PHARMACOGNOSTIC, ANTIOXIDANTAND ANTIMICROBIAL STUDIES OF THE LEAVES OF *AMARANTHUS VIRIDIS* LINN.(AMARANTHACEAE)

By

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DEPARTMENT OF PHARMACOGNOSY AND DRUG DEVELOPMENT, FACULTY OF PHARMACEUTICAL SCIENCES, AHMADU BELLO UNIVERSITY,

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DECEMBER, 2015

DECLARATION

I declare that the work reported in this dissertation entitled Pharmacognostic, Antioxidant and Antimicrobial Studies of the Leaves of *Amaranthus viridis* Linn. (Amaranthaceae)**,**has been performed by me in the Department of Pharmacognosy and Drug Development, Ahmadu Bello University, Zaria- Nigeria. The information derived from the literature were duly acknowledged in the text and a list of references provided. No part of this dissertationwas previously presented for another degree or diploma at this or any other institution.

DanielChristopher KAGARU ---------- ----------

Name of Student Signature Date

CERTIFICATION

This dissertation entitled “PHARMACOGNOSTIC, ANTIOXIDANTAND ANTIMICROBIAL STUDIES OF THE LEAVES OF *AMARANTHUS VIRIDIS* LINN.(AMARANTHACEAE)” by Daniel Christopher KAGARU meets the regulations governing the award of the degree of Master of Science in Pharmacognosy of the Ahmadu Bello Universityand is approved for its contribution to knowledge and literary presentation.

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DEDICATION

This work is dedicated, firstly,to God Almighty, the reason for my being, and to my wife, Mrs. Veronica D. Kagaru, my children: Imubarsa Ruth-Florence Kagaru, Kima Gabrielle Kagaru and Reze Danielle Kagaru, and finally, to my mother, Mrs. Vou Angelina Musa and my siblings.

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ABSTRACT

Amaranthus viridis (Amaranthaceae), an annual herb, used in traditional medicine as diuretic and emollient, in poultices on inflammations, boils or abscesses, as well as in the treatment of gonorrhea, piles, fever, filaria, dysentery etc. This study focussed on the evaluation of various pharmacognostic parameters including microscopic, physicochemical and phytochemical properties of the leaves of this plant, as well as the antioxidant and antimicrobial properties. The 70% aqueous ethanol leaf extract was used for the antioxidant and antimicrobial studies. The microscopy of the leaves revealed the presence of slightly wavy-walled epidermal cells on the upper surface and larger wavy-walled epidermal cells on the lower surface, with both containing anisocytic stomata; uniseriate glandular trichomes with unicellular head on both surfaces; rosettes of calcium oxalate crystals within the cells. A transverse section through the midrib of the leaf revealed it to be a dorsiventral type and a round type of vascular bundles, which possess spiral and annular types of xylem vessels. Chemomicroscopically, the presence of lignins, tannins and starch grains at different specific locations within the leaf; and the absence of fats and oils are worthy of note. The physicochemical properties were determined as follows: moisture content of 12.19 ± 0.06% w/w; total ash value of 19.92 ± 0.10% w/w; acid-insoluble ash value of 1.07 ± 0.08% w/w; water-soluble ash value of 5.97 ± 0.06% w/w; ethanol – soluble extractive value of 1.71 ± 0.26% w/w and water – soluble extractive value of 2.7 ± 0.36% w/w. Phytochemical screening of the 70% aqueous ethanol leaf extract of the plant revealed the presence of alkaloids, saponins, tannins, flavonoids, steroids and cardiac glycosides. The evaluation of the antioxidant activity by the free radical scavenging effect on DPPH showed that the extract has significant ($P < 0.05$) antioxidant activity which is comparable to that of ascorbic acid. The aqueous ethanol leaf extract of the plant showed significant ($P < 0.05$)

broad spectrum antibacterial activity, compared to the standard Gentamicin, against both gram positive and gram negative bacteria, including *Escherichia coli, Pseudomonas aeruginosa, Salmonella typhi, Staphylococcus aureus* and *Bacillus subtilis*, with the minimum inhibitory concentrations; 62.5 mg/ml, 125 mg/ml, 15.63 mg/ml, 31.25 mg/ml and 31.25 mg/ml respectively. Determination of the minimum bactericidal concentrations revealed that the extract has no cidal effect against *E. coli, S. typhi* and *B. subtilis*, while it has against *P. aeruginosa* and *S. aureus* with 125 mg/ml as MBC in both cases. The evaluation of the antifungal activity of the crude leaf extract of the plant revealed its inability to inhibit the growth of *Penicillium notatum* and *Candida albicans* as compared to the standard drug, Griseofulvin. The findings in this work were able to establish important pharmacognostic standards for correct identification of the plant, as well as its potentials for possible development of yet another antioxidant and antimicrobial agents.

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Abbreviations and Glossary

DPPH = 1,1-diphenyl-2-picrylhydrazyl radical

TLC = Thin Layer Chromatography

EE = 70% Aqueous Ethanol Extract

AA = Ascorbic Acid

SEM = Standard Error of Mean

MBC = Minimum Bactericidal Concentration

MIC = Minimum Inhibitory Concentration

EP1 = Upper Epidermis

EP2 = Lower Epidermis

UV = Ultraviolet

HPLC = High Performance Liquid Chromatography

LD_{50} = Lethal Dose to 50% of the subject population

CHAPTER ONE

1.0 INTRODUCTION

1.1 Medicinal and Allied Uses of *Amaranthus viridis*

Amaranthus viridis Linn. is widely used in traditional medicine as diuretic and emollient, in poultices on inflammations, boils or abscesses and in the treatment of gonorrhea and orchitis as well as in enemas and suppositories for piles (leaves). An infusion of the whole plant is used in Nigeria to purify the blood and as a cooling medicine of value in eye complaints. The leaf-sap is used in Ivory Coast as an eyewash for ophthalmias and for treating convulsions in children, attacks of epilepsy and fits of insanity (Burkill, 1985).

In Congo, it is known to be vermifugal and effective against filaria in the eyelid or eyeball and as emmenagogue to relieve tummy and heart troubles. Preparations of the leaves are given to persons suffering from fever in parts of West Africa, while in Congo, a decoction of the whole plant is used as a bath for feverish children and for washing newborn babies. The powdered dried leaves, also, are applied to the pustules of yaws and smallpox in Congo, and the powdered charred plant is put into sores in Ivory Coast. The pounded root is given for dysentery in Nigeria (Burkill, 1985). In Indian Ayurvedic medicine, it's known to be emollient and vermifugal, and called 'Tanduliya' in Sanskrit (Chopra *etal.*, 1986). A decoction of the entire plant is used to treat dysentery and inflammation in China (Duke and Ayensu, 1985).

Species of Amaranthus are widely used by native Americans for food, livestock forage, medicine and as face and body paint (Zipcodezoo, 2010).

1.2 Medicinal Plant Research and Standardization of Herbal Drugs

These are scientific procedures that lead to the establishment of the botanical identity and usefulness of medicinal plants as well as their content of bioactive agents (Agrawal and Paridhavi, 2007).

There are a number of crude drugs whose plant sources have not yet been correctly and scientifically identified. In some cases, more than one plant species, sometimes with widely different morphological and taxonomic characters are considered as the source of a particular drug. The true source of the crude drug in such cases can only be ascertained after detailed physical, chemical and pharmacological studies (Agrawal and Paridhavi, 2007).

Pharmacognostic studies have been done on many important drugs, and the resulting observations have been incorporated in various pharmacopoeias. These findings, which should, also, be defined properly in terms of chemical and physicochemical standards, since morphological standards alone may not always be sufficient to ensure their quality, are used to check adulteration; which is the use of other plants or parts than that mentioned in official texts or pharmacopoeias. In recent years, a physiochemical procedure such as chromatography, has become a highly useful tool in determining the quality and genuineness of a drug. Among the chromatographic techniques, TLC has been widely employed for the analysis of extractives obtained from crude drugs. On account of its extreme rapidity and ease of visual evaluation, TLC has become the most ideal analytical method for the

systematic identification and control of purity in plant drugs (Agrawal and Paridhavi, 2007).

The existence of varieties of the same plant species, due to agro-climatic conditions and locality factors has become well known because of continuing research. As a result, various varieties of the same plant originating from different parts of the world, continent, region, country etc, could vary in chemical composition and therapeutic indications. The resolution therefore, lies in the scientific determination of the major chemical constituents responsible for the pharmacological actions observed (Agrawal and Paridhavi, 2007).

The concentrations of the required chemical constituents in a plant are influenced by the stage of growth (age) and season. This however, has to be substantiated with scientific proof based on detailed chemical examination of samples collected under different conditions of growth and maturity, and a suitable time of harvest will then be determined. Also, an understanding of the physical and chemical properties of the constituents will enhance the specification of the conditions for drying and storage as well as the shelf-life, which will influence the post-harvest processing (Agrawal and Paridhavi, 2007).

Screening of plants for bioactive agents which involves the extraction of active constituents from plants remains one of the most intensive areas of natural product research today. Only around 10% of all plants had been investigated in detail for bioactive agents, creating so much room for further research. Also, in an analysis of data on worldwide research on plant derived drugs, it has been reported that

1650 compounds of novel structure were reported in the year 1975 alone, while 3077 compounds of known structure were also reported from higher plants in the same year. Also, in the same year, more than 400 patents were given for substances isolated from higher plants. This analysis implies that this type of research can lead to the discovery of patentable and industrially exploitable compounds for drug development. In addition, the isolation of bioactive agents gives room for demonstrating the reality of the reported physiological activity of any plant, and provides chemotaxonomic evidence for the classification of genera or species, especially those whose classification on morphological grounds alone is unclear (Sofowora, 2008).

1.2.1 Pharmacognostic Evaluation

This is the detailed process of evaluating the quality and purity of crude drugs in order to establish the standard pieces of information that will form the monographs to be used for their correct identification. This follows the collection, confirmation/authentication and preparation of the plant sample, and includes microscopic, phytochemical and physicochemical investigations (Evans, 2002).

Microscopic evaluation involves observations beyond the naked eyes and aims to achieve the following (Evans, 2002);

i) The determination of the size, shape and relative positions of the cells and tissues.

ii) The determination of the chemical nature of the cell walls.

iii) The determination of the form and chemical nature of the cell contents.

Phytochemical analyses follow extraction of chemical constituents with suitable solvents, and involves the use of general and specific reagents to confirm the presence of various classes of bioactive agents (secondary metabolites), as well as solvents for separation and isolation of compounds with confirmatory TLCs.

Physicochemical evaluations involve the determination of various physical and chemical constants such as extractive values, ash values, moisture content etc.

1.2.2 Biological Evaluation of Vegetable Drugs

Extracts are used in tests for the evaluation of the antimicrobial properties of crude drugs. Usually, the effects of the various concentrations of the extracts, by either causing the death or inhibiting the growth of bacteria and fungi, are comprehensively evaluated. Hence, an agent that is found to exhibit a killing activity can then be described as bactericidal or fungicidal or virucidal, if bacteria or fungi or viruses, respectively, were killed. But, it will be bacteriostatic or fungistatic, if it only inhibits the growth of the respective organisms (Denyer *et al.*, 2004).

When evaluating the activity of antibacterial agents, the terms Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal/Fungicidal Concentration (MBC/MFC) are commonly used. MIC refers to the minimum concentration of an antimicrobial agent that inhibits growth of the micro organism under test and is generally recorded in mg or µg/ml. With most cidal antimicrobials, the MIC and MBC/MFC are often near or equal in value, although with essentially static agents (e.g. tetracycline), the lowest concentration required to kill the micro organism (i.e. the

MBC/MFC) is invariably many times the MIC and clinically unachievable without damage to the human host (Denyer *et al.*, 2004).

Free radical reactions involving reactive oxygen species are known to contribute to the overall metabolic perturbations that result in tissue injury and disease. These reactive oxygen species are generated in specific organelles of cells under normal physiological conditions. The defence mechanisms against these reactive oxygen species include radical scavenging enzymes and cellular antioxidants. A critical balance exists between the generation and detoxification of reactive oxygen species in cells (Tringali, 2001).

However, diseases, aging and chemical environments such as drugs, pesticides, herbicides and various pollutants can disrupt this balance by inhibition of the cellular antioxidant defences and/or by stimulation of the formation of reactive oxygen species. These reactive oxygen species can damage DNA, so as to cause mutation and chromosomal damage, oxidize cellular thiols (resulting in inhibition of key enzymes), and abstract hydrogen atoms from unsaturated fatty acids to initiate the peroxidation of membrane lipids. It is suggested that lipid peroxidation may be a common pathogenic mechanism because it is considered a basic mechanism involved in reversible and irreversible cell and tissue damage. Thus, antioxidative plant constituents would protect mitochondrial enzyme activities against oxidative stresses and scavenge reactive oxygen species in microsomes (Tringali, 2001).

The antioxidant activity of any plant constituent can, therefore, be evaluated based on its ability to scavenge the free radicals generated from a system (e.g. DPPH = 1,1-diphenyl-2-picrylhydrazyl), in comparison with a standard antioxidant agent like

ascorbic acid or rutein or tocopherol. Ultimately, this happens in a redox reaction where the radical gets reduced, while the antioxidant agent gets oxidized (Tringali, 2001).

DPPH is a stable nitrogen – centered free radical, the color of which changes from violet to yellow upon reduction by either hydrogen or electron donating. Substances that are able to perform this reaction can be considered as antioxidants and therefore radical scavengers (Brand-Williams *et al*., 1995).

1.3 Statement of the Research Problem

There are so many closely related species of the genus *Amaranthus*, as well as varieties of *Amaranthus viridis*, which pose great challenges with respect to correct identification.

The existence of numerous traditional claims of medicinal uses of various plants including *Amaranthus viridis,* as well as the issues surrounding imprecise diagnosis and dosing, and improper handling, collection and preparation of medicinal plants, have left so much room for scientific research.

1.4 Justification for the Study

Pharmacognostic evaluation, which includes; macroscopic, organoleptic, microscopic, phytochemical and physicochemical measurements/studies, is necessary to solve the problem associated with the accurate identification of the plant, *Amaranthus viridis*, as well as, provide understanding of the basis for the observed medicinal effects

and uses, and pointers to the discovery of new chemical compounds (drugs) with successful separation and isolation of purer compounds.

Since this plant abounds in our environment, tests for antimicrobial activities could indicate its potential as a source of another natural alternative to synthetic orthodox antimicrobials which are prone to the problems of high cost, microbial resistance and dangerous side effects.

An antioxidant study of the extract of the leaves of *A. viridis*, would provide scientific proof of its traditional use in skin and eye inflammations. It could also satisfy the growing interest in the food industry and in preventive medicine in the development of natural antioxidants from botanical sources.

1.5 Aim and Objectives of the Study

1.5.1 Aim

To establish pharmacognostic standards for the proper identification of *Amaranthus viridis*, with a view to ascertaining its ethnomedicinal uses as an antimicrobial and antioxidant agent.

1.5.2 Objectives of the Study

a) To establish some microscopic and physical constants of the leaves of *A. viridis*.

b) To conduct phytochemical screening of 70% aqueous ethanol leaf extract of the plant.

c) To establish the antioxidant and antimicrobial properties of the aqueous ethanol leaf extract of the plant.

1.6 Statement of the Research Hypotheses

A. viridis contains unique diagnostic microscopic and physical characters, as well as phytochemical constituents with antioxidant and antimicrobial activities.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Description of the Family *Amaranthaceae*

This family consists of about 70 genera and 900 species worldwide. Members are herbs, subshrubs or shrubs (Zipcodezoo, 2010).

The leaves are alternate or opposite and entire or exstipulate.Flowers are small, bisexual or unisexual, solitary or aggregated in cymes. Inflorescences are elongated or condensed spikes (heads). The fruit is a dry utricle or a fleshy capsule, indehiscent and irregularly bursting or circumscissile. Seeds are lenticular, reniform, subglobos or shortly cylindrical and smooth (Zipcodezoo, 2010).

Morphology of the androecium, perianth (sepals) and the inflorescence has traditionally been used to circumscribe genera and tribes. Units of dispersal vary considerably (capsules opening with lower part persistent or flower and bracteoles falling together or cymose or partial inflorescences breaking off above bract) and can be characteristic for genera. Several genera possess long trichomes serving the purpose of dispersal at the base of the sepals (Zipcodezoo, 2010).

Widespread in the *Amaranthaceae* is the occurrence of betalain pigments. In phytochemical research, several methylenedioxyflavonols, saponins, triterpenoids, ecdysteroids, and specific rootlocated carbohydrates have been found (Muller and Borsch, 2005). Other chemical analyses have demonstrated, also, the occurrence of

anthraquinones, aurone, betacyanins, betalains, betaxanthins, chromoalkaloids, ecdysteroids, flavonoids, protoalkaloids, saponins, steroids and triterpenes (Salvador *et al,* 2006).

2.2 Description of the Genus *Amaranthus*

There are approximately 70 species of this genus, found mostly in tropical, subtropical and warm-temperate zones, but some species also occur in temperate zones. Some taxa are at present almost worldwide as introduced and naturalized weeds. Some subgenera of *Amaranthus*, have been proposed and are sometimes recognized. These are subg. *Acnida*, subg. *Amaranthus*, and subg. *Albersia*. But, currently, *Amaranthus* is accepted in its broad sense (Zipcodezoo, 2010).

The species here are herbs, usually annual, rarely perennial, monoecious (subg. *Amaranthus* and *Albersia*) or dioecious (subg. *Acnida*), glabrous or pubescent. Stems are erect, ascending, decumbent, or prostrate, usually branched, occasionally simple or nearly so; without nodal spines (except in *A. spinosus*)**.** Leavesare alternate, petiolate; blade is rhombic-ovate, ovate, obovate, spatulate, lanceolate, oblanceolate, or orbiculate to linear, base is rounded to narrowly cuneate, margins are usually entire, usually plane, slightly undulate, or crispate, rarely undulate-erose, apex is acute, obtuse, or emarginate, usually mucronulate. Inflorescencesare terminal and/ or axillary or exclusively terminal. Flowersare unisexual. Pistillate flowers:sepals are absent or 1-5; stamens are absent (rudimentary); pistil 1; ovule 1; style 0.1-1 mm, or absent; stigmas are 2-5 and slender. Staminate flowers: sepals are 3-5, equal or

subequal; stamens are 3-5, filaments are distinct, anthers are 4-locular, pseudostaminodes are absent; pistils are absent or rudimentary. Seedis sub-globose or lenticular, usually smooth, shiny, sometimes indistinctly puncticulate or reticulate and embryo is annular (Zipcodezoo, 2010). Specimens of *Amaranthus* are often difficult to identify by someone not familiar with the group. When using the key, look closely at the tips of pistillate inflorescence branches for staminate flowers to determine whether the plant is monoecious or dioecious. This is especially important for some monoecious species that produce few staminate flowers. Also, pistillate plants of dioecious species are usually required for positive identification (Zipcodezoo, 2010).

2.3 Taxonomy and Description of *Amaranthus viridis* Linn.

Domain: Eukaryota

Kingdom: Plantae

Subkingdom: Viridaeplantae

Phylum: Magnoliophyta

Subphylum: Euphyllophytina

Infraphylum: Radiatopses

Class: Magnoliopsida

Subclass: Caryophyllidae

Superorder: Caryophyllanae

Order: Caryophyllales

Suborder: Chenopodiineae

Family: Amaranthaceae

Subfamily: Amaranthoideae

Tribe: Amarantheae

Genus*:* *Amaranthus* [Greek *amarantos*, unfading, nonwithering]

Specie: *viridis* (Zipcodezoo, 2010)

Amaranthus viridis Linn., syn., *gracilis* Desf., belongs to the family, Amaranthaceae. It is an annual herb, nearly one meter high, occurring throughout the West African region (Burkill,1985). It is a palatable vegetable in parts of Africa and Southern India; where it is called "*kuppacheera*" (Chopra *et al.*, 1986).

It is commonly known, in English, as Slender or Green Amaranthus, and locally, in various Nigerian languages as follows (Dalziel,1937; Burkill,1985):

Fulani-Fulfulde - Haakondiyam

Hausa – Malan- Kotshi, Namijin-Za'akii-banza'a, Za'akii-banza, Zarangade, Labashi and Tatsanya

Igbo – Inine' Ojii, Inine' ofia olo, Ininembekwu and Ininemme

Kanuri – Lekir

Yoruba – Tete, Tete atele and Tete kekere

The stem is erect, simple or with lateral branches (especially distally), and about 0.2 - 1 m high. Leaveshave petiole 0.5 - 1.5 cm as long as blade; blade is rhombic-ovate or ovate and 1 - 7 × 0.5 - 5 cm; base isrounded, cuneate, or attenuate; margins are entire or plane, and apex is obtuse, rounded, emarginate or

mucronate. Seedsare black or dark brown, subglobose to thick-lenticular, 1 mm in diameter and minutely punctulate (Zipcodezoo, 2010).

