COMPARATIVE PHARMACOGNOSTIC AND BIOLOGICAL STUDIES ON THE LEAVES OF *VERNONIA AMYGDALINA* DEL. AND *VERNONIA KOTSCHYANA* SCH. BIP. (ASTERACEAE)

BY

GARBA IBRAHIM

DEPARTMENT OF PHARMACOGNOSY AND DRUG DEVELOPMENT
AHMADU BELLO UNIVERSITY, ZARIA - NIGERIA

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BY

GARBA IBRAHIM
B. Sc. APPLIED BIOLOGY (B.U.K.);
M. Sc. PHARMACOGNOSY (A.B.U.)
(Ph. D. /PHARM. Sc./35904/2001-2002)

BEING

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FACULTY OF PHARMACEUTICAL SCIENCES,
AHMADU BELLO UNIVERSITY,
ZARIA – NIGERIA

FEBRUARY, 2009

DECLARATION

I declare that the work in the dissertation entitled C**omparative Pharmacognostic and Biological Studies on the Leaves of *Vernonia amygdalina* Del. and *Vernonia kotschyana* Sch. Bip. (Asteraceae)** has been performed by me in the Department of Pharmacognosy and Drug Development under the supervision of Prof. E. M. Abdurahman, Dr. H. Ibrahim and Prof. N. D. G. Ibrahim.

The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this dissertation was previously presented for another degree or diploma at any university.

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

CERTIFICATION

This dissertation entitled **COMPARATIVE PHARMACOGNOSTIC AND BIOLOGICAL STUDIES ON THE LEAVES OF *VERNONIA AMYGDALINA* DEL. AND *VERNONIA KOTSCHYANA* SCH. BIP. (ASTERACEAE)** by **Mal. Garba Ibrahim** meets the regulations governing the award of the degree of Doctor of Philosophy in Pharmacognosy of Ahmadu Bello University and is approved for its contribution to knowledge and literary presentation.

_____ _____
Prof. E. M. Abdurahman; B. Pharm. Sc.; M. Sc.; Ph. D. Date
Chairman, Supervisory Committee,
Department of Pharmacognosy
and Drug Development,
Ahmadu Bello University, Zaria.

_____ _____
Dr. H. Ibrahim; B. Pharm.; M. Sc.; Ph. D. Date
Member, Supervisory Committee,
Department of Pharmacognosy
and Drug Development,
Ahmadu Bello University, Zaria.

_____ _____
Prof. N. D. G. Ibrahim; DVM; M. Sc.; Ph. D. Date
Member, Supervisory Committee,
Department of Veterinary Pathology
and Microbiology,
Ahmadu Bello University, Zaria.

_____ _____
Dr. K. Y. Musa; B. Sc.; M. Sc.; Ph. D. Date
Head, Department of Pharmacognosy
and Drug Development,
Ahmadu Bello University, Zaria.

_____ _____
Prof. S. A. Nkom Date
Dean, Postgraduate School,
Ahmadu Bello University, Zaria.

DEDICATION

This work is dedicated to my parents Alh. Ibrahim Lawal and Malama Halimah Idris; my beloved wife: Zakiyya; our daughter: Halimatu Sa'adiya (Ummi) and son: Ibrahim (Khalifah).

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ABSTRACT

Vernonia amygdalina Del. and *V. kotschyana* Sch. Bip. (Asteraceae) are respectively shrub or tree and annual herb found commonly in tropical and subtropical countries. They are employed in ethno-medicine in the treatment of many ailments namely stomach ache, gingivitis, rheumatism, schistosomiasis, fevers and cough, which are associated with pains and inflammations. Macroscopic, microscopic, chemo-microscopic, quantitative evaluative and thin layer chromatographic studies by using standard pharmacognostic methods of evaluations were carried out on both plant species. Elemental analysis by using Energy Dispersive X-ray Fluorescence (EDXRF) technique; analgesic and anti-inflammatory studies by respectively using acetic acid-induced writhing in mice and carrageenan-induced hind paw oedema in rats' methods were carried out on the two plants.

Macroscopically, leaves of the two plant species were found to have some similar and differing diagnostic features. Leaves of both plant species were alternately arranged with acute apices, symmetrical bases and pubescent surfaces. Leaves of *V. amygdalina* were relatively smaller (12.0 x 5.7 cm) than those of *V. kotschyana* (17.0 x 6.7 cm) and both were petiolate. Leaves of the former plant were lanceolate in shape with serrated margin while those of the latter one were elliptical in shape with dented margin. Organoleptically, leaves of *V. amygdalina* were more greenish in colour and bitter in taste than those of *V. kotschyana*, although, both plants had distinct odour.

Microscopically, leaves of *V. amygdalina* had epidermal cells with slightly beaded wavy anticlinal walls while those of *V. kotschyana* had deeply wavy anticlinal walls. Numerous anomocytic stomata (abaxially and adaxially), unicellular and few uniseriate multicellular covering trichomes were identified in both plants. Few uniseriate multicellular glandular trichomes with warty surface were found only in *V. kotschyana* leaves.

Transversely, the leaves of both plant species were dorsiventral and had multilacunar vascular bundles. Calcium oxalate crystals (prism and rosette), starch grains (oval-shaped), xylem vessels (reticulate and spiral) and fibres were found in the fresh and powdered leaves of both plants.

Quantitative-leaf microscopy revealed that, palisade ratio, vein-islet and veinlet termination number were higher in *V. kotschyana*, 5.2, 12.7 and 16.5, than those in *V. amygdalina*, 4.1, 6.2 and 10.7 respectively. Stomatal numbers and indices were higher in the latter plant: 265.2 and 19.1 (upper epidermis) and 312.1 and 38.1 (lower epidermis) than those in the former one: 118.3 and 17.1 and 227.3 and 28.3 respectively.

Physical constants (%w/w) showed that *V. kotschyana* had higher moisture content (5.0%) than *V. amygdalina* (4.5%). However, the latter plant had higher total ash value (11.0%) than the former one (8.0%), although, both plants had similar acid-insoluble ash value (1.5%). Water-soluble ash, alcohol and water extractive values were higher in *V. amygdalina*: 4.5, 14.0 and 22.5% than those in *V. kotschyana*: 4.2, 13.5, and 18.5% respectively.

Mineral element concentrations in milligram (mg) per 1.0 g of the leaf powders of the two plant species vary considerably. The *V. kotschyana* was found to contain more mineral elements than *V. amygdalina.* Uranium (0.007±LDL mg) was found only in the latter plant. Potassium, calcium, manganese and iron (although with varied concentrations) were of common occurrence in both plants.

The yield of extracts per 800 g leaf powders of the two plants show that, the petroleum ether extract was more in *V. kotschyana* (98.72 g) than in *V. amygdalina* (92.61 g). However, the ethanol extract was more in the latter plant (221.94 g) than in the former one (195.25 g). Phytochemical investigation on the extracts of the plants showed that, both plants contained similar constituents namely tannins, flavonoids and saponins. Fixed oils and alkaloids were detected in *V. kotschyana* only.

The acute toxicity study on the ethanol leaf extracts of the two plants show that *V. amygdalina* was relatively more toxic (288.5 mg/kg) than *V. kotschyana* (471.2 mg/kg) body weight *i.p.* to the experimental mice.

Fractionated crude flavonoids and saponins per 150.0 g of the ethanol leaf extracts of the two plants show that, *V. amygdalina* had higher percentage yield of crude flavonoids (1.43%) than *V. kotschyana* (1.35%). However, the latter plant specie had higher percentage yield of crude saponins (2.97%) than the former one (2.21%). The thin layer chromatographic analysis carried out on the crude ethanol extracts, flavonoids and saponins of *V. amygdalina* and *V. kotschyana* showed the presence of different types of flavonoids and saponins in both plant species. The crude extracts of both plants gave brown coloured spots when exposed to iodine vapour. The crude extracts and flavonoids fraction were found to have range of colour (yellow, purple, brown and green) spots and R_f values (0.55 – 0.95) when they were visualized by exposure to concentrated ammonia vapour. However, the crude extracts and saponins were found to have brown, purple, blue, yellow and green colours and R_f values (58 - 0.95) when sprayed with Liebermann-Burchard's reagent followed by heating at 105^{o}C.

The analgesic studies on the ethanol leaf extracts of both plants, show that *V. kotschyana* (V. K. E. E.) produced significantly ($p<0.05$) higher inhibition (74.6%) at 50 mg/kg than that (71.9%) of *V. amygdalina* (V. A. E. E.) at 100 mg/kg against acetic acid-induced writhing in mice when compared with the negative control (normal saline) group. The effects were comparable to that produced by ketoprofen (67.5%) at 10 mg/kg that was used as positive control group.

The crude flavonoids of both plants show significant ($p<0.05$) and dose-dependant analgesic activities. The *V. kotschyana* crude flavonoids (V. K. C. F.) offered higher inhibition (99.0%) than that (97.0%) of *V. amygdalina* (V. A. C. F.) both at 100 mg/kg against acetic acid-induced writhing in mice when compared with the negative control group. The activities were comparable to that produced by the control ketoprofen (58.0%) group at 10 mg/kg.

The significant ($p<0.05$) and dose-dependant analgesic activities produced by the crude saponins from the two plants show that, the crude saponins of *V. kotschyana* (V. K. C. S.) had higher inhibitory effect (97.0%) than that (78.0%) of *V. amygdalina* (V. A. C. S.) both

at 100 mg/kg against acetic acid-induced writhing in mice when compared with the negative control group. The control ketoprofen group produced (58.0%) inhibitory effect at 10 mg/kg.

The anti-inflammatory studies on the ethanol extracts of the two plants show that *V. kotschyana* (V. K. E. E.) and *V. amygdalina* (V. A. E. E.) offered significantly ($p<0.05$) similar inhibition (45.5%) at 50 and 100 mg/kg, against carrageenan-induced oedema in rats when compared with the negative control (normal saline) group. The activity of both plant extracts was lower than that produced by ketoprofen (63.6%) at 10 mg/kg that was used as the positive control.

The crude flavonoids of *V. amygdalina* (V.A.C.F) offered significantly ($p<0.05$) higher dose-dependant inhibitory effect (45.5%) at 100 mg/kg than that (36.7%) of *V. kotschyana* (V. K. C. F.) at 50 mg/kg against carrageenan-induced oedema in rats when compared with the negative control group. This effect was lower than that produced by the control ketoprofen group (50.0%) at 10 mg/kg.

The crude saponins of *V. amygdalina* (V. A. C. S.) produced significantly ($p<0.05$) higher dose-dependant inhibition (50.0%) than that (40.9%) of *V. kotschyana* (V. K. C. S.) both at 100 mg/kg against carrageenan-induced oedema in rats when compared with the negative control group. The control ketoprofen group produced similar effect (50.0%) at 10 mg/kg.

The various diagnostic features identified from the results of pharmacognostic studies on *V. amygdalina* and *V. kotschyana* leaves, would significantly aid in identifying and differentiating them. The significant analgesic and anti-inflammatory activities of the crude ethanol leaf extracts, flavonoids and saponins of both plant species had greatly supported the traditional claims of using the plant species in the management of tooth ache, stomach ache, rheumatism and gingivitis, which are associated with pains and inflammations. The two plant species could therefore, serve as potential sources of both analgesic and anti-inflammatory drugs from medicinal plants.

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ABBREVIATIONS

μm = Micrometer

CINC = Cytokine Induced Neutrophil Chemo-attractant

Conc. = Concentration

COX-2 = Cyclooxygenase-2

CX3C = Chemokine 3C

CXC = C-X- Chemokine

Df = Degree of Fredom

DFd = Difference Frequency Distortion

DFn = Data File Number

ELAM = Endothelial Leukocyte Adhesion Molecule

F = Fisher's F Ratio (statistics; usually written in italics)

Ft. = Feet

HUVEC = Human Umbilical Vein Endothelial Cell

i.p. = Intraperitoneal

IC_{50} = Median Inhibition Concentration

ICAM = Intercellular Adhesion Molecule

IFN = Interferon Nucleus

IL = Interleukin (cytokine)

iNOS = Inducible Nitric Oxide Synthase (enzyme)

JNK = Jun N-terminal Kinase

kB (NF-kB) = kappa B (Nuclear Factor-kappa B)

kB = kappa B

Kg = Kilogram

LDL = Least Detectable Limit

LOD = Limit of Detection

LPS = Lipoprotein Polysaccharide

MCP = Monocyte Chemotactic Protein

mg = Milligram

mg/kg = Milligram per kilogram

MIP = Macrophage Inflammatory Protein

ml = Milliliter

mm = Millimeter

mm^2 = Millimeter Square

MMP = Matrix Metalloproteinase

mRNA = Messenger Ribonucleic Acid

NF-kB = Nuclear Factor kappa B

NO = Necrotic Obsession

NOx = Necrotic Obsession-x

NRK = Normal Rat Kidney or Non-neoplastic Rat Kidney

NSAIDs = Non-steroidal Anti-inflammatory Drugs

PGE = Prostaglandin E

PMA = Progressive Muscular Atrophy

RAW = Renal Atrophied Wall

RBL = Rat Basophilic Leukemia

S.C. = Subcutaneous

SDF = Strong Cell-derived Factor

SW = Stab Wound

THP = Thyroid Hormone-binding Protein

TLC = Thin Layer Chromatography

TNF = Tumour Necrosis Factor

V. amygdalina = *Vernonia amygdalina*

V. kotschyana = *Vernonia kotschyana*

V.A.C.F. = *Vernonia amygdalina* Crude Flavonoids

V.A.C.S. = *Vernonia amygdalina* Crude Saponins

V.A.E.E. = *Vernonia amygdalina* Ethanol Extract

V.K.C.F. = *Vernonia kotschyana* Crude Flavonoids

V.K.C.S. = *Vernonia kotschyana* Crude Saponins

V.K.E.E. = *Vernonia kotschyana* Ethanol Extract

V/v = Volume by volume

VCAM = Vascular Cell Adhesion Molecule

W/v = Weight by volume

CHAPTER ONE

1.0 INTRODUCTION

1.1 Traditional Medicines

Traditional medicines are defined as “the total combination of knowledge and practices, whether explicable or not, used in diagnosing, preventing or eliminating a physical, mental or social disease which may rely exclusively on past experience and observation handed down from generation to generation, verbally or in writing” (Sofowora, 1993).

The desire to take medicines is one feature which distinguishes man from his fellow creatures (Bean, 1968). Medicinal herbs were found in the personal effects of an "ice man," whose body was frozen in the Swiss Alps for more than 5000 years (Capasso, 1998). However, the uncertain composition of many herbal products raises questions about their safety. There is also the lack of evidence suggesting that herbs may have harmful interactions with prescription drugs. The result is that, such adverse effects of herbs are probably underreported. Meanwhile, systematic studies, such as those identifying adverse reactions to drugs, are hindered because herbal preparations are often not standardized (Peter, 2001).

The effectiveness of a modern drug is ultimately judged by the results of clinical trials. Ordinarily, such trials are designed to test the assumption that a drug's pharmacologic activity will favourably affect a disease process, which in turn is viewed in terms of a physiologic model. Clinical trials yield convincing results, however, only if they are conducted in accordance with certain principles, for instance, to ensure elimination of bias

and reduce the possibility that results occurred merely by chance. Trials must also use drug preparations with consistent pharmacologic properties. These principles apply to all drugs, whether they originate as traditional remedies or in precepts of molecular biology. Indeed, such principles have successfully guided Digitalis from medicinal plant to modern drug and these principles apply to the evaluation of today's herbal medicines (Peter, 2001).

By the early 20th century, it was understood that activities of medicines derived from medicinal plants such as Digitalis (foxglove) were influenced by certain factors. These include, the time when the leaves are gathered, climatic and soil conditions as well as the manner in which the drug is prepared for the market. Clearly, plants have ingredients with therapeutic activity, but their preparations must be standardized to yield consistent products. These will therefore, be given in doses that are maximally safe and effective (Linden-Baum *et al.*, 1971). Bioassays must be based on biological models such as toxicity tests which are not available for the health claims made by traditional herbalists on many popular herbs. Since, their active ingredients responsible for the plants' activities have not been identified and their chemical analysis has limited value. In addition, if the active ingredients of an herb were known, it would remain unclear whether the crude herb would be preferable to its purified active principle or otherwise. In the absence of definitive information in this regard, such traditional herbal preparations as Digitalis leaf and Opium have been replaced by such drugs as digoxin and codeine, respectively (Gold *et al*., 1941).

1.2 Medicinal Plants

The quest for research on medicinal plants and their constituents was first used by the Austrian physician; Schmidt in 1811. During the 19th century and the beginning of the 20th century the research encompassed the study of the physical, chemical, biochemical and biological properties of drugs. This comprises drug substances or potential drugs or drug substances of natural origin and the search for new drugs from other natural sources. The importance of plants in synthesizing large variety of chemical constituents beside their basic metabolites needs to be emphasized. Chemical constituents namely alkaloids, terpenes, glycosides, and a host of others are believed to be of no apparent importance to the plants' own life. However, they are believed to possess prominent effects on animal systems and some important therapeutic properties. These resulted in their uses in the treatment and prevention of many human and animal diseases for thousands of years. On this basis, a plant is regarded as medicinal if, in one or more of its organs, it contains substances that can be used for therapeutic purposes or which are precursors for the synthesis of useful drugs (Sofowora, 1993).

Medicinal plants have contributed immensely to the development of orthodox medicine. These include quinine from Cinchona bark, reserpine from Rauwolfia roots, digitoxin from Digitalis leaves, atropine from Belladonna roots and leaves, hyoscyamine from Hyoscyamus and Datura leaves and roots. Others are coniine from Conium fruits, morphine from Opium capsule, sennosides from Cassia leaves, colchicine from Colchicum corm and vincristine and vinblastine from Catharanthus roots (Sofowora, 1981).

