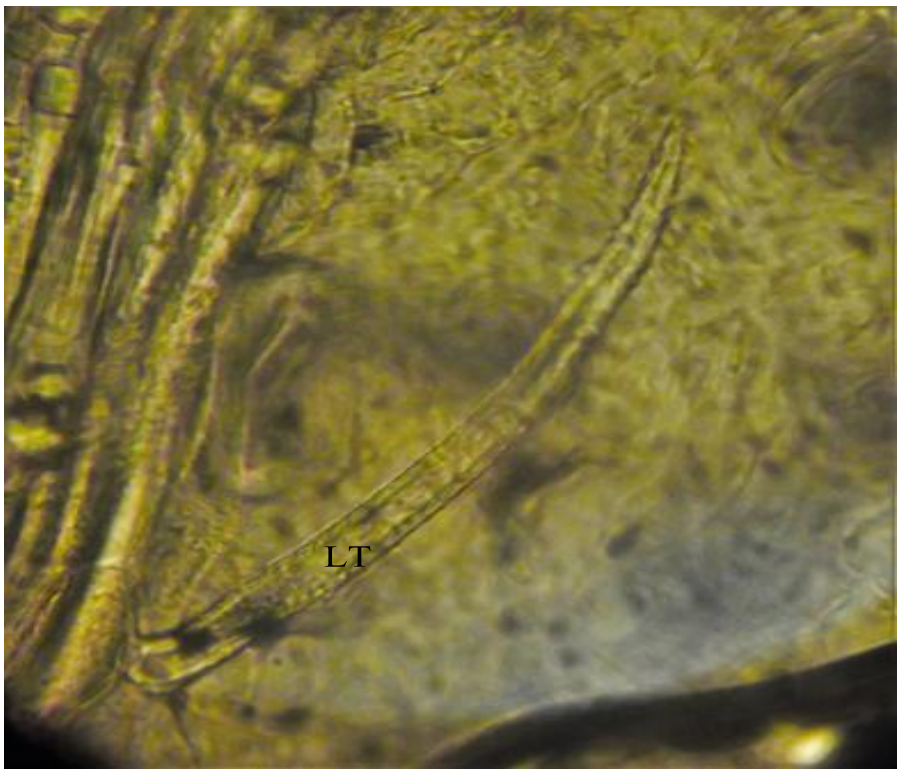


Plate IV: Upper Epidermal Layer of *A. chevalieri* Leaf showing lignified trichome (x400); LT- lignified trichome

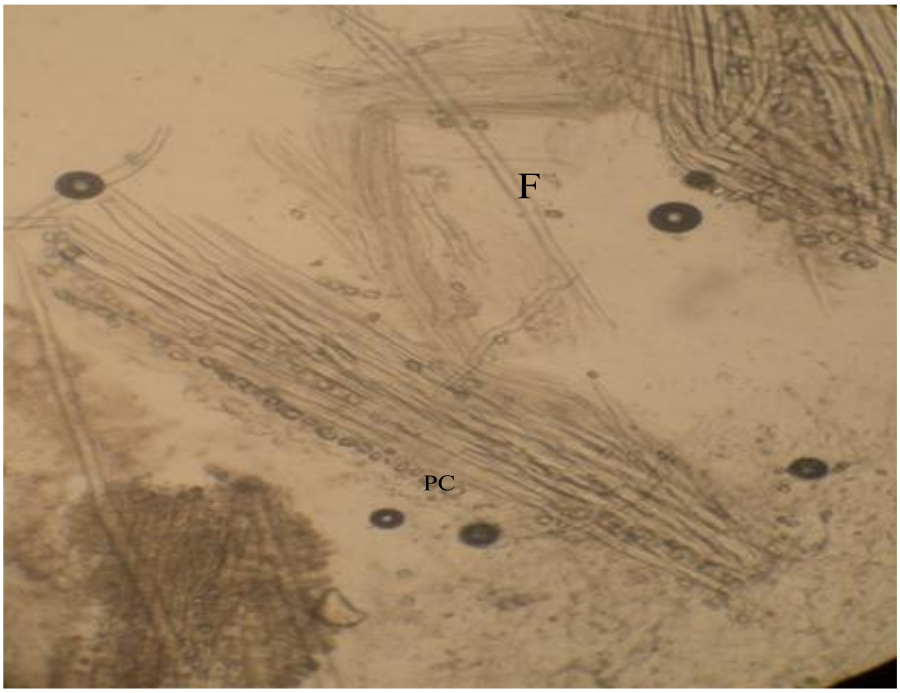
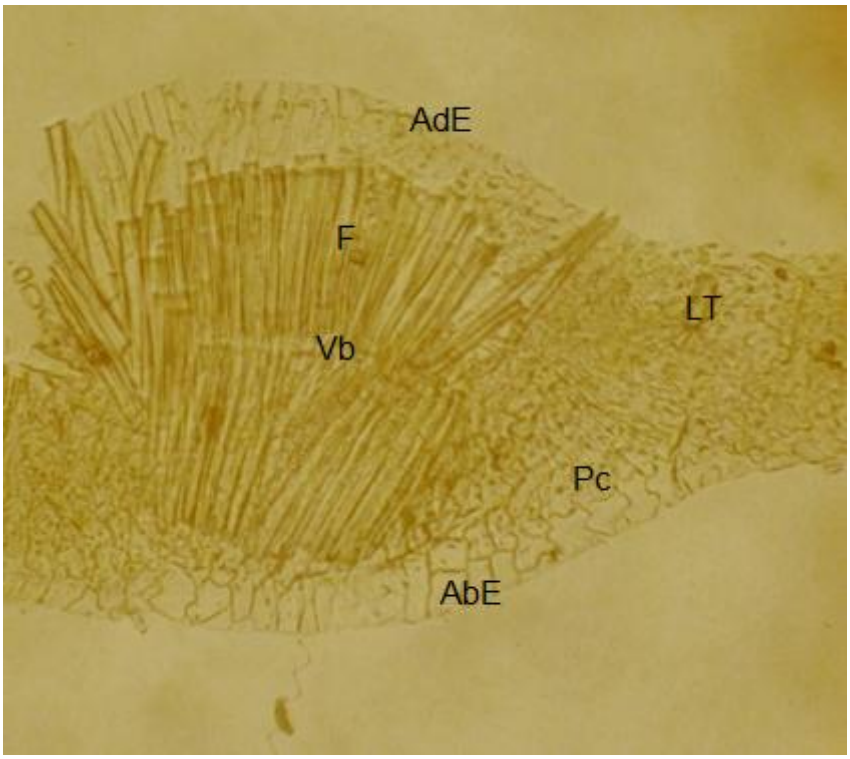