The image of the plant is shown below, taken with a digital camera after the identity was authenticated and favourably compared with established literature.

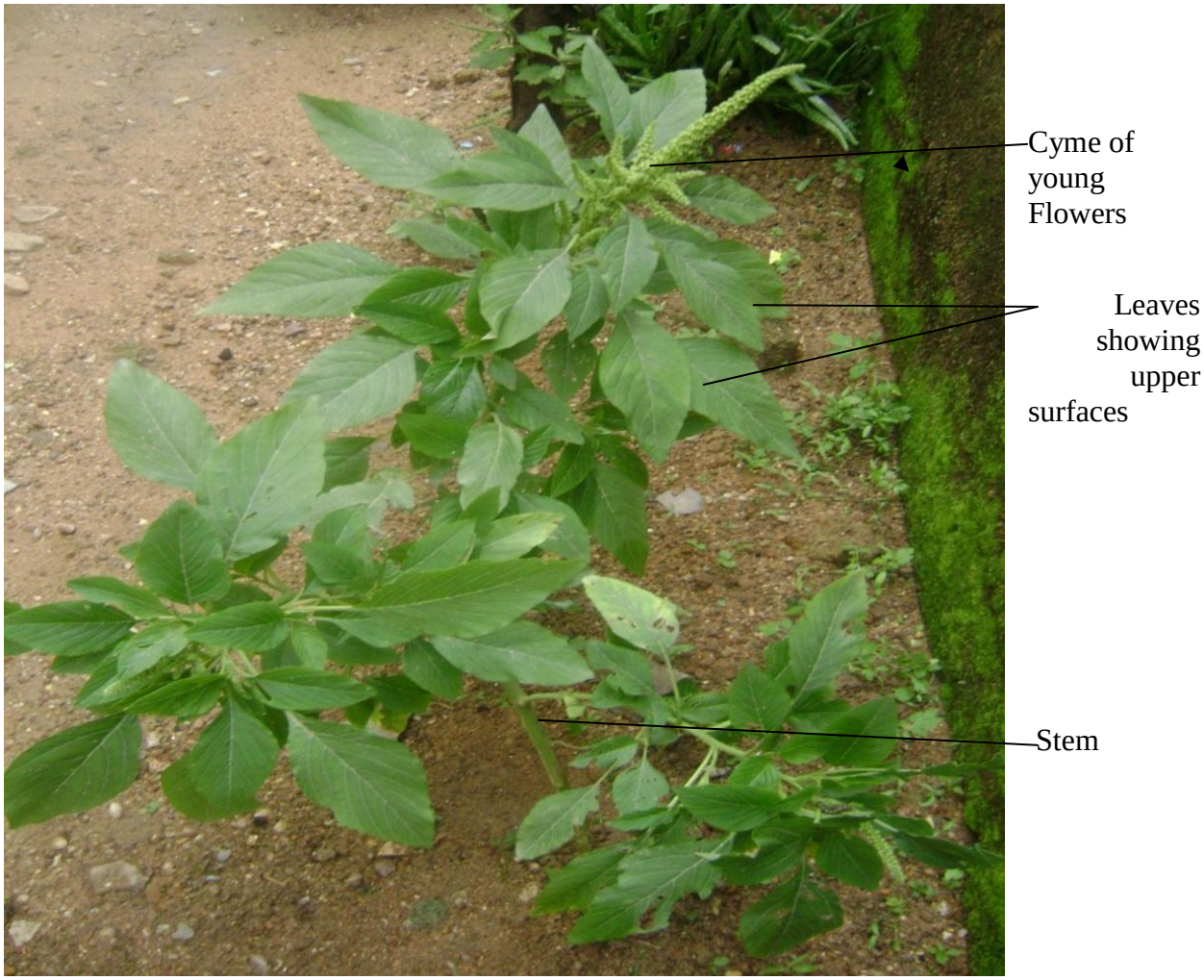


Plate I: *Amaranthus viridis* Linn. in Jos, Plateau State, Nigeria

2.4 Some Reported Works on *Amaranthus viridis*

2.4.1 Pharmacognostic Evaluation of *A. viridis*

Khan *et al* (2011) reported that: Leaf epidermal cells, of both sides have polyhedral to hexagonal shapes and smooth walls. Size of epidermal cells; adaxial -137 × 45 μm. Stomata are of anisocytic type, with the length of 85 μm at aperture size of 12 μm. Vein termination number ranges from 44.65 to 57.25 per unit area, vein islet number ranges from 14.56 to 23.57 per unit area and the palisade ratio ranges from 15.62 to 24.42 per unit area. Stomatal index of the upper surface of leaf is 21.25 to 24.62 and of the lower surface of the leaf of the plant is 42.54 to 43.47. Phytochemical and chemomicroscopical screenings indicated that alkaloids, saponins, starch, fat, protein and cellulose were present in all parts of the plant. Mucilage and calcium oxalate were present in the stem only and were absent from other parts of the plant. Anthraquinone derivatives and lignin were present in the root and stem and were absent from leaf and flower of the plant. Tannins were absent from all parts of the plant. Cutin was present in stem and leaf and was absent from root and flower.

2.4.2 Rutin and Quercetin Content of *A. viridis*

Rutin and Quercetin, which are flavonoids, have been qualitatively and quantitatively shown to be present in the methanolic leaf extractof *A. viridis* by Ashok Kumar *et al* (2009) by High Performance Liquid Chromatography (HPLC). The retention times of standards, rutin and quercetin were found to be 4.072 and 19.104, which matched the retention times of rutin and quercetin in *A. viridis*, 4.715 and 19.051, respectively. Then the amount of rutin and quercetin in *A. viridis* were found to be 58.52 and 9.12 % w/v, respectively.

2.4.3 Acute Toxicity Studies of the Methanol Extract of *A. viridis* Leaves

In their work, Krishnamurthy *et al* (2011) carried out acute oral toxicity study according to the guidelines set by Organization for Economic Co-operation and Development (OECD), using Healthy Wistar rats. They reported that the treated rats showed normal behavior and no toxic effects were observed at a higher dose of 5 g/kg body weight.

2.4.4 Anthelmintic Property of *A. viridis* Ashok Kumar *et al* (2010) investigated the anthelmintic activity of *A. viridis* using the methanolic extract of the whole plantagainst *Pheritima pasthuma* (an earthworm). The methanol extract of *A. viridis* showed concentration dependent paralysis which led to death, compared to the standard, piperazine.

2.4.5 Antioxidant Activity of Betalains obtained from members of the Amaranthaceae Family

Cai *et al* (2003) evaluated the antioxidant activity of betalain pigments (seven pure compounds and four combined fractions) from plants of the family Amaranthaceae, using the modified DPPH (1, 1-diphenyl-2-picrylhydrazyl) method. All tested betalains exhibited strong antioxidant activity. **2.4.6 Use of *A. viridis* in Pain Management** Agunu *et al* (2009) verified the use of *A. viridis*, among other medicinal plants, in pain management around the Jos area in Plateau state, North Central Nigeria. In the acetic acid – induced writhing assay and the hot-plate method Ethanol seed extract of *A. viridis* showed significant activity ($P < 0.05$) compared to aspirin and morphine.

2.4.7 Antioxidant and Antimicrobial Properties of Methanol Extracts of *A. viridis* Leaf and Seed

Methanol extracts of the dried leaves and seeds of *A. viridis* were reported to contain tannins (6.07% - 5.96%), saponins (53% - 32%), alkaloids (13.14% - 11.42%), proteins (16.76% - 24.51%) and glycosides (63.2% - 32.3%). The extracts also showed appreciable levels of total phenolic contents (2.81 - 3.61 GAE, g/100g), total flavonoid contents (18.4 - 5.42 QE, g/100g) and DPPH free radical scavenging activity, showing IC_{50} (83.45 - 75.95 µg/mL). The MIC of extracts ranged 178 - 645 µg/mL (Ahmed *et al*, 2013).

2.4.8 *In Vivo* Antioxidant Activity of the Methanol Extract of the Whole *A. viridis*

Ashok Kumar *et al* (2011) have reported, as part of their work, that pretreatment with methanol extract of the whole plant significantly reversed the oxidative changes (increase in lipid peroxidation (MDA) and decrease in GSH (glutathione), CAT (catalase) and TT (Total thiols) levels) caused by paracetamol in the hepatic tissue.

2.4.9 Physicochemical and Antioxidant Properties of the Whole *A. viridis* Sharma *et al* (2012), in their work have reported that the ash value for a whole herb of *A. viridis* (4g) is 1.85%, moisture content (100g of fresh ground material) is 87.90% and antioxidant activity based on DPPH method, IC_{50} µg mL^{-1} is 28.92.

2.4.10 Antiproliferative, Antifungal and Antiviral Properties of *A. viridis* A novel antiproliferative and antifungal lactin and ribosome inactivating protein, β-carotene were isolated from *A. viridis* (Kaur *et al*, 2006, Kwon *et al*, 1997 and Sena *et al*, 1998). Obi *et al* (2006) also reported the antiviral activity of *A. viridis*.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Materials

3.1.1 Equipment

Blender (Philips®),

Compound Microscope (Leica®),

Graduated ruler,

Microscope Digital Camera (FotoWinJoe®),

Razor blade,

Rotary evaporator and

Rotary shaker (Stuart Scientific®)

3.1.2 Test Animals, Microbes and Plant materials

Bacteria (*Staphylococcusaureus, Bacillussubtilis, Salmonellatyphi, Pseudomonas aeruginosa* and *Escherichiacoli*),

Blood diluted in normal saline,

Fungi (*Penicillium notatum* and *Candida albicans*),

Leaves of *Amaranthusviridis* (fresh and dry powder) and

Mice (12, adult, male and female)

3.1.3 Solvents, Extract, Reagents, Drugs and Culture Media

Acetone,

Ammonia solution,

Aqueous Ethanol (70%) Extract,

Ascorbic acid,

Barfoed's reagent (Copper acetate solution in lactic acid),

Benzene,

Bromine water,

Chloral hydrate solution,

Chloroform,

Dilute Glycerol,

Distilled water,

DPPH (1, 1-Diphenyl-2-picrylhydrazyl solution),

Dragendorff's reagent (Potassium Bismuth Iodide solution),

Ethanol (70%; 90%),

Fehling's solution A (Copper sulphate Solution),

Fehling's solution B (Solution of potassium hydroxide and potassium sodium tartrate),

Ferric ammonium citrate solution,

Ferric Chloride solution,

Gentamicin (Gentalek®) injection,

Griseofulvin (Fulcin®) tablet,

Hydrochloric acid,

Kedde's reagent (2 % solution of 3, 5-dinitrobenzoic acid in methanol),

Lead acetate solution,

Lead sub-acetate solution,

Magnesium ribbon,

Mayer's reagent (Potassium Mercuric Iodide solution),

Methanol,

Millon's reagent (Mercury nitrate-Nitric acid solution),

Molisch's reagent (5% 1-Naphtol in alcohol),

Mueller Hinton culture media,

N/50 Iodine,

Nitric acid (Concentrated),

Phloroglucinol,

Potassium chlorate crystals,

Potassium dichromate,

Potassium hydroxide,

Pyridine,
Sabouraud Dextrose Agar (SDA),
Selivanoff's reagent (0.05 % w/v resorcinol dilute (1:2) hydrochloric acid),
Sodium carbonate,
Sodium hydroxide,
Sodium nitroprusside,
Sudan IV and
Sulphuric acid

3.2 Collection and Identification of the Plant

The plant material was collected in April, 2009 in its whole form, containing the leaves, stems and aerial parts, using descriptions from literature, and with the assistance of a traditional medicine practitioner from the Babale area of Jos North Local Government Area, Plateau State, Nigeria. The plant was later taken for authentication at the Herbarium unit of the Department of Biological Sciences, Ahmadu Bello University (A.B.U.), Zaria. The plant was identified and authenticated to be Amaranthus viridis Linn. by Mallam U. S. Galla on the 28th of April, 2009 with the specimen voucher number given as 335.

Furthermore, a herbarium specimen of the plant was prepared and deposited at the herbarium of the Department of Pharmacognosy, University of Jos.

3.3 Preparation of the Leaves of *A. viridis* for Studies

The leaves were then separated from the other parts of the plant and dried under the shade for about two weeks, by spreading them well on a flat drying surface in the laboratory, with adequate aeration, to prevent fungal growth.

The well-dried leaves were collected and pulverized using a blender. The powder was then stored in an airtight container, for further use.

3.4 Extraction of the Powdered Leaves of *A. viridis*

Powdered leaves (150g) was extracted by cold maceration in 500 ml of 70% Ethanol. Extraction was carried out with the aid of a rotary shaker for about 48 hours. Afterwards, the extract was concentrated with the aid of a rotary evaporator and kept in the refrigerator for further use.

3.5 Microscopical Examinations of *A. viridis* Leaf

3.5.1 Fresh Leaf Sections

Carefully derived tiny sections of the fresh leaf, including: transverse sections of the lamina with the midrib, other portions of the whole leaf as well as the leaf margin and surface preparations of both epidermises, were used. They were, at various points, placed on clean microscope slides, few drops of chloral hydrate solution added, covered with cover slips and gently boiled until they were cleared. These were then mounted in dilute glycerol and observed/studied under a compound microscope, using suitable magnifications (Evans, 2002; Wallis, 1985). The images of diagnostic features were taken using a microscope digital camera (FotoWinJoe®).

3.5.2 Examination of Powdered Leaf of *A. viridis*

Here, a small quantity of the leaf powder was put on a clean microscope slide and covered with a cover slip. Few drops of chloral hydrate solution were added and a gentle boiling was done to clear it. This was then mounted in dilute glycerol and observed under the microscope, with suitable magnifications (Evans, 2002; Kokate, 1994). The images of diagnostic features were also taken using a microscope digital camera (FotoWinJoe®).

3.5.3 Chemomicroscopical Examination of Fresh and Powdered leaf of A. viridis

Anatomical sections of leaf and powder were variously treated (mounted) with appropriate chemical reagents on microscope slides and observed under the microscope for the presence or otherwise of chemical substances such as lignin (phloroglucinol + conc. hydrochloric acid), tannins (ferric chloride), oil (sudan iv), starch (N/50 iodine), proteins (millon's reagent) and calcium oxalate (chloral hydrate + hydrochloric acid) using standard procedures as outlined in Evans (2002) and Kokate (1994).

3.6 Evaluation of the Physicochemical Constants of *A. viridis* Leaf Powder

3.6.1 Determination of Moisture Content

A crucible was heated to a constant weight in an oven, and the weight was noted. 2 g of the leaf powder was accurately weighed and placed in the crucible. The crucible with the drug content was put in the oven at 105 ^{o}C and the content dried to a constant weight, with checks at 30 min. intervals after an initial drying period of one hour. The observation of two consecutive same weights confirmed a constant weight. The total loss in weight (i.e. the weight of the moisture) was determined by subtracting the constant weight of the crucible and leaf powder

after heating from the weight of the crucible and the content before heating. The percentage of the moisture content relative to the initial weight of the leaf powder used was then calculated by dividing the weight of the moisture by the weight of the drug taken and multiplying by 100%. Three different determinations were done and the average ± SEM taken as the moisture content of the drug (Trease and Evans, 1983).

3.6.2 Determination of Ash Values

A) Determination of Total Ash: Six silica crucibles were heated for 30 min., allowed to cool in a desiccator and weighed. In each crucible, 2 g of the leaf powder was evenly spread and dried 105 ^{o}C for one hour. Afterwards, the crucibles and their contents were ignited to constant weights in a muffle furnace at 575^{o} to 625^{o} until the contents became white, indicating the absence of carbon. The crucibles were allowed to cool in a desiccator after each ignition, and weighed until constant weights were obtained. For each, the total ash was calculated by subtracting the weight of the crucible from the final constant weight of the crucible and ash. The percentage of total ash was then calculated with reference to the air-dried leaf powder. The average of the six determinations was taken (B. P., 1993).

B) Determination of Acid-insoluble Ash: Three of the above crucibles and their ash contents were used for this experiment. In each case, 25 ml of hydrochloric acid was added to the crucible containing the total ash and covered with a watch glass followed by gentle boiling for 5 min. The watch glass was then

rinsed exhaustively with 5ml of hot water and the liquid added to the crucible. The insoluble matter was collected on an ashless filter paper and washed with distilled water until the filtrate was neutral to litmus. The filter paper with its content of insoluble matter was then transferred to the crucible, dried on a hot plate and ignited to a constant weight. The final constant weight was obtained after the crucible and its content were allowed to cool. The weight of the insoluble ash was then determined by subtracting the predetermined constant weight of the crucible and its content (insoluble ash). The content of acid-insoluble ash was calculated as a percentage of ash with reference to the air-dried leaf powder used. The average of the three determinations was taken (B. P., 1993; Agrawal and Paridhavi, 2007).

C) Determination of Water-soluble Ash: The remaining three crucibles and their ash contents were used in this experiment. For each, the ash in the crucible was boiled with 25 ml of water for 5 min. and the insoluble matter collected on ashless filter paper, washed with hot water and ignited for 15 min. at 450^{o}. The weight of the residue was then subtracted from that of the ash, and the difference taken as the water-soluble ash value. The percentage of water-soluble ash was calculated with reference to the air-dried leaf powder used. The average of the three determinations was taken (B. P., 1993).

3.6.3 Determination of Extractive Values of A. viridis Leaf Powder

A) Ethanol-soluble Extractive Value : 5 g of the air-dried leaf powder was macerated with 100 ml of 90 % ethanol in a closed flask for 24 hours; shaken frequently

during the first 6 hours and then allowed to stand for the remaining 18 hours. It was then filtered rapidly, to guard against loss of ethanol. 20 ml of the filtrate was then evaporated to dryness in a weighed beaker at 105 ^{o}C, until a constant weight was obtained. The percentage of ethanol-soluble extractive was then calculated with reference to air-dried leaf powder used. The average of three determinations was taken (B. P., 1993; Agrawal and Paridhavi, 2007).

B) Water-soluble Extractive Value: The procedure was the same with that of ethanol-soluble extractive, but chloroform-water (1:40) was used in place of ethanol. Also, the average of three determinations was taken.

3.7 Phytochemical Screening

The 70% aqueous ethanol leaf extract was subjected to preliminary chemical tests as follows: ***Test for Carbohydrates***

A) Molisch's Test (General Test): Two drops of Molisch's reagent (5 % 1-naphthol in alcohol) were added to about 2 ml of test solution (extract) and mixed well in a test tube. The tube was then inclined and about 1ml of concentrated sulphuric acid was added along the sides of the tube. The colour formed at the junction of the two liquids was then observed (Evans, 2002).

B) Fehling's Test (for Free Reducing Sugars) : To a heated solution of the extract, a mixture of equal parts of Fehling's solution 'A' andFehling's solution'B' was added drop by drop, and the formation of a brick-red precipitate was looked out for (Evans, 2002).

C) Barfoed's Test (for Monosaccharides) : To 1 ml of the test solution was added about 2 ml of Barfoed's reagent. This was then boiled for one minute and allowed to stand for a few minutes (Sadasivam and Balasubramanian, 1985).

D) Test for Pentoses : A solution of the extract, with an equal volume of hydrochloric acid containing a little phloroglucinol, was heated in a test-tube, and formation of a red colour was noted (Evans, 2002).

E) Selivanoff's Test (for Ketoses) : A crystal of resorcinol was added to the test solution and warmed on a water-bath with an equal volume of concentrated hydrochloric acid. The production of a deep red or rose colour was then noted (Evans, 2002).

F) Test for Combined Reducing Sugars : 1 ml of the extract was hydrolyzed by boiling with 5 ml of dilute hydrochloric acid, and neutralized with sodium hydroxide solution. The Fehling's test was then carried out (as in 'C' above). It was then observed to see if a brick-red colour results (Evans, 2002).