United Nations Commission for Trade and Development (UNCTAD) reported that about 33 per cent of modern drugs in developing countries are derived from medicinal plants. However, if microbes are added, according to Sofowora (1981), 60 per cent of the modern drugs would be of natural origin. Some literatures claim that over 80 per cent of the present-day medicines are either obtained directly or indirectly from medicinal plants (Myers, 1982). Hence, medicinal plants remain the primary source of many drugs used in orthodox medicine in the developed countries (Sofowora, 1993).

1.3 Phytochemistry of Glycosides

These are complex organic molecules occurring in plants in conjugation with sugar moieties, mostly monosaccharide. The sugar molecules may be one or more. They exert therapeutically important effects on humans and animals. Glycosides, upon enzymatic or acid hydrolysis, give one or more sugar moieties and non sugar moiety. The sugar moiety is called glycone and the non sugar moiety is the aglycone or genin. The sugars involved in glycosides are of different types but the most commonly occurring one is β-D-glucose. Other sugars involved are galactose, mannose, rhamnose, digitoxose and cymarose (Kokate *et al.,* 2002).

1.3.1 Types of Glycosides

(a) According to chemical nature of aglycone moiety, they are grouped into the following:

(i) Anthraquinone or Anthracene Glycosides: Examples are sennosides A and B found in *Cassia angustifolia* (Leguminosae) used as purgative; hypericin from *Hypericum perforatum* (Leguminosae) used as anti-depressant.

(ii) Sterol or Cardiac Glycosides: Examples are digitoxin from *Digitalis purpurea* (Scrophulariaceae) used as cardiotonic; strophanthin from *Strophanthus kombe* (Apocynaceae) used as cardiotonic.

(iii) Saponin Glycosides: Examples are glycyrrhizin from *Glycyrrhiza glabra* (Leguminosae) used as expectorant and in the treatment of peptic ulcer; senegin from *Polygala senega* (Polygalaceae) used as stimulants.

(iv) Cyanogenetic or Cyanophoric Glycosides: Examples are amygdalin from *Prunus amygdalus* (Rosaceae) used as demulcent and sedative; prunasin from *Prunus serotina* (Rosaceae) used as mild sedative and flavouring agent.

(v) Isothiocyanate Glycosides: Example is sinigrin from *Brassica nigra* (Cruciferae) used as counter irritant and emetic.

(vi) Flavonol Glycosides: Examples are rutin from *Fagopyrum esculentum* (Polygonaceae) used in the treatment of capillary bleeding; silybin from *Silybus marianum* (Asteraceae) used in the treatment of liver disorder.

(vii) Coumarin and Furanocoumarin Glycosides: Examples are visnagin from *Ammi visnaga* (Umbelliferae) used as smooth muscle relaxant; cantharidin from *Cantharis vasicatoria* (Meloidae) used as counter irritant.

(viii) Aldehyde Glycosides: Example is gluco-vanillin from *Vanilla planifolia* (Orchidaceae) used as flavouring agent.

(ix) Phenol Glycosides: Example is arbutin from *Aritostaphylos uvaursi* (Ericaceae) used as diuretic in urethritis.

(x) Steroidal Glycoalkaloids: Example is solasodine from *Solanum khasianum* (Solanaceae) used for steroidal synthesis.

(xi) Miscellaneous Bitter Glycosides: Examples are gentiopicrin from *Gentiana lutea* (Gentianaceae) used as bitter and stomachic tonic; lawson from *Lawsonia inermis* (Lythraceae) used as antifungal (Kokate *et al.,* 2002).

(b) Glycosides may also be classified on the basis of their therapeutic activity into different groups such as cathartics, cardiotonics, laxatives, analgesics, anti-rheumatics and anti-ulcers. Examples are digitoxin and strophanthin are used as cardiotonics; sennosides are used as laxatives and salicin is used as analgesic.

(c) Glycosides are sometimes classified on the basis of their glycone moiety as glucosides with glucose; rhamnosides with rhamnose; pentosides with pentose like ribose.

(d) Another classification is by considering the linkage between glycone and aglycone portions. Basically, glycosidic linkages occurred by interaction of –OH group of glycone and hydrogen (H) coming from any of the radicals such as CH, -OH, -SH and –NH present on the aglycone part; examples and chemical structures of the different classes (Figure 1.1) are as follows:

(i) C-glycosides: Some of the anthraquinone glycosides example cascarosides: Glycone-OH + HC-aglycone $\xrightarrow{\text{glycone-C-aglycone}}$ + H_2O

(ii) N-glycosides: Typical representative is nucleoside:

Glycone-OH + HN-aglycone $\longrightarrow$ glycone-N-aglycone + H_2O

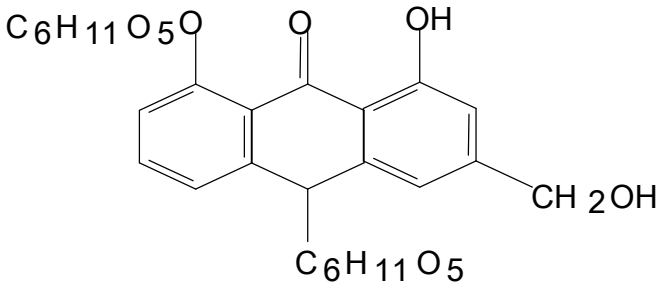
(iii) O-glycosides: Common among higher plants example sennosides:

Glycone-OH + HO-aglycone $\longrightarrow$ glycone-O-aglycone + H_2O

(iv) S-glycosides: Restricted to isothiocyanate glycosides such as sinigrin:

Glycone-OH + HS-aglycone $\longrightarrow$ glycone-S-aglycone + H_2O

(Kokate *et al.,* 2002).



C - Glycoside: Cascaroside A

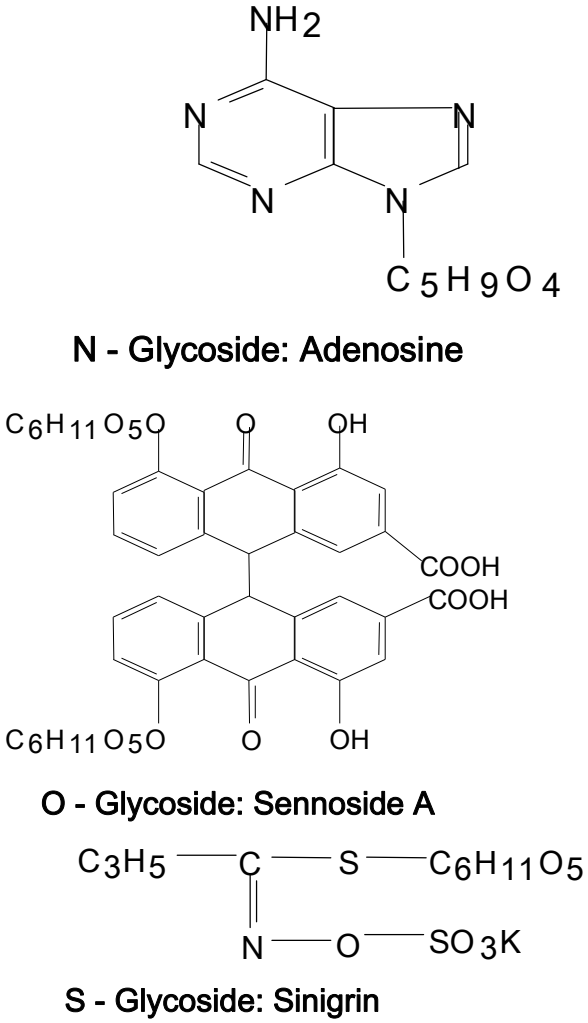


Figure 1.1: Examples of the Different Classes of Glycosides (Balbaa *et al.*, 1976; Kokate *et al.*, 2002)

1.3.2 Properties of Glycosides

Glycosides contain sugars but their physical, chemical and therapeutic properties are dictated by the aglycone portion. The sugar moiety facilitates absorption of glycosides and helps in the transportation of aglycone portion to the site of action. Glycosides are crystalline or amorphous substances which are soluble in water and dilute alcohol. They

are insoluble in organic solvents like chloroform or ether. The aglycone portion is soluble in non-polar solvents such as benzene or ether. Glycosides are easily hydrolyzed by water, mineral acids and enzymes. They show optical activity, normally with laevo rotatory effects. Glycosides do not reduce Fehling's solution until they are hydrolyzed. They are believed to participate in growth regulation and protection of plants (Kokate *et al.,* 2002).

1.3.3 Flavonoids

Flavonoids are the largest group of naturally occurring phenols in plants in both free and combined (glycosidic) states. They occur mostly in higher plants and in young tissues in their cell sap. Flavonoids occur commonly in flowers, leaves and fruits of plant families such as Polygonaceae, Rutaceae, Asteraceae (Compositae), Leguminosae and Umbelliferae (Balbaa *et al.,* 1976).

They are derivatives of Benzo-γ-pyrone and their basic structure is composed of C_{15} (C_6-C_3-C_6) body of flavone. They differ from other phenolic substances in the degree of oxidation of their central pyran ring and very fundamentally, in their biological properties. In some classes of flavonoids (the flavanones, for example) members are colourless. However, members of other classes (the anthocyanins, for example) are always coloured and known as pigments of flowers or other plant parts. Anthocyanins are normally red or yellow; their colour is pH-dependent. Blue pigments are achieved by chelate formation with certain metal ions such as Fe^{3+} or Al^{3+} (Sengbusch, 2003).

The variability of the flavonoids is largely based on the hydroxylation and/ or methylation pattern of the three ring systems. A correlation between two flavonoids points often to a

relationship between the producing plant species. The different classes within the group are distinguished by the additional oxygen, hetrocyclic ring and hydroxyl groups distribution pattern. They have therefore proven to be suitable traits for the study of the phylogenetic relations between higher plants (Sengbusch, 2003). Emerenciano *et al.* (2001) reported that, flavonoids such as flavones have been shown to be good taxonomic markers for Asteraceae (Compositae).

They are generally soluble in water and alcohol but insoluble in organic solvents. The genins are only sparingly soluble in water but soluble in ether. Flavonoids dissolve in alkalis such as sodium, potassium or ammonium hydroxides giving yellow solutions, which turn colourless upon addition of acid (Balbaa *et al.,* 1976).

Flavonoids have various medicinal and pharmaceutical uses that include the following:

(a) Rutin from *Fagopyrum esculentum* is used in the treatment of capillary bleeding.

(b) Hesperidin from unripe green citrus fruits is used against capillary fragility.

(c) Silybin and silycristin from *Silybus marianum* are used in liver disorders.

(d) Gingkolides from *Gingko biloba* are used in vascular disorders.

(e) Luteolin and nobiletin are used as analgesic and anti-inflammatory agents (Kokate *et al.,* 2002).

As far back as 1975, the number of identified flavonoids was estimated to be more than 2000. Some important representatives of flavonoids and their biological significance are

given in Table 1.1. Some of their chemical structures are given in Figure 1.2 (Sengbusch, 2003).

Table 1.1: Some Important Classes of Flavonoids and their Biological Significance

CLASS	NUMBER OF MEMBERS	BIOLOGICAL SIGNIFICANCE
Anthocyanin(s)	250	Red and blue pigments
Chalcones	60	Yellow pigments
Aurones	20	Yellow pigments
Flavones	350	Cream-coloured pigments of flowers
Flavonols	350	Feeding repellents in leaves
Dihydrochalcones	10	Some taste bitter
Proanthocyanidins	50	Astringent substances
Catechins	40	Some have properties like those of tannins
Isoflavonoids	15	Oestrogen effect, toxic for fungi

(Sengbusch, 2003)

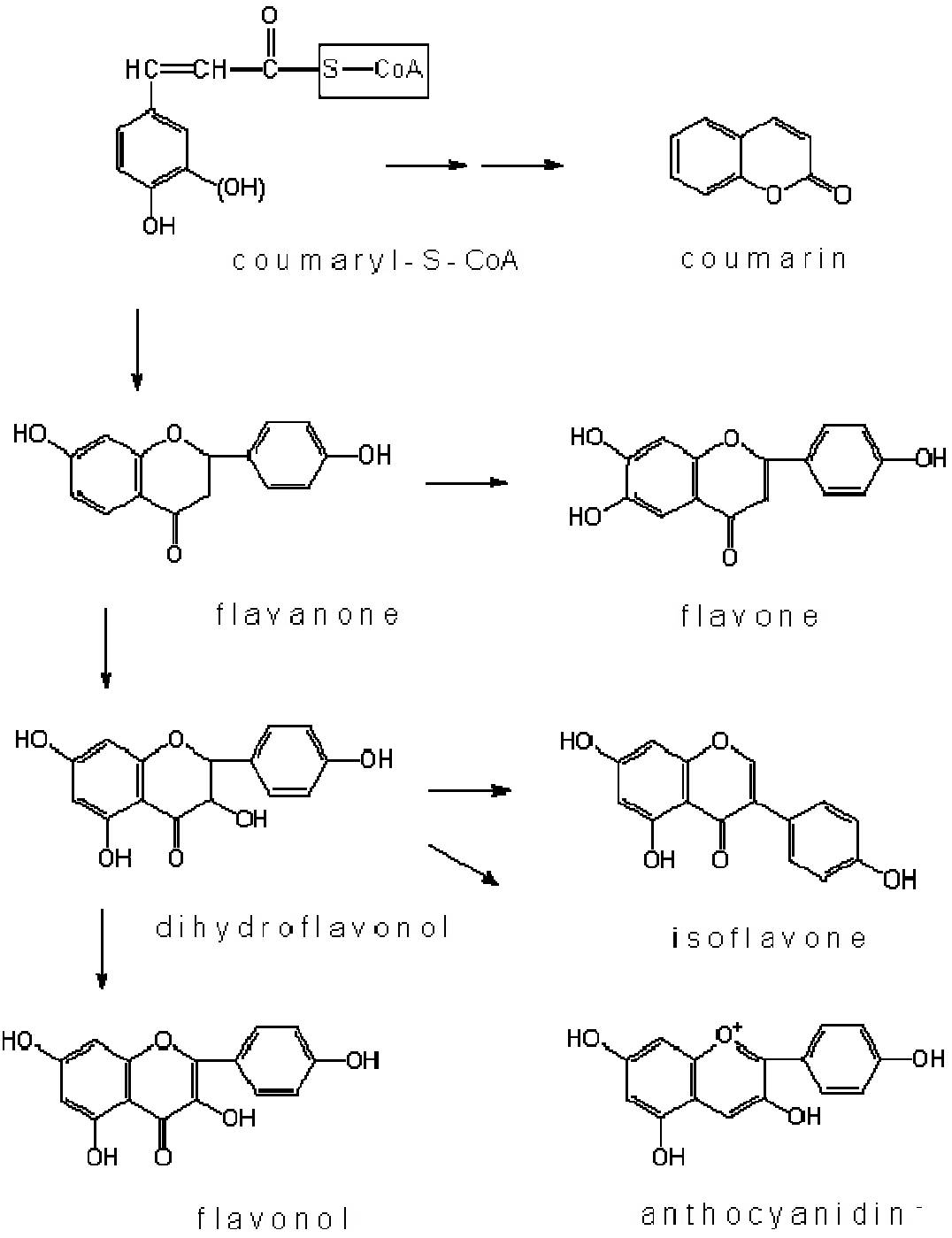


Figure 1.2: Biosynthetic Pathways of Various Classes of Flavonoids (Sengbusch, 2003)

1.3.4 Saponins

Saponins are a group of plant glycosides which share, in varying degrees, two common properties, namely:

(a) They foam in aqueous solution.

(b) They cause haemolysis of red blood cells.

The aglycones of the saponins are collectively called sapogenins and more poisonous saponins are referred to as sapotoxins. Saponins occur in cell sap of plant families such as Asteraceae (Compositae), Rosaceae, Polygalaceae, Scrophulariaceae and Caryophyllaceae. They also occur in monocotyledonous plant families such as Dioscoreaceae, Amaryllidaceae and Liliaceae (Balbaa *et al.,* 1976).

Basically, there are two types of saponins namely, the steroids ($C\text{-}_{27}$) and triterpenoids ($C\text{-}_{30}$). They are characterized by a skeleton derived of the 30-carbon precursor oxidosqualene or are derivatives of isoprene units to which glycosyl residues are attached. Carbohydrate chains are usually attached at C3 and/or C17 atom which may consist of 1-8 units (Vincken *et al*., 2007). The kind and positions of the substituents do not seem to be plant order-specific. The various classes of saponins are given in Figure 1.3 (Balbaa *et al.,* 1976).

They are differentiated by the fact that, upon dehydrogenation by selenium at 360^{o}C, steroids give Diel's hydrocarbon (Methyl cyclopentanophenanthrene) whereas triterpenoids give naphthalene or picene (Dibenz phenanthrene). The steroidal saponins are

less widely distributed in plants than the triterpenoid ones (Balbaa *et al.,* 1976; Kokate *et al.,* 2002).

Saponins have bitter acrid taste and are usually irritating to mucus membrane of eyes and nose. They are highly toxic when injected into the blood stream but are comparatively harmless if taken orally. They are toxic to cold blooded animals and so are often used as fish poisons. Saponins are usually extracted from plants by hot water and alcohol. They are precipitated by diethyl ether after concentrating the extract (Balbaa *et al.,* 1976).

Steroid saponins include the followings:

(a) Sarsaponins from *Salix* species

(b) Digitonin from seeds of *Digitalis purpurea* and *D. lanata*

(c) Dioscin from *Dioscorea* species

Triterpenoid saponins include the followings:-

(a) Calendula saponins from *Calendula officinalis*

(b) Glycyrrhizic acid from *Glycyrrhiza* species

(c) Quillaia saponins from *Quillaia saponaria*

(d) Primula saponins from *Primula* species (Kokate *et al.,* 2002).