Plate V: Powdered microscopy of *A. chevalieri* Leaf showing some Features (x100); F- fibre, Pc- prism crystals.



`Plate VI: Transverse Section of the Leaf of *A. chevalieri* showing some Features (×400); AdE- adexial epidermis, F- fibre, LT- lignified trichome, VB- vascular bundle, Pc- palisade cell, AbE- abaxial epidermis.

4.1.3 Qualitative Microscopical Values for the Leaf of *A. chevalieri*

On the average, stomatal number (22.33), and index (21.54), palisade ratio (17.25), vein termination numbers (6.65) and vein islets (5.00) were determined and recorded (Table 4.4).

Table 4.4: Qualitative Microscopical Values for the Leaf of *A. chevalieri*

Evaluative Parameter	**Values***
Stomatal number	18.98 - **22.33** - 25.68
Stomatal index	18.31 - **21.54** - 24.77
Palisade ratio	14.66 - **17.25** - 19.84
Veinlet termination number	5.67 - **6.67** - 7.67
Vein islet number	4.25 - **5.00** - 5.75

***** Average Value of 5 count

4.1.4 Chemomicroscopical Examination of the powdered Leaf *A. chevalieri*

Chemomicroscopical examination of the powdered leaf of *A. chevalieri* revealed the presence of cellulose cell wall, lignified cell wall, tannins, starch, calcium oxalate prisms like, and suberin or cutin while calcium carbonate was found to be absent (Table 4.5)

Table 4.5: Chemomicroscopical Features of *A. chevalieri* Powdered Leaf.

Constituents	Detecting reagents	Observation	Inference
Starch	N/50 iodine	Blue-black colour on grains within the cell.	Starch present
Lignin	Phloroglucinol	Red-pink colour on the walls of lignified cell.	Lignin present
Tannins	5 % $FeCl_3$	Greenish-black colour in some parenchyma cells.	Tannins present
Calcium oxalate	HCl	Dissolution of shining crystals on the anatomical sections of the leaf.	Crystal present (Prism type)
Calcium carbonate	HCl	No Effervescence in the cell.	$CaCO_3$ absent
Cellulose	Chlor-Zinc- Iodine	Blue coloration of the cell wall	Cellulose present
Suberin	Sudan red	Orange red colour on cell wall	Suberin present

4.1.5 Some Physicochemical Parameters of Leaf of *Albizia chevalieri*

The results of the physicochemical of the leaf of *Albizia chevalieri* is presented below and also in Table 4.6

Table 4.6: Physicochemical Parameters of plant leaf of *Albizia chevalieri*

Parameter	**Value obtained ± SED (%w/w)**
Moisture contents	9.11 ± 0.29
Total Ash value	4.83 ± 0.33
Water soluble ash	1.75 ± 0.25
Acid insoluble ash	1.50 ± 0.00
Water soluble extractive value	15.67 ± 0.88
Alcohol soluble extractive value	21.00 ± 1.00

*Values are expressed as mean% ± SEM. n= 3

Key: SEM= standard error of mean

4.2 DNA Fingerprint Profile of *A. chevalieri*

4.2.1 DNA Extraction from Leaf *A. chevalieri*

DNA was extracted from *Albizia chevalieri* leaf, quality and concentration of the extracted DNA were obtained using gel electrophoresis and NanoDrop spectrophotometer. Table 4.7 showed the concentration and quality of the extracted genomic DNA. Plate VII depicts the size of the extracted genomic DNA.