Tests for Alkaloids

About 5g of the dry extract was moistened with sufficient quantity of a strong ammonia solution and allowed to stand for about 10 minutes. The mixture was then stirred with or extracted by boiling with 10% sulphuric acid on a water bath for 30 minutes. It was then filtered and the filtrate was divided into two portions and treated separately with few drops of Mayer's reagent (Potassium mercuric iodide solution) and Dragendorff's reagent (Potassium bismuth iodide). Turbidity or precipitation with either or both of the reagents, red to brown in colour, indicated the presence of alkaloids (Evans, 2002; Sofowora, 2008).

Tests for Saponins

To about 0.1g of the dry exract was added 20 ml of distilled water and boiled. The mixture was then filtered hot, and cooled (Sofowora, 2008).

A) *Frothing Test :* 5 ml of the filtrate was diluted with water and shaken vigorously, in a test tube. The persistence of a honey-comb froth, on warming, was then taken as evidence for the presence of saponins (Sofowora, 2008).

B) *Blood Haemolysis Test:* In each case, 1 ml of a 10% dilution of blood (suspension of red blood cells) in normal saline, was put in each of two test tubes, and to one of them was added 1 ml of the extract from above. The tubes were then shaken gently and observed by holding them up against light. By comparison, a clear red solution indicated the haemolysis of red blood cells, as further evidence of the presence of saponins (Sofowora, 2008).

Tests for Tannins

A) *Ferric Chloride Test :* A portion of the crude extract was mixed with 10 ml of distilled water, and a few drops of 10% ferric chloride solution was added. The formation of a blue-black, green or blue-green precipitate was then taken note of (Evans, 2002; Sofowora, 2008).

B) *Lead Sub-acetate Test :* A few drops of lead sub-acetate solution was added to 1 ml of the extract. The formation of a coloured precipitate was then observed (Ibrahim, 1990).

C) *Ferric Ammonium Citrate Test:* To 1 ml of the water extract was added 0.25% ferric ammonium citrate solution; followed by addition of sufficient quantities of solid sodium acetate to adjust the pH of the solution to 8, with the aid of

a pH indicator. This was boiled on a water bath and filtered while hot. A coloured precipitate indicates the presence of tannins (Ibrahim, 1990).

D) *Gelatin-salt test:* 10 mg of the extract was dissolved in 6 ml of hot deionized distilled water, filtered and the solution divided into three test tubes. To the first was added a 1% solution of NaCl, to the second was added a 1% NaCl and 5% gelatin solution, and to the third was added a $FeCl_3$ solution. Formation of a precipitate in the second treatment suggests the presence of tannins, and a positive response after addition of $FeCl_3$ to the third portion supports this inference (Sarker *etal*, 2006).

E) Test for Phlobatannins: To a small portion of the aqueous extract was added 1% dilute hydrochloric acid and boiled. A red precipitate deposition indicates the presence of phlobatannins (condensed tannins) (Sofowora, 2008; Ibrahim, 1990).

Tests for Flavonoids

The dry crude extract was completely detanned with acetone. The residue, after evaporating the acetone on a water bath, was then extracted with warm water, and the mixture filtered while hot. The filtrate was cooled and used for the following tests (Ibrahim, 1990).

A) Lead Acetate Test: 10% Lead acetate solution was added to 5 ml of the detanned aqueous extract. A coloured precipitate indicates the presence of flavonoids (Ibrahim, 1990).

B) *Sodium Hydroxide Test :* 5 ml of 10% sodium hydroxide was added to an equal volume of the detanned aqueous extract. A yellow solution indicates the presence of flavonoids (Ibrahim, 1990; Agrawal and Paridhavi, 2007).

C) *Ferric Chloride Test:* A few drops of 10% $FeCl_3$ solution was added to about 2 ml of the detanned aqueous extract solution. A green or blue to black coloured solution indicates the presence of phenolic nucleus (Ibrahim, 1990;Agrawal and Paridhavi, 2007).

D) *Magnesium Hydrochloride Reduction Test (Shinoda Test):* To the solution of the ethanol extract, was added a mixture containing pieces of magnesium ribbon and concentrated hydrochloric acid. A pink or red colour indicates the presence of flavonoids (Agrawal and Paridhavi, 2007).

Tests for Steroids

Salkowski's Test: The ethanol extract was dissolved in anhydrous chloroform and then filtered. A small volume of the filtrate was then carefully mixed with 2 ml of conc. sulphuric acid, such that the acid formed a lower layer. A reddish-brown colour at the interface indicates the presence of steroidal ring (Sofowora, 2008).

Tests for Anthraquinones

A) Borntrager's Test (for Free Anthraquinones): The aqueous ethanol extract was shaken with 10 ml benzene. This was then filtered and 5 ml of 10% ammonia solution added. The mixture was then shaken and the presence of a pink, red or

violet colour in the ammoniacal (lower) phase indicates the presence of free anthraquinones (Ibrahim, 1990; Sofowora, 2008).

B) Test for Combined (bound) Anthraquinones: 0.5 g of the dry ethanol extract was boiled with 10 ml of aqueous sulphuric acid for about 2 to 3 minutes and filtered while hot. The filtrate was allowed to cool, and then mixed with an equal volume of benzene. The benzene layer was then separated, and half its volume of 10% ammonia solution added to it and shaken. A pink, or violet coloration in the ammonia phase (lower layer) indicates the presence of anthraquinone glycosides (Evans, 2002; Sofowora, 2008).

Tests for Cardiac Glycosides

A) Lieberman-Burchard's Test: 0.5 g of the extract was dissolved in 2 ml of glacial acetic acid. Sulphuric acid was then carefully added. A colour change from violet to blue to green will indicate the presence of a steroidal nucleus (i.e. aglycones portion of the cardiac glycoside) (Sofowora, 2008).

B)Keller- Kiliani's Test (for Digitoxose): 0.5 g of the extract was dissolved in 2 ml of glacial acetic acid containing one drop of ferric chloride solution. This was then underlayed with 1 ml of concentrated sulphuric acid. A reddish-brown ring at the interface will indicate the presence of deoxy sugars. Also, a violet ring may appear below the brown ring (in the acid layer) while, in the acetic acid layer a greenish ring may form just above the brown ring (Evans, 2002; Sofowora, 2008).

C) Kedde Test: Solution of the extract in methanol was mixed with 1ml of a 2 % solution of 3, 5-dinitrobenzoic acid in methanol and 1ml of a 5.7% aqueous sodium hydroxide. An immediate violet colour will indicate the presence of cardenolides in the extract (Sofowora, 2008).

C) Legal Test: The extract was dissolved in pryridine and a few drops of 2% sodium nitroprusside together with a few drops of 20 % NaOH were added. A deep red colour which fades to a brownish yellow indicates the presence of cardenolides (Sofowora, 2008).

3.8 Acute Toxicity Studies (LD_{50})

Source and Maintenance of Experimental Animals

Healthy male and female adult mice, weighing between 15 and 20 g were obtained from the Animal House of the University of Jos, Nigeria. The animals were housed under standard environmental conditions, with feed and water provided *ad libitum*. Proper handling and using of the animals were in accordance with the guidelines and regulations, monitored and approved by the Ethical Committee on Animal use, Department of Pharmacology, University of Jos, Nigeria.

Procedure

LD_{50} determination was conducted using the Lorke's method. Nine mice were divided into three groups of three mice each. The first group received the extract, intraperitoneally (*i.p.*) at a dose of 1000 mg/Kg; group 2 received the extract

at a dose of 100 mg/Kg (*i.p.*), while the last group received the extract at the dose of 10 mg/Kg body weight. The animals were observed for general signs and symptoms of toxicity including mortality over a period of 24 hours. In the second phase, three mice were divided into 3 groups of one mouse each. The extract was administered at the dose of 1500, 2900 and 5000 mg/Kg (*i.p.*) to groups 1, 2 and 3, respectively based on the result of the first phase. The final LD_{50} was calculated as the square root of the geometrical mean of the highest non-lethal dose and the lowest lethal dose (Lorke, 1983).

3.9 Test for Antioxidant Activity

The radical scavenging activity/effect on 1,1-Diphenyl-2-picrylhydrazyl (DPPH), a stable radical (red in colour), which is equivalent to the quantitative antioxidant activity of the ethanol extract was determined. The method was adopted from Brand-Williams *et al*, 1995 and Onocha *et al*, 2011.

DPPH (7.5mg) was dissolved in 100 ml of methanol to give a 75µM solution. To 3.0 ml of the methanol solution of DPPH was added 1.5 ml of each of the concentrations of the extract ranging from 11.88 to 385 µg/ml, prepared by serial dilutions from stock solutions of both the crude extract of the leaf and the standard/reference drug (ascorbic acid), with concentration of 25 mg of drug/10ml of methanol. The mixtures were then shaken or homogenized and allowed to stand (incubated) for 20 min.; for reaction to occur. Afterwards the various absorbance were

measured at 517 nm. The DPPH radical-scavenging activity (% inhibition) was calculated according to the following equation:

DPPH· scavenging activity (% inhibition) = [(A_B –A_T)/A_B] x *100*

Where A_B was the absorbance of the blank i.e no sample, DPPH solution only (negative control) and A_T was the absorbance in the presence of the test compound.

These determinations were done in triplicates for both the test extract and ascorbic acid (standard/reference antioxidant) and averaged. The absorbance measurements were then expressed as mean absorbance $\pm$ SEM via statistical analysis.

3.10 Test for Antimicrobial Activity

Clinical laboratory isolates of some Gram positive (*Staphylococcusaureus* and *Bacillussubtilis*) and Gram negative (*Salmonellatyphi*, *Pseudomonasaeruginosa* and *Escherichiacoli*) bacteria as well as fungal strains (*Penicillium notatum* and *Candida albicans*) obtained from the Pharmaceutical Microbiology Laboratory, University of Jos, Nigeria, were used.

The antibacterial and antifungal activities of the 70% aqueous ethanol extract of the leaves of *A.viridis* were tested.

3.10.1 Antibacterial Activity

A) Antibacterial Susceptibility Test: The agar well diffusion method was used. Different stock solutions of the ethanol extract at the concentrations 125, 250 and 500 mg/ml were prepared, but 0.2 ml of each were introduced into the wells, bringing down

the concentrations to 25, 50 and 100 mg/ml respectively. Standard broad antibacterial agent (Gentamicin) at a concentration of 4 µg/ml was also used against all the bacterial species and its zones of inhibition compared with those of the different concentrations of the extract.

Mueller Hinton Media (MH-Media) (38 g) was mixed with 1 litre of distilled water and then 20 ml of the preparation, in universal bottle sterilized in the autoclave at 121 ^{0}C for 15 minutes. The sterilized media was allowed to cool to a suitable temperature (45 ^{0}C). Using the seeding method, 0.1 ml of overnight culture was inoculated into the cool sterile molten agar, mixed properly and poured into sterile petri dishes and allowed to solidify. After solidifying, 4 wells (3 for the different extract concentrations and 1 for the standard drug) were bored in the agar with sterile 6mm cork borer. The extract and standard concentrations were introduced into the wells using sterile syringe and left on the bench for 1 hour to allow for agar diffusion followed by incubation of the plates at 37 ^{0}C for 24 hours. After which the plates were observed for the zone clearance (zones of inhibition) around the wells. The zone of inhibition was determined by measuring the diameter of the inhibition zone around the well (in mm) including the well diameter. The readings were taken in three different fixed directions in all 3 replicates and the average values tabulated as mean ± SEM (mm) (Onaolapo, 2006; Gandhiraja *et al*, 2009 ; Aji *et al*, 2010).

*B) Determination of Minimum Inhibitory Concentration (MIC) :*Serial dilution of the lowest concentration of the extract that was effective in the susceptibility test was done to obtain solutions of six different concentrations (125,62.5, 31.25,15.625,

7.8125 and 3.90625mg/ml) which were used to determine the MIC. 250 mg/ml was the highest concentration used for *P. aeruginosa*, as 125 mg/ml did not show activity in susceptibility test. 5 ml of each concentration of the extract was added aseptically to 5 ml of double strength medium (tube 1), prepared by dissolving 14 g of nutrient broth in 1 litre of distilled water, and shaken to mix. With a fresh pipette, 5 ml of the mixture was transferred to tube 2 which contains 5 ml of single strength medium, prepared by dissolving 14 g of nutrient broth in 500 ml of distilled water, and shaken to mix. The procedure was repeated until all the six serial dilutions were done. 0.1 ml inoculum of the respective test bacterial species were added to each tube, properly shaken to mix and then incubated at 37 ^{0}C for 48 hours. After which they were observed for turbidity (growth) or clarity (no growth). The MIC was taken as the least concentration of the extract that completely inhibited the growth of the test organisms, in each case (Onaolapo, 2006; Aji, *et al*, 2010).

C)Determination of Minimum Bactericidal Concentration (MBC): The tubes with concentrations that did not show visible growth in the MIC determination were subcultured in respective nutrient agar plates at 37 ^{0}C overnight to determine bactericidal or bacteriostatic activity. The lowest concentrations that did not show visible growth, afterwards, were taken to be cidal to the different bacterial species and recorded as the MBCs (Onaolapo, 2006; Aji *et al*, 2010).

3.10.2 Antifungal Activity

The aqueous ethanol extract of *A. viridis* at concentrations of 125, 250 and 500 mg/ml were tested against fungal species; *Penicillium notatum* and *Candida albicans*,

in comparison with a standard broad spectrum antifungal agent (Griseofulvin), at a concentration of 12.5 mg/ml, using the agar well diffusion assay method.

Sabouraud Dextrose Agar (SDA) was used as the culture medium. It was prepared by suspending 65 g of the medium in one litre of distilled water and heating with frequent agitation, followed by boiling for one minute to completely dissolve the medium. It was then autoclaved at 121 ^{0}C for 15 minutes and allowed to cool to a suitable temperature (45 ^{0}C). Using the seeding method, 0.1 ml of overnight culture was inoculated into the cool sterile molten agar, mixed properly and poured into sterile petri dishes and allowed to solidify. After solidifying, 4 wells (3 for the different extract concentrations and 1 for the standard drug) were bored in the agar with sterile 6mm cork borer. The extract and standard concentrations were introduced into the wells using sterile syringe and left on the bench for 1 hour to allow for agar diffusion followed by incubation of the plates at 25 ^{0}C for 7 days. After which the plates were observed for the zone clearance (zones of inhibition) around the wells. The zone of inhibition was determined by measuring the diameter of the inhibition zone around the well (in mm) including the well diameter. The readings were taken in three different fixed directions in all 3 replicates and the average values tabulated as mean $\pm$ SEM (mm) and the zones of inhibition determined afterwards (Onaolapo, 2006; Gandhiraja *et al,* 2009 ; Aji *et al,* 2010).

3.11 Statistical Analysis

Data were recorded as mean ± SEM. The statistical significance of differences between the groups was determined by analysis of variance (ANOVA) using the SPSS Statistical tool (version 16). Differences of $P < 0.05$ were considered statistically significant.

CHAPTER FOUR

4.0 RESULTS

4.1 Microscopy

4.1.1 Microscopic Characters

Microscopic examination of different preparations of the leaf of *A. viridis,* which included fresh upper and lower surfaces, transverse sections and powdered leaf preparation, revealed different diagnostic features of the leaf as presented in plates II through.

The epidermal cell walls in the upper epidermis are slightly wavy in nature while the wavy nature are more conspicuously shown on the lower epidermis. The stomatal arrangement was anisocytic and the stomata are more on the lower epidermis than the upper epidermis. The trichomes are glandular and non-glandular and multicellular in nature with uniseriate cells containing 4 cells (Plates II - V).

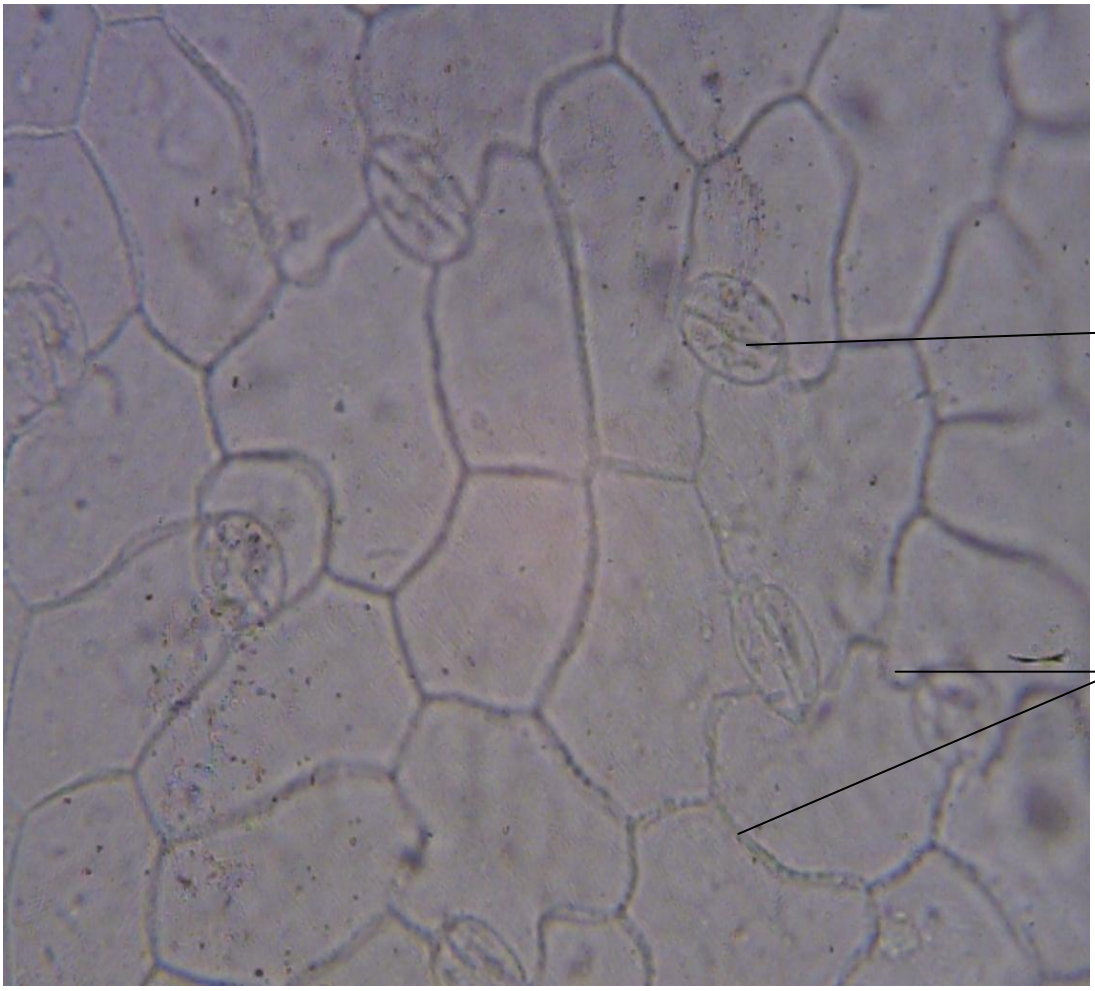


Plate II: Upper epidermal (EP1) surface of *A. viridis* L. leaf showing the stomata (anisocytic) and slightly wavy epidermal cell walls, cleared with chloral hydrate, mounted in dilute glycerol and viewed under the compound microscope (Leica®) (x 400)