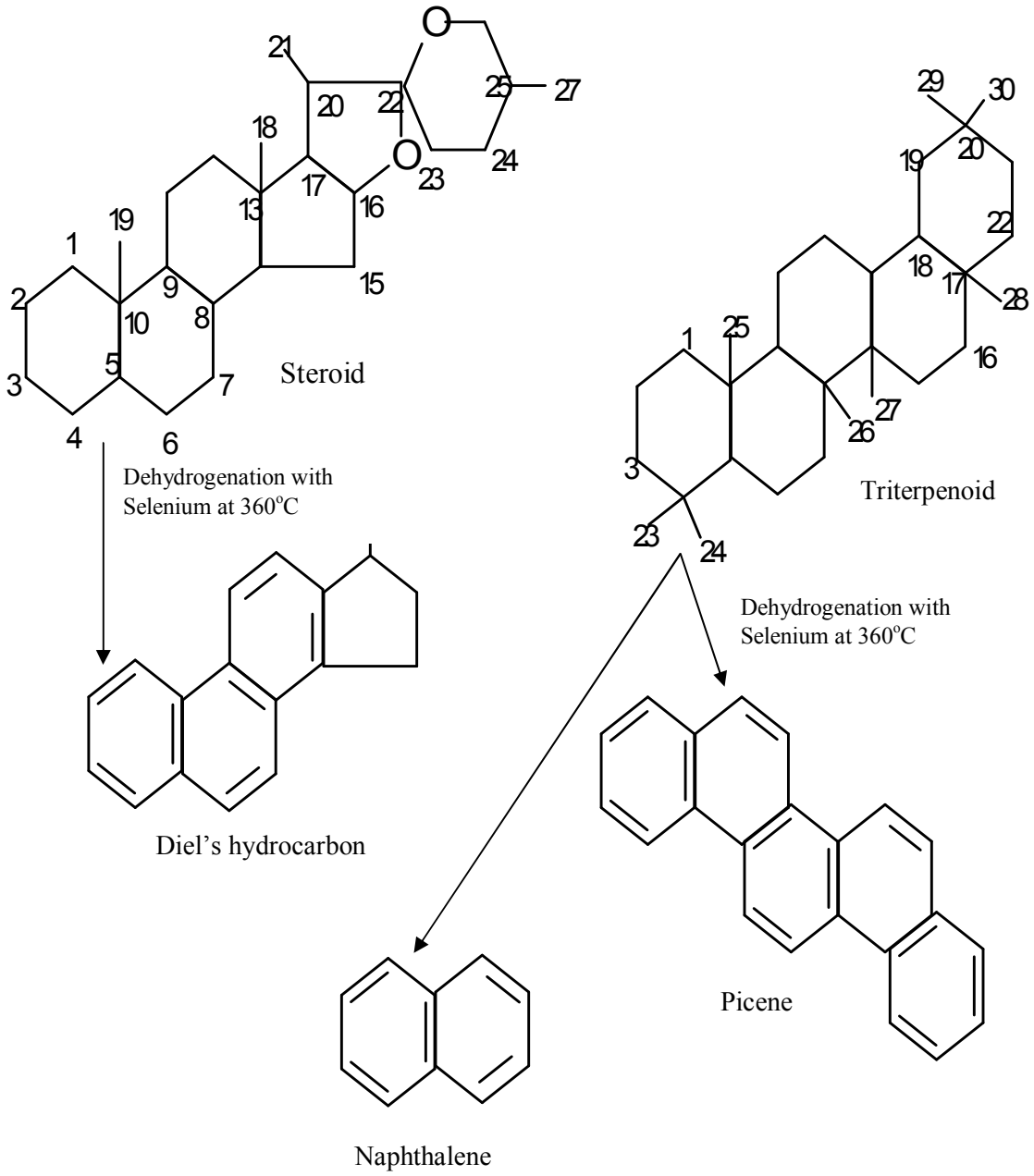


Figure 1.3: Chemical Structures of the Two Classes of Saponins and Their Dehydrogenation Derivatives (Balbaa *et al.*, 1976).

1.4 Toxicity

Toxicity is the degree to which a substance is poisonous (Martin, 2002). Plant crude drug evaluation must encompass the study of its possible toxicity and/or safety margin. The toxicity of a substance may be one of the following types.

(a) Acute toxicity: A subject is exposed to a single or multiple (large enough) doses of toxic agents for over a period of 24 hours and observation for effects may continue for 7 to 14 days.

(b) Sub-acute toxicity: Arises from frequent repeated exposure of subject to small doses of an agent which is sufficient in any of the single doses to produce deleterious effects over a period of 30 to 90 days.

(c) Chronic toxicity: Arises from repeated and continuous exposure of a subject to small doses of a toxic agent, which may not be eliminated from the body, accumulate to toxic level and last for over a period of 90 days (Bucks, 1976).

1.5 Nociception (Pain)

1.5.1 Concepts of Nociception (Pain)

Nociception or pain is an unpleasant sensation, which ranges from mild discomfort to agonized distress associated with real or potential tissue damage. It is a response to

impulses from the peripheral nerves in damaged tissues. The impulses pass to the nerves in the spinal cord where they are subjected to a 'gate control'. The gate modifies the subsequent passage of the impulses according to descending controls from the brain (Martin, 2002). Anti-nociception is synonymous to analgesia, which implies the loss of pain impression without loss of tactile sense (Stewart and Brooks, 1963).

The knowledge of the genesis of pain has led to the understanding that, certain pain mediators such as hydrogen and potassium ions, kinins, biogenic amines, histamine, 5-hydroxytryptamine (serotonin) and metabolites of arachidonic acid (prostaglandins and leocotrienes) evoke pain when liberated in the tissues. The generation of pain is closely related to other tissue reactions such as inflammation and allergy (Katzung, 2001).

Pain may have different causes but variety of peripheral and central neuronal structures might contribute to its processing information. However, a noxious stimulus when applied to some parts of the body, serves as its initial event. Apparently, a subgroup of afferent nerve fibres transfer the information to the central nervous system and these units are referred to as nociceptors (Bessou and Perl, 1969).

Three classes of nociceptors have been found in the mammalian skin (including man) and these include the following:

(a) Unmyelinated C fibres: These are excited by noxious heat and mechanical stimulus in the noxious range. These units are referred to as polymodal nociceptors or C-MH units and

are defined by their responses to mechanical and heat stimuli and their conduction velocities (<2m/s).

(b) Myelinated: These are slowly conducting A-delta units that are excited by high threshold mechanosensitive (HTM) A-delta units. Their conduction velocities are usually <20m/s in rats and <30m/s in humans.

(c) A-delta units: These have similar conduction velocities to those found in group (b) and are excited by strong mechanical stimuli and heat. They are referred to as A-MH units (Andriaensen *et al.*, 1983).

1.5.2 Mechanism of Pain Sensation and Sites of Action of Analgesics

The sequence of events that underpin pain sensation is basically presented in the order as follows (McQuay (1992).

(a) Noxious stimuli are directed by receptors in pain-sensitive tissues.

(b) The signal generated by these receptors is transferred by sensory nerves, through the dorsal root ganglia, to the dorsal horn of the spinal cord.

(c) The signal received through the peripheral sensory mechanisms is processed by the spinal cord segment and transferred via ascending spinal cord pathways to various parts of the brain.

(d) The signals received in the thalamic nuclei, periventricular grey matter, and brain-stem reticular formations are processed and passed on to the sensory cortex, giving off the sensation of pain.

Signals received in several sites, but particularly in the reticular formation and medullar, descend through polysynaptic pathways, to the dorsal horn of the spinal cord, where they may either facilitate or inhibit activity. The schematic diagram (Figure 1.4) is given below in accordance with McQuay (1992).

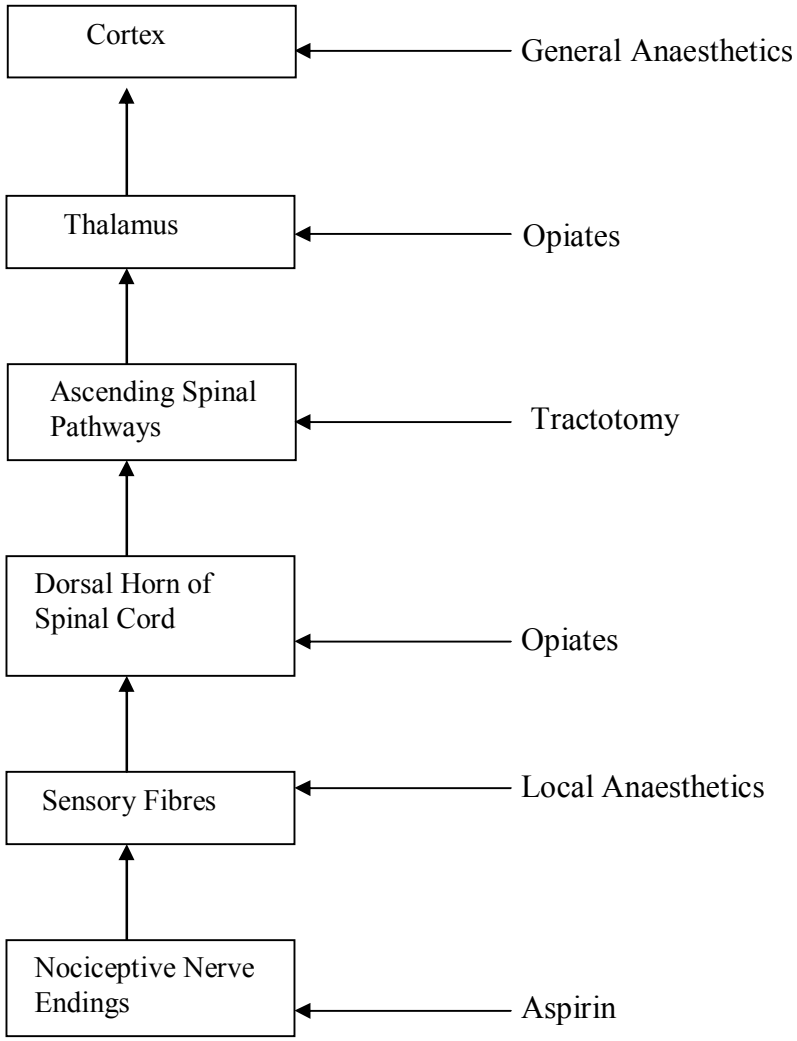


Figure 1.4: Nervous Pathways Mediating Pain and the Sites of Action of Analgesics (McQuay, 1992)

1.6 Inflammation

1.6.1 Concept of Inflammation

Inflammation is a body defensive response of the local microcirculation to tissue injury. This arises from cell damages due to mechanical trauma, chemical, physical and thermal injury, antigen-antibody reactions and infections. Redness, heat, swelling, pain and loss of function of the affected areas are the cardinal signs of inflammation (Bowman and Rand, 1980). It is an attempt by the body to restore and maintain homeostasis after injury and is an integral part of body defense. Most of the body defense elements are located in the blood. Inflammation is the means by which body defense cells and defense chemicals leave the blood and enter the tissue around the injured or infected site. Inflammation is essentially beneficial; however, excess or prolonged inflammation can cause harm (Kaiser, 2001).

The primary physical effect of the inflammatory response is for blood circulation to increase around the affected area. The blood vessels around the site of inflammation dilate, allowing increased blood flow to the area. Gaps appear in the cell walls surrounding the area, allowing the larger cells of the blood, i.e. the immune cells, to pass through. As a

result of the increased blood flow, the immune presence is strengthened. All the different types of cells that constitute the immune system congregate at the site of inflammation, along with a large supply of proteins, which fuel the immune response. There is an increase in body heat, which can itself have an anti-biotic effect, swinging the balance of chemical reactions in favour of the host (Bessou and Perl, 1969).

The main symptoms of the inflammatory response are therefore described as follows in accordance with Kaiser (2001).

(a) The tissues in the area are red and warm, as a result of the large amount of blood reaching the site.

(b) The tissues in the area are swollen, due to the increased amount of blood and proteins that are present.

(c) The area is painful, due the expansion of tissues, causing mechanical pressure on nerve cells, and also due to the presence of pain mediators (Kaiser, 2001).

Inflammatory responses occur in three distinct phases, which are apparently mediated by different mechanisms. Gallin *et al.* (1992) reported that many different mechanisms are involved in the inflammatory process. Phases of inflammatory response include the following:

(a) An acute transient phase, which is characterized by local vasodilation and increased capillary permeability.

(b) A delayed, sub-acute phase, which is most prominently characterized by infiltration of leucocytes and phagocytes.

(c) A chronic proliferative phase, characterized by tissue degeneration and fibrosis, which leads to total loss of tissues.

Basically, there are two types of inflammation: acute and chronic inflammation. Acute inflammation is essential to body defense. Chronic inflammation, however, can result in considerable tissue damage and scarring. With prolonged increased capillary permeability, neutrophils continually leave the blood and accumulate in the tissue at the infected or injured site. As they discharge their lysosomal contents and oxidizing agents, surrounding tissue is destroyed and eventually replaced with scar tissue (Kennedy, 2001).

1.6.2 Mechanism of Inflammation

Following injury or infection, proinflammatory cytokines (a wide variety of intercellular regulatory proteins that control every aspect of body defense) and chemokines (cytokines that promote inflammation by enabling white blood cells to adhere to the inner surface of blood vessels and be chemo-tactically attracted to the injured or infected tissues) are released. Mast cells in the connective tissue as well as basophils, neutrophils and platelets leave the blood from injured capillaries, and stimulate the synthesis of vasodilators such as histamine, leukotrienes, bradykinins, and prostaglandins (Vane and Botting, 1987).

Certain products of the complement pathways (two biochemical pathways involved in non-specific body defense) complement proteins produced. They promote inflammation, phagocytosis and lysis of membrane-bound cells that are recognized by the immune system. The products: C5a and C3a can also trigger mast cells to release their vasodilators. Vasodilation is a reversible opening of the junctional zones between endothelial cells and consequently leads to macroscopic manifestation of inflammation (Kaiser, 2001) (Figure 1.5).

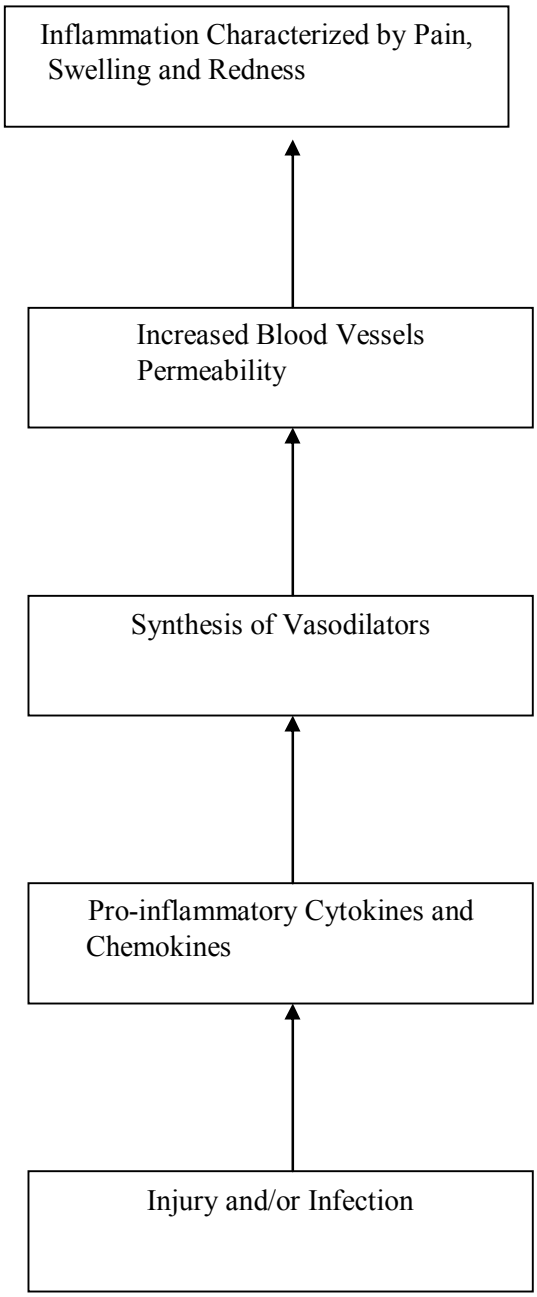


Figure 1.5: Schematic Illustration of Inflammatory Mechanism (Vane andBotting, 1987)

1.7 Some Analgesics and Anti-inflammatory Drugs

Analgesics and non-steroidal anti-inflammatory drugs (NSAIDs) are either classified as non selective cyclooxygenase (Cox) inhibitors or cyclooxygenase-2 (Cox-2) inhibitors. Cyclooxygenases are of two types (i) cyclooxygenase-1 (Cox-1) that is found in normal cells and tissues and (ii) cyclooxygenase-2 (Cox-2) that are induced in setting of inflammation by cytokines and inflammatory mediators (Seibert *et al.*, 1997). Chemical classification of these drugs is given as:

1.7.1 Non selective Cyclooxygenase (Cox) Inhibitors

(a) Salicylic acid derivatives: aspirin, sodium salicylate, choline magnesium trisalicylate, salsalate, diflunisal, sulfasalazine, olsalazine.

(b) Para-aminophenol derivatives: acetaminophen (paracetamol).

(c) Indole and indene acetic acids: indomethacin, sulindane.

(d) Hetroaryl acetic acids: tolmetin, diclofenae, ketorolae.

(e) Arylpropionic acids: ibuprofen, naproxen, flurbiprofen, ketoprofen, fenoprofen, oxaprozin.

(f) Anthracitic acids (fenamates): mefenamic acid, meclofenamic acid.