Table 4.7: NanoDrop of extracted genomic DNA from *A. chevalieri* Leaf

Nucleic Acid (ng/µl)	A260 (Abs)	A280 (Abs)	260/280	260/230	Factor
161.1	3.222	1.931	1.67	1.92	50

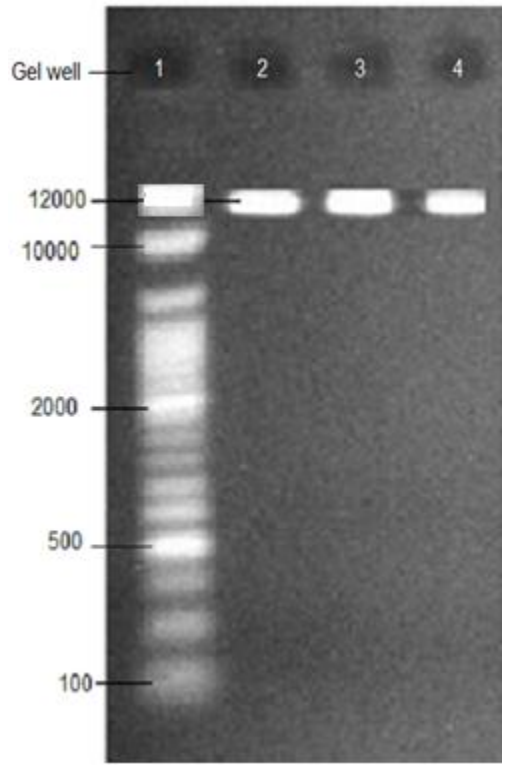


Plate VII: Electrophoresis of extracted genomic DNA from *A. chevalieri*. Lane 1: 1kb plus DNA ladder; Lane 2, 3, 4: genomic DNA with molecular weight (12000bp)

4.2.2 PCR Amplification of *A. chevalieri*

Plate VIII depicts the size of amplified rbcL gene of the plant sample on gel electrophoresis of forward and reverse *rbcL* primers.

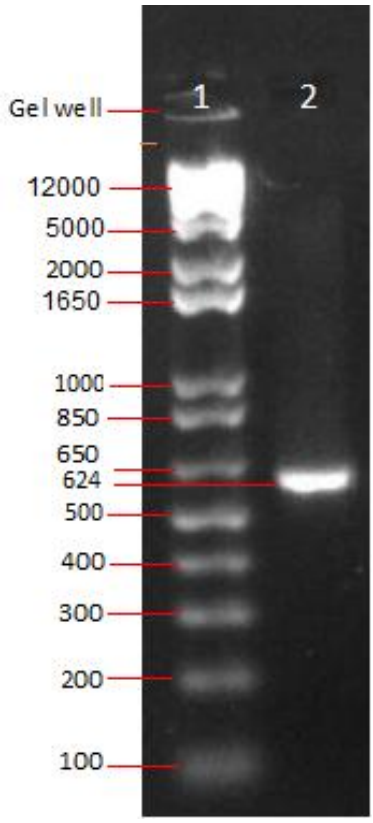


Plate VIII: Electrophoresis of PCR products of amplified rbcl gene from *A. chevalieri*. Lane 1: 1kb plus DNA ladder; Lane 2: amplified product with molecular weight (624bp)

4.2.3 DNA Sequence of *A. chevalieri*

Chromatogram of the sequence amplified rbcL gene (Figure II) showed the order of nucleotide sequence and the quality of the sequence. The length of the analysed rbcL sequences with forward primer is about 616 bp and 559 bp with reverse primer