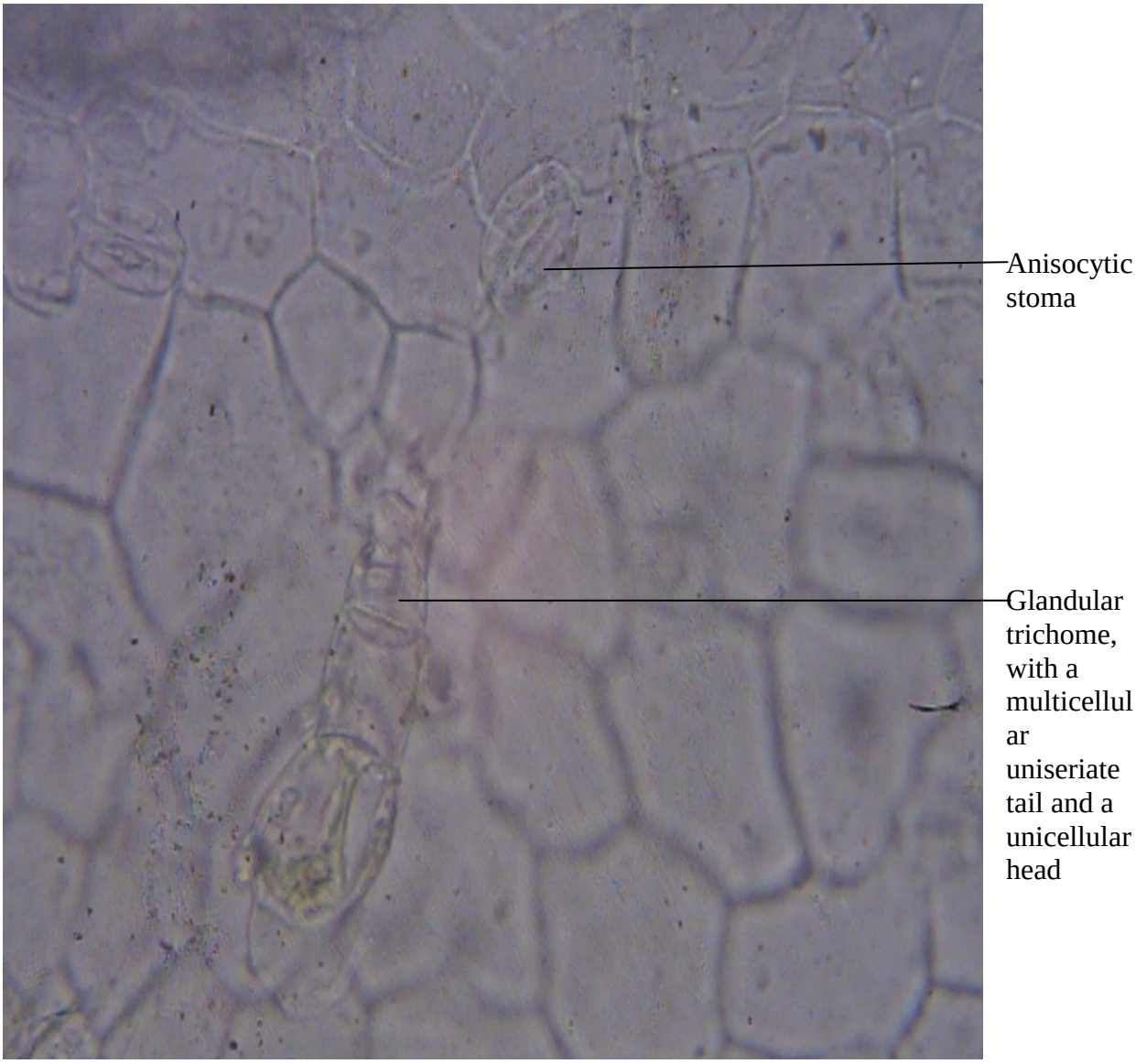


Plate III: Upper epidermal (EP1) surface of *A. viridis* L. leaf showing the stomata (anisocytic) and glandular trichome, with a multicellular uniseriate tail and a unicellular head, cleared with chloral hydrate, mounted in dilute glycerol and viewed under the compound microscope (Leica®) (x 400)

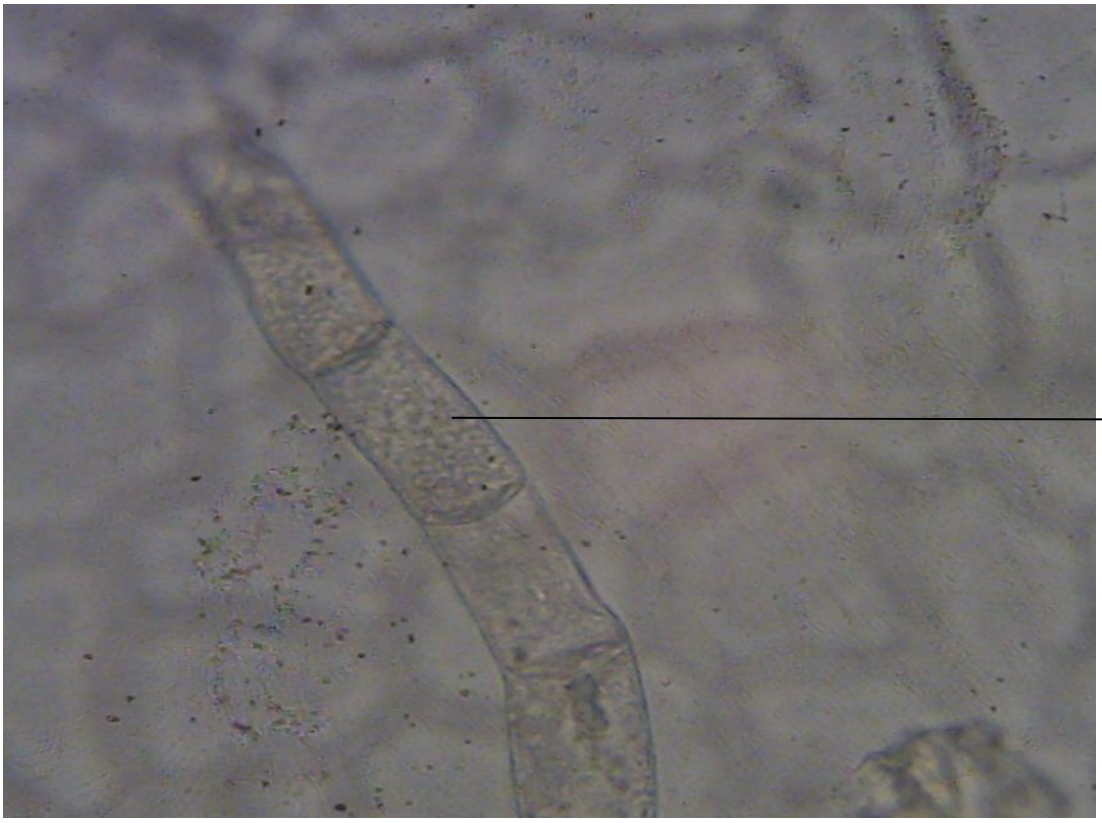


Plate IV: Upper epidermis of *A. viridis* L. leaf showing showing non-glandular multicellular uniseriate trichome containing 4 cells with warty surface, cleared with chloral hydrate, mounted in dilute glycerol and viewed under the compound microscope (Leica®) (x 400)

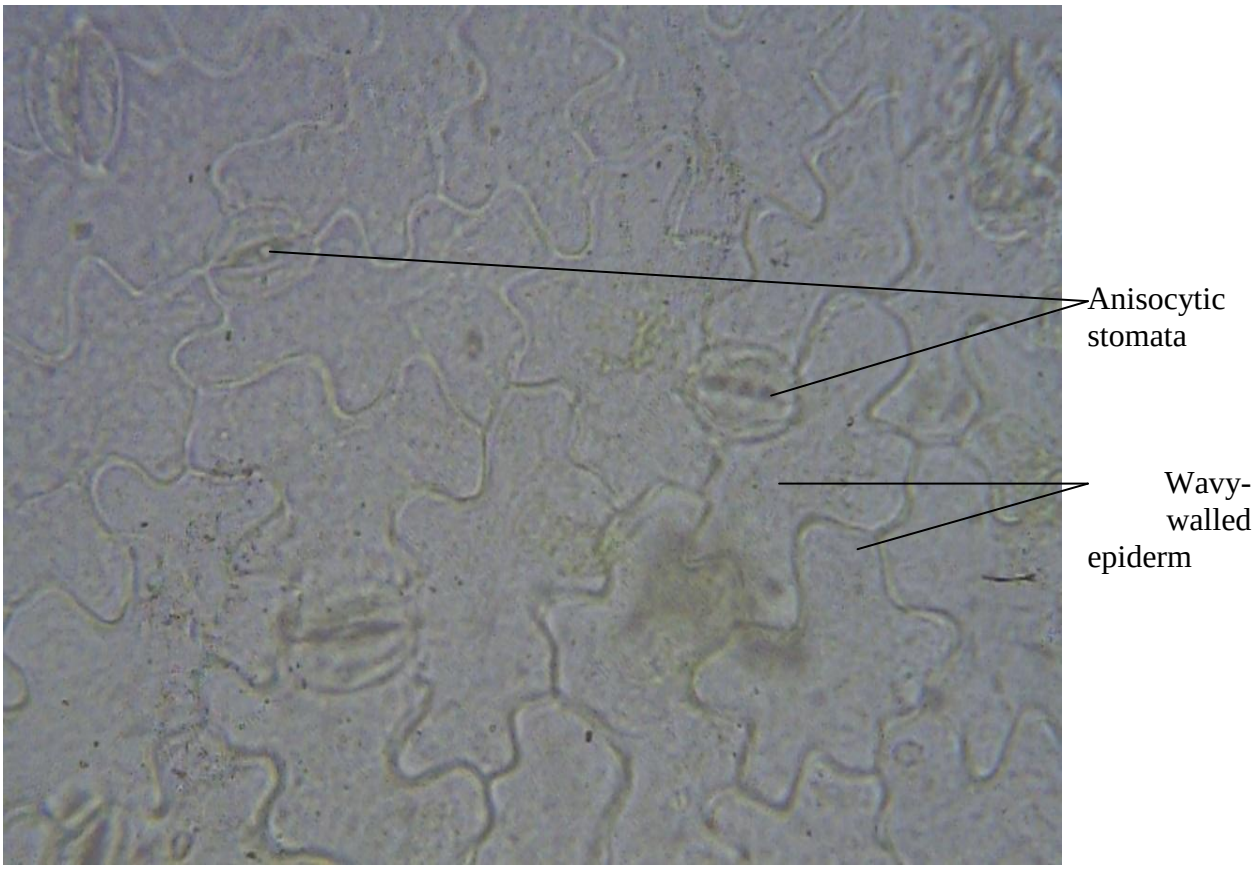


Plate V: Lower epidermis (EP2) of *A. viridis* L. leaf showing more numerous anisocytic stomata, surrounded by wavy-walled epidermal cells, cleared with chloral hydrate, mounted in dilute glycerol and viewed under the compound microscope (Leica®) (x 400)

The transverse section of the leaf through the midrib and sections of the lamina revealed the presence of cuticle, trichomes, upper epidermal layer, palisade layer, parenchyma, vascular bundle, xylem vessels of spiral and annular types, collenchyma, spongy tissue and lower epidermal layer, showing the leaf type to be dorsiventral (Ep1P1SmEp2). The microscopical features examined, with respect to the transverse section of the leaf of *A. viridis* are shown below (Plates VI - X).

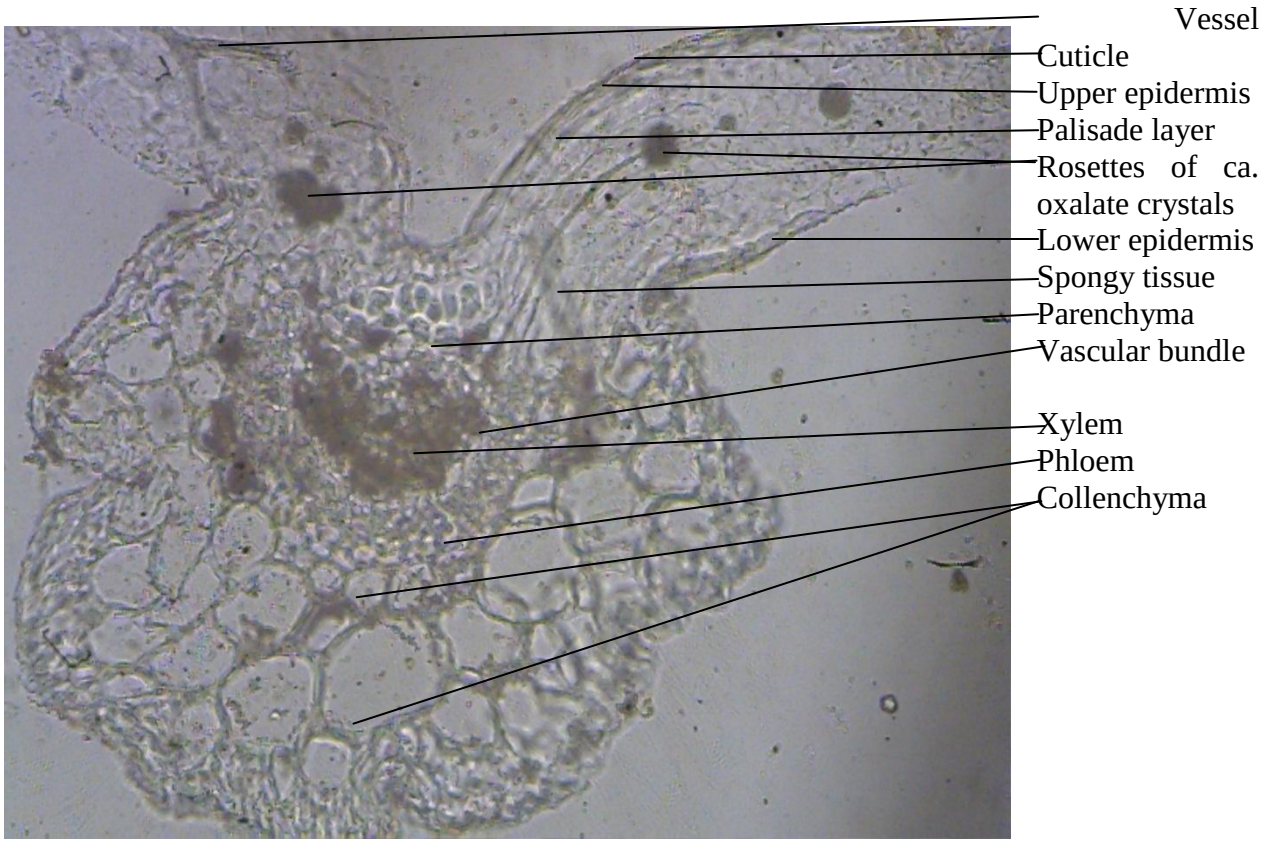


Plate VI: Transverse section of the leaf through the lamina and midrib, showing a dorsiventral leaf type with cuticle, upper epidermis, upper palisade layer, spongy mesophyll and lower epidermis (Ep1P1SmEp2), also including xylem and phloem vessels as well rosettes of calcium oxalate crystals, cleared with chloral hydrate, mounted in dilute glycerol and viewed under the compound microscope (Leica®) (x 100)

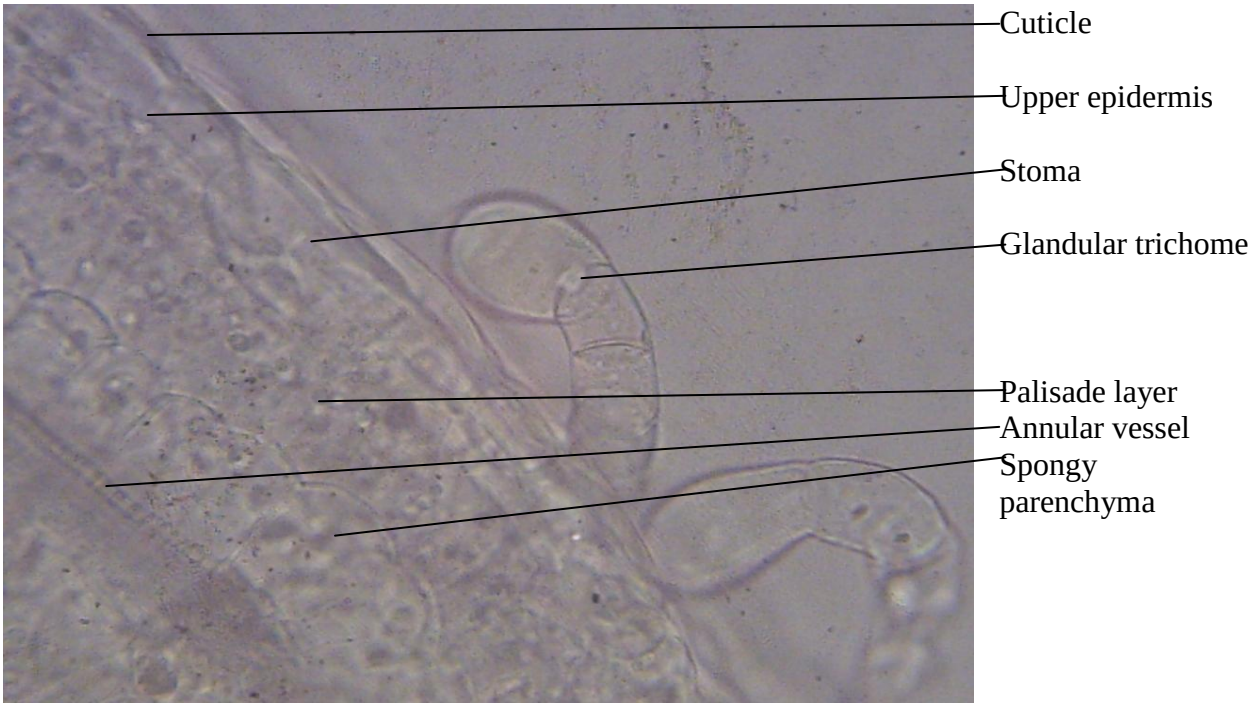


Plate VII: Transverse section of leaf showing upper part of lamina with glandular trichomes, a stoma, layer of palisade cells, spongy parenchyma and an annular vessel, cleared with chloral hydrate, mounted in dilute glycerol and viewed under the compound microscope (Leica®) (x 400)

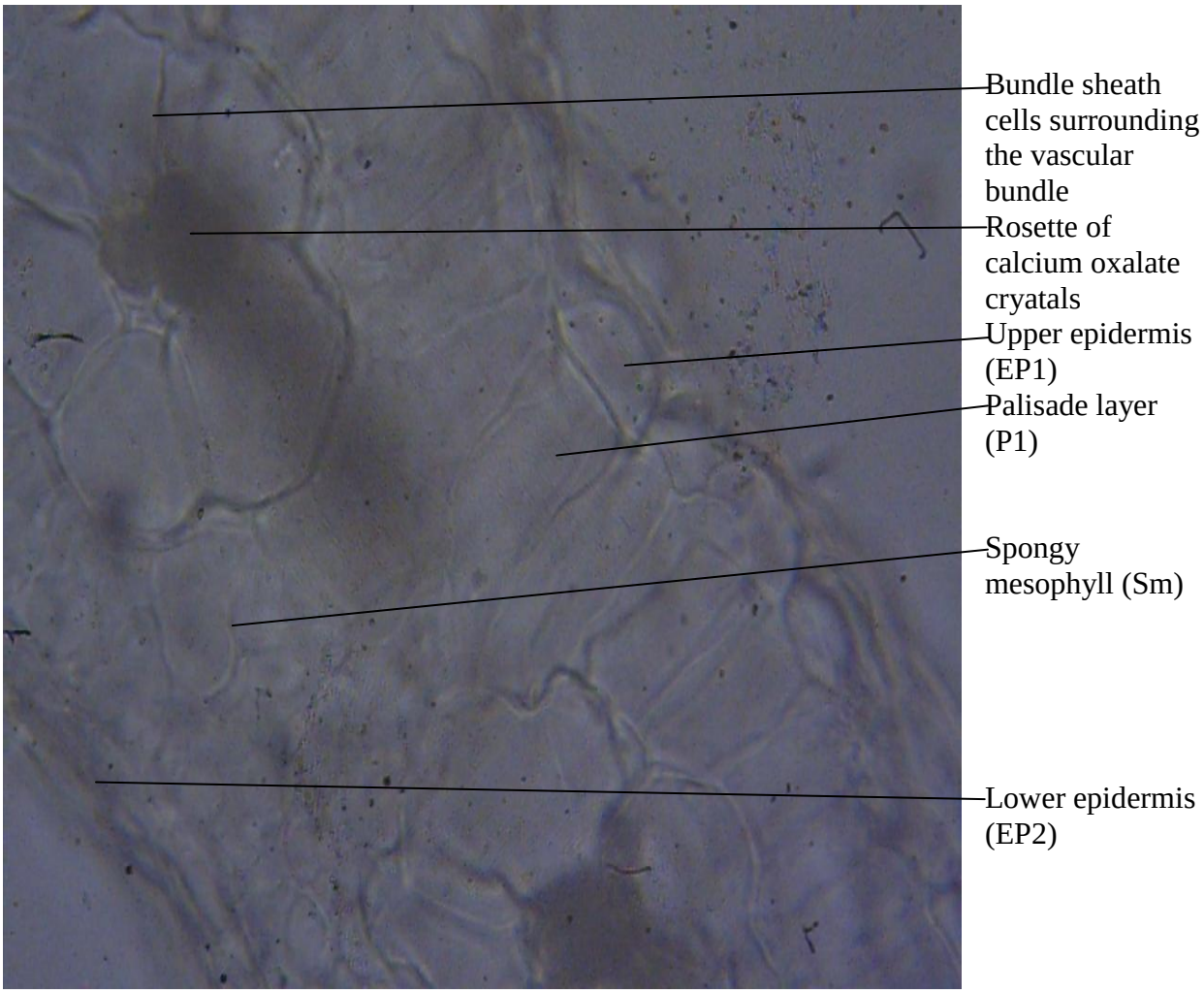


Plate VIII: Transverse section of the leaf through the lamina showing a typical dorsiventral leaf containing upper epidermis, upper palisade, spongy mesophyll and lower epidermis (Ep1P1SmEp2), cleared with chloral hydrate, mounted in dilute glycerol and viewed under the compound microscope (Leica®) (x 400)