(g) Enolic acids: oxicam (piroxicam, meloxicam).

(h) Alkanones: nabumetone (Robert and Morrow, 2001).

1.7.2 Selective Cyclooxygenase-2 (Cox-2) Inhibitors

(a) Diaryl substituted furanones: rofecoxib.

(b) Diaryl substituted pyrozoles: celecoxib.

(c) Indole acetic acids: etodolae.

(d) Sulfonanilides: nimesulide (Robert and Morrow, 2001).

Analgesics and non-steroidal anti-inflammatory drugs (NSAIDs) have some side effects. They have the potential of inducing gastric and intestinal ulcer. This effect can sometimes be accompanied by anaemia due to blood loss. Users of non selective NSAIDs on a chronic basis have about three time greater risk for serious adverse gastrointestinal events compared to nonusers. However, NSAIDs vary considerably in their tendency to cause such erosion and ulcer (Gabriel *et al.,* 1991).

1.8 Carrageenan as the Inflammatory Agent

Carrageenan is synonymously called Chondrus extract, Irish moss extract (Figure 1.6). It is a sulphated polysaccharide extract of sea weed called carrageen or irish moss (red algae) known as *Chondrus crispus* (Rhodophyceae). The algae were commonly found growing on the North Atlantic coast of Europe and North America. It is found in the intercellular

matrix and cell wall of the algae and constitutes about 60-80% of its dry weight. It is a water soluble colloid, dried to form free flowing powder. Its molecular weight is 10,000-50,000 and is stable at pH 9.0. Depending on the position of sulphate and the presence or absence of anhydrogalactose; two types of carrageenan are known, namely the kappa (k) and the lambda (λ) carrageenan. The k-carrageenan contains D-galactose, 3, 6 anhydrous D-galactose and ester sulphate group whereas the λ-carrageenan contains D-galactose and its mono and disulphate ester. The k-carrageenan is a good gelling agent while the λ-carrageenan type is non-gelling but a good thickener (Kokate *et al.,* 2002).

Carrageenan induces inflammation, first by causing the release of histamine and serotonin and secondly by causing the release of bradykinin, protease, prostaglandin and lysosome (Crunckhon and Meacock, 1971).

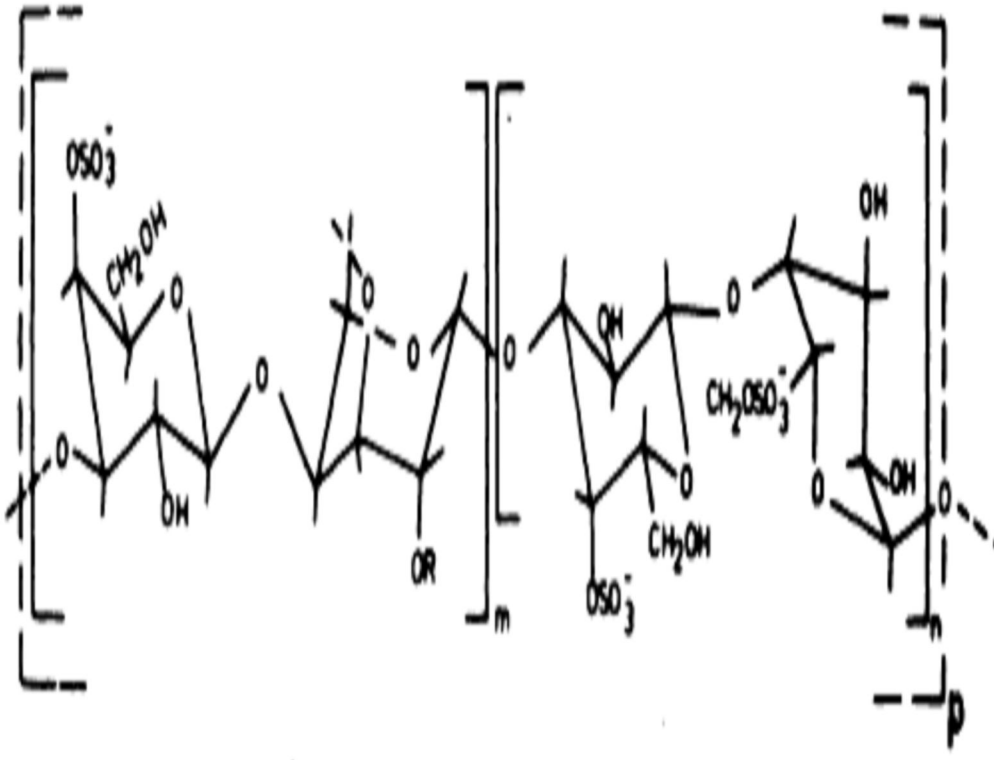


Figure 1.6: Chemical structure of Carrageenan (Rees, 1981)

Note: R = H for kappa carrageenan and R = SO_3^- for lambda carrageenan

1.9 Ketoprofen as the Standard Analgesic and Anti-inflammatory Drug

Ketoprofen also called Ketonal® (Figure 1.7) is a phenyl propionic acid derivative: 2-(3-benzoylphenyl)-propionic acid ($C_{16}H_{14}O_3$). It is one of the propionic acids with analgesic and antipyretic effects. It is most commonly used for muscoskeletal pain, joint problems, and soft tissue injury (Kantor, 1986). Ketoprofen belongs to a group of medicines called non-steroidal anti-inflammatory drugs (NSAIDs). It acts by blocking or inhibiting the production of a chemical (prostaglandin) which the body produces in response to injury or certain diseases. This prostaglandin would otherwise go on to cause swelling, pain and inflammation (Chishinan, 2006).

It inhibits both cyclooxygenase (nonselectively) and lipoxygenase. It is indicated in rheumatoid arthritis, osteoarthritis, gout, dysmenorrhoear and other painful conditions but it negatively affects the central nervous system (Katzung, 2007).

Its side effects include gastrointestinal ulcers, drop in red blood cell count (a result of gastrointestinal bleeding), and rarely kidney damage, protein loss, and bleeding disorders. It should therefore be used with caution in cases of liver or kidney disease, or gastrointestinal problems (Forney, 2007).

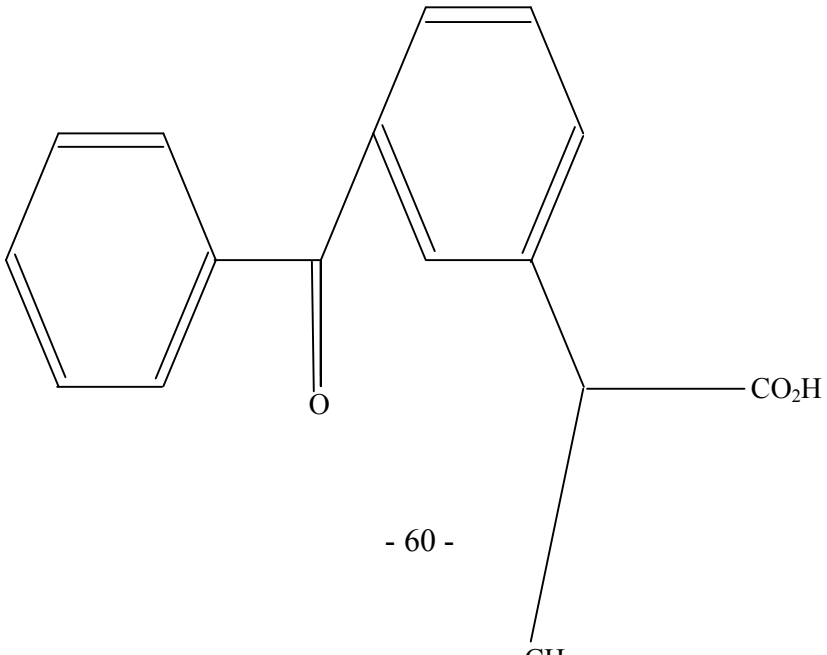


Figure 1.7: Chemical Structure of Ketoprofen (Kantor, 1986)

1.10 Some Medicinal Plants/Constituents with Analgesic and/or Anti-inflammatory Activities

A substantial body of evidence obtained from *in-vivo* and *in-vitro* studies show that, various plant-derived compounds with anti-inflammatory properties exert their effects through the modulation of the cytokine system (Habtemariam, 2000). For instance, flavonoids, a class of compounds widely distributed throughout the plant kingdom, possess interesting anti-inflammatory actions (Middleton, 1998).

Xagorari *et al*. (2001) reported that luteolin, quercetin, luteolin 7-glucoside and the isoflavonoid genistein inhibited LPS (lipopolysaccharide)-stimulated TNFα and interleukin-6 release in RAW 264.7 macrophages. However, eriodictyol and hesperetin only inhibited TNFα release. Luteolin also inhibited the production of TNFα *in-vivo* and was capable of decreasing both PMA and oxazolone-induced allergic ear oedema. Luteolin significantly reduced LPS-stimulated ICAM-1 expression in the liver of LPS (*Salmonella enteriditis*)-treated mice.

A study demonstrated that chronic administration of luteolin significantly attenuated ovalbumin-induced airway bronchoconstriction and bronchial hyper-reactivity. Moreover, the same treatment with luteolin was capable of reducing the levels of both IL-4 and IL-5. However, it induced an increase in IFNγ in the broncheoalveolar lavage fluid of sensitized mice (Das *et al*., 2003).

It has been demonstrated that a citrus polymethoxyflavonoid, nobiletin (5, 6, 7, 8, 3′, 4′-hexamethoxyflavone) effectively inhibits the production of PGE_2 and proMMP-9 in rabbit synovial fibroblasts (Ishiwa *et al.,* 2000). Lin *et al.* (2003) have shown that nobiletin suppressed IL-1β-induced production of PGE_2 in human synovial fibroblasts cells. This decreased the expression of IL-1α, IL-1β, TNFα and IL-6 mRNAs in J774A.1 macrophages (at a concentration of 32 μM). These results allowed the authors to suggest that nobiletin could be a candidate for the development of a novel anti-inflammatory or immunomodulatory drug.

Baicalin, baicalein and wogonin are flavonoids present in *Scutellaria baicalensis* G. (Lamiaceae), a plant used in the treatment of a variety of inflammatory diseases such as bronchitis, nephritis, hepatitis, asthma, and atopic dermatitis (Kubo *et al.,* 1984). The anti-inflammatory activities of these flavonoids have been attributed firstly, to their antioxidant properties and secondly, to their ability to inhibit LPS-induced NO production and iNOS gene expression, as well as the increase in TNFα levels by RAW 264.7 cells (Chen *et al.,* 2001).

Li *et al.* (2000) demonstrated that baicalin significantly inhibited the binding of several chemokines. These include CXC, [stromal cell-derived factor (SDF)-1α and IL-8]; CC [macrophage inflammatory protein (MIP)-1β, and monocyte chemotactic protein (MCP)-2). It also inhibits C lymphotactin (Ltn)] to human leukocytes or cells transfected with chemokine receptors.

Baicalein prevented eotaxin production and the mRNA eotaxin expression in human fibroblasts stimulated with IL-4 plus TNFα. Confirming the *in-vitro* studies, the co-injection of baicalein with interleukin-8 (IL-8) significantly inhibited IL-8-elicited neutrophil infiltration in rat skin. However, baicalin failed to block CX3C chemokine fractalkine/neurotactin or other cytokines, such as TNFα or IFN-γ. Most flavonoids, including baicalein inhibit IL-1β-, TNFα- and thrombin-induced endothelial leukocyte adhesion molecule-1 (ELAM-1) and ICAM-1 expression in cultured human umbilical vein endothelial cells (HUVEC) (Kimura *et al.,* 2001).

Sartor *et al.* (2002) reported that different flavonoids such as flavones (baicalein), anthocyanidins (delphinidin and pelargonidin), flavanols [epigallocatechin-3-gallate], flavonols [morin and quercetin] and other compounds with antioxidant properties were highly effective in inhibiting the activities of leukocyte elastase, MMP-2 and MMP-9. Such actions might explain the anti-inflammatory, antiangiogenic, anti-invasive and antimetastatic properties described for these compounds.

Kang *et al.* (2001) reported that hypericin, an active component of *Hypericum perforatum* L. (Hypericaceae), significantly inhibited in a concentration-dependent manner IL-12 production in LPS-activated macrophages. Hypericin potently inhibited the activation of the IL-12 gene promoter, suggesting that hypericin negatively regulated IL-12 production at the transcription level. Bork *et al.* (1999) have demonstrated that hypericin inhibits PMA- and TNFα-induced activation of NF-κB by a mechanism not involving antioxidant pathways. These results might explain some known biological activities of hypericin, including its reported antirheumatic effects.

It has been demonstrated that a methanolic extract of the root cortex of *Paeonia suffruticosa* A. (Ranunculaceae), known as mudanpi, inhibited in a concentration-related manner the secretion of IL-8 and MCP-1 induced by PMA in human monocytic U937 cells (Oh *et al.,* 2003).

Chou (2003) demonstrated that the main active compound of *Paeonia suffruticosa*; paeonol (2′-hydroxy-4′-methoxyacetophenone), exhibited analgesic and anti-inflammatory effects in the rat model of carrageenan-evoked thermal hyperalgesia. The analgesic and

anti-inflammatory effects of paeonol are associated with its ability to inhibit in a concentration-dependent manner the formation of several pro-inflammatory cytokines (such as TNFα, IL-1-β and IL-6), as well as the over-production of NO and PGE_2. In addition, the effects of paeonol are also associated with an increase in the anti-inflammatory cytokine IL-10, with inhibition of neutrophil infiltration and iNOS and COX-2 protein expression. Thus, paeonol represents a potential candidate for the development of a new anti-inflammatory therapy.

Tsuda *et al.* (2002) reported that oral administration of a typical anthocyanin, cyanidin 3-O-β-D-glucoside, suppressed the zymosan-induced inflammatory response in rats. Treatment of cyanidin 3-O-β-D-glucoside also reduced the elevation of NO_x, TNFα, IL-1β, IL-6 and CINC-1 concentrations. Furthermore, cyanidin 3-O-β-D-glucoside normalised the levels of several acute phase proteins, including α_2-macroglobulin, albumin and transferrin in the serum of rats treated with zymosan.

Rhizome of *Curcuma longa* L. (Zingiberaceae) was found to contain curcumin (polyphenols) that presents a high interest as a lead compound to develop new clinically relevant anti-inflammatory drugs. Apart from its effect on inflammatory events, this compound significantly blocked IL-12 mediated T cell proliferation and Th_1 differentiation, an action that possibly implies its ability to reduce the production of pro-inflammatory cytokines (Natarajan and Bright, 2002).

Curcumin was found to significantly down-regulate the TNFα-induced increase in MMP-13 mRNA and protein expression in primary human chondrocytes and SW1353 cells by a

mechanism involving the inhibition of NF-κB, c-jun and JNK (Liacini *et al.,* 2003). Moreover, curcumin significantly inhibited the increase of both IL-1β and TNFα in a chronic model of inflammation in rats (Banerjee *et al.,* 2003).

Hematein is a compound isolated from *Caesalpinia sappan* Linn (Leguminosae), a plant employed in oriental medicine as an analgesic or anti-inflammatory (Oh *et al.,* 1998). Hematein was efficacious in reducing the expression of VCAM-1 in the aorta of hypercholesterolemic New Zealand rabbits. It has been reported that an extract of *Tripterygium wilfordii* Hook (Celastraceae) markedly inhibited mRNA synthesis and protein expression of MMP-3 and MMP-13, induced by the pro-inflammatory cytokines IL-1β, IL-17 and TNFα as assessed in primary osteoarthritic human or normal bovine chondrocytes. The extract could be useful as a source and template for novel antiarthritic and cartilage-protective drugs (Oh *et al.,* 2001).

Celastrol decreased the production of the pro-inflammatory cytokines TNFα and IL-1β in human monocytes and macrophages. Celastrol (3 mg/kg) has also been reported to inhibit rat adjuvant-induced arthritis, thus confirming its reported *in-vitro* anti-inflammatory properties. It has been proposed that celastrol might be useful for improving performance in memory, learning and psychomotor activities, and common features of the neurodegenerative diseases accompanied by inflammation, such as Alzheimer's disease. The terpenic saponin kalopanaxsaponin A isolated from *Kalopanax pictus* Nakai (Araliaceae) prevented the formation of TNFα by RAW 264.7 macrophages stimulated with LPS at concentrations up to 5 μM. The compound was found to be a useful substance in pain and inflammation (Kim *et al.,* 2002).

Sesquiterpene pyridine alkaloids of *T. wilfordii*, named *wilfornines* A, B and C, as well as several other related compounds, showed significant inhibitory effects on the production of a range of pro-inflammatory cytokines, including TNFα, IL-1β, IL-4, IL-2 and IFN-γ in human peripheral mononuclear cells (Duan *et al.,* 2001).

Sesquiterpene lactones including cynaropicrin, reynosin and santamarine from roots of *Saussurea lappa* Rad. (Compositae) inhibited TNFα production in LPS-activated RAW 264.7 cells. They are found to be potential sources of analgesic and anti-inflammatory agents (Cho *et al.,* 1998).

Jung *et al*. (1998) reported that reynosin exhibited a concentration-dependent inhibition of CINC-1 formation in NRK-52E rat kidney epithelial cells stimulated with LPS. Moreover, the diterpenes casearinols A and B and casearinones A and B isolated from *Casearia guianensis* J. (Flacourtiaceae) were found to be effective in reducing the expression of ICAM-1 and VCAM-1 in THP-1 human monocytes (Hunter *et al.,* 1997).

Terpinen-4-ol, the main constituent of the essential oil of *Melaleuca alternifolia* (Maiden and Betche) Cheel (Myrtaceae) (tea tree oil) suppressed the formation of TNFα, IL-1β, IL-8, IL-10 according to an assessment in LPS-activated human peripheral blood monocytes (Hart *et al.,* 2000).