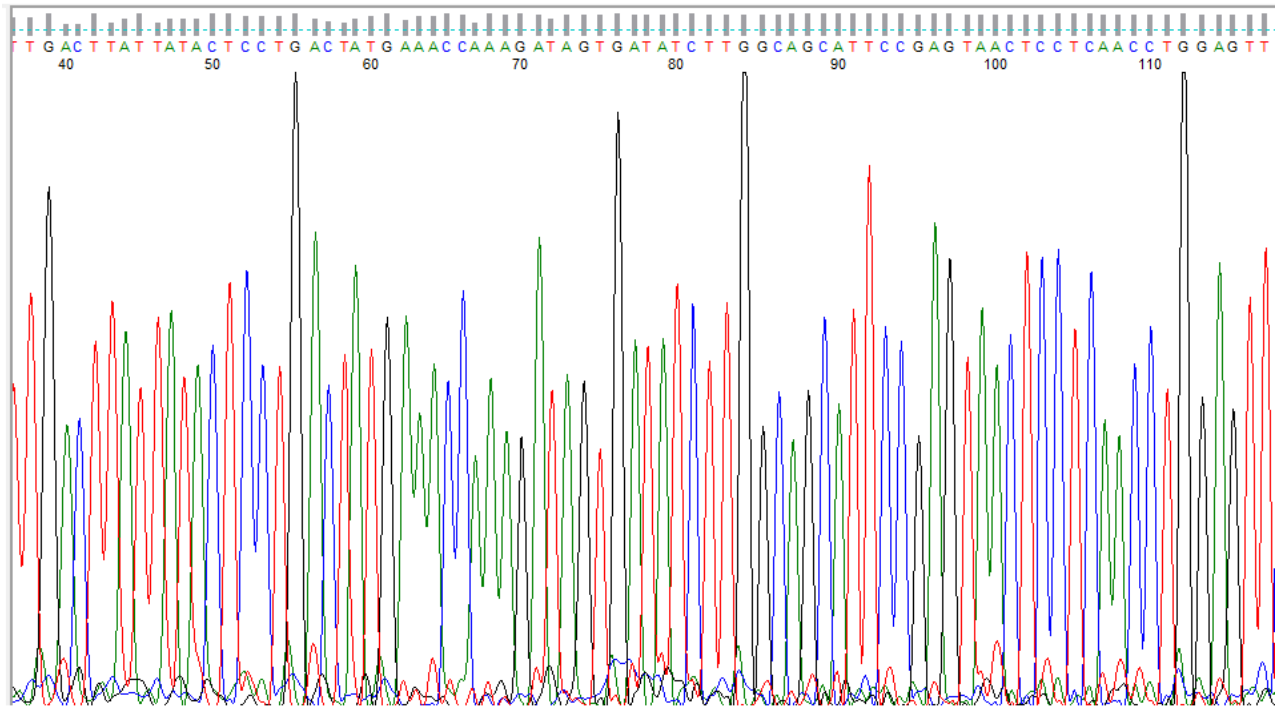


Figure II: Portion of chromatogram of sequences DNA from *A. chevalieri*

TTGACTTATTATACTCCTGACTATGAAACCAAAGATAGTGATATCTTGGCAGCAT
TCCGAGTAACTCCTCAACCTGGAGTTCCGCCTGAAGAAGCAGGTGCCGCGGTAG
CTGCTGAATCTTCTACCGGTACATGGACAGCTGTGTGGACCGATGGGCTTACCAG
TCTTGATCGTTACAAAGGACGATGCTACCACATCGAGCCCGTTGCTGGAGAAGA
AAGTCAATTTATTGCTTATGTAGCTTATCCCTTAGACCTTTTTGAAGAAGGTTCTG
TTACTAACATGTTTACTTCGATTGTGGGTAATGTATTTGGGTTCAAGGCCCTGCGC
GCTCTACGTCTGGAAGATTTGCGAATCCCCCCTTCTTATTCTAAAACTTTCCAAGG
TCCGCCTCACGGCATCCAAGTTGAGAGAGATAAATTGAACAAGTACGGCCGTCC
CCTATTGGGATGTACTATTAAACCAAAATTGGGGTTATCCGCGAAGAATTACGGT
AGAGCGGTTTATGAATGTCTCCGTGGTGGACTTGATTTTACCAAAGATGATGAGA
ATGTGAATTCCCAACCATTTATGCGTGGGA

Figure III: DNA sequence data found in chromatogram of *A. chevalieri*

***keys: T-Thymine, A-Adenine, G-Guanine, C-Cytosine**

4.2.4 Sequence Alignment & Data analysis:

Conducted BLAST database-search determined the approximate identification and related taxa of the studied specimen. Percent similarity was recorded for the closest matches (graphic presentation) (Figure IV). Dendrogram shows the position of *A. chevalieri* and similarity with order genera in figure V.