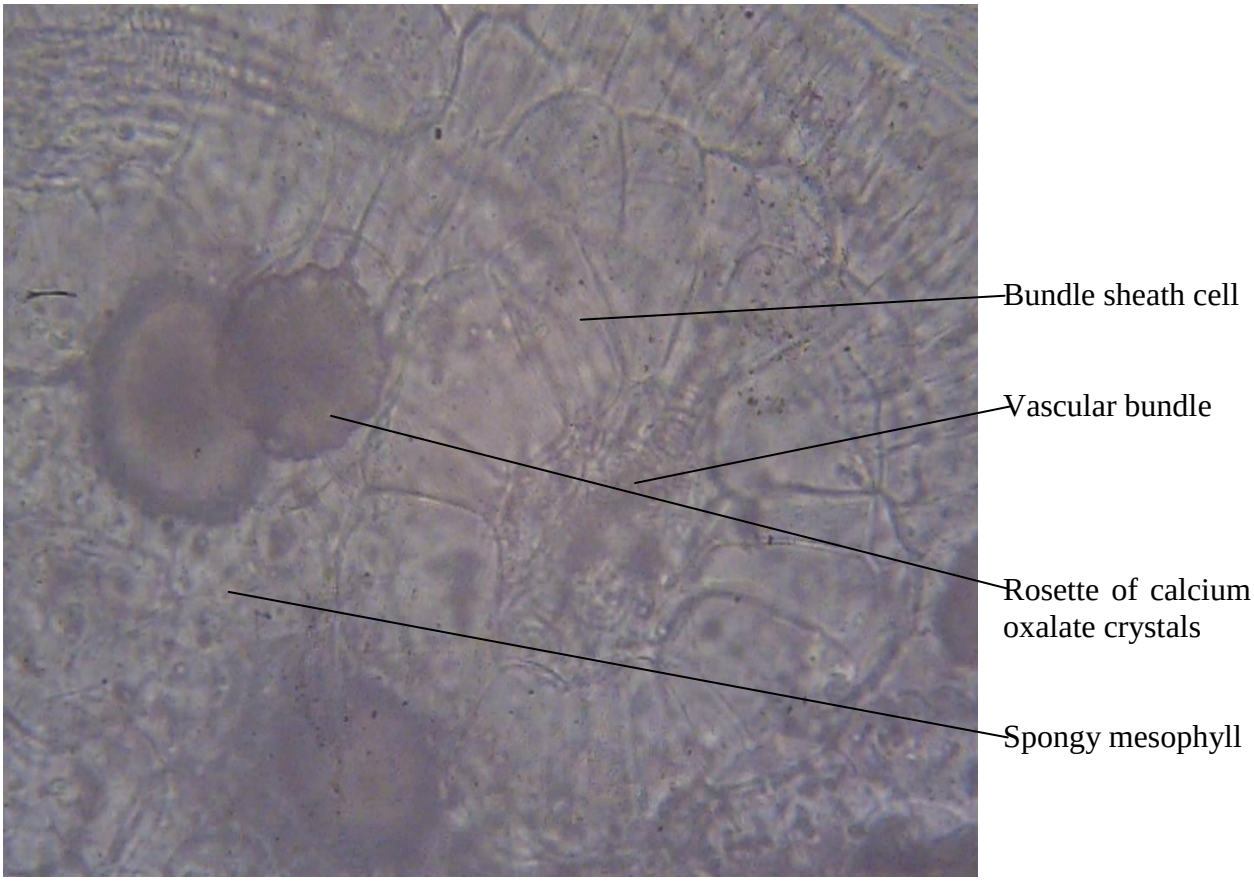


Plate IX: Transverse section of the leaf around the lamina showing upper epidermal layer, spongy parenchyma, spiral vessel surrounded by bundle sheath cells and rosettes of calcium oxalate crystals, cleared with chloral hydrate, mounted in dilute glycerol and viewed under the compound microscope (Leica®) (x 400)

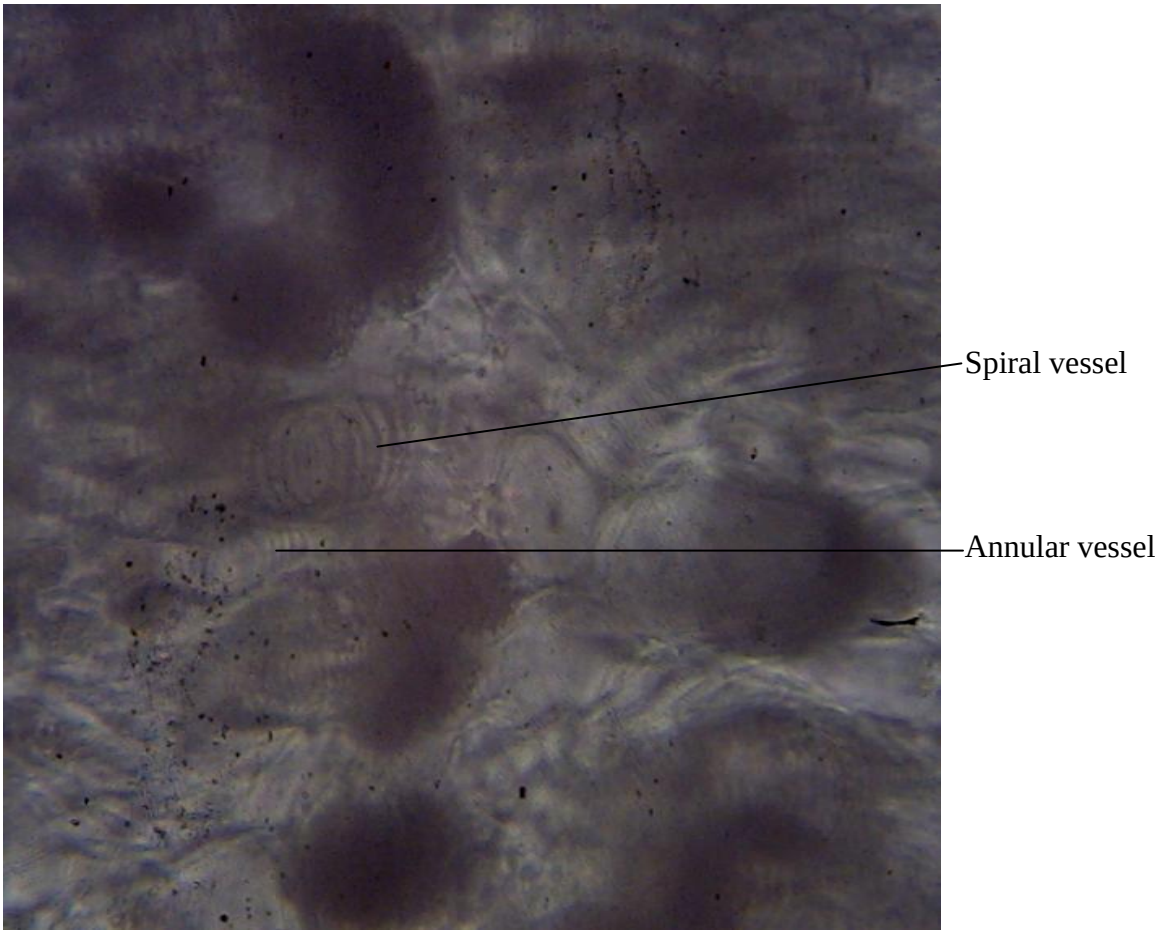


Plate X: Transverse section of the leaf showing vascular bundles with both spiral and annular vessels, cleared with chloral hydrate, mounted in dilute glycerol and viewed under the compound microscope (Leica®) (x 400)

The powder characteristics of the leaf of *A. viridis* were determined microscopically, and showed that it contains fragments of the leaf lamina indicating a dorsiventral leaf type, both fibres with sharp pointed ends and thick walls occurring singly and in bundle, stomata and rosettes of calcium oxalate crystals (Plates XI-XIV)

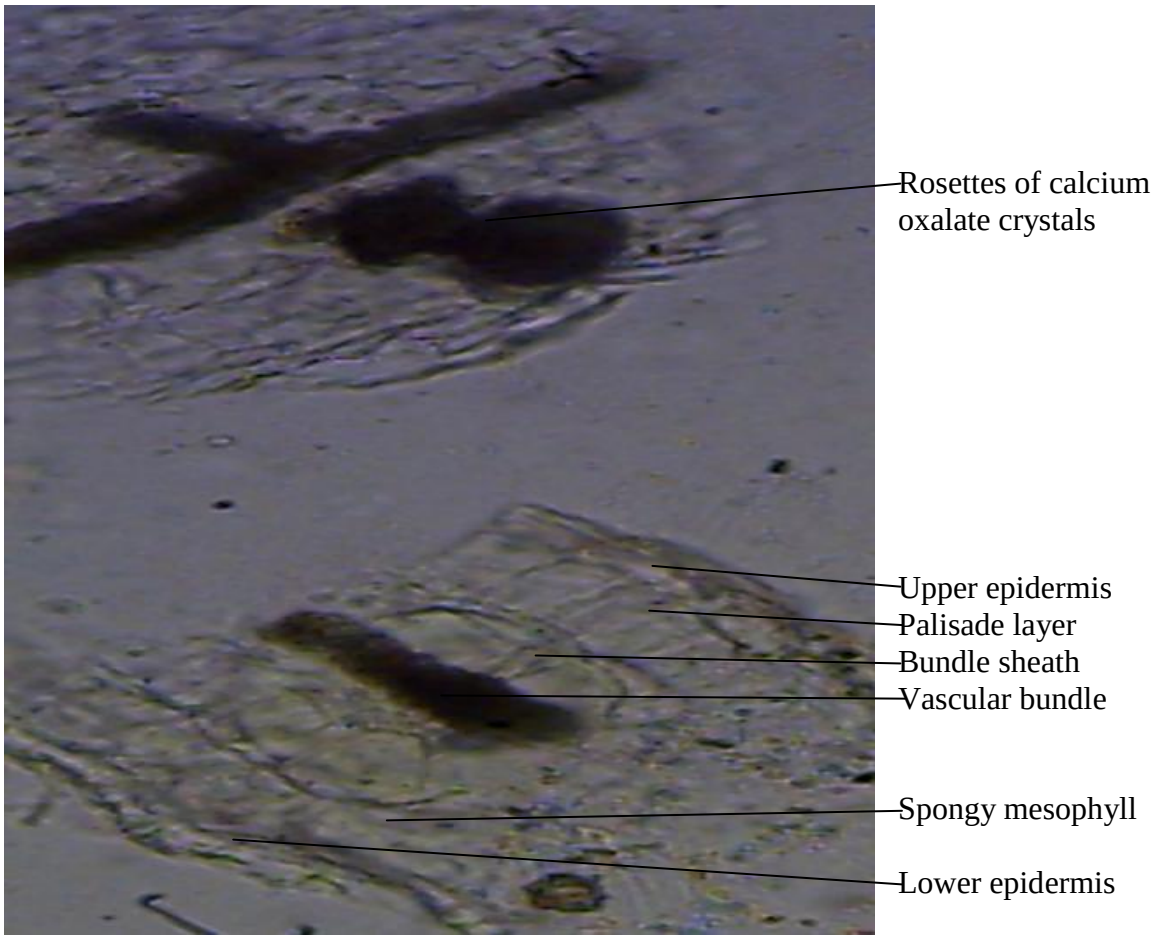


Plate XI: Microscopy of the leaf powder showing fragments of the lamina showing the presence of layers of cell types, in transverse section, that make up a dorsiventral leaf type (EP1P1SmEP2), as well as rosettes of calcium oxalate crystals, cleared with chloral hydrate, mounted in dilute glycerol and viewed under the compound microscope (Leica®) (x 400)

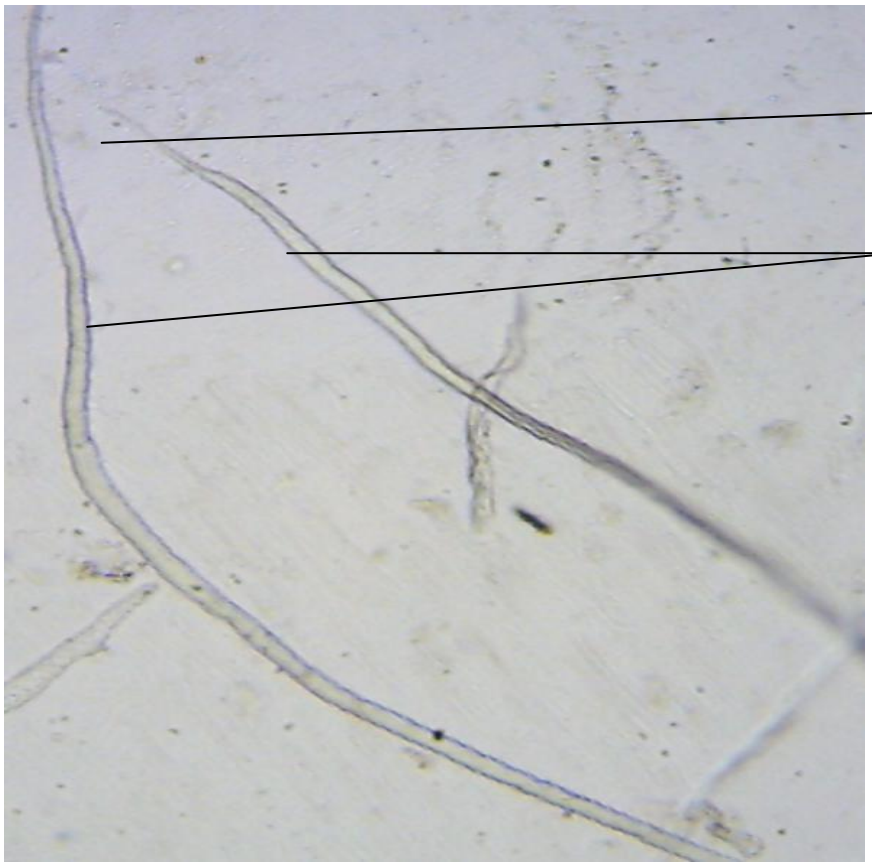


Plate XII: The leaf powder microscopy showing single fibres with pointed ends and thick walls, cleared with chloral hydrate, mounted in dilute glycerol and viewed under the compound microscope (Leica®) (x 400)

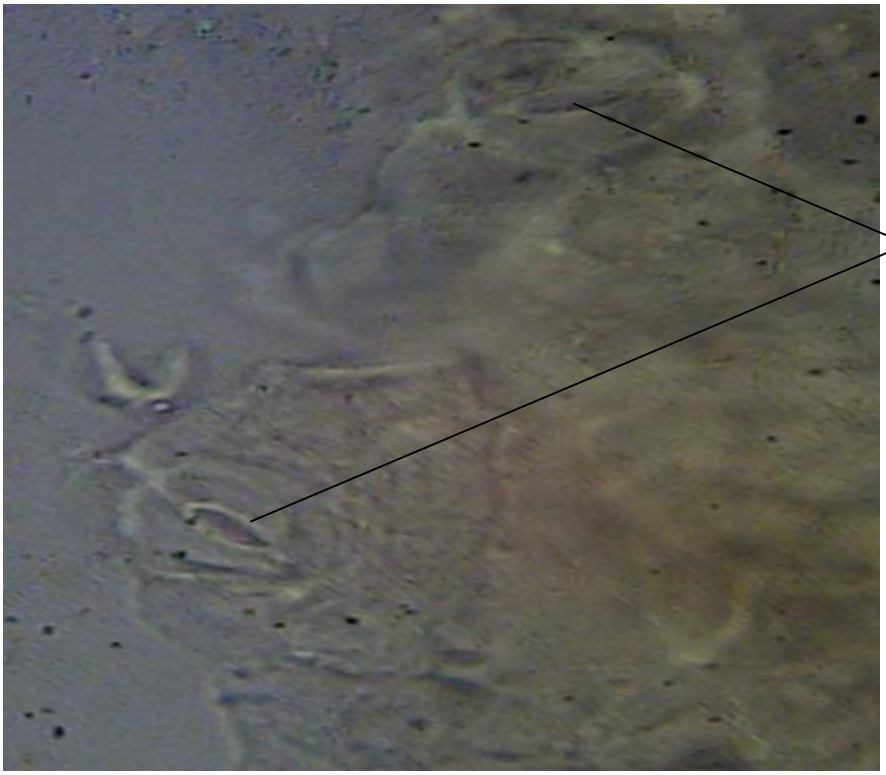


Plate XIII: Leaf powder microscopy showing epidermal fragments with stomata,cleared with chloral hydrate, mounted in dilute glycerol and viewed under the compound microscope (Leica®) (x 400)



Plate XIV: The leaf powder microscopy showing bundle and single fibres, cleared with chloral hydrate, mounted in dilute glycerol and viewed under the compound microscope (Leica®) (x 400)

4.1.2 Chemomicroscopic Evaluation

Microscopic examination of the leaf powder and sections stained (mounted) with various test reagents revealed the following as contained in the table below:

A) *Test for Lignin:* A positive result of red colouration was observed around the vessels and fibres.

B) *Test for Tannins:* A greenish colouration, positive for tannins, was observed around the calcium oxalate crystals and fibres.

C) *Test for Starch:* Blue colouration was observed to be scattered throughout the structures.

D) *Test for Proteins:* Red colouration was observed, mostly around the vessels.

E) *Test for Calcium oxalate Crystals:* Shiny structures were observed, after clearing with chloral hydrate, mostly within the spongy mesophyll. These remained after treatment with hydrochloric acid and were the rosette type of crystals.

F) *Test for Oils:* Pink stain was not observed anywhere within the powder structures; which confirmed the absence of oils.

The summary of the results is presented in Table 4.1 below.

Table 4.1:Chemomicroscopic features of *A. viridis* L. leaf

Constituent	Result	Distribution
Lignin	Present	Vessels and fibres
Tannins	Present	Calcium oxalate crystals and fibres
Starch	Present	All through
Proteins	Present	Mostly in vessels
Calcium oxalate	Present	Scattered, but mainly in spongy mesophyll
Oils	Absent	-

4.2 Physicochemical Properties of A. viridis Leaf

The quantitative determinations of the various physicochemical properties of the *A. viridis* leaf including moisture content, total ash value, acid-insoluble ash value, water-soluble ash value, ethanol-soluble extractive value and water-soluble extractive value, were done based on standard procedures and the results obtained recorded as mean ± SEM. These are summarized in Table 4.2 below.

Table 4.2: Physicochemical Properties of *A. viridis* Dry Leaf Powder

S/N	Quantitative Parameters	Values (Mean ± SEM % w/w)
1	**Moisture content**	12.19 ± 0.06
2	**Total ash value**	19.92 ± 0.10
3	**Acid-insoluble ash value**	1.07 ± 0.08
4	**Water-soluble ash value**	5.97 ± 0.06
5	**Ethanol-soluble extractive value**	1.71 ± 0.26
6	**Water-soluble extractive value**	2.17 ± 0.36

4.3 Phytochemical Screening of the Aqueous Ethanol Leaf Extract of *A. viridis*

(i) Tests for Carbohydrates

a) General Test (Molisch's Test): The formation of reddish ring at the junction of the solution of the extract and the reagent was obtained as a positive result for the presence of carbohydrates.

b) Tests for Sugars: Positive results were obtained for monosaccharides (Barfoed's test), free reducing sugars (Fehling's test) and pentoses, while those of ketoses (Selivanoff's test) and combined reducing sugars were negative.