Crocus sativus L. (saffron) aqueous and ethanolic maceration extracts of stigma and petals were evaluated for analgesic and anti-inflammatory activities. The extracts exhibited antinociceptive activity against acetic acid-induced writhing. Naloxone partially blocked only the antinociceptive activity of the stigma aqueous extract. Only the stigma extracts showed weak to moderate effect against acute inflammation. In chronic inflammation, both aqueous and ethanolic stigma extracts, as well as ethanolic petal extract of *C. sativus* L. (saffron) exerted anti-inflammatory effects. Phytochemical screening of the extracts indicated the presence of flavonoids, tannins and anthocyanins in the petal aqueous and ethanolic extracts. Alkaloids and saponins were found in the stigma aqueous and ethanolic extracts (Hosseinzadeh and Younesi, 2002).

Sanguinarine is found in the root of *Sanguinaria canadensis* that is commonly called bloodroot (Papaveraceae) that was used extensively by numerous Native American societies for blood tonification and purification, pain relief, wound healing, fevers, and numerous other purposes. The use of a medicinal botanical as a tonic usually indicates that the botanical has the ability to enhance certain immune responses. It is uncertain what is meant by "blood purification" in terms of modern medicine (Chaturvedi *et al,* 1997).

In-vitro, sanguinarine suppressed human peripheral blood neutrophil function, including chemotaxis, adhesion, oxidative burst, degranulation, and phagocytosis, and was nontoxic at all concentrations tested. It also strongly inhibited the activation of nuclear transcription factor κB (NF-κB), which is involved in the induction of numerous proinflammatory mediators. Sanguinarine suppressed NF-κB activation by preventing phosphorylation and

degradation of inhibitory κB, which prevents entry of NF-κB into the nucleus (Chaturvedi *et al,* 1997).

Preparations of *Hamamelis virginiana* (commonly called witch hazel) of the family Hamamelidaceae were taken by Native Americans for pain relief, colds, and fevers. A crude alcohol and water extract of *H. virginiana*, as well as fractions in which the hamamelitannin content was reduced by ultra-filtration, were assessed for their anti-inflammatory activities in several *in-vitro* and *in-vivo* experimental systems. *In-vitro*, elastase, a proteolytic enzyme participating in the inflammatory response, was inhibited most potently by the fraction containing the highest concentration of hamamelitannin, and this fraction exhibited the strongest antioxidant activity (Erdelmeier *et al.,* 1996).

Sambucus nigra (black elder; Caprifoliaceae family) is one of many *Sambucus* species used by Native Americans for rheumatism and fever. A methanol, and particularly butanol and chloroform extracts of *S. nigra* leaves, had significantly inhibited lipopolysaccharide (LPS)-induced synthesis of tumor necrosis factor α(TNF-α) by human peripheral blood mononuclear cells but had minimal effect on interleukin (IL)-1α and IL-1ß production. Oral administration of an aqueous extract of the aerial parts of *S. nigra* before a carrageenan injection significantly inhibited hind-paw edema in mice (Yesilada *et al.,* 1997).

Echinacea angustifolia Linn. (Compositae) the narrow-leafed purple coneflower, has long been used by Native Americans for pain relief and wound treatment, as an antidote against various poisons, and for symptoms associated with the common cold (Moerman, 1986).

1.11 Statement of Research Problems

Many Nigerian medicinal plants like their counterparts world wide possess various similar and confusing macro-morphological and cyto-morphological features in common. These posed great difficulties to their proper identification and differentiation from the closely related ones.

Pain and inflammation are manifestations of a number of diseases. These diseases may be as a result of dietary imbalance, biochemical reactions, microbial infections and many other infectious diseases that are manifested in form of pain and inflammatory responses. Infectious diseases such as Acquired Immunodeficiency Syndrome (AIDS), anthrax, cancer, arthritis, gingivitis, diarrhea account for millions of deaths every year (Dara, 2004). Although the great majority of these deaths occur in developing countries, infectious diseases are not confined by international borders and therefore, present a substantial threat to populations in all parts of the world. As such pains and inflammatory responses of varied degrees are seen as major threats to both animal and human health worldwide.

1.12 Justification of the Study

African countries especially Nigeria are blessed with enormous medicinal plant resources. However, only few plants have been evaluated as potential analgesic and/or anti-inflammatory agents in the whole African continent. Even for the few plants that have been studied, pharmacognostic standards for their identities and their mineral element content have not been established. The active principles responsible for the analgesic and/or anti-inflammatory properties were not identified.

Research on botanicals used by indigenous populations has generally been confined to *in-vitro* screenings of individual plants or their constituents for their antibacterial, antiviral, analgesic or anti-inflammatory activities. The fact that a botanical was traditionally used for wound healing, fever, infection, edema, or rheumatic disease is taken as an indicator that the plant should be tested for its analgesic and/or anti-inflammatory properties. Although several *in-vitro* assays can be used to test for anti-inflammatory activities, most screening procedures include inhibition of cyclooxygenase and 5-lipoxygenase. These 2 enzymes are central to the pathways producing thromboxanes, prostaglandins, and leukotrienes (Borchers *et al.,* 2000).

Further research in these areas will go a long way in revealing the potential of the commonly available plants to possibly serve as cheap and efficient sources of analgesic and/or anti-inflammatory drugs of international standards.

Therefore, the search for phytochemical constituents that possess antinociceptive and/or anti-inflammatory activities is worthy of consideration in drug discovery from medicinal plants worldwide.

1.13 Aims and Objectives of the Study

(i) To provide macroscopic, microscopic, quantitative-leaf/chemo-microscopic and solvent extractive standards for the identification of these plants.

(ii) To determine and compare some of the mineral element concentrations of the two plants.

(iii) To determine and compare the median lethal dose (LD_{50}) values of the two plants in laboratory mice.

(iv) To evaluate and compare the analgesic and/or anti-inflammatory activities of the crude extracts of the two plants in laboratory mice and rats respectively.

(v) To fractionate the crude extracts of the two plants into crude flavonoids and saponins fractions.

(vi) To evaluate and compare the analgesic and/or anti-inflammatory activities of each of the crude flavonoids and saponins of the two plants.

(vii) To provive scientific evidence for the ethno-medical uses of *Vernonia amygdalina* and *V. kotschyana* in alleviating pains and inflammations.

(viii) To statistically analyze and compare the results obtained from the analgesic and anti-inflammatory studies on the two plants by using Student *t*-test in GraghPad Prism software.

1.14 Statement of Research Hypothesis

Vernonia amygdalina and *V. kotschyana* possess similar and differing macroscopical, microscopical, quantitative/chemo-microscopical and elemental diagnostic features. The crude ethanol extracts, flavonoids and saponins of the two plants have analgesic and/or anti-inflammatory activities that support their traditional uses in the management of diseases such as stomach ache, head ache, pains of the joints and gingivitis, which are associated with pains and inflammations.

1.15 Statement of Null Hypothesis

Vernonia amygdalina and *V. kotschyana* do not possess similar and differing macroscopical, microscopical, quantitative/chemo-microscopical and elemental diagnostic features. The crude ethanol extracts, flavonoids and saponins of the two plants have no analgesic and/or anti-inflammatory activities that do not support their traditional uses in the management of diseases such as stomach ache, head ache, pains of the joints and gingivitis, which are associated with pains and inflammations.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 The Family Asteraceae

The largest family of flowering plants that contains about 900 genera and some 13,000 species. Members are herbs, shrubs or rarely small trees or climbers. Leaves are alternate or opposite, simple or variously divided. Flowers are crowded into heads with capitula single or few-flowered, which can be unisexual, hermaphrodite or neuter. Corolla is sympetalous, stamen epipetalous and filaments/ anthers are free. Ovary is inferior and Fruits (achene) are sessile, sometimes beaked (Hutchinson and Dalziel, 1963).

Members are known to store carbohydrate as polyfructose, notably inulin and commonly producing polyacetylenes which are borne in the resin-canals. Polyacetylenes are characterized by the presence of cyclic, aromatic or heterocyclic end-group; bitter sesquiterpenes (especially sesquiterpene lactones), terpenoid volatile essential oils and often one or another sort of alkaloids, notably the pyrrolizidine alkaloids, but without iridoid compounds. They are usually not tanniferous, or at least not strongly so. They generally lack ellagic acid and proanthocyanins but occasionally cyanogenic, crystals of calcium oxalates seldom present in some of the cells of parenchymatous tissues; nodes trilacunar to multilacunar (Cronquist, 1981). Asteraceae are known to contain pyridine, quinoline alkaloids and occasionally diterpenes. Glycosides, saponins, flavonoids and tannins are their chief constituents and flavonoids are used as taxonomic markers (Evans, 1999).

2.2 The Genus *Vernonia* Schreb.

The genus has about 60 members that are trees, shrubs or woody climbers. They are characterized by involucral bracts with coloured or whitish appendages; capitula 1.3-3cm or more diameters; achenes often black and ribbed; pappus-setae more or less uniform. They are usually deciduous and buff in colour and leaves are often toothed (Hutchinson and Dalziel, 1963).

2.3 The Species: *Vernonia amygdalina* Del. and *V. kotschyana* Sch. Bip.

Vernonia amygdalina is a shrub or small tree 6-15 feet high with striate, pubescent stems, branchlets becoming glabrous. Its florets were sweet-scented, white; heads are broad, pappus buff or reddish. This plant specie is common and often planted. On the other hand *V. kotschyana* is a shrub with erect, pithy, striate, sparsely pubescent stems. It is 2-5 feet high; florets pale mauve in heads, and are short (Hutchinson and Dalziel, 1963).

Both species are commonly found in the tropical and subtropical countries. In Nigeria, they are commonly found in the Northern and southern parts. Although, it is only the *V. amygdalina* that is commonly planted and cultivated, the other specie is wildly available. They are classified in according to Sanogo (2005) as follows:

Kingdom: Plantae

Division: Magnoliophyta

Class: Magnoliopsida

Order: Asterales

Family: Asteraceae

Genus: Vernonia

Species: *Vernonia amygdalina* Del. and *V. kotschyana* Sch. Bip.

2.4 Ethno-medical Uses of *Vernonia amygdalina* and *V. kotschyana*

2.4.1 Ethno-medical Uses of *Vernonia amygdalina*

Vernonia amygdalina is used for whooping cough, improvement of contraction during labour, schistosomiasis, abortion, and infertility in barren women (Bullough and Leary, 1982). It is also used for diabetes, fever reduction, and a non-pharmaceutical solution in form of infusion to persistent fever. Infusion of this plant specie is also used for head ache, and joint pains associated with AIDS (Senyonga and Brehony, 1993). Its roots have been used for gingivitis and tooth ache due to its proven antimicrobial activity (Elujoba *et al.,* 2005).

2.4.2 Ethno-medical Uses of *Vernonia kotschyana*

Vernonia kotschyana is used for stomach ache, tuberculosis, head ache, gastric ulcers, gingivitis and gastrointestinal disorders (Germano *et al.,* 1996).

The roots from *V. kotschyana* Sch. Bip. are used in Malian folk medicine for the treatment of gastritis, gastroduodenal ulcers, as an aid to ameliorate digestion and as a wound healing remedy (Isawumi *et al.*, 2004).

2.4.3 Traditional Hausa-Fulani Uses of *Vernonia amygdalina* and *V. kotschyana*

Some Hausa-Fulani medical traditional herberlists in rowan-sanyi district of Malumfashi local government area of Katsina state, when consulted reported some of the traditional medicinal uses of *Vernonia amygdalina* (Shiwaka) and *V. kotschyana* (Kumbura Fage). Leaf decoction of the two plant species is used in the management of fever, head ache,

pains of the joints and stomach ache. It is also used as a mouth wash in cases of bleeding and swollen gums. Developing stems of *V. amygdalina* are excellent substitute to tooth paste. Leaves of plants especially those of *V. amygdalina* are used as vegetables. They are used as antidiabetic agents and as fish poisons in the eastern part of Nigeria (Ibrahim *et al.,* 2002).

2.5 Previous Research on *Vernonia amygdalina* and *V. kotschyana*

2.5.1 Previous Research on *Vernonia amygdalina*

A chemical study carried out by Oke (1965) on the leaves of *V. amygdalina* reported the presence of oxalate, cyanide and minerals. The content of oxalate and cyanide was very low but alluminium was significantly high and could cause toxicity. The calcium content was found to be about 4 – 7 per cent and as such could provide the daily requirement for this element /mineral. Thus the plant could be an important dietary component; for which its leaves are used as vegetable in Nigeria and many other countries.

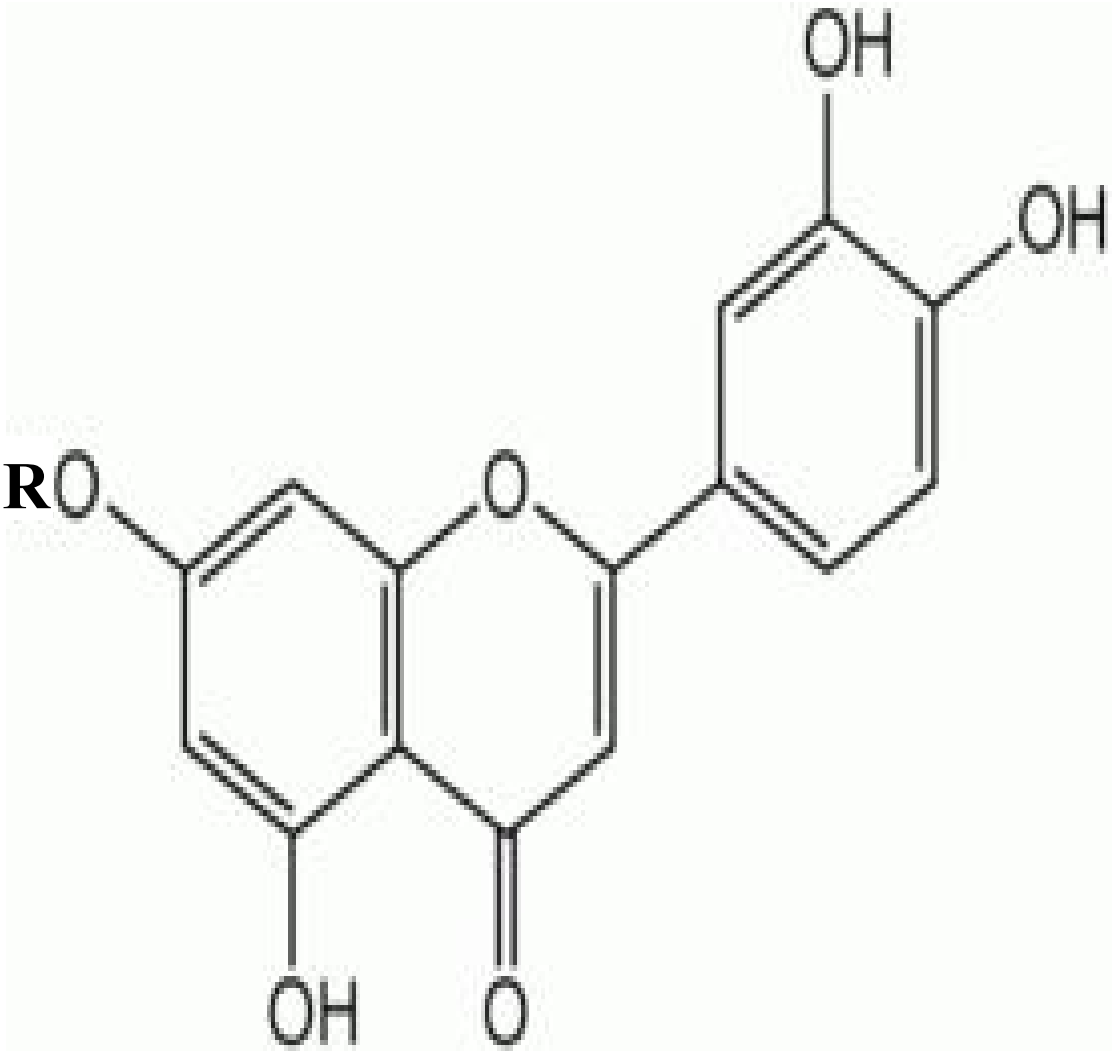
Chloroform extract of *V. amygdalina* was found to show a significant inhibitory activity *in-vitro* against cells derived from human carcinoma of the nasopharynx carried out in tissue culture. Isolation and structure elucidation revealed the extract to contain two new cytotoxic sesquiterpene lactones – Vernodalin and Vernomygdin. Mass spectrometry and elemental analysis showed that $C_{19}H_{20}O_7$ and $C_{19}H_{25}O_7$ were their molecular formulae respectively (Kupchan *et al.,* 1969).

It was found that aqueous/ methanol extracts of *V. amygdalina* showed antibacterial activity against *Staphylococcus aureus, S. albus, Proteus species* and *Escherichia coli* and even against *Pseudomonas aeruginosa* (Ogunlana and Ramstad, 1975).

Acute toxicity assessment on *V. amygdalina* methanol and aqueous extracts show the plant to have LD_{50} values of 1000 and 2000 (mg / kg) *i.p.* respectively in mice. However, the aqueous extract in rabbits has an LD_{50} value of 1,012 mg /kg body weight *i.p.* (Rwangabo *et al.,* 1986).

Phytochemical studies on *V. amygdalina* revealed three new stigmastane-type steroid glycosides. They were identified as Vernonioside A_4, B_2 and B_3 as well as the aglycone of A_4. Vernonioside A_4 and its aglycone were bitter, whereas others were not (Jisaka *et al.,* 1993).