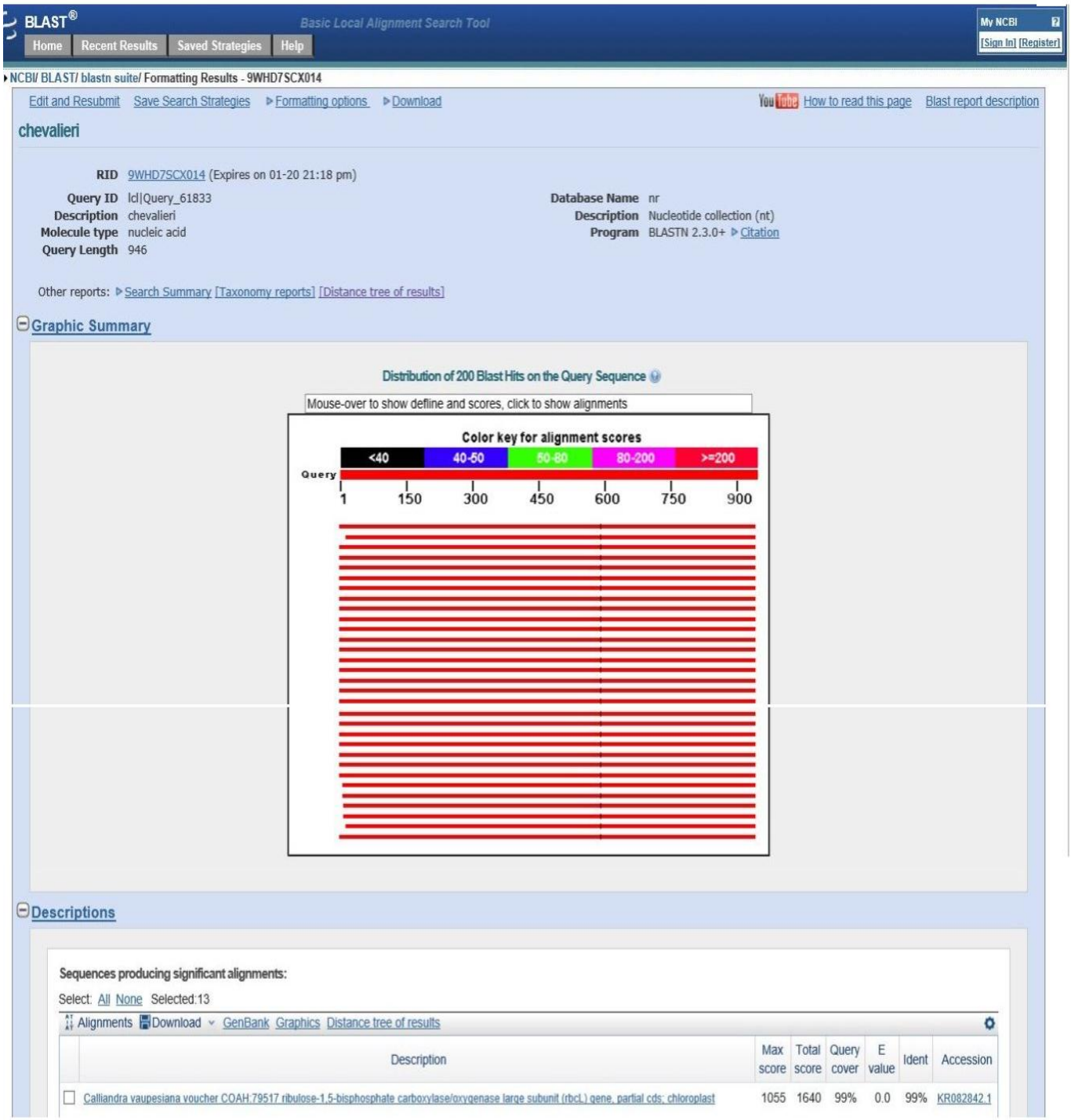


Figure IV: BLAST search of DNA sequence of *A. chevalieri*

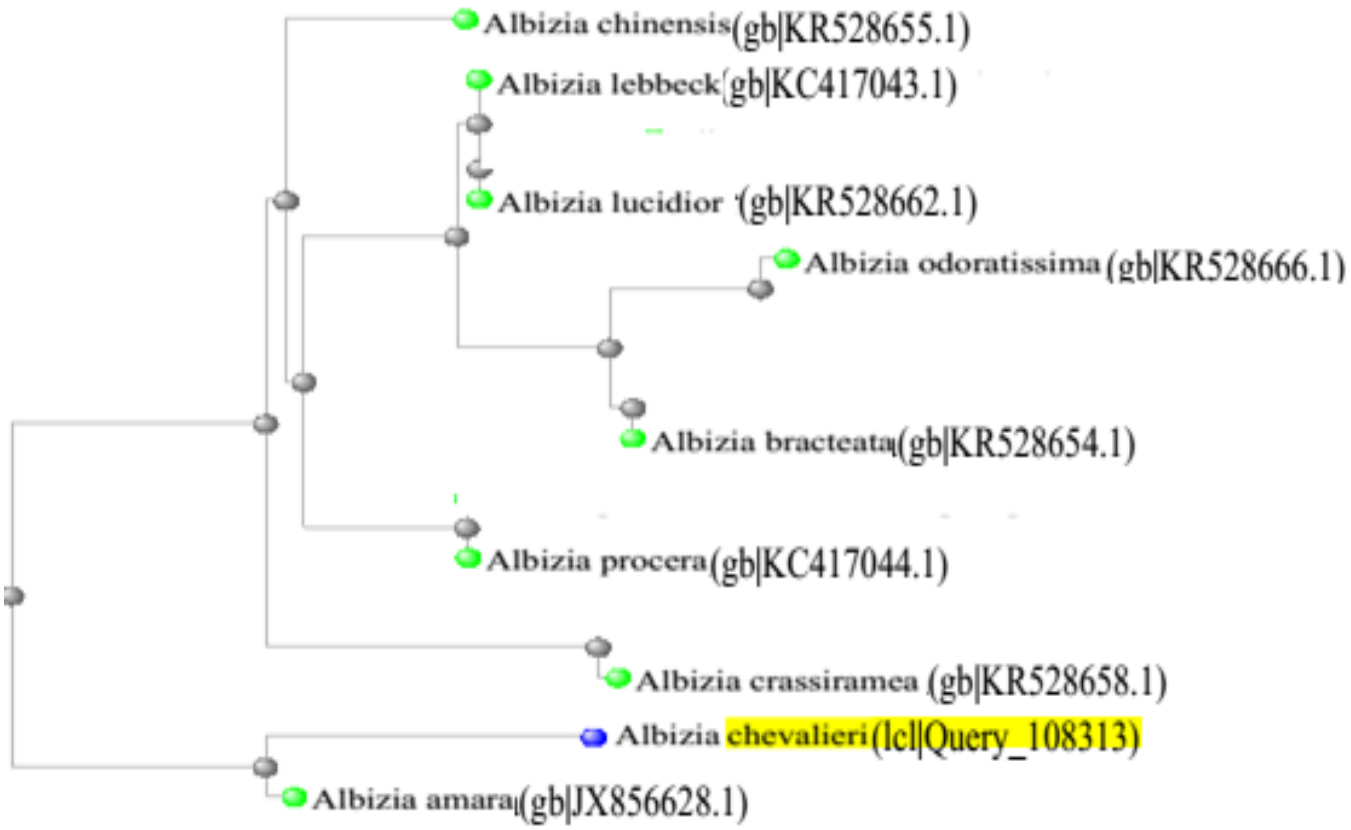


Figure V: The Dendrogram showing the relationship of *A. chevalieri* with the related genera. Query numbers of the corresponding taxa are written in parentheses with a p-distance of 0.0003

CHAPTER FIVE

5.0 Discussion

In this report, various macroscopical, microscopical, chemomicroscopy, physicochemical standards and DNA sequencing have been developed, that will help for proper identification and standardization of *A. chevalieri.*

One of the simplest and fastest methods to establish the accurate identity of plant material is DNA sequencing (Anonymous, 2015) and also cheapest method is microscopy analysis (Singh *et al.,* 2010). Microscopically, the leaf was observed to be hypostomatic distribution with numerous paracytic type of stomata, with lignified trichomes on the abaxial (lower) surface only. The epidermal cells are wavy or irregular in shape on both surface with straight anticlinal walls. It also reveals the presence of calcium oxalate which are prism in nature along the veins. Study of transverse section of the leaf across the midrib showed hypostomatic leaf arrangement with palisade cells and fibre found at the vascular bundle. The occurrence of the above mentioned characteristic features was observed among members of the Fabaceae (Rahul *et al.,* 2010). Anatomical features of the internal structures of plant drugs provides an important diagnostic features for the identification of fresh and powdered crude drugs and detection of adulterants in plant materials (Ghani, 1990). Determination of qualitative physical leaf constants is of great value in the identification of crude drugs. The average for stomatal number, stomatal index, veinlet termination number and vein islets number determined.

The stomatal number for the abaxial (lower) epidermis of the leaf was found to be (22.33). 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 (Sunita, *et al.,*

2010). Evans stated that early investigation by Timmerman indicated that stomatal numbers are useless in distinguishing between closely allied species (Evans, 2009).

The stomatal index of lower surface was found to be (21.54). The stomatal index is 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 (Sunita, *et al.,* 2010). The vein islets number (5.00) and veinlet termination number (6.65) 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 (17.25) 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 features of powdered leaf of *A. chevalieri* revealed