(ii) Tests for Alkaloids

The extract treated with Mayer's and Dragendorff's reagents and positive results as cream and red precipitates were obtained implying that the plant material contains alkaloids.

(iii) Tests for Saponins

Preliminary frothing test was positive. Confirmatory blood haemolysis test was also positive.

(iv) Tests for Tannins

The solutions of the extract gave the following results:

a) Ferric Chloride Test: Green coloured precipitate was observed.

b) Lead Sub-acetate Test: A pale yellowish (buff) precipitate was observed when the crude extract was treated with lead sub-acetate reagent.

c) Gelatin – Salt Test: Precipitate was obtained in the second test tube and a positive test with $FeCl_3$ in the third test tube. *d) Test for Phlobatannins:* Deposition of a red precipitate was observed when aqueous solution of the extract was boiled with 1 % aqueous hydrochloric acid.

(v) Tests for Flavonoids

Positive results were obtained as follows:

a) Lead Acetate Test: Buff coloured precipitate was obtained, indicating the presence of flavonoids.

b) Sodium Hydroxide Test: Yellow solution was formed to indicate the presence of flavonoids.

c) Ferric Chloride Test: A greenish-black coloured solution was observed, indicating the presence of phenolic nucleus.

d) Shinoda Test: Pink to red precipitate was observed confirming the presence of flavonoids.

(vi) Test for Steroids

Salkowski's test for steroidal ring was positive, by the formation of a reddish-brown ring at the interphase between sulphuric acid and chloroform filtrate of the drug.

(vii) Tests for Anthraquinones

Both the *Borntrager's test* for free anthraquinones and the test for bound /combined anthraquinones were negative implying that the plant material lacks anthracene derivatives.

(viii) Tests for Cardiac Glycosides

a) Lieberman-Burchard's Test: A colour change from violet to blue to green which indicates the presence of a steroidal nucleus, was positive for the crude extract.

b)Keller- Kiliani's Test (for Digitoxose): A reddish-brown ring at the interface indicative of the presence of deoxy sugars, was observed for the crudeextract. *c)Kedde Test*: An immediate violet colour formation indicative of the presence of cardenolides was observed for the extract.

d)Legal Test: A red colour fading to brownish yellow indicating the presence of cardenolides was also observed for the extract.

The summary of all the phytochemical tests are as expressed in the following Table 4.3 below.

Table 4.3: Results of Preliminary Phytochemical Screening of EtOH of the Leaves of *A. viridis* L.

Constituent/Test	Observation	Inference
Carbohydrates		
Molisch's	+	Carbohydrates present
Barfoed's	+	Monosaccharides Present
Fehling's	+	Free reducing sugars Present
Combined reducing sugars	-	Combined reducing sugars absent
Pentose	+	Pentose Present
Selivanoff's	-	Ketoses absent
Alkaloids		
Mayer's	++	Alkaloids Present
Dragendorff's	++	Alkaloids Present
Saponins		
Frothing	++	Saponins Present
Haemolysis	++	Saponins Present
Tannins		
Ferric chloride	++	Phenols present
Lead subacetae	++	Tannins present
Gelatin-salt	++	Tannins present
Phlobatannins	++	Condensed tannins present
Flavonoids		
Lead acetate	++	Flavonoids present
Sodium hydroxide	++	Flavonoids present
Shinoda	++	Flavonoids present
Steroids		
Salkowski's	+	Steroids present
Anthraquinones		
Borntrager's	-	Anthraquinones absent
Combined anthraquinones	-	Anthraquinones absent
Cardiac glycosides		
Lieberman-Burchard's	++	Steroidal nucleus present
Keller-Kiliani's	++	Deoxy sugars present
Kedde's	++	Cardenolides present
Legal's	++	Cardenolides present

Key:

++ = Present in high amount
+ = Present in low amount
- = Absent
EtOH = Aqueous Ethanol extract

4.4 Acute Toxicity Studies

The aqueous ethanol extract of the A. viridis leaf was subjected to an acute toxicity test according to Lorke's method of LD_{50} determination in mice, the results of which showed it to be relatively as expressed in Table 4.4 below.

Table 4.4: LD_{50} Determination for 70% Ethanol Extract of *A. viridis* Linn. Leaves

Treatment(mg/kg)	Number of mice used	Number of mice dead
	Phase 1	
10	3	0
100	3	0
1000	3	0
	Phase 2	
1500	1	1
2900	1	1
5000	1	1

From the results above, the LD_{50} was determined as 1224.74 mg/kg from the expression;

LD_{50} = √minimum toxic dose x maximum tolerated dose

= √1500 x 1000

= √1500000

= 1224.74 mg/kg

4.5 Antioxidant Activity of Aqueous Ethanol Extract of *A. viridis* Leaves

The scavenging activities directly proportional to percentage inhibition but inversely proportional to absorbance of the extract and standard at various concentrations expressed as mean ± SEM are shown in Table 4.5 below. The absorbance of DPPH alone (Control) at 517 nm was measured as 1.454.

Statistical Analysis: Using one way ANOVA (SPSS) analysis, there was statistical significant difference at **P < 0.05**, between the activities observed.

Table 4.5 : Scavenging Activities of 70% Ethanol Extract of *A. viridis* Leaves and Ascorbic Acid on DPPH Absorbance at 517 nm

	Sample/Absorbance	
Concentration(µg/ml)	**Aq. Ethanol Extract**	**Ascorbic Acid**
12.03	0.532 ± 0.006	0.014 ± 0.0005^{a}
24.06	0.481 ± 0.013	0.013 ± 0.0003^{a}
48.13	0.413 ± 0.010	0.011 ± 0.0003^{a}
96.25	0.306 ± 0.002	0
192.5	0.103 ± 0	0
385	0.011 ± 0.0002^{a}	0

***** Absorbances with (^{a}) show similar activity.

From the absorbances measured the percentage inhibitions were derived from the mathematical expression;

Radical scavenging activity (% inhibition) = [($A_{control} - A_{sample}$)/$A_{control}$] x *100*

Statistical Analysis : Using one way ANOVA (SPSS) analysis, there was statistical significant difference at **P < 0.05**, between the activities observed.

The results are as shown in Table 4.6 below.

Table 4.6: Percentage (%) Inhibitions of 70% Aqueous Ethanol Extract of *A. viridis* Leaves and Ascorbic Acid on DPPH Absorbance at 517 nm

Sample/Percentage inhibition

Concentration(µg/ml)	Ethanol Extract (%)	Ascorbic Acid (%)
12.03	63.4	98.1
24.06	66.9	99.1
48.13	71.6	99.2
96.25	79.0	100
192.50	92.9	100
385	99.2	100

4.6 Antimicrobial Activity of *Amaranthus viridis* Leaves

4.6.1 Antibacterial Activity

The aqueous ethanol extract of the *A. viridis* leaf inhibited the growth of the bacterial species at the various concentrations used in a concentration-dependent manner. While at 125 mg/ml *P. aeruginosa* was not susceptible to the extract, the growths of the other bacteria were inhibited to varying extents. Gentamicin inhibited the growths of all the bacterial species (Table 4.7).

Table 4.7: Antibacterial Efficacy of 70% Aqueous Ethanol Extract of *A. viridis* Leaves

		Antibacterial activity/Zone of inhibition (mm)				
Extract/ Antibiotic	**Conc. (mg/ml)**	***E. coli***	***P. aeruginosa***	***S. typhi***	***S. aureus***	***B. subtilis***
Ethanol	125	12.7±1.0	-	13.5±0.6	14.3±0.4	13.2±0.8
	250	16.2±0.8	6.2±5.0	9.2±3.7	15±0.2	17.5±1.0
	500	21.8±1.21	21.2±3.9	15.5±1.3	18.2±0.9	20.2±0.6
Gentamicin	4µg/ml	41.2±1.1	41.2±2.2	39.5±1.3	37.5±1.2	40.7±1.2

Key:

- = No inhibition

The serial dilutions of the lowest concentrations of the aqueous ethanol extract of the A. viridis leaf that inhibited growth in the susceptibility test above were used to determine the Minimum Inhibitory Concentration (MIC*)* against the various bacterial species and indicated that *P. aeruginosa* has the highest MIC of 125 mg/ml, followed by *E. coli* with MIC of 62.5 mg/ml, *S. aureus* and *B. subtilis* with same MIC of 31.25 mg/ml and *S. typhi* with MIC of 15.63 mg/ml (Table 4.8).

Table 4.8: Minimum Inhibitory Concentration (MIC) of the Aqueous Ethanol Leaf Extract of *A. viridis* Linn. against Some Bacterial Organisms

	Concentration of ethanol extract (mg/ml)						
Organism	**250**	**125**	**62.5**	**31.25**	**15.63**	**7.81**	**3.91**
E. coli		-	-	+	+	+	+
P. aeruginosa	-	-	+	+	+	+	+
S. typhi		-	-	-	-	+	+
S. aureus		-	-	-	+	+	+
B. subtilis		-	-	-	+	+	+

Key:

+ = Growth

- = No growth (Inhibition)

For Minimum Bactericidal Concentration (MBC), subculturing of the bacterial contents of tubes that were clear or inhibited growth in MIC determination showed that the aqueous ethanol extract of *A. viridis* leaf has cidal or killing effect against *P. aeruginosa* and *S. aureus* at 125 mg/ml (MBC), while it has static or growth inhibiting effect only, against the other bacterial species at all the concentrations used (Table 4.9).

Table 4.9: Minimum Bactericidal Concentration (MBC) of the Aqueous Ethanol Leaf Extract of *A. viridis* Linn. against Some Bacterial Species

	Concentration of ethanol extract (mg/ml)				
Organism	**250**	**125**	**62.5**	**31.25**	**15.63**
E. coli	+	+	+	+	+
P. aeruginosa	-	-	+	+	+
S. typhi	+	+	+	+	+
S. aureus	-	-	+	+	+
B. subtilis	+	+	+	+	+

Key:

+ = Growth(Bacteriostatic)

- = No growth (Death) (Bactericidal)

4.6.2 Antifungal Activity of *Amaranthus viridis* Leaves

The aqueous ethanol extract of the *A. viridis* leaf did not show any inhibition of growth against the fungal species, *Penicillium notatum* and *Candida albicans* at all the concentrations used, while the standard Griseofulvin inhibited the growths of both species at 12.5mg/ml following the susceptibility test by agar well diffusion method, and the results are as shown in Table 4.10 below.

Table 4.10: Antifungal Efficacy of Aqueous Ethanol Extract of *A. viridis* Leaves

		Antifungal activity/Zone of inhibition(mm)	
Extract/ Antifungal	**Conc. (mg/ml)**	***P. notatum***	***C. albicans***
Ethanol extract	500	-	-
	250	-	-
	125	-	-
Griseofulvin	12.5	18±0.2	12±0.4

Key:

- = Resistance (No inhibition)

CHAPTER FIVE

5.0 DISCUSSION

The results of the microscopic examinations provide vital data to aid the correct identification and description of the plant. Hence, microscopy of the upper epidermis of the leaf, as revealed by the photomicrographs, showed the presence of slightly wavy–walled epidermal cells, glandular and non-glandular trichomes having uniseriate (multicellular) stalk containing four cells with warty surfaces and unicellular head, as well as anisocytic type of stomata, being surrounded by about three epidermal cells with one markedly smaller (Plates II - IV). Examination of the lower epidermis also revealed the presence of anisocytic type of stomata, but it is composed of epidermal cells with wavy anticlinal walls, that seem to have larger surface areas than those of the upper epidermis (Plate V). The lower epidermis also seem to contain more stomata than the upper epidermis. These were reflected in the work by Alege and Daudu (2014), in Nigeria, who reported for *A. Viridis,* the mean stomatal number and stomatal indices for the upper and lower epidermises as, 8.8000 and 10.0839, and 10.8330 and 16.9266, respectively. Also Khan *et al* (2011), in Pakistan, reported that the leaf has epidermal cells on both sides, having polyhedral to hexagonal shapes with smooth walls, as well as stomata of anisocytic type and so affirming the fact that locational or climatic differences do not necessarily vary these features.

The microscopic examination of the transverse section (T. S.) of the leaf through the midrib and sections of the lamina revealed the presence of glandular trichomes with unicellular head on both surfaces, cuticle, upper epidermis, vascular bundle

containing xylem and phloem vessels, dark deposits of rosettes of calcium oxalate crystals, a stoma, layer of palisade cells, spongy parenchyma and annular vessel as well as spiral vessels surrounded by bundle sheath cells, and collenchyma at the base of the midrib (Plates VI - X). The occurrence of upper epidermis (EP1), upper palisade layer (P1), Spongy mesophyll (Sm) and lower epidermis (EP2) (EP1P1SmEP2) in that order of transverse arrangement, specifically characterizes the leaf of *A. viridis* as a dorsiventral type.

Microscopy of the powder characteristics of the leaves showed the presence of bundle and single fibres, having narrow lumen and pointed ends, as well as fragments of the lamina showing a typical transverse arrangement of a dorsiventral leaf, rosettes of calcium oxalate crystals and epidermal fragments showing the stomata (Plates XI - XIV). No previous published report on the powder characteristics of the leaf was seen. Hence, these findings will aid in the identification and standardization of the plant to prevent adulteration and substitution, especially as the plant material could be used in the powdered form for medicinal purposes.

The chemomicroscopy of the prepared leaf powder samples, treated with various test reagents provide information with respect to the distribution of important chemical components within the leaf as a morphological part of the plant, and can be diagnostic. This showed the presence of lignin, mostly within and around vessels and fibres, for fortification. Tannins were also shown to be present, mostly around the calcium oxalate crystals and fibres. Tannins are known to be some of the most abundant secondary metabolites occurring in plants, across

the families, for mostly defensive and protective purposes. Starch was observed to be present almost throughout the leaf structure, while proteins were indicated mostly within and around the vessels; but oil was not seen. These attest to the nutritional value of *A. viridis* as a food vegetable. The presence of rosettes of calcium oxalate crystals was also confirmed, and were mostly within the spongy mesophyll (Plates VI, VIII, IX and XI). The crystallization of excess calcium in this form, occurs to a large extent in plants generally, and the nature and type are often diagnostic. Khan *et al* (2011), in Pakistan, reported that the leaves of the Pakistani variety of *A. viridis* also contain starch, protein and fat, but lacks lignin. The difference may be attributed to differing climatic and soil conditions, which can lead to phytochemical variations in plants, within families, genera or even species, especially, quantitatively. The findings here will be useful in building a standard monograph for the plant.

Physicochemical evaluations carried out for *A. viridis* leaves will be expected to be relatively constant, at least for the variety grown in this region. Hence, they are diagnostic for the purposes of correct identification and prevention of adulteration or substitution. The moisture content (loss on drying) for the crude leaf drug of *A. viridis* was determined as 12.11 ± 0.06 % w/w. The moisture content for the whole herb (100g) of the plant was reported to be 87.90% (Sharma *et al*, 2012). Baral *et al* (2011) have reported a moisture content of 0.3g for *Amaranthus spinosus*. The pharmacopoeial limit for dry herbal drugs is 14 % (African Pharmacopoeia, 1986). This implies that the moisture content of this drug is minimal and could guarantee the stability of active chemical constituents, as well as minimize the

tendency of microbial spoilage during storage. As a result, this drug can be said to have the potential of remaining in stable conditions on long term storage. In addition, the retention of moisture by this crude drug could be corroborated by the fact that the water – soluble extractive value (2.17 ± 0.36 % w/w) has been found to be higher than the ethanol – soluble extractive value (1.71 ± 0.26 % w/w). This could be related to the presence of phytochemical constituents that water can dissolve, indicating that the bulk of such constituents are phenolic and highly polar. This could also be related to the probable antioxidant activities, as polyphenols have been reported to possess such effects. The value of the moisture content will be expected to be relatively constant for the drug grown in this region.

Ash values are useful in determining the quality and purity of crude drugs, especially in the powdered forms (Kokate, 1994). In this work, the total ash value was determined as 19.92 ± 0.10 % w/w, which represents both 'physiological ash' (i. e. ash from the plant tissue) and 'non – physiological ash' (i. e. ash from extraneous matter) (African Pharmacopoeia, 1986). The total ash value of a crude drug reflects the degree of care taken in its preparation. The physiological ash usually consists of the carbonates, phosphates, silicates, nitrates, sulphates and chlorides of metal elements taken up by the plant during its growth. Elements taken up by plants from the soil include sodium, potassium, calcium, magnesium, cobalt, iron, manganese, carbon, phosphorus, nitrogen, sulphur, oxygen, chlorine and silicon (Shellard, 1958). The result here means that, 2 g of this plant crude drug (leaves) contains only about 20 % of residual substances, including impurities, that did not volatilize when ignited. Sharma *et al* (2012) have

reported that the ash content of the whole herb (100g) of *A. viridis*, in their work was found to be 1.85%. Baral *et al* (2011) also reported a total ash value for *Amaranthus spinosus* leaves as 14.9% w/w. The water – soluble ash value was found to be 5.97 ± 0.06% w/w, which is composed of physiological aspects or parts of the plant tissue where the active constituents reside. That of *Amaranthus spinosus* was found to be 7.3% (Baral *et al*, 2011). The acid – insoluble ash value was determined as 1.07 ± 0.08 % w/w, while that of *Amaranthus spinosus* was found to be 2.4% (Baral *et al*, 2011). A high acid – insoluble ash in leaf drugs indicates contaminations with earthy material (impurities). The result obtained is favourable, because it shows that impurities make up only about 1 % of the 2 g of the drug powder; which becomes very significant, especially where the drug is to be used directly as the powder (Evans, 2002).

The determination of extractive values helps in measuring the amount of chemical constituents in a drug, that are extractable by a chosen solvent (water or alcohol) under specified conditions. It helps to also tell the chemical nature of the contained constituents (their degree of polarity) and the choice of the most suitable solvent for extraction and phytochemical studies. The evaluation is important as it aids to guard against wrong identification and adulteration or substitution with other drugs. The result of this work shows that, the ethanol – soluble extractive and the water – soluble extractive values were 1.71 ± 0.26 % w/w and 2.17 ± 0.36 % w/w, respectively. This shows that the leaves of *A. viridis* contain more of water – soluble (very polar) constituents, and could explain the probable antioxidant activity due the content of polyphenolic

compounds. Baral *et al* (2011) reported that the ethanol – soluble extractive and the water – soluble extractive values for *Amaranthus spinosus* (its Genus relative) are 1.601 and 1.811, respectively. In both cases water appears to be a better solvent for the constituents. No previous published report of the physicochemical properties of *Amaranthus viridis* leaves was seen. These findings will be useful for a standard monograph for the plant, which will also aid in the detection of adulteration or substitution.

Phytochemical screening, which involved preliminary chemical tests of the crude aqueous extract of the leaves of *A. viridis*, would confirm the chemotaxonomic linkage with other members of the Amaranthaceae family, and also establish the phytochemical profile for its identity and standardization, as well as provide the basis for its traditional medicinal applications. Preliminary screening showed that carbohydrates, alkaloids, saponins, tannins, flavonoids, steroids and cardiac glycosides present in the crude 70% aqueous ethanol leaf extract. Ashok Kumar *et al* (2010), in their work, reported that preliminary phytochemical analysis showed the presence of carbohydrates, steroids, alkaloids, phenolic compounds, flavonoids, saponins and amino acids in the methanol extract of the whole *A. viridis* plant. Also, Krishnamurthy *et* al (2011) and Ahmed *et al* (2013) have reported the presence of tannins, saponins, alkaloids, proteins, glycosides and appreciable levels of total phenols in the methanol extract of the leaves. This shows insignificant variations in the occurrence of phytochemical contituents, the use of different solvents of similar polarities and/or plant parts and locality difference notwithstanding. It has also been reported by other workers that flavonoids and saponins commonly

occur in this plant and its relatives of the Amaranthaceae (Ashok Kumar *et* al, 2009; Muller and Borsch, 2005; Ghani, 2003; Olufemi *et al*, 2003).