Igile *et al.,* (1994), reported that chemical and spectroscopic studies on the leaves of *V. amygdalina* revealed the presence of three flavones: luteolin, luteolin 7-0-β-glucuronoside and luteolin 7–0-β-glucoside (Figure 2.1) and luteolin 7–0-β-glucuronoside was the most abundant. They were found to be antioxidants, with luteolin being a more significantly potent antioxidant than the synthetic butylated hydroxy-toluene at the same concentration of 15 mg / L. The two others have lower activity than that of luteolin and butylated hydroxy-toluene.



Luteolin 7–0-β-glucoside: R = β-D-glucopyranosyl
Luteolin 7–0-β-glucuronoside: R = β-D-glucuronopyranosyl

Figure 2.1: Chemical Structures of Some Flavonoids from *Vernonia amygdalina* (Igile *et al.*, 1994)

Elemental analysis and laboratory experiment by Igile *et al.* (1995) with mice fed diets containing alcohol extract or purified saponins of *V. amygdalina* was carried out. It was observed to have no negative effect on the feeding performance of mice fed amended diets. However, it significantly reduced the body weight and increased urinary and fecal output of the mice. At necropsy, liver weights were reduced and stomach and small intestines were enlarged in treated groups.

Comparative anatomical studies on *V. amygdalina* and *Talinum triangulare* showed the plant to have numerous trichomes on the epidermis of young stems. The outer cortical part is composed of strands of sclerenchymatous fibers alternating with strands of phloem toward periphery. Leaves were dorsiventrally flattened and slightly pubescent with ranunculaceous stomata and medullated vascular cylinder (Nwosu, 1999).

Ogboli *et al.* (2000) reported that, *V. amygdalina* petroleum ether and ethanol extracts were found to have potential curative effect on experimental schistosomiasis in mice.

Ibrahim (2001) discovered that *V. amygdalina* has a spermatogenic stimulating and melanin depigmentation effect in albino rats (wistar strains).

Ethanol leaf and root-bark extracts of *V. amygdalina* were found to possess significant antimalarial activities against drug-sensitive *Plasmodium berghei* in mice *in-vivo* (Abosi and Roseroka, 2003).

The aqueous extract of *V. amygdalina* was found to elicit *in-vivo* hepatoprotectivity through antioxidant activity on acetaminophen-induced hepatic damage in mice (Iwalokun *et al.*, 2006).

The aqueous leaf extract of *V. amygdalina* Del. (Compositae) given *i.p*. produced a dose-related fall in blood sugar. A dose of 80 mg/kg body weight in adult rabbit produced a maximum lowering of blood sugar in both fasted normal and alloxanized rabbits. The fasting blood sugar in normoglycaemic rabbits was reduced from 96 mg% to 48 mg% in 4 h. In alloxanized rabbits, the blood sugar was reduced from the mean value of 520 mg% to 300 mg% in 8 h. The hypoglycaemic effects were compared with those of tolbutamide. The blood sugar lowering effect of the plant extract may involve a mechanism not related to insulin secretion (Akah and Okafor, 2006).

The ethanol leaf extract of *V. amygdalina* was found to be safe and active for use in ethno-medicine, especially in ethno-therapy of diabetes mellitus in rats (Ekpo *et al.*, 2007).

The aqueous leaf extracts of *V. amygdalina* was found to have significant *in-vitro* and *in-vivo* anthelmintic activities against the chicken parasite *Ascaridia galli* and can be integrated in indigenous chicken health management system (Siamba *et al.*, 2007).

Aqueous extract of *V. amygdalina* infused intragastrically at a dose of 10 mg/ml to rats induced a significant increase in acid output which was reduced by, ranitidine (5 mg/kg b.w.) and atropine (1.2 µMol/kg b.w.). Moreover the extract evoked a dose-dependent contraction of the guinea pig ileum at a dose range of 0.6 mg/ml to 66 mg/ml which was inhibited by atropine at $2.4 \times 10^{-8} - 2.4 \times 10^{-6}$ M (Owu *et al.*, 2008).

The methanol extract of *Vernonia amygdalina* leaves was found to have lipid-lowering effects in rats fed a high cholesterol diet when compared with a standard hypolipidemic drug, questran and probably the plant might serve as a new potential natural product for the treatment of hyperlipidemia (Oluwatosin *et al.*, 2008).

Possible combined efficacy of ethanol extracts of *V. amygdalina* and *Azadirachta indica* in management of diabetes were studied. *V. amygdalina* extract alone was most potent in blood glucose reduction mechanism while A. indica extract was most potent in protecting the liver against damage in diabetic states. However, combination of the two plant extracts provides the wholistic efficacy desired in the management of diabetes (Ebong *et al.*, 2008).

2.5.2 Previous Research on *Vernonia kotschyana*

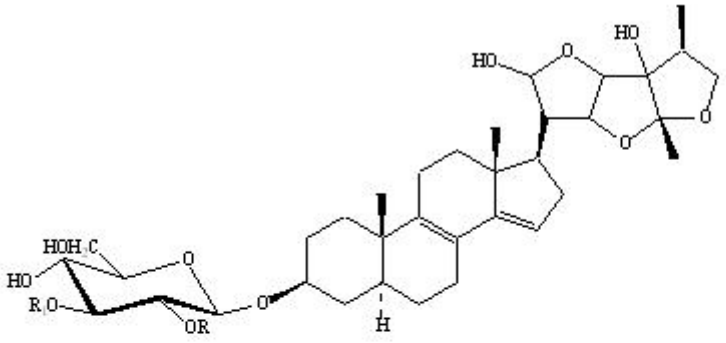
According to Deeni and Hussain (1994), *V. kotschyana* was found to possess varied antimicrobial activities against a wide range of microbes notably bacteria, yeast and fungi at various concentrations of its extracts.

The ethanol extract of *V. kotschyana* was reported to be active against a wide variety of bacterial species including *Salmonella* species and *Staphylococcus aureus.* Its extract was found to contain alkaloids (Deeni and Hussain, 1994).

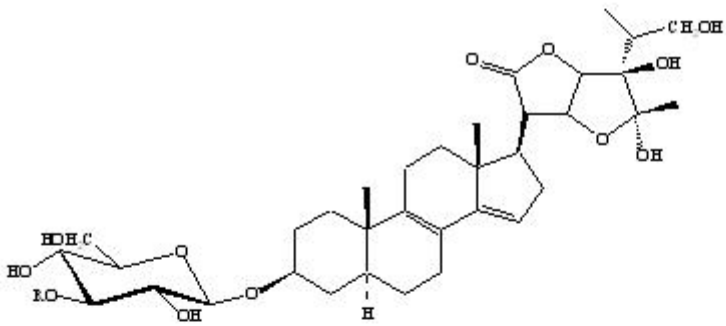
The aqueous extract of *V. kotschyana* was found to possess significant antiulcer activity in male rats (Sanogo *et al.,* 1996).

Phytochemical constituents identified from both aerial and underground parts of *V. kotschyana* include, flavonoid glycosides, sesquiterpene lactones, steroid glycosides and alkaloids (Sanogo *et al.,* 1998).

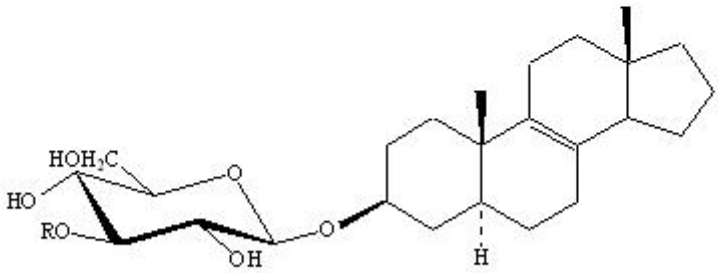
The roots of *V. kotschyana* were reported to contain various types of steroidal glycosides such as vernoniosides D_1, D_2, D_3, F_1 and F_2 (Figure 2.2). Aqueous root extract of *V. kotschyana* was found to have gastro-protective properties against ethanol-induced ulcers *in-vivo* in rats. It also contain acidic polysaccharide fraction with complement fixing ability and T-cell independent induction of B-cell proliferation *in-vitro* (Sanogo *et al.,* 1998).



Vernonioside D_1: R = R_1 = H
Vernonioside D_2: R = H, R_1 = β-D-xylopyranosyl
Vernonioside D_3: R = β-D-glucopyranosyl, R_1 = H



Vernonioside F_1: R = H
Vernonioside F_2: R = β-D-xylopyranosyl



Vernonioside S: R = β-D-glucopyranosyl

Figure 2.2: Chemical Structures of Some Saponins from *Vernonia amygdalina* and *V. kotschyana* (Jisaka *et al.*, 1993; Sanogo *et al.*, 1998)

Fractions isolated from both the 50°C and the 100°C water extracts of *V. kotschyana* were found to have immunomodulating activities. The active principles were identified as acidic polysaccharide fractions, containing pectic arabinogalactan type II structures, which showed both complement fixing ability and T-cell independent induction of B-cell proliferation *in-vitro*. Some activity was also observed on macrophages. This might provide additional support for the popular use of the plant to improve intestinal health (Nergard *et al.*, 2004).

Structure and immunological characteristics of the pectic arabinogalactan Vk2a from the roots of *V. kotschyana* Sch. Bip. ex Walp. were investigated, after enzymatic digestion of the galacturonan moiety and the side chains of the rhamnogalacturonan structure of Vk2a. All the enzyme resistant fractions expressed potent complement fixation and induction of B-cell mitogenic activity, which indicates that, there may be several and possibly structurally different active sites involved in the bioactivity of Vk2a (Nergard *et al.*, 2005).

Structures of three pectic arabinogalactans, one from *V. kotschyana* (Vk2a) and two from *Cochlospermum tinctorium* (Ct50A1 and Ct50A2), their complement fixation and induction of B-cell proliferation *in-vitro* were compared. The polysaccharide Vk2a expressed potent biological activity in both assays compared with Ct50A1 and Ct50A2. The Vk2a possessed a very high molecular weight (1150 ± 20 kDa) compared with Ct50A1 and Ct50A2 which both showed a polydisperse nature with the highest molecular weight polymers in each fraction estimated at ~105 kDa (Ct1a) and 640 ± 100 kDa (Ct2a), respectively (Cecilie *et al.*, 2006).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Equipments, Solvents and Reagents/ Solutions

3.1.1 Equipments

The following equipments were used for the research.

Sieve size no. 0.6 mm

Cardboard papers

Slides and cover slips

Beakers

Compound microscope

Camera lucida

Micrometers

Measuring cylinders

Weighing balance

Mechanical shaker

Razor blades

Funnels

Vernier caliper

Conical flasks

Photographic camera

Filter papers

Ashless filter papers

Water bath

Desiccators

Cages /Feeding bottles

3.1.2 Solvents

All solvents used for this research were of analytical grades (B.P).

Ethyl acetate

n-Butanol

Xylene

Chloroform

Water

Ether

Ethanol

Petroleum ether

Methanol

3.1.3 Reagents / Solutions

All reagents and/ or solutions used for this research were of analytical grade.

Ammonia solution (10 and 25 percent)

Chloral hydrate Solution (70 percent)

Concentrated hydrochloric acid

Hydrochloric acid (10 percent)

Hydrochloric acid (2N)

Methylene blue

Potassium hydroxide (5 percent)

Sodium hydroxide (10 percent)

Acetic acid (0.6% v/v)

Ketonal® (Ketoprofen)

Carrageenan (1% w/v)

All reagents / solutions were prepared according to Ciulei (1994) as follows:

(a) Carl Price's Reagent (Antimony (iii) Trichloride)

This was prepared by dissolving 25 g of antimony (iii) trichloride in 75 ml chloroform.

(b) Dragendorff's Reagent:

This was prepared by dissolving 0.85 g of basic bismuth nitrate in a mixture of 40 ml of water and 10 ml of acetic acid. A solution of 8 g potassium iodide dissolved in 20 ml of water was added and homogenized. This, when kept in a dark flagon, may be used for 2-3 months.

(c) Fehling's Solution (Cupric–Alkaline solution)

(1) A 34.66 g of copper (ii) sulphate dissolved in 200 ml of water and diluted to 500 ml.

(2) A 173 g of sodium and potassium tartrate and 100 g of sodium hydroxide dissolved in 300 ml of water and then diluted to 500 ml, after cooling. Equal volumes of solution 1 and 2 were mixed immediately before use.

(d) Mayer's Reagent

This was prepared by dissolving 1.35 g of mercuric chloride in 60 ml of water and then 5 g of potassium iodide in 10 ml of water added and then diluted to 100 ml with water.

(e) Molisch's Reagent

This contained (i) not less than 17 per cent v/v of sulphuric acid (H_2S0_4) and (ii) 20 percent thymol in alcohol.

(f) Phloroglucinol (1 per cent) in Alcohol

This was prepared by dissolving 1 g of phloroglucinol in 100 ml of alcohol.

3.2 Collection and Identification of *Vernonia amygdalina* Del. and *V. kotschyana* Sch. Bip.

Sample of *Vernonia amygdalina* Del. and *V. kotschyana* Sch. Bip. were collected during their flowering stages in January and October, 2002 respectively. They were collected from Ruwan-sanyi of Malumfashi Local Government area of Katsina State. They were identified first in the field based on their botanical and taxonomic characteristics as described earlier by Hutchinson and Dalziel (1958; 1963) and Irvine (1961); secondly, by direct comparison with authentic samples at the Herbarium by the Taxonomists / Herbarium keeper (Mr. U. S. Gallah) of the Department of Biological Sciences, Ahmadu Bello University, Zaria.

Fresh leaves of both plants were used for some microscopic studies while the bulk were carefully air dried at room temperature, powdered, sieved and then kept in a cool and dry place, protected from sun, for experimental purposes.

3.3 Preparation of Herbarium Specimens of *Vernonia amygdalina* and *V. kotschyana*

The collected samples of the plants were separately arranged between pieces of newspapers. These were then inserted between more sheets of papers and placed on flat

boards. Other boards were placed on top and these were weighed down with building blocks. The wet outer newspapers were change on alternate days until the specimens were completely dried. The dried samples were carefully removed from the drying papers. These were placed and fastened on thick white plain mounting sheets. Information cards indicating locality, botanical and vernacular names, habitat and date of specimen collection were affixed on the herbarium sheets. The herbarium sheets were later put in folded cardboard papers and these were occasionally placed under the sun to prevent mould growth. They were preserved with methylated spirit and mercury chloride (Walters, 1963).

3.4 Preparation of *Vernonia amygdalina* and *V. kotschyana* Leaves

The bulk and air dried leaves of each of the two plants were separately size-reduced with pestle and mortar. They were sieved with 0.6 mm mesh sieve size to obtain the powdered crude plant materials. They were labeled appropriately, stored in tightly closed containers and kept in a good place for use during the reseach.

3.5 Macroscopic Studies on *Vernonia amygdalina* and *V. kotschyana* Leaves

Macroscopic observations carried out on the leaves of the two plant species; include observations of the macroscopic appearance of the leaves, such as surface appearance, dimension, and point of attachment, lamina structure in terms of shape, composition, incision, venation, margin, apex, and base. Their organoleptic properties, namely taste, odour and colour were determined. Examinations were made with naked eyes/hand lens and the results were recorded (Brain and Turner, 1975; Evans, 1996).

3.6 Calibration of Eyepiece Micrometer

Eyepiece micrometer was calibrated by using the standard stage micrometer, under x40 objective in accordance with Brain and Turner (1975). Microscopic measurements of the diagnostic features of *V. amygdalina* and *V. kotschyana* were carried out by using the calibrated eyepiece micrometer.

3.7 Microscopic/ Chemo-microscopic / Quantitative-leaf Microscopic Studies on *Vernonia amygdalina* and *V. kotschyana* Leaves

3.7.1 Anatomical Sections and Powder of Leaves

Microscopic examinations on the fresh and powdered leaves of the two plant species to analytically assess their microscopic features were carried out. Surface preparations were made by peeling off the upper and lower epidermis of the fresh leaves of both plants. Transverse sections of their leaves were also prepared with the aid of razor blades. Small amounts of the finely ground powdered leaves were used for the microscopy. They were cleared in 70% chloral hydrate solution on a microscope slide (sections from fresh leaves) and in test-tubes (finely ground leaves) by heating on Bunsen flame and boiling on water bath respectively. They were mounted by using diluted glycerol and examined with the microscope (Brain and Turner, 1975).

Microscopic features of these plants, such as the type, nature and size of the epidermal cells, trichomes, stomata, fibres, xylem elements, and even calcium oxalate crystals were observed. Measurements were done with the aid of calibrated eyepiece micrometer (Evans, 1996).

3.7.2 Chemo-microscopic Studies

These were carried out on the powdered leaves of the two plant species. Small amount of the finely ground powdered leaves of each of the two plants was cleared in a test-tube containing 70% chloral hydrate solution. They were boiled on a water-bath for about thirty minutes to remove obscuring materials. The cleared sample was mounted on a microscope slide, using dilute glycerol. Using various detecting reagents, the presence of some cell inclusions and cell wall materials were detected in accordance with Brain and Turner (1975); Evans (1996) as follows:

(a) Test for Cellulose

To a small amount of the cleared leaf powder of each of the two plants, chlor-zinc iodine was added. The occurrence of blue colour would be considered positive for cellulose on cell walls of epidermal cells.

(b) Test for Lignins

To a small amount of the cleared leaf powder of each of the two plants, phloroglucinol solution and concentrated HCl were added. Red stain of lignified walls would be considered positive for lignins.

(c) Test for Tannins

To a small amount of the cleared leaf powder of each of the two plants, 5% ferric chloride solution was added. Greenish black colour in some parenchyma cells would be considered positive for tannins.

(d) Test for Starch

To a small amount of the cleared leaf powder of each of the two plants, N/50 iodine was added. Blue-black colouration on some grains would be considered positive for starch.