the presence of cellulose cell wall, lignified tissues, mucilage, tannins, starch, suberin and calcium oxalate crystals while calcium carbonates was found to be absent, this result is in agreement with the finding of Agboola and coworkers who detected the presence of calcium oxalate crystals on the leaf of *A. altissimum*, there were prismatic and located along the veins (Agboola *et al*., 2012). The microscopic structures are most valuable in the identification of powdered drug as their identification is largely based on the form, the presence or absence of certain cell types and cell inclusions (Eggeling *et al*., 2000).

Physico-chemical parameters such as moisture content/loss on drying, total ash content, water soluble ash content, acid insoluble ash content and extractable matter content serve an important role in standardization and quality control by means of purity, stability and

phytochemical composition of plant drugs (Bharat and Parabia, 2010). The moisture content in the *A. chevalieri* powdered leaf was found to be 9.11%. The general requirement of moisture content in crude drug is that, it should not be greater than 14% (*British Herbal Pharmacopeia*, 1990) and the value observed in this research work was within the accepted range. Determination of the moisture content helps prevent degradation of drug during storage. The lower the value, the less likelihood of degradation of drug and suggests better stability of product. Moisture is considered an adulterant because of its added weight as well as the fact that excess of it promotes mould and bacterial growth (WHO, 2011). Ash values are used to determine purity and quality of crude drug. It indicates the presence of various impurities such as carbonate, oxalate and silicate. The water soluble ash (1.75%) contains mainly silica, particularly in sand and it indicates contamination with earthy material. The acid insoluble ash value obtained in this study was 1.5%. The total Ash value (4.83%) represents both the physiological and non-physiological ash from the plant. The non-physiological ash is an indication of inorganic residue after the plant drug is incinerated. Total ash value is a reliable aid for detecting adulteration in drugs (WHO, 1996). Extractive values are useful to estimate the chemical constituents present in the crude drug and are a measure to determine the solubility of phytoconstituents from the crude drug in a given solvent (Thomas*et et al.*, 2008). This study showed that ethanol had high extractive value of 21.00 %w/w compared to water which had extractive value of 15.61 % w/w.