A major concern with respect to medicinal products, apart from effectiveness and potency, is their safety. Usually, the less toxic a drug is, the more tolerated and safer it is for the user. The acute toxicity test (LD_{50}) is useful in determining the safety of the drug (Lorke, 1983). The results of the acute toxicity test of the aqueous ethanol leaf extract of the plant (*A. viridis*) showed an LD_{50} of 1224.74 mg/Kg body weight in mice (*ip*). Krishnamurthy *et al* (2011), in India, via the oral route in wistar rats and using the guidelines set by Organization for Economic Co-operation and Development (OECD), reported that the rats showed normal behavior and no toxic effects and lethality were observed at a higher dose of 5000 mg/kg of the methanol extract of the leaves of the plant. Ashok Kumar *et al* (2011), reported that via the OECD, using rats, the methanol extract of the whole plant was devoid of any toxicity at the dose of up to 2000 mg/kg orally. It should be noted that the determination of safety or toxicity is dependent on the route of administration of the drug as well as the type of animal subjects used. The observed differences can be attributed to the fact that mice are usually smaller and more sensitive than the rats and the intraperitoneal route is more sensitive than the oral route of drug administration. But, this serves as a basis for further experiments with the drug in mice and extrapolations to other animals. This also implies that the use of doses greater or equal to the LD_{50} in mice and their extrapolations in other animals and humans, could lead to various toxic effects, including death. The extract can therefore, be considered to be

relatively safe, giving an insight to the non- toxic nature of the plant in its use for food and medicinal purposes.

The antioxidant activity (free radical scavenging activity) of the aqueous ethanol extract of the leaves of *A. viridis* was evaluated based on its action on the stable radical 1, 1 – diphenyl – 2 – picrylhydrazyl (DPPH) as compared with the standard antioxidant agent (Ascorbic acid). It is based on the principle of a redox reaction between the extract constituents (reducing agent) (AH) and the radical (oxidizing agent) (DPPH);

$$AH + DPPH^{-}\ (Purple) \longrightarrow A^{-} + DPPH{:}H\ (No\ colour/Yellow)$$

The charged radical loses its colour and absorption power at 517 nm in visible spectroscopy, as it gets reduced (inhibited) by the antioxidant agent. This means that there is an inverse relationship between the concentration of the antioxidant /percentage inhibition and absorbance/colour/concentration of the radical. From the results obtained in this work, both the aqueous ethanol leaf extract of the plant and the standard drug (ascorbic acid), decreased the absorbance by DPPH with increase in concentration of the samples, while the percentage inhibition (free radical scavenging activity) also increases. At the lowest concentration (12.03 µg/ml), both the test and standard samples exhibited percentage inhibitions above 50 %; 63.41 % for the extract and 98.08 % for ascorbic acid. At the highest concentration (385µg/ml), the activities of both samples were highly comparable; 99.24 % for the extract and 100 % for ascorbic acid. On the whole, at every concentration the standard (ascorbic acid) showed

better antioxidant activity than the test extract, as exhibited by the chart, and the statistical analysis that showed there is significant difference ($P < 0.05$) between the activities of the two samples.

The best way to compare the activity of both samples is to determine the concentrations of the different samples that gave the same activity. Usually, IC_{50}, the concentration of each of the samples that scavenges or inhibits 50 % of the radical (DPPH) is determined graphically and used for comparison, to make inference with respect to the antioxidant activity of the test sample. In this case, the corresponding activities, even from the lowest concentration were greater than 50 % , but the same activity was recorded at 99.24 % for both samples. Thus, instead of IC_{50}, the $IC_{99.24,}$ which is 385 µg/ml (by mathematical extrapolation, IC_{50} is equal to 193.97 µg/ml) for the aqueous ethanol extract and 48.13 µg/ml (by mathematical extrapolation, IC_{50} is equal to 22.25 µg/ml) for ascorbic acid, were used. This shows that the standard (ascorbic acid) has better antioxidant activity than the crude ethanol extract.

But, it should be noted that, from the performances of the higher concentrations of the aqueous ethanol extract of the *A. viridis* leaves, the antioxidant activity, which is concentration dependent, is highly significant and comparable to that of the standard, and could be far better if purer isolates of the extract were used. Ashok Kumar *et al* (2011) in India, have also shown that the methanol extract of the whole plant is able to reverse the oxidative hepatotoxicity induced by paracetamol. This reinforces its use as a dietary vegetable. The antioxidant activity of the aqueous

ethanol leaf extract of *A.viridis* could be attributed to the presence of polyphenols, especially flavonoids, and tannins (Ahmed *et al*, 2013). This is also corroborated by many other workers (Ashok Kumar, 2010; Motlhanka *et al*, 2008; Salvador *et al*, 2006; Riedl and Hagerman, 2001; Tringali, 2001). Hence, the plant is found useful in the traditional treatment of free radical mediated inflammatory diseases like cancer (e.g. skin cancer), eczema, bronchitis, piles etc. (Ashok Kumar, 2009; Burkill, 1985; Duke and Ayensu, 1985).

The antibacterial activity screening results help to explain the traditional medicinal application of the leaves or whole plant in the treatment of various bacterial infections. The results reveal that all the five gram positive and gram negative bacteria were sensitive to the different concentrations of the aqueous ethanol extract and the standard (Gentamicin). However, there was resistance by *P. aeruginosa* at 125 mg/ml. But, in all respects, the standard at 4 µg/ml showed the highest activity, and statistical analysis revealed significant difference between the activities recorded, at $P < 0.05$. The order of antibacterial activity observed for the extract was as follows :

- At 125 mg/ml; *S. aureus* > *S. typhi* >*B. subtilis* >*E. coli*
- At 250 mg/ml; *B. subtilis* > *E. coli* > *S. aureus* > *S. typhi* > *P. aeruginosa*
- At 500 mg/ml; *E. coli* > *P. aeruginosa* > *B. subtilis* > *S. aureus* > *S. typhi*

The order of activity for gentamycin was observed as follows :

- At 4 µg/ml; *E. coli* ≥*P. aeruginosa* > *B. subtilis* > *S. typhi* > *S. aureus*

The very significant antibacterial activity exhibited by the crude aqueous ethanol extract of the leaves of *A. viridis* could imply that if purer and more specific fractions or isolates are used, much better activity comparable to that of the standard could be observed.

The determination of the minimum inhibitory concentration (MIC) of the aqueous ethanol leaf extract via impregnation of the serial dilutions of the lowest effective doses in the experiment above into nutrient broth cultures of the respective bacterial organisms, and observing for the lowest doses that will prevent growth (turbidity) was conducted. The observations made as shown in Table 4.8 revealed the following MICs : 125 mg/ml for *P. aeruginosa* , 62.5 mg/ml for *E. coli,* 15.63 mg/ml for *S. typhi,* 31.25 mg/ml for *S. aureus* and 31.25 mg/ml for *B. subtilis*. This shows the more resistant bacterial specie to the constituents of the aqueous ethanol leaf extract to be *P. aeruginosa.*

The bacterial contents of the clear/non – turbid tubes (extract concentrations) were sub – cultured to observe for growth (bacteriostatic activity) or no growth/clarity (bactericidal/killing activity), in the determination of the minimum bactericidal concentration (MBC). The results as in Table 4.9, reveal that the aqueous ethanol leaf extract is bacteriostatic at the concentrations employed to *E. coli, S. typhi* and *B. subtilis*, but bactericidal to *P. aeruginosa* and *S. aureus* with MBCs at 125 mg/ml.

Ahmed *et al* (2013) using the disc diffusion method and only two bacterial strains (S. *aureus* and *E. coli*) have reported antibacterial activity for 100% methanol leaf extract of the plant (25 mm) than for 80% methanol leaf extract of the plant (23 mm). In

comparison to this work, any observed variations in activity could be attributed to the type and concentration of extract used. This confirms the antibacterial potential of the leaves or whole of the plant, and shows why it is traditionally used to treat infective ailments such as abscesses, gonorrhea, sores, eye infections, leprosy and dysentery (Ashok Kumar, 2009; Burkill, 1985; Duke and Ayensu, 1985).

The evaluation of the antifungal activity of the crude leaf extract of the plant via the well diffusion method and measuring the zones of inhibition of the growth of *P. notatum* and *C. albicans* was done using the same concentrations as in antibacterial sensitivity testing. The results, as in Table 4.10, showed that while the standard antifungal agent (griseofulvin) at 12.5 mg/ml gave some activity in both cases, all the various concentrations of the extract were resisted by the two fungi. This implies that the aqueous ethanol leaf extract of *A. viridis* lacks antifungal activity, but increasing the concentrations may raise safety concerns. Ahmed *et al* (2013) also reported that, unlike for the bacterial strains, there was less activity or sensitivity for 100% and 80% methanol leaf extract against fungal strains (*Fussarium solani* and *Rhizopus oligosporus*). The variations observed could also be due to the type and concentration of extracts and fungal strains used in the different cases.

CHAPTER SIX

6.0 SUMMARY, CONCLUSIONS AND RECOMMENDATIONS

6.1 Summary

Pharmacognostic parameters, which included microscopic and chemomicroscopic properties were determined and the findings revealed, most importantly, that the leaves of *A. viridis* are composed of: slightly wavy-walled epidermal cells on the upper surface and larger wavy-walled epidermal cells on the lower surface, with both containing anisocytic stomata; uniseriate glandular trichomes with unicellular head on both surfaces; rosettes of calcium oxalate crystals within the spongy mesophyll and around the vessels; spiral and annular vessels and a transverse section that shows it to be a dorsiventral (with distinct upper and lower surfaces) leaf type. The chemomicroscopy revealed the presence of lignin, tannins, starch, proteins and calcium oxalate crystals in different specific locations within the leaf, but the absence of fat/oil. The moisture content was determined as 12.19 $\pm$ 0.06% w/w of the air – dried leaf powder, which is comparable to the pharmacopoeial limit 14 %. The total, acid – insoluble and water – soluble ash values, were determined as 19.92 $\pm$ 0.10% w/w, 1.07 $\pm$ 0.08% w/w and 5.97$\pm$ 0.06% w/w, respectively. The ethanol – soluble extractive value (1.71 $\pm$ 0.26% w/w) and water – soluble extractive value (2.17 $\pm$ 0.36% w/w) as determined show that water is a better solvent for the extraction than ethanol (alcohol). Phytochemical screening of the crude ethanol leaf extract of the plant affirmed the presence of diverse active secondary metabolites, including: carbohydrates (free reducing, monosaccharides and pentose sugars), alkaloids,

saponins, tannins, flavonoids, steroids and cardiac glycosides. The findings above are diagnostic with respect to the medicinal plant's correct identification and useful for standardization, particularly for the Nigerian or African pharmacopoeia, as well as explain the basis for the various local traditional medicinal uses of the plant.

The acute toxicity studies was carried out by Lorke's method and the LD_{50} determined as 1224.74 mg/Kg in mice (*ip*), which shows the crude drug to be relatively safe and not prone to easy toxicities; especially because the intraperitoneal (parenteral) route and mice (very sensitive animal subjects) were used. In the test for antioxidant activity by evaluation of the free radical scavenging effect on DPPH, indicated by the varying absorbance measured at 517 nm in a UV spectrophotometer, the aqueous ethanol leaf extract of *A. viridis* significantly inhibited (scavenged) the free radical and decreased its light absorption (absorbance). Thus, it has significant antioxidant activity which is comparable to that of the standard/known antioxidant agent, ascorbic acid with statistical significant difference at $P < 0.05$. This activity can be attributed to its content of antioxidant polyphenols (flavonoids and tannins) and saponins.

With respect to antimicrobial activity, the aqueous ethanol leaf extract of the plant was found to show significant broad spectrum antibacterial activity against both gram positive and gram negative bacteria, including : *E. coli, P. aeruginosa, S. typhi, S. aureus* and *B. subtilis*, with the following respective MICs; 62.5 mg/ml, 125 mg/ml, 15.63 mg/ml, 31.25 mg/ml and 31.25 mg/ml. Further determination of the MBCs revealed that the drug is bacteriostatic against *E. coli*, *S. typhi* and *B. subtilis*, while it is bactericidal against *P. aeruginosa* and *S. aureus* , with 125

mg/ml as MBC in both cases. In comparison to the standard Gentamicin, there was statistical significant difference at $P < 0.05$. This confirms the basis for the effectiveness of the plant in the traditional medicinal treatment of infectious diseases that are predominantly bacterial in origin. The 70% aqueous ethanol leaf extract did not show any antifungal activity at the concentrations applied, and increasing the concentrations would increase the tendency for toxicities.

6.2 Conclusion

This work has been able to establish pharmacognostic characters of the leaves of *Amaranthus viridis* growing in Nigeria, including microscopic features of upper and lower epidermises, transverse section and drug powder, as well as diagnostic physicochemical properties, to serve as useful standards for proper identification of the plant. The antioxidant and antimicrobial effects of the plant were consistent with the results of other researchers conducted on similar species as reported in the literature.

6.3 Recommendations

The crude 70% aqueous ethanol leaf extract of *Amaranthus viridis* was found to have antibacterial and antioxidant activities; hence, further work should be done to ascertain the specific fractions or pure isolates responsible for these activities via well regulated bioassay-guided separation techniques, with structural elucidations in order to advance the course of new drug discovery and development.

REFERENCES

African Pharmacopoeia (1986). Determination of Ash Values, Extractives and Swelling Index. First Edition. Organization of African Unity/Scientific, Technical and Research Commission, Scientific Publication No. 3. Lagos, Nigeria; pp 78 – 142.

Agrawal, S. S. and Paridhavi, M. (2007). *Herbal Drug Technology*. University Press (India) Private Limited, Hyderabad, India; pp 625 – 627.

Agunu, A., Dafam, D. G. and Kagaru, D. C. (2009). Medicinal Plants Used In Traditional Pain Management In Jos, Nigeria. *Planta Medica*, 75 (9): 877–1094.

Ahmed, S. A., Hanif, S. and Iftkhar, T. (2013). Phytochemical Profiling with Antioxidant and Antimicrobial Screening of *Amaranthus viridis* L. Leaf and Seed Extracts. *Open Journal of Medical Microbiology*, 3: 164 – 171.

Aji, S. B., Auwal, M. S., Onyeyili, P, A. and Dawurung, C. J. (2010). Phytochemical Screening and Antibacterial Evaluation of the Leaves of *Olea hochstetteri* Bak. (Oleaceae). *Research Journal of Pharmacology*, Vol. 4 (2): 26 – 30.

Akiyama, H., Fujii, K., Yamasaki, O., Oono, T. and Iwatsuki, K. (2001). Antibacterial Action of Several Tannins Against *Staphylococcus aureus*. Journal of Antimicrobial Chemotherapy, 48 (4): 487 – 491.

Alege, G. O. and Daudu, S. M. (2014). A Comparative Foliar Epidermal and Morphological Study of Five Species of *Amaranthus*. *European Journal of Experimental*, 4 (4): 1 – 8.

Ashok Kumar, B. S., Lakshman, K., Jayaveera, K. N., Nandeesh, R., Mani Tripathi, S. N.,

Vamshi Krishna, N., Manjunath, M. and Suresh, M. V. (2009). Estimation of Rutin and Quercetin in *Amaranthus viridis* L. by High Performance Liquid Chromatography (HPLC). *Ethnobotanical Leaflets*, 13: 437 – 442.

Ashok Kumar, B. S., Lakshman, K., Jayaveera, K. N., Ranganayakulu, D. and Manoj, B. (2010). Invitro Anthelmintic Property of Methanolic Extract of *Amaranthus viridis* Linn. *Electronic Journal of Environmental, Agricultural and Food Chemistry*, 9 (6): 1093 – 1097.

Ashok Kumar, B. S., Lakshman, K., Jayaveera, K. N., Sheshadri Shekar, D. and Vivek, C. (2010). Antinociceptive and Antipyretic Activities of *Amaranthus viridis* Linn. in Different Experimental Models. *Archive of Biological Sciences*, Belgrade, 62 (2): 397 – 402.

Ashok Kumar, B. S., Lakshman, K., Narayan Swamy, V. B., Arun Kumar, P.A., Sheshadri Shekar, D., Manoj, B. and Vishwantha, G. L. (2011). Hepatoprotective and Antioxidant Activities of *Amaranthus viridis* Linn. *Macedonian Journal of Medical Sciences*,10.3889/MJMS.1857-5773.2011.0163

Baral, M., Datta, A., Chakraborty, S. and Chakraborty, P. (2011). Pharmacognostic Studies on Stem and Leaves of *Amaranthus spinosus* Linn. *International Journal of Applied Biology and Pharmaceutical Technology*, Volume 2, Issue 1: ISSN 0976 – 4550.

Brand – Williams, W., Cuvelier, M. E. and Berset, C. (1995). Use of Radical Scavenging Method to Evaluate Antioxidant Activity. *Lebensmittel Wissenschaf and Technologie*, 28: 25 – 30.

British Pharmacopoeia (1993), Vol. II. Total Ash. Appendix XI. Her Majesty's Stationary Office, London.

British Pharmacopoeia (2009), Vol. III. Herbal Drugs and Herbal Drug Preparations. The Stationery Office on behalf of the Medicines and Healthcare products, Regulatory Agency (MHRA), Market Towers, 1 Nine Elms Lane, London SW8 5NQ.

Burkill, H. M. (1985). *The Useful Plants of West Tropical Africa. Vol. 1, Families A – D.* Royal Botanical Gardens, Kew: pp 52 – 53.

Cai, Y., Sun, M. and Corke, H. (2003). Antioxidant Activity of Betalains from Plants of the Amaranthaceae. *Journal of Agricultural and Food Chemistry*, 51 (8): 2288 – 94.

Chopra, R. N., Nayar, S. L. and Chopra, I. C. (1986). *Glossary of Indian Medicinal Plants*. Council of Scientific and Industrial Research, New Delhi, India.

Dalziel, J. M. (1937). *Useful Plants of West Tropical Africa.* Crown Agent for Overseas Government and Administration, London.

Denyer, S. P., Hodges, N. A. and Gorman, S. P. (2004). *Hugo and Russell's Pharmaceutical Microbiology*. Seventh Edition. Blackwell Publishing Company, Blackwell Science Inc., 350 Main Street, Malden, Massachusetts, USA. Pp 187 – 188.

Duke, J. A. and Ayensu, E. S. (1985). *Medicinal Plants of China*. Reference Publications Inc. ISBN 0 – 917256 – 20 – 4.

Evans, W. C. (2002). *Trease and Evans Pharmacognosy*. Fifteenth Edition. (© Harcourt Publishers Limited). Elsevier, 17 – A/1, Main Ring Road, Lajpat Nagar – IV, New Delhi – 110 024, India. Pp 98 – 99, 137, 516, 538.

Gandhiraja, N., Sriram, S., Meena, V., Srilakshmi, J. K., Sasikumar, C. and Rajeswari, R. (2009). Phytochemical Screening and Antimicrobial Activity of the Plant Extracts of *Mimosa pudica* L., against Selected Microbes. *Ethnobotanical Leaflets*, 13: 618 – 24.

Ghani, A. (2003). *Medicinal Plants of Bangladesh With Constituents and Uses*, 2nd Edition. Asiatic Society of Bangladesh, 5 Old Secretariat Road, Nimtali, Dhaka, Bangladesh.

Ibrahim, H. (1990). Pharmacognostic and Evaluative Studies of the Stem Bark of *Cissus populnea* Gull and Perr. Unpublished M. Sc. Thesis. Ahmadu Bello University, Zaria, Nigeria.

Kaur, N., Dhuna, V., Kamboja, S. S., Agrewala, J. N. and Singh, J. (2006). A Novel Antiproliferative and Antifungal Lectin from *Amaranthus viridis* Linn. Seeds. *Protein Pept Letter*, 13 (9): 897 – 905.

Khan, M., Musharaf, S., Ibrar, M. and Hussain, F. (2011). Pharmacognostic Evaluation of *Amaranthus viridis* L. *Research in Pharmaceutical Biotechnology*, Vol. 3 (1):11-16.

Kokate, C. K. (1994). *Practical Pharmacognosy*. Fourth Edition. Vallabh Prakashan, SU – 221, Pitampura, Delhi – 110088, India: pp 18 – 22, 123.

Krishnamurthy, G., Lakshman, K., Pruthvi, N. and Chandrika, P. U. (2011). Antihyperglycemic and hypolipidemic activity of methanolic extract of *Amaranthus viridis* leaves in experimental diabetes. *Indian Journal of Pharmacology;* 43(4): 450–454.

Kwon, S. Y., An, C. S., Liu, J. R. and Pack, K. H. (1997). A Ribosome – Inactivating Protein from *Amaranthus viridis. Biosciences Biotechnology Biochemistry*, 61 (9):

1613 – 14.

Lorke, D. (1983). A New Approach to Practical Acute Toxicity Testing. *Archives of Toxicolgy*, 54: 275 – 287.