(e) Test for Mucilages

To a small amount of the cleared leaf powder of each of the two plants, ruthenium red was added. Pink colouration in mucilage-containing cells within the epidermis would be considered positive for mucilaginous walls.

(f) Test for Calcium Oxalate Crystals

To a small amount of the cleared leaf powder of each of the two plants, 80% H_2SO_4 acid was added. Brightly coloured crystals which disappeared upon addition of acid would be considered positive for calcium oxalate crystals.

(g) Test for Calcium carbonates ($CaCO_3$)

To a small amount of the cleared leaf powder of each of the two plants, 80% H_2SO_4 acid was added. Calcium carbonate deposit dissolve upon addition of acid with effervescence. This would be considered positive for calcium carbonates.

(h) Test for Fixed Oils and Fats

To a prepared transverse section of the leaf of each of the plants separately, few drops of Sudan IV solution were added and observed for few minutes. The appearance of a red

colouration indicates the presence of fixed oils and fats (Brain and Turner, 1975; Evans, 1996).

3.7.3 Quantitative-leaf Microscopic Studies

Determination of the various physical constants was carried out on the leaves of the two plants. These include stomatal number, stomatal index, palisade ratio, vein–islet number and vein-let termination number. Sections of fresh leaves from the two plant species were cleared in boiling 70% chloral hydrate solution and mounted with diluted glycerol on microscope slides and examined. Counting of microscopic features such as stomata, palisade cells were done with the aid of a Camera lucida, as described by Evans (1996).

(a) Palisade Ratio

The average number of palisade cells beneath the upper epidermis for *V. amygdalina* and *V. kotschyana* leaves was determined. Section from the upper epidermis was cleared with boiling 70% chloral hydrate solution and mounted on a clean microscope slide with diluted glycerol and examined with x40 objective. A Camera lucida was arranged, the epidermal cells and palisade cells lying beneath them were traced. Groups of 4 epidermal cells were traced, and their outlines linked to make them conspicuous. Palisade cells lying beneath each group were focused, traced and counted. Those included were ones that are more than half covered by epidermal cells. The figure obtained was divided by 4 and this gave the palisade ratio of that group (Brain and Turner, 1975).

(b) Stomatal Number

The average number of stomata per square millimeter of epidermis of upper and lower sides of the leaves of *V. amygdalina* and *V. kotschyana* was determined. Sections from the upper and lower epidermis of the leaves of the plants were cleared with 70% chloral hydrate solution and mounted on a microscope slides with diluted glycerol.

A Camera lucida was set up, with the aid of stage micrometer, a paper was divided into squares of 1 mm^2 using a X10 objective. The stage micrometer was replaced by the cleared preparations or sections. The stomata were traced and counted in the fields on a single section of the leaves of each of the two plants and the average number of stomata per mm^2 of epidermis was calculated (Evans, 1996).

$$\text{Stomatal Number} = \frac{\text{Average Number of Stomata}}{\text{Square Millimeter of Epidermis}}$$

(c) Stomatal Index

The percentage proportion of the ultimate divisions of the epidermis of a leaf, which have been converted into stomata, of each of the two plants was determined. Sections of the epidermal portion of the leaves were mounted and examined as described in (a) earlier. It was only that in each field viewed, both stomata and epidermal cells were counted. The stomatal index was calculated in accordance with Evans (1996).

$$\text{Stomatal Index} = \frac{\text{Number of Stomata}}{\text{No. of Epidermal Cells + No. of Stomata}} \times 100$$

(d) Vein–islet Number

The number of vein–islet (the minute area of photosynthetic tissues encircled by the ultimate division of conducting strands) per mm^2 was calculated in four contagious millimeter squares, in the central part of the lamina of *V. amygdalina* and *V. kotschyana* leaf midway between the midrib and the margin. It was determined by boiling pieces of leaf of the plants in a test-tube containing 70% chloral hydrate solution. This was followed by treatment with hydrochloric acid (10% v/v) to remove calcium oxalate crystals that will consequently enhance visibility.

A camera lucida was set up and by means of a stage micrometer; the paper was divided into 1 mm^2 using X10 objective. The stage micrometer was then replaced by cleared preparations of the leaf and the veins were traced in 4 contagious squares. That is a rectangle of 1 mm x 4 mm. Each vein was traced and areas which were completely enclosed by veins were counted and those that were not completely enclosed were excluded (Evans, 1996).

(e) Vein-let Termination Number

The number of vein-let termination per mm^2 of *V. amygdalina* and *V. kotschyana* leaf surface was determined. A camera lucida was set up as described earlier and paper was divided into squares of mm^2 and vein-let terminations in each square were counted to get the vein-let termination number (Evans, 1996).

3.8 Determination of Physical Constants on *Vernonia amygdalina* and *V. kotschyana* Leaves

Physical constants namely, extractive (water and alcohol) values, moisture content, total ash, acid–insoluble ash and water-soluble ash were determined on the leaf powder of the plants (Appendix I).

(a) Moisture Content

The moisture content of the powdered leaves of each of *V. amygdalina* and *V. kotschyana* was determined. In a crucible 2 g of the powdered leaves of the plants was weighed separately. It was then heated for 1 hour at 105^{o}C, cooled in a desiccator and re-weighed. This was repeated for five times until a constant weight was obtained. This was because the method adopted was based on loss on drying. The moisture content was determined as percentage for each of the two plants (Brain and Turner, 1975) as:

$$\text{Moisture Content (\%)} = \frac{\text{Weight of Water Lost}}{\text{Original Weight of Sample}} \times 100$$

(b) Total Ash

The total ash of the powdered leaves of *V. amygdalina* and *V. kotschyana* was determined. In a crucible 2 g of the powdered leaves of the plants was weighed separately. It was heated gently until the material is charred at a temperature of 450^{o}C. It was heated until all the carbon has been removed, cooled and weighed. The total ash value of each of the two plant species as percentage was determined (Brain and Turner, 1975) as follows:

$$\text{Total Ash Value (\%)} = \frac{\text{Weight of Residual Ash}}{\text{Initial Weight of Sample}} \times 100$$

(c) Acid- Insoluble Ash

The acid-insoluble ash value for the powdered leaves of *V. amygdalina* and *V. kotschyana* was determined separately. The ash obtained in (d) above together with the crucible, were quantitatively and carefully transferred into a beaker, containing 25 ml of diluted hydrochloric acid. It was boiled for 5 minutes and the insoluble ash was collected on an ashless filter paper. The beaker containing the acid and crucible were washed with hot water. The washings were passed through the ashless filter paper continuously until it was free from acid. The residue and filter paper were dried gently in an oven. It was ignited in a tarred crucible, cooled and weighed. The acid-insoluble ash as percentage for each of the two plants was determined (Brain and Turner, 1975) as follows:

$$\text{Acid-Insoluble Ash (\%)} = \frac{\text{Weight of Residual Ash}}{\text{Initial Weight of Sample}} \times 100$$

(d) Water-Soluble Ash

The water-soluble ash value of the powdered leaves of *V. amygdalina* and *V. kotschyana* was determined separately. The ash obtained following method described in (b) was used. It was determined, following the same procedure in (c) except that water was used instead of diluted hydrochloric acid. The water-soluble ash as percentage for each of the two plants was determined (Brain and Turner, 1975) as follows.

$$\text{Water-Soluble Ash (\%)} = \frac{\text{Int. Wt. of Ash–Wt. of Res. Ash}}{\text{Initial Weight of Sample}} \times 100$$

(e) Alcohol-Soluble Extractive Value

This is the amount of extractive in percentage of a plant sample with alcohol. To 10 g of powdered leaves of *V. amygdalina* and *V. kotschyana* separately in a stoppard flask, 100 ml ethanol was added and macerated for 24 hours. The mixture was frequently shaken during the first 6 hours using a flask shaker. The mixture was filtered and 20 ml of the filtrate was evaporated to dryness in a beaker in each case. It was dried to a constant weight on a boiling water bath. The percentage of alcohol-soluble extractive value was determined for each of the two plants (Brain and Turner, 1975) as:

$$\text{Alcohol-Soluble Extractive Value (\%)} = \frac{\text{Wt. of Residue x 5}}{\text{Weight of Sample}} \times 100$$

(f) Water-Soluble Extractive Value

This is the amount of extractive in percentage of a plant sample with water. To 10 g of powdered leaves of *V. amygdalina* and *V. kotschyana* separately in a stoppard flask, 100 ml chloroform-water was added and macerated for 24 hours. The mixture was frequently shaken, during the first 6 hours, using a flask shaker. The mixture was filtered and 20 ml of the filtrate was evaporated to dryness in a beaker on a boiling water-bath for each of the two plants. These were dried to constant weight and the percentage water-soluble extractive value was determined in accordance with Brain and Turner (1975) as:

$$\text{Water–Soluble Extractive Value (\%)} = \frac{\text{Wt. of Residue x5}}{\text{Weight of Sample}} \times 100$$

3.9 Elemental Analysis on *Vernonia amygdalina* and *V. kotschyana* Leaf Powders

Method outlined by Idris (1999) was adopted. This was done on the leaf powder of the plants separately by using an Energy Dispersive X-ray Fluorescence (EDXRF) transmission emission technique (at the Centre for Energy Research and Training, Ahmadu Bello University, Zaria). The EDXRF system consists of a 925 MBq^{109}Cd annular isotopic source with a Canberra Si (Li) detector having a resolution of 170 eV at 5.9 KeV. The X-ray spectra were acquired with a computer based MCA card (Trump 8k). The set-up provides for dead-time correction and pile-up rejection. Sensitivity calibration of the system was performed using thick foils of pure metals (Ti, Fe, Co, Ni, Cu, Zn, Zr, Nb, Mo, Sn, Ta, Pb) and of stable chemical compounds (KCo_3, $CaCo_3$, Ce_2O_3, Wo_3, Tho_2, U_3O_8). Concentration of some mineral elements per gram of the leaf powder was determined and expressed in milligram (mg) for each of the plants.

3.10 Extraction of *Vernonia amygdalina* and *V. kotschyana* Leaf Powders

Petroleum ether (60-80) was used to extract 800 g of the dried powdered plant material of *V. amygdalina* and *V. kotschyana* separately, continuously by using Soxhlet for a period of 18 hours. The marcs of the two plant leaf powders were later exhaustively extracted with ethanol (96%v/v) separately. The extracts obtained were evaporated to dryness on a boiling water bath to obtain the crude extract of the two plants (Ciulei, 1994; Brain and Turner, 1975).

3.11 Phytochemical Studies on *Vernonia amygdalina* and *V. kotschyana* Ethanol Leaf Extracts

Phytochemical investigation was carried out on the extracts from the powdered leaves of *V. amygdalina* and *V. kotschyana* separately in order to test for the presence of some chemical constituents in these plants. This was carried out following standard procedures.

(a) Test for Alkaloids

To 1 g of the powdered leaves of *V. amygdalina* and *V. kotschyana* separately in a test tube, 10 ml of acid alcohol was added and boiled on a water bath. It was cooled and filtered. To 3 ml of the filtrate 3 drops of Dragendorff's reagent was added. The occurrence of an orange-red precipitate indicates the presence of alkaloids. When no precipitate was observed, then to a small amount of fresh filtrate, a small quantity of diluted ammonia was added to make it alkaline by testing with litmus paper. Five (ml) of chloroform was then added, layers were allowed to separate and chloroform layer was pipetted into another test-tube.

The chloroform layer was extracted with 10 ml of diluted hydrochloric acid and the chloroform was discarded. The extract was divided into five portions, 3 drops of Dragendorff's, Mayer's, Wagner's and Hager's reagents were respectively added to the first, second, third and forth portions of the extract. A turbidity or precipitate formed compared with the fifth untreated portion indicates the presence of alkaloids (Mohammed, 2002).

(b) Test for Phenolic Compounds

To 2 ml of ethanol extract of the powdered leaves of *V. amygdalina* and *V. kotschyana* separately in a test-tube, 2 ml of water was added. To it 5 drops of 5% ferric chloride solution were added. Blackish- green colour would be considered positive for phenolic compounds (Evans, 1996).

(c) Test for Tannins

(i) Lead Acetate Test

To 2 ml of ethanol extract of the powdered leaves of *V. amygdalina* and *V. kotschyana* separately in a test-tube, 2 ml of water was added. To it 5 drops of lead acetate solution were added. Coloured precitates indicate the presence of tannins (Kokate *et al.,* 2002).

(ii) Bromine Solution Test

To 0.2 g of the ethanol extract of the powdered leaves of *V. amygdalina* and *V. kotschyana* separately, 3 ml of water was added and shaken. To the solution 1 ml bromine solution was added. Appearance of blue colour indicates the presence of condensed tannins (Mohammed, 2002).

(iii) Ammonia Solution Test

To 0.2 g of the ethanol extract of the powdered leaves of *V. amygdalina* and *V. kotschyana* separately, 3 ml of water was added, shaken and filtered. To the filtrate, 3 ml of 25% ammonia solution was added and the solution was exposed to the air. The appearance of a slowly forming green colouration indicates the presence of chlorogenic acid (Kokate, 2002).

(d) Test for Carbohydrates

(i) Molisch's Test

To 0.2 g of each of the ethanol extract of *V. amygdalina* and *V. kotschyana* separately in a test tube, 5 ml of boiling water was added and cooled. To it 5 drops of Molisch's reagent were added. Small amount of concentrated H_2SO_4 was carefully added from the side of the test tubes to form lower layers. Formation of purple colouration indicates the presence of carbohydrates (Evans, 1996).

(ii) Fehling's Test

To 1 ml of ethanol extract of the powdered leaves of *V. amygdalina* and *V. kotschyana* separately in a test tube, 2 ml of water was added. To it 1 ml of Fehling's solutions A and B were added and heated on a boiling water bath for 15 minutes. A brick–red precipitate indicates the presence of reducing compounds (Ciulei, 1994).

(iii) Methylene Blue Test

To 2 ml of aqueous extract of the powdered leaves of *V. amygdalina* and *V. kotschyana* separately in a test tube, 10 ml of acetone was added drop wise. The thick precipitate formed was separated by filtration. It was washed with alcohol and stained with methylene blue (specific stains). The occurrence of a violet or blue precipitate denotes the presence of mucilage (Cuilei, 1994).

(e) Test for Anthracenes (Borntrager's Reaction)

To 2 ml concentrated ethanol extract of the powdered leaves of *V. amygdalina* and *V. kotschyana* separately in a test- tube, 2 ml of 10% HCl solution was added. To it 2 ml of 25 per cent ammonia solution was added with gentle shaking. A cherish red colour of the alkaline solution indicates the presence of emodols - aglycones of anthracenosides in an oxidized form (Ciulei, 1994).

(f) Test for Coumarins

To 0.2 g of ethanol extract of the powdered leaves of *V. amygdalina* and *V. kotschyana* separately in a test-tube, 2 ml of water was added and heated. The aqueous solution was divided into two equal volumes in two test-tubes. To one of the test-tubes, 0.5 ml of 10% ammonia solution was added. The other test-tube served as reference control. The occurrence of a blue or green fluorescence under U.V. light in the alkaline solution indicates the presence of coumarins (Ciulei, 1994).

(g) Test for Flavonoids

(i) Ferric Chloride Test

To 0.2 g of the ethanol extract of the powdered leaves of *V. amygdalina* and *V. kotschyana* separately in a test tube, 8 ml of distilled water and 5 drops of 10% ferric chloride solution were added together. Green or blue precipitates indicate the presence of phenolic nucleus (Musa, 2005).

(ii) Lead Acetate Test

To 5 ml of the ethanol extract of the powdered leaves of *V. amygdalina* and *V. kotschyana* separately, a solution of lead acetate solution was added. A yellow precipitate indicates presence of flavonoids (Musa, 2005).

(iii) Sodium Hydroxide Test

To an equal volume of the ethanol extract of the powdered leaves of *V. amygdalina* and *V. kotschyana* separately, 5 ml of 10% NaOH was added. Yellow colouration indicates presence of flavonoids (Musa, 2005).

(iv) Shinoda Test

To 0.1 g of the ethanol extract of the powdered leaves of *V. amygdalina* and *V. kotschyana* separately, 5 ml water was added and warmed on water bath for five minutes, filtered and cooled. To the filtrate, few pieces of magnesium turnings and five drops of hydrochloric acid were added. A pink colouration indicates the presence of flavonoids (Balbaa *et al.,* 1976).

(v) Amyl Alcohol Test

To 0.2 g of the ethanol extract of the powdered leaves of *V. amygdalina* and *V. kotschyana* separately, 5 ml of water was added. This was shaken with 4 ml of amyl alcohol. Yellow colour indicates the presence of flavonoid nucleus (Musa, 2005).

(h) Test for Saponins (Steroids /triterpenoids)

(i) Frothing Test

To 0.2 g of ethanol extract of the powdered leaves of *V. amygdalina* and *V. kotschyana* separately in a test- tube, 5 ml of water was added. It was shaken for 15 minutes. The occurrence of a frothing column of at least 1 cm in height, persisting for at least an hour was taken as a preliminary evidence for the presence of saponins (Sofowora, 1993).

(ii) Haemolysis Test

To 0.2 g of the ethanol extract of the powdered leaves of *V. amygdalina* and *V. kotschyana* separately, 2 ml of distilled water was added. To two test tubes, 2 ml of 1.8 per cent sodium chloride solution was put. To one of the test-tubes, 2 ml distilled-water was added and to the other one, 2.0 ml of crude extract was added. The concentration of sodium chloride in each test-tube is now isotonic with blood-serum. Five drops of a freshly collected and preserved (5% v/v of 3.8% w/v sodium citrate solution in blood) blood were then added to each of the test tubes. The test-tubes were inverted gently to mix the contents and allow standing for sometimes. Haemolysis in the test-tube containing the crude extract but not in the control indicates the presence of saponins (Brain and Turner, 1975).

(iii) Liebermann-Burchard's Reaction

To 0.2 g of ethanol extract of the powdered leaves of *V. amygdalina* and *V. kotschyana* separately in a test-tube, 2 ml acetic anhydride and 2 ml chloroform were added. The solution was transferred to a dried test-tube and 2 ml of concentrated sulphuric acid (H_2SO_4) was added with a pipette from the bottom. At the junction of the two liquids a

formation of a reddish brown or violet brown ring, the upper layer being bluish green or violet indicates the presence of sterols and triterpenes espectively (Ciulei, 1994).

(iv) Salkowski's Test

To 0.2 g of ethanol extract of the powdered leaves of *V. amygdalina* and *V. kotschyana* separately in test-tube, 2 ml chloroform was added. Sulphuric acid was carefully added from the side of the test-tube to form a lower layer. A reddish-brown colour at the interface indicates the presence of a steroidal ring (Sofowora, 1993).

(i) Test for Carotenoids (Carl Price's Reaction)

To 0.2 g of petroleum ether extract of the powdered leaves of *V. amygdalina* and *V. kotschyana* separately, 5 ml of a saturated solution of antimony trichloride in chloroform was added. On addition of concentrated sulphuric acid, the carotenoids usually turn deep blue or bluish green (Ciulei, 1994).

(j) Test for Fixed Oils and Fats

To 1 ml of the petroleum ether extract of the leaves of *V. amygdalina* and *V. kotschyana* separately in test-tube, 1 ml of 1% copper sulphate solution was added and mixed. Five drops of 10% sodium hydroxide solution was then added. Formation of a clear blue solution shows the presence of glycerine (Kokate *et al.,* 2002).

(k) Test for Resins

To 0.5 g of the ethanol extract of *V. amygdalina* and *V. kotschyana* separately in a test tube, 5 ml of 10% potassium permanganate solution was added and heated. Odour of

benzaldehyde due to oxidation of benzoic acid indicates the presence of resins (Brain and Turner, 1975).

(l) Test for Cyanophore Glycosides (Guignard's reaction)

Strips of white filter paper were dipped in to 1 per cent solution of picric acid, drained and then dipped in to 10 per cent of sodium carbonate solution and drained. Bruised and moistened fresh leaves of each of *V. amygdalina* and *V. kotschyana* were separately put in to small flasks. Strips of the prepared sodium picrate paper were suspended above the plant materials by trapping them with corks. Hydrocyanic acid liberation would be detected by a change in the colour of sodium picrate paper from yellow to brick red due to formation of sodium isopurpurate (Balbaa *et al*., 1976).

3.12 Fractionation of the Ethanol Extracts of *Vernonia amygdalina* and *V. kotschyana* Leaves

This was aimed at obtaining the crude flavonoids and saponins from the ethanol extracts of *V. amygdalina* and *V. kotschyana* (i.e. V. A. E. E. and V. K. E. E.). One hundred and fifty (150) g of the crude ethanol extract was used for each of the plants by using the slightly modified method of Woo *et al.* (1980) as shown in the schematic chart (Figure 3.1).

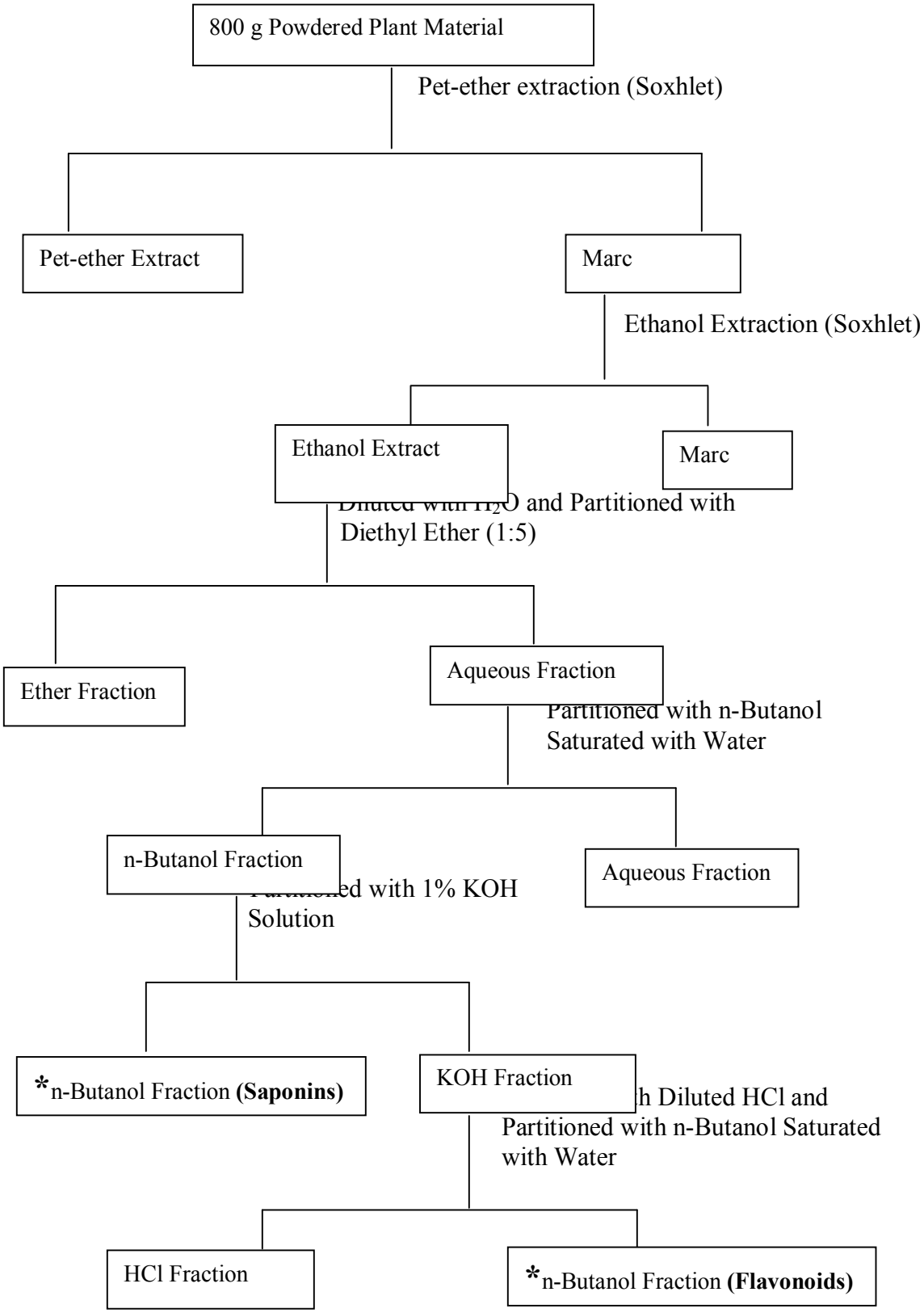

800 g Powdered Plant Material
Pet-ether extraction (Soxhlet)
Pet-ether Extract
Marc
Ethanol Extraction (Soxhlet)
Ethanol Extract
Marc
Diluted with H_2O and Partitioned with
Diethyl Ether (1:5)
Ether Fraction
Aqueous Fraction
Partitioned with n-Butanol
Saturated with Water
n-Butanol Fraction
Aqueous Fraction
ed with 1% KOH
Solution
*n-Butanol Fraction (Saponins)
KOH Fraction
h Diluted HCl and
Partitioned with n-Butanol Saturated
with Water
HCl Fraction
*n-Butanol Fraction (Flavonoids)

Figure 3.1: Schematic Chart for Fractionation of Flavonoids and Saponins (Woo *et al.*,1980)

3.13 Chemical Identification of the Crude Flavonoids and Saponins from *Vernonia amygdalina* and *V. kotschyana* Ethanol Leaf Extracts

(a) Identification of Flavonoids

This was carried out on the crude flavonoids obtained from the fractionation procedures on the ethanol extracts of *V. amygdalina* and *V. kotschyana* as follows:

(i) Ferric Chloride Test

To 0.2 g of the crude flavonoids, 8 ml of distilled water was added. Five drops of 10% ferric chloride solution was added together. Green or blue precipitates indicate the presence of phenolic nucleus (Musa, 2005).

(ii) Lead Acetate Test

To 5 ml of the crude flavonoids aqueous solution, 2 ml of lead acetate solution was added. Yellow precipitates indicate the presence of flavonoids (Musa, 2005).

(iii) Sodium Hydroxide Test

To an equal volume of the crude flavonoids, 5 ml of 10% NaOH was added. Yellow colouration indicates presence of flavonoids (Musa, 2005).

(iv) Shinoda Test

To 0.1 g of the crude flavonoids ethanol was added and boiled on water bath for 5 minutes, filtered and cooled. To the filtrate, few pieces of magnesium turnings and few drops of

hydrochloric acid was added. A pink colouration indicates the presence of flavonoids (Balbaa *et al.,* 1976).

(v) Amyl Alcohol Test

To 0.3 g of the crude flavonoids 10 ml water was added. To 5 ml of the solution 4 ml of amyl alcohol was added and shaken. Yellow colour indicates the presence of flavonoid nucleus (Balbaa, 1976; Musa, 2005).

(b) Identification of Saponins

This was carried out on the crude saponins obtained from the fractionation procedures on the ethanol extracts of both *V. amygdalina* and *V. kotschyana* as follows:

(i) Frothing Test

To 0.2 g of the crude saponins in a test tube, 2 ml of water was added and shaken vigorously. Appearance of a copious froth persisting for at least an hour would be considered positive for saponins (Sofowora, 1993).

(ii) Haemolysis Test

To 0.2 g of the crude saponins in a test tube, 2 ml of distilled water was added. To two test tubes, 2 ml of 1.8 per cent sodium chloride solution was added. To one of the test tubes, 2 ml distilled-water was added and to the other one, 2 ml of crude saponins was added. The concentration of sodium chloride in each test tube is now isotonic with blood – serum. Five drops of a freshly and preserved (5% v/v of 3.8 w/v sodium citrate solution in blood) collected blood was then added to each of the test-tubes. The test-tubes were inverted

gently to mix the contents and allow standing for some times. Haemolysis in the test tube containing the crude saponins but not in the control one indicates saponins (Brain and Turner, 1975).

(iii) Liebermann-Burchard's Reaction

To 0.2 g of the crude saponins in to a dry test tube, 2 ml acetic anhydride and 2ml chloroform were added and gently shaken. By means of a pipette, 1 ml concentrated sulphuric acid was added at the bottom. At the separating level of the two liquids, a reddish green or violet-brown ring will be formed. Bluish superior layer indicates sterols while violet indicates triterpenes (Ciulei, 1994).

(iv) Salkowski's Test

To 0.2 g of of the crude saponins in a dry test-tube, 2 ml chloroform was added. Sulphuric acid was carefully added from the side of the test-tube to form a lower layer. A reddish-brown colour at the interface indicates the presence of a steroidal ring (Sofowora, 1993).

3.14 Thin Layer Chromatographic Analysis on *Vernonia amygdalina* and *V. kotschyana* Crude Ethanol Leaf Extracts, Flavonoids and Saponins

This was carried out on the ethanol extracts, crude flavonoids and saponins of the two plant species.

Methods of Igile *et al.* (1994; 1995) were slightly modified and used for the analysis. Crude ethanol extracts, flavonoids and saponins (0.3 g) from the plants were separately dissolved in methanol. These were spotted on silica gel precoated Thin Layer Chromatographic plates (DC Fertigflatten, Merck) and co-chromatographed. Developing

solvent used was n-Butanol: Glacial acetic acid: Water (50:20:30) in a developing chromatographic tank, by dipping spotted and dried plates into the tank and covered over a period of 40 minutes. Developed plates were brought out and allowed to dry.

Visualization was carried out by using general and specific detecting reagents. Plates of the crude ethanol extacts of both plants were separately exposed to iodine vapour, concentrated ammonia vapour and Liebermann-Burchard's reagents. Plates from the crude flavonoids and saponins of the two plant species were respectively visualized by exposure to concentrated ammonia vapour and spraying with Liebermann-Burchard's reagent: chloroform: acetic anhydride: concentrated sulphuric acid (5:1:1) followed by heating at 105^{o}C. Number of spots, colours and retardation factors (R_f values): Distance moved by the solute from point of origin/Distance traveled by the solvent front, for each of the spots were determined and recorded.

3.15 Experimental Animals

Two hundred and fifty adult Wister rats and Swiss albino mice of both sexes were obtained from the Animal house of the Department of Pharmacology and Clinical Pharmacy, Ahmadu Bello University, Zaria and used for the experiments. They were kept in well-ventilated room and fed with standard ECWA grower mash. The animals were allowed access to food and water *ad libitum*. The animals' care and handling were conducted in compliance with the National Regulations for Animal Research. These regulations are consistent with Ahmadu Bello University Animal Research and Ethics Guidelines.

3.16 Acute Toxicity Studies (LD_{50}) on *Vernonia amygdalina* and *V. kotschyana* Ethanol Leaf Extracts

These were carried out on the ethanol extract of *V. amygdalina* and *V. kotschyana* (i.e. V.A.E.E. and V.K.E.E.) separately. Method of Lorke (1983) was used for the experiment by using Swiss albino mice. In the first phase, mice were divided into 3 groups of 3 mice each. They were administered 10, 100 and 1000 mg extract/ kg body weight *i.p*. and were observed for 24 hours. In the second phase, mice were divided into 4 groups of 1 mouse each and were administered 140, 225, 370 and 600 mg extract/kg body weight *i.p.* consequent upon the results of the first phase. Final LD_{50} was calculated by determining the geometric mean of the doses from the groups for which 0/1 and 1/1 death was observed.

3.17 Analgesic Studies on *Vernonia amygdalina* and *V. kotschyana* Crude Ethanol Leaf Extracts, Flavonoids and Saponins

These were carried out on the ethanol extract of *V. amygdalina* and *V. kotschyana* (i.e. V.A.E.E. and V.K.E.E.), the crude flavonoids (i.e. V.A.C.F. and V.K.C.F.) and the crude saponins (i.e. V.A.C.S. and V.K.C.S.) extracted from the two plant species separately.

The method outlined by Koster *et al.* (1959) which is also called the acetic acid-induced writhing in mice, was used for the experiments. Swiss albino mice were divided in 5 groups of 5 mice each. Group I (negative control) received normal saline (0.9% w/v NaCl solution); Groups II, III and IV were respectively administered 25, 50 and 100 mg extract or crude saponins or crude flavonoids /kg body weight *i.p*; Group V (positive control) received ketoprofen at a dose of 10 mg/kg body weight *i.p*. Thirty (30) minutes later, all the groups were treated with acetic acid (0.6% v/v *i.p*). They were placed in individual cages and observed. The numbers of abdominal constrictions were counted 5 minutes later

after acetic acid injection for a period of 10 minutes. Percentage inhibition of writhing was calculated by using the formula:

$$\text{Inhibition (\%)} = \frac{\text{Mean No. of Writhing (-ve control)} - \text{Mean No. of Writhing (treated)}}{\text{Mean No. of Writhing (-ve control)}} \times 100$$

3.18 Anti-inflammatory Studies on *Vernonia amygdalina* and *V. kotschyana* Crude Ethanol Leaf Extracts, Flavonoids and Saponins

These were carried out on the ethanol extracts of *V. amygdalina* and *V. kotschyana* (i.e. V.A.E.E. and V.K.E.E.), the crude flavonoids (i.e. V.A.C.F. and V.K.C.F.) and the crude saponins (i.e. V.A.C.S. and V.K.C.S.) extracted from the two plants separately.

The method described by Winter and Nuss (1963) was used for the experiment in which carrageenan was used as the inflammatory agent. Wister rats were grouped into 5 groups of 5 rats each. The first group (negative control) received normal saline (0.9% w/v NaCl solution). The II, III and IV groups were administered 25, 50 and 100 mg extract or crude saponins or crude flavonoids /kg body weight *i.p.* respectively. The V group (positive control) received 10 mg/kg of ketoprofen. Thirty (30) minutes later, all the groups were administered 0.1 ml of 1% w/v carrageenan solution in the sub-plantar of the right hind paw limb. Oedema as paw diameter (cm) was measured at 0, 1, 2, 3, 4, and 5 hours after carrageenan injection by using vernier caliper. Percentage inhibition of the oedema was determined by using the formula:-

$$\text{Inhibition (\%)} = \frac{\text{Mean Paw Diameter (-ve control)} - \text{Mean Paw Diameter (treated)}}{\text{Mean Paw Diameter (-ve control)}} \times 100$$

3.19 Statistical Analyses

The results obtained from the experiments on the two plants were expressed as Mean ± Standard Error of Mean (Mean ± SEM). They were analyzed by using student's *t*-test in Graphpad Prism Software. Differences between control and treated groups (i.e. Df. = 4) at probability level ($p<0.05$) were considered significant (Crosland, 1980).

CHAPTER FOUR

4.0 RESULTS

4.1 Collection and Identification of *Vernonia amygdalina* Del. and *V. kotschyana* Sch. Bip.

Vernonia amygdalina and *V. kotschyana* plants were collected from Ruwan-Sanyi area of Malumfashi Local Government Area, Katsina State, in January and October, 2002 respectively. They were identified by the Taxonomist (Mr. U. S. Gallah) of the Department of Biological Sciences, Ahmadu Bello University, Zaria.

4.2 Preparation of Herbarium Specimens of *Vernonia amygdalina* and *V. kotschyana*

Herbarium specimens of *V. amygdalina* and *V. kotschyana* (Plates I and II) respectively were prepared, preserved (with methylated spirit and mercury chloride) and stored. Their voucher specimen numbers were 675 and 2624 respectively.