Genomic DNA was extracted successfully and the quality and concentration of extracted genomic DNA was verified. The PCR product of *rbcL* was sequenced successfully using forward and reverse primers. The electrophoresis of the PCR product (amplicon) of amplified *rbcl* region from *A. chevalieri* reveals a bond with no shearing or contamination and molecular weight of about 624 bp.

On the basis of *rbcl region* analysed sequence alignment (BLAST) database-search of the plant *A. chevalieri* with Query id (lcl|Query_108313) in order to determine the approximate identification and related taxa, search showed 98-99% sequence similarities with multiple plant species (*Albizia amara,* gb|JX856628.1; *Albizia bracteata,* gb|KR528654.1; *Albizia lucidior,* gb|KR528662.1. etc) and 99% sequence similarity with *A. amara* retrieved from the related sequences from the GenBank database and to determine the phylogenetic position of the species. Here the correct identification means that the highest BLAST % identity of the query sequence was from the expected species or the species belonging to the expected genera; ambiguous identification means that the highest BLAST % identity for a query sequence was found to match several genera of the expected family; incorrect identification means that the highest BLAST % identity of the query sequence was not from the expected species/expected genera/expected family (Arif *et al.,* 2010). All the species that were inferred from partial *rbcL* gene sequence of the studied specimen and related taxa demonstrated a distinct lineage of the studied specimen.

In our study of *Albizia chevalieri*, we found that *rbcL* could be amplified using a set of primers reported by kress and Erickson, 2007. This therefore could be used to distinguish between closely related genera or species and clearly delineate their medicinal use and appropriate selection of the right species for optimum and desired medicinal efficacy (Zabta *et al.,* 2014).

CHAPTER SIX

6.0 Summary, Conclusion and Recommendation

6.1 Summary

Pharmacognostic studies of the leaves of *Albizia chevalieri* revealed that it contain; paracytic stomata located on the lower epidermis of the leaves, lignified trichomes and calcium oxalate are found on the upper and lower epidermis and wavy or irregular epidermal cells with straight anticlinal walls. The physical constant revealed an alcohol soluble extractive value of 21.00 %, moisture content of 9.11 %, total ash value of 4.83 %, water soluble ash value of 1.75 % and alcohol soluble ash value of 1.5 %. The plant contains cellulose, lignin, starch, tannins, proteins, mucilages and calcium oxalate. These findings are useful information in preparation of the plant monograph.

The DNA sequence reveals length of about 624 bp of forward and reverse reaction. The *A. chevalieri* with Query id (lcl|Query_108313) BLAST showed, a 98-99% sequence similarities hit with multiple plant species (*Albizia amara,* gb|JX856628.1; *Albizia bracteata,* gb|KR528654.1; *Albizia lucidior,* gb|KR528662.1. etc) and the position of *A. chevalieri* in dendrogram with a p-distance of 0.0003 of the neighbouring joining tree. The molecular phylogeny of *A. chevalieri* showed closest similarity with *A. amara.*

6.2 Conclusion

This study pharmacognostic (macroscopy. microscopy, chemomicroscopy and physicochemical constant) features and DNA fingerprint of the leaf of *A. chevalieri* has provided information which are useful in preparation of the monograph of the plant. Genomic fingerprinting which can differentiate between individuals, species and populations in characterization of sample homogeneity and detection of adulterants. It is a work that offers powerful new tools and entry points for measures aimed at quality control and quality assurance in medicinal plant research of herbal medicines.

On the basis of morphology and phylogeny the given plant belong to family Mimosoideae.

6.3 Recommendations

It is recommended that the information obtain from this research should be included in the Nigeria/African Pharmacopoeia which will assist in standardization of the plant as tool for quality control. Further research on how to use these DNA fingerprint profile to understand the principles governing the biosynthesis of secondary metabolites, provide scientific bases for high quality production, in plant breeding & conservation and to improve the image of herbal medicine.