Motlhanka, D. M. T., Habtemariamc, S. and Houghton, P. (2008). Free Radical Scavenging Activity of Crude Extracts and 4´-O-Methylepigallocatechin Isolated From Roots of *Cassine Transvaalensis* Burtt-Davy from Botswana. *African Journal of Biomedical Research*, Vol. 11: 55 – 63.

Muller, K. and Borsch, T. (2005). Phylogenetics Using MatK/TrnK Sequence Data – evidence from Parsimony, Likelihood and Bayesian Approaches. *Annals of the Missouri Botanical Garden*, 92: pp 66 – 102.

Obi, R. K., Iroagba, I. I., and Ojiako, O. A. (2006).Virucidal Potential of Some Edible Nigerian Vegetables. *African Journal of Biotechnology*, 5 (19): 1785 – 1788.

Olufemi, B. E., Assiak, I. E., Ayoade, G. O. and Onigemo, M. A. (2003). Studies on the Effects of *Amaranthus spinosus* Leaf Extract on the Haematology of Growing Pigs. *African Journal of Biomedical Research*, Volume 6; 149 – 150.

Onaolapo, J. A. (2006). *Practical Applied Pharmaceutical Microbiology*. 400 Level Practical Manual, Department of Pharmaceutics and Pharmaceutical technology, Faculty of Pharmaceutical Sciences, University of Jos, Jos, Nigeria.

Onocha, P. A., Oloyede, G. K. and Afolabi, Q. O. (2011). Cytotoxicity and Free Radical Scavenging Activities of Hexane Fractions of Nigerian Specie of African Pear (*Dacryodes edulis*). *International Journal of Biological Chemistry*, 5(2):143 – 149.

Riedl, K. M. and Hagerman, A. E. (2001). Tannin Protein Complexes as Radical Scavengers and Radical Sinks. *Journal of Agricultural and Food Chemistry*; 49: 4917 – 4923.

Sadasivam, S. and Balasubramanian, T. (1985). *Practical Manual* (*Undergraduate*), Tamil Nadu Agricultural University, Coimbatore, India: pg 2.

Salvador, M. J., Ferreira, E. O., Mertens – Talcott, S. U., Victor De Castro, W., Butterweck, V., Derendorf, H. and Dias, D. A. (2006). Isolation and HPLC Quantitative Analysis of Antioxidant Flavonoids from *Alternanthera tenella* Colla. *Z. Naturforsch*, 61c: 19 – 25.

Sarker, S. D., Latif, Z. and Gray, A. I. (2006). *NaturalProductsIsolation*. Second Edition. Humana Press Inc., 999 Riverview Drive, Suite 208, Totowa, New Jersey 07512: pp 265, 269 – 285, 330, 341.

Sena, L. P., Vanderjagt, D. J., Rivera, C., Tsin, A. T. C., Muhamadu, I., Mahamadou, O., Millson, M., Pastuszyn, A. and Glew, R. H. (1998). Analysis of Nutritional Components of Eight Famine Foods of the Republic of Niger. *Plant Foods and Human Nutrition*, 52 (1): 17 – 30.

Sharma, N., Gupta, P.C. and Rao, Ch.V. (2012). Nutrient Content, Mineral Content and Antioxidant Activity of *Amaranthus viridis* and *Moringa oleifera* Leaves. *Research Journal of Medicinal Plant*, 6: 253-259.

Shellard, E. J. (1958). *Exercises in the Evaluation of Drugs and Surgical Dressings*. First Editon. Pitman Medical Publishing Co. Ltd., London: pp 81, 86, 88, 89, 95.

Sofowora, A. (2008). *Medicinal Plants and Traditional Medicine in Africa*. Third

Edition. Spectrum Books Limited, Ibadan.

Tasdemir, D. (2009). Natural Products for the Treatment of Infectious Neglected Diseases. *Planta Medica*, 75 (9): 877 – 1094.

Trease, G. E. and Evans, W. C. (1983). *Pharmacognosy*. Twelfth Edition. Bailliere Tindall, London.

Tringali, C. (2001). *Bioactive Compounds from Natural Sources*. E-Library Edition. Taylor and Francis, 11 New Fetter Lane, London EC4P4EE and 29 West 35th Street, New York, NY 10001; pp 339 – 341, 401.

Wallis, T. E. (1985). *Textbook of Pharmacognosy*. Fifth Edition. CBS Publishers and Distributors, 485, Jain Bhawan, Bhola Nath Nagar, Shahdara, Delhi – 110032, India: pp 114, 584 – 585.

ZipcodeZoo Encyclopedia (2010). *Amaranthus viridis* (Tropical Green Amaranth). https:// zipcodezoo .com/Plants/A/Amaranthus viridis.

APPENDICES

Appendix A:

Determination of Moisture Content of the dry leaf powder of the drug by Loss on drying method:

Using the expression> weight of moisture lost (Weight of crucible/drug powder before heating minus Weight of crucible/drug sample after heating) (g), divided by weight of drug used before heating (2g) and multiplying by 100%, in triplicate i.e.

i) $\frac{34.8627g - 34.6205g}{2g}$ x 100% = $\frac{0.2422g}{2g}$ x 100% = 12.11%

ii) $\frac{34.5144g - 34.2679g}{2g}$ x 100% = $\frac{0.2465g}{2g}$ x 100% = 12.33%

iii) $\frac{28.6018g - 28.3594g}{2g}$ x 100% = $\frac{0.2424g}{2g}$ x 100% = 12.12%

Mean of the three (3) observations (n) using calculator in statistic mode is 12.19% and standard deviation (SD) is 0.10 > standard error of mean (SEM) = SD/√n = 0.10/√3 = 0.0577≈ 0.06. Therefore, mean moisture content ±SEM = 12.19 ± 0.06% w/w

Determination of the Total Ash Value:

This is given by weight of ash (Weight of crucible/drug powder ash after incineration minus Weight of empty crucible) (g), divided by weight of drug used before heating (2g) and multiplying by 100%, for six measurements. i.e.

i) $\frac{21.4623g - 21.0572g}{2g}$ x 100% = $\frac{0.4051g}{2g}$ x 100% = 20.26%

ii) $\frac{11.2533g - 10.8508g}{2g}$ x 100% = $\frac{0.4025\ g}{2g}$ x 100% = 20.13%

iii) $\frac{32.3573g-31.9551g}{2g}$ x 100% = $\frac{0.4022\ g}{2g}$ x 100% = 20.11%

iv) $\frac{31.4378g-31.0429g}{2g}$ x 100% = $\frac{0.3949g}{2g}$ x 100% = 19.75%

v) $\frac{27.6225g-27.2287g}{2g}$ x 100% = $\frac{0.3938\ g}{2g}$ x 100% = 19.69%

vi) $\frac{10.9372g-10.5452g}{2g}$ x 100% = $\frac{0.3920g}{2g}$ x 100% = 19.60%

Mean of the six(6) observations (n) using calculator in statistic mode is 19.92% and standard deviation (SD) is 0.25 > standard error of mean (SEM) = SD/√n = 0.25/√6 = 0.1021 ≈ 0.10. Therefore, the total ash value ±SEM = 19.92 ± 0.10% w/w

Determination of Acid-insoluble Ash value:

This is obtained from dividing the weight of insoluble matter (Constant Weight of crucible/ash residue after acid treatment minus Weight of empty crucible)(g) by weight of drug used before heating (2g) and multiplying by 100%, in triplicate. i.e.

i) $\frac{31.0663g-31.0429g}{2g}$ x 100% = $\frac{0.0234g}{2g}$ x 100% = 1.17%

ii) $\frac{27.2463g-27.2287g}{2g}$ x 100% = $\frac{0.0176g}{2g}$ x 100% = 0.88%

iii) $\frac{10.5684g-10.5452g}{2g}$ x 100% = $\frac{0.0232g}{2g}$ x 100% = 1.16%

Mean of the three (3) observations (n) using calculator in statistic mode is 1.07% and standard deviation (SD) is 0.13 > standard error of mean (SEM) = SD/√n = 0.13/√3 = 0.0751 ≈ 0.08. Therefore, the acid-insoluble ash value ±SEM = 1.07 ± 0.08% w/w

Determination of Water-soluble Ash value:

This is obtained from dividing the weight of insoluble matter (Constant Weight of

crucible/ash residue after acid treatment minus Weight of empty crucible)(g) by weight of drug used before heating (2g) and multiplying by 100%, in triplicate. i.e.

i) $\frac{21.4623g-21.3403g}{2g}$ x 100% = $\frac{0.1220g}{2g}$ x 100% = 6.1%

ii) $\frac{11.2533g-11.1364g}{2g}$ x 100% = $\frac{0.1169g}{2g}$ x 100% = 5.85%

iii) $\frac{32.3573g-32.2384g}{2g}$ x 100% = $\frac{0.1189g}{2g}$ x 100% = 5.95%

Mean of the three (3) observations (n) using calculator in statistic mode is 5.97% and standard deviation (SD) is 0.10 > standard error of mean (SEM) = SD/√n = 0.10/√3 = 0.0577 ≈ 0.06. Therefore, the water-soluble ash value ±SEM = 5.97 ± 0.06% w/w.

Determination of the Ethanol-soluble Extractive value:

This is obtained from dividing the weight of dry extract (weight of beaker/extract minus weight of empty beaker)(g) by weight of drug used for extraction (5g) and multiplying by 100%, in triplicate. i.e.

i) $\frac{27.4020g-27.3328g}{5g}$ x 100% = $\frac{0.0692g}{5g}$ x 100% = 1.38%

ii) $\frac{48.1363g-48.0190g}{5g}$ x 100% = $\frac{0.1173g}{5g}$ x 100% = 2.35%

iii) $\frac{51.1124g-51.0427g}{5g}$ x 100% = $\frac{0.0697g}{5g}$ x 100% = 1.39%

Mean of the three (3) observations (n) using calculator in statistic mode is 1.71% and standard deviation (SD) is 0.45 > standard error of mean (SEM) = SD/√n = 0.45/√3 = 0.2598 ≈ 0.26. Therefore, ethanol-soluble extractive value ±SEM = 1.71 ± 0.26% w/w.

Determination of Water-soluble Extractive value:

This is obtained from dividing the weight of dry extract (weight of beaker/extract minus weight of empty beaker) (g) by weight of drug used for extraction (5g) and multiplying by 100%, in triplicate. i.e.

i) $\frac{32.9989g-32.8627\ g}{5g}$ x 100% = $\frac{0.1362g}{5g}$ x 100% = 2.72%

ii) $\frac{32.6384g-32.5144g}{5g}$ x 100% = $\frac{0.124g}{5g}$ x 100% = 2.48%

iii) $\frac{26.6657g-26.6018g}{5g}$ x 100% = $\frac{0.0639g}{5g}$ x 100% = 1.30%

Mean of the three (3) observations (n) using calculator in statistic mode is 2.17% and standard deviation (SD) is 0.62 > standard error of mean (SEM) = SD/√n = 0.62/√3 = 0.3580 ≈ 0.36. Therefore, water-soluble extractive value ± SEM = 2.17 ± 0.36% w/w.

Appendix B:

Determination of the Scavenging Activity/Percentage Inhibition of the Crude Aqueous Ethanol Extract (EE) and the Standard, Ascorbic Acid (AA), which shows Antioxidant Activity:

Using the absorbance values (A) from table 20 by the following expression;

Radical scavenging activity (% inhibition) = $[(A_{control} - A_{sample})/A_{control}] \times 100$

The absorbance (A) of DPPH alone (Control) at 517 nm was measured as 1.454.

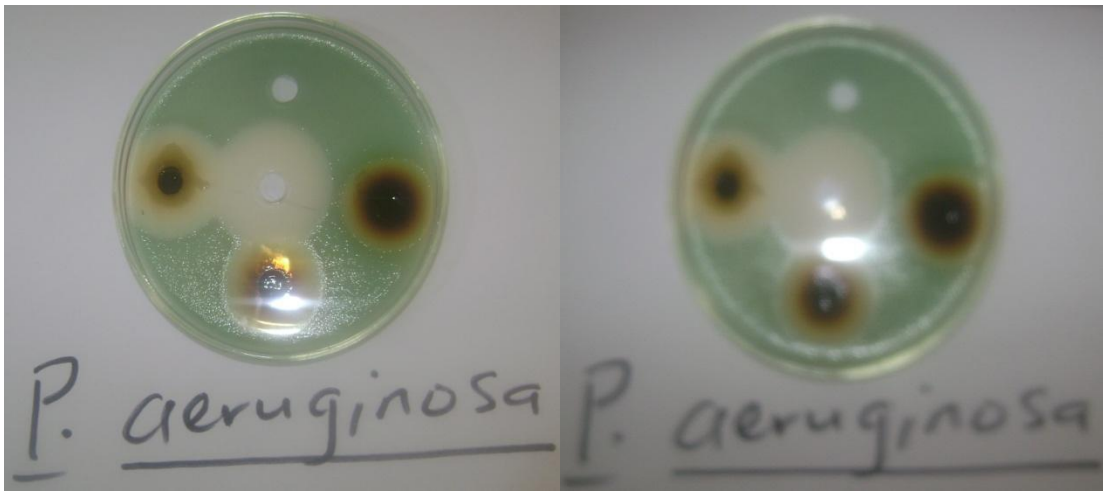
At 12.03 µg/ml: For EE, % Inhibition = $\frac{1.454-0.532}{1.454}$ x 100% = $\frac{0.922}{1.454}$ x 100% = 63.4%

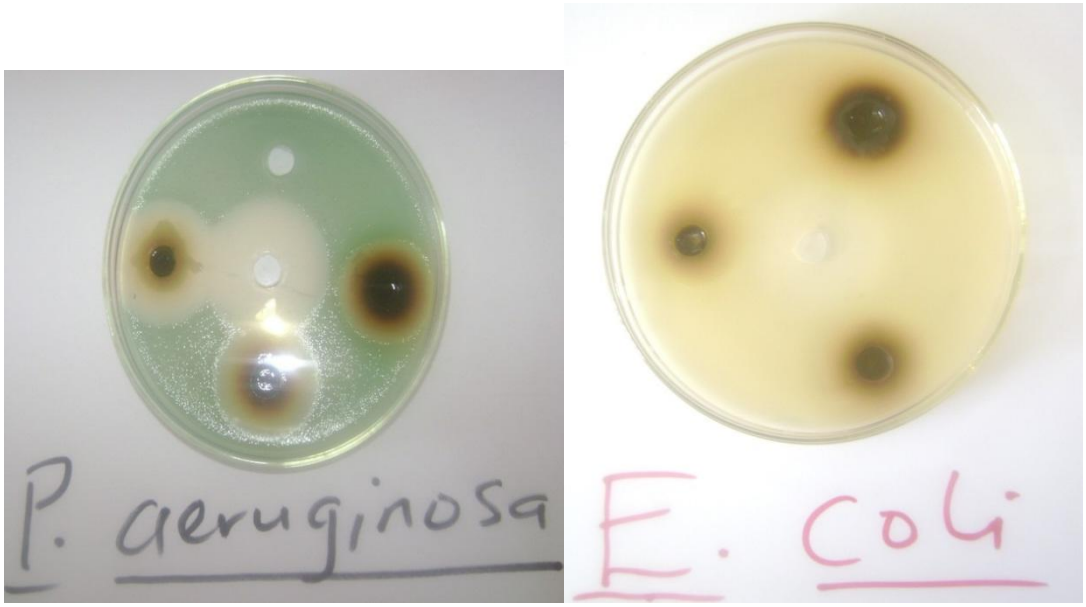
For AA, % Inhibition = $\frac{1.454-0.014}{1.454}$ x 100% = $\frac{1.44}{1.454}$ x 100% = 99%

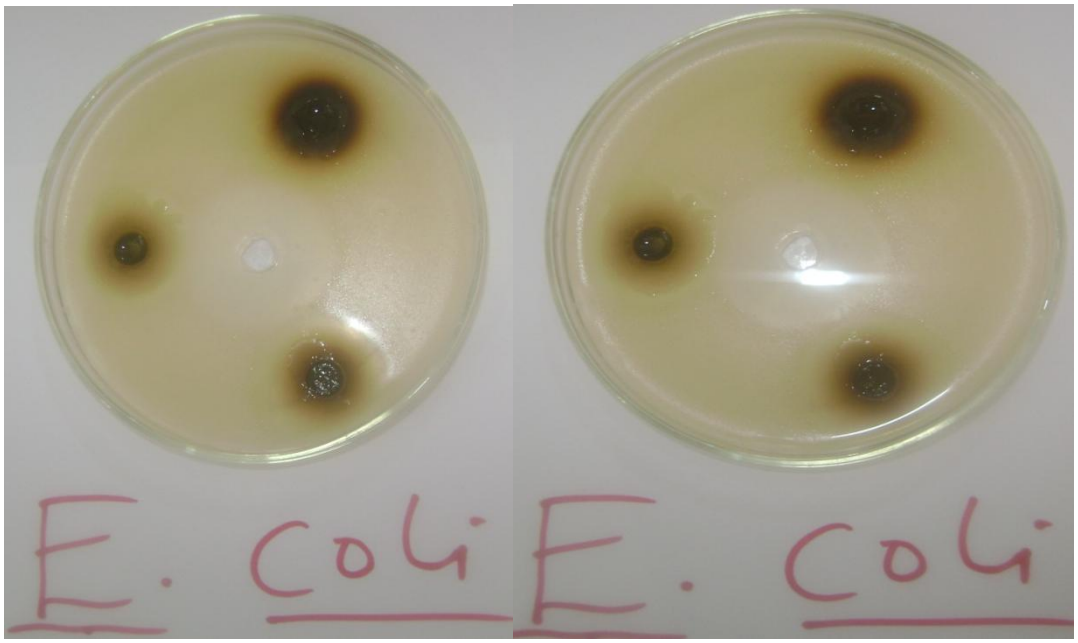
The same expression was used to determine the remaining values at various concentrations or doses for both EE and AA.

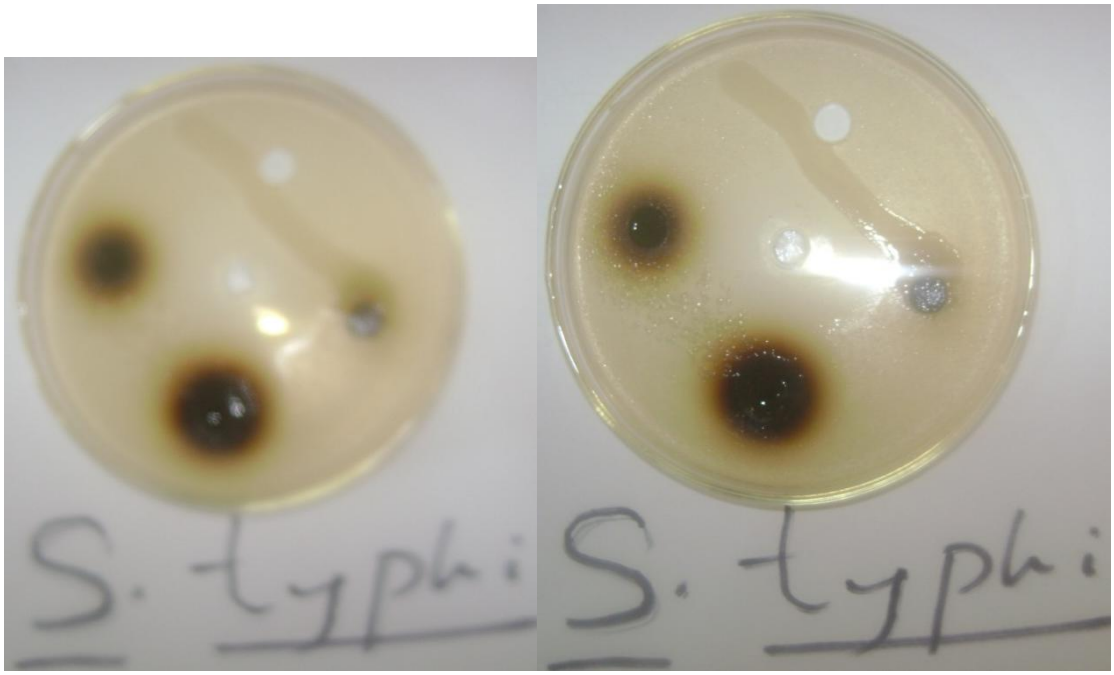
Appendix C:

Plates showing Antibacterial Susceptibility Test by the Agar Well Diffusion Method:






E. coli
E. coli


S. typhi
S. typhi


S. typhi


B. subtilis


B. subtilis


S. aureus


S. aureus