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APPENDIX A

a). Determination of moisture content of powdered leaf of *A. chevalieri*

3g of the powdered plant material was used

Description	1	2	3
Constant weight of crucible (g)	51.61	44.6	46.73
Initial weight of crucible + powdered drug	54.61	47.60	46.73
weight of crucible + powdered drug (g)	54.32	47.33	49.47
Total Loss in weight (g)	0.29	0.27	0.26
Moisture content (%)	9.67	9	8.67
Average mean (%)		9.11	

Sample calculation

Total loss in weight = initial weight of crucible & powder – final weight of crucible & powdered drug

Total loss in weight = 54.61 - 54.32

= 0.29

$$\% \text{ Moisture content} = \frac{Initial\ Weight\ of\ Powder - Final\ Weight\ of\ Powder}{Initial\ Weight\ of\ Powder} X\ 100$$

$$\% \text{ Moisture content} = \frac{(50.18 - 49.92)}{3} X\ 100$$

= 0.26 %w/w

APPENDIX B

b). Determination of Ash Value of powdered leaf of *A. chevalieri*

Description	1	2	3
Constant weight of crucible (g)	50.69	39.21	38.30
Weight of powdered drug (g)	2	2	2
Weight of crucible and content (g)	52.69	41.21	40.30
Weight of crucible and Ash (g)	50.78	39.32	38.39
Weight of Ash (g)	0.09	0.11	0.09
Ash Value (%)	4.5	5.5	4.5
Average mean (%)		4.83	

Sample calculation

Weight of residue = final weight of crucible + residue – weight of crucible

Weight of Residue = 50.78 – 50.69

= 0.09

$$\text{Ash value} = \frac{\text{weight of Ash}}{\text{Initial weight of drug}} X\ 100$$

$$\text{Ash Value} = \frac{0.09}{2} X\ 100$$

= 4.5 %w/w

APPENDIX C

c) Determination of Acid insoluble Ash of powdered leaf of *A. chevalieri*

Description	1	2	3
Constant weight of crucible (g)	50.69	39.21	39.63
Weight of crucible and residue ash (g)	50.72	39.24	39.66
Weight of residue ash (g)	0.03	0.03	0. 03
Acid Insoluble Ash Value (%)	1.5	1.5	1.5
Average mean (%)		1.5	

Sample calculation

Weight of residue = final weight of crucible + residue – weight of crucible

Weight of Residue = 50.72 – 50.69

= 0.03

Acid Insoluble Ash value = $\frac{\text{weight of residue ash}}{\text{Initial weight of drug}} X\ 100$

Acid Insoluble Ash value = $\frac{0.03}{2} X\ 100$

= 1.5 %w/w

APPENDIX D

d). Determination of water soluble Ash of powdered leaf of *A. chevalieri*

Description	1	2	3
Constant weight of crucible (g)	38.30	21.30	39.63
Weight of crucible and residue Ash (g)	38.36	21.35	39.90
Weight of residue Ash (g)	0.06	0.05	0.27
Weight of total ash value (g)	0.09	0.09	0.25
Water soluble Ash Value (%)	1.5	2.00	0.50
Average mean (%)		1.75	

Sample calculation

Weight of residue = final weight of crucible + residue – weight of crucible

Weight of Residue = 38.36 – 38.30

= 0.06

$$\text{Water soluble Ash value} = \frac{\text{Wt of total ash} - \text{Wt of Water Insoluble Ash}}{\text{Initial weight of drug}} X\ 100$$

$$\text{Water soluble Ash value} = \frac{(0.09 - 0.06)}{2} X\ 100$$

= 1.5 %w/w

APPENDIX E

e). Determination of water–soluble extractive value of leaf of *A. chevalieri*

4 g of the powder was used in 100 ml of water.

Description	1	2	3
Constant weight of dish (g)	144.73	84.28	64.80
Weight of dish and Residue (g)	144.90	84.42	64.96
Weight of Residue (g)	0.17	0.14	0.16
Water extractive Value (%)	17	14	16
Average mean (%)		15.67	

Sample calculation

Weight of residue = weight of dish & residue – weight of dish

Weight of Residue = 144.90 – 144.73

= 0.17

Water extractive value = $\frac{\text{Weight of residue (g) X 4}}{\text{Initial weight of drug}} X\ 100$

Water extractive value = $\frac{(017)\ \text{X}\ 4}{4} X\ 100$

= 17 %w/w

APPENDIX F

f). Determination of alcohol – soluble extractive value of leaf of *A. chevalieri*

4 g of the powdered was used in 100 ml of 90% ethanol

Description	1	2	3
Constant weight of dish (g)	64.86	84.28	64.86
Weight of dish and residue (g)	65.06	84.48	65.09
Weight of residue (g)	0.2	0.2	0.23
Alcohol extractive Value (%)	20	20	23
Average mean (%)		21	

Sample calculation

Weight of residue = weight of dish & residue – weight of dish

Weight of Residue = 65.06 – 64.86

= 0.2

Alcohol extractive value = $\frac{\text{Weight of Residue (g) X 4}}{\text{Initial weight of drug}} X\ 100$

Alcohol extractive value = $\frac{(0.2)\ \text{X}\ 4}{4}\ X\ 100$

= 14.0 %w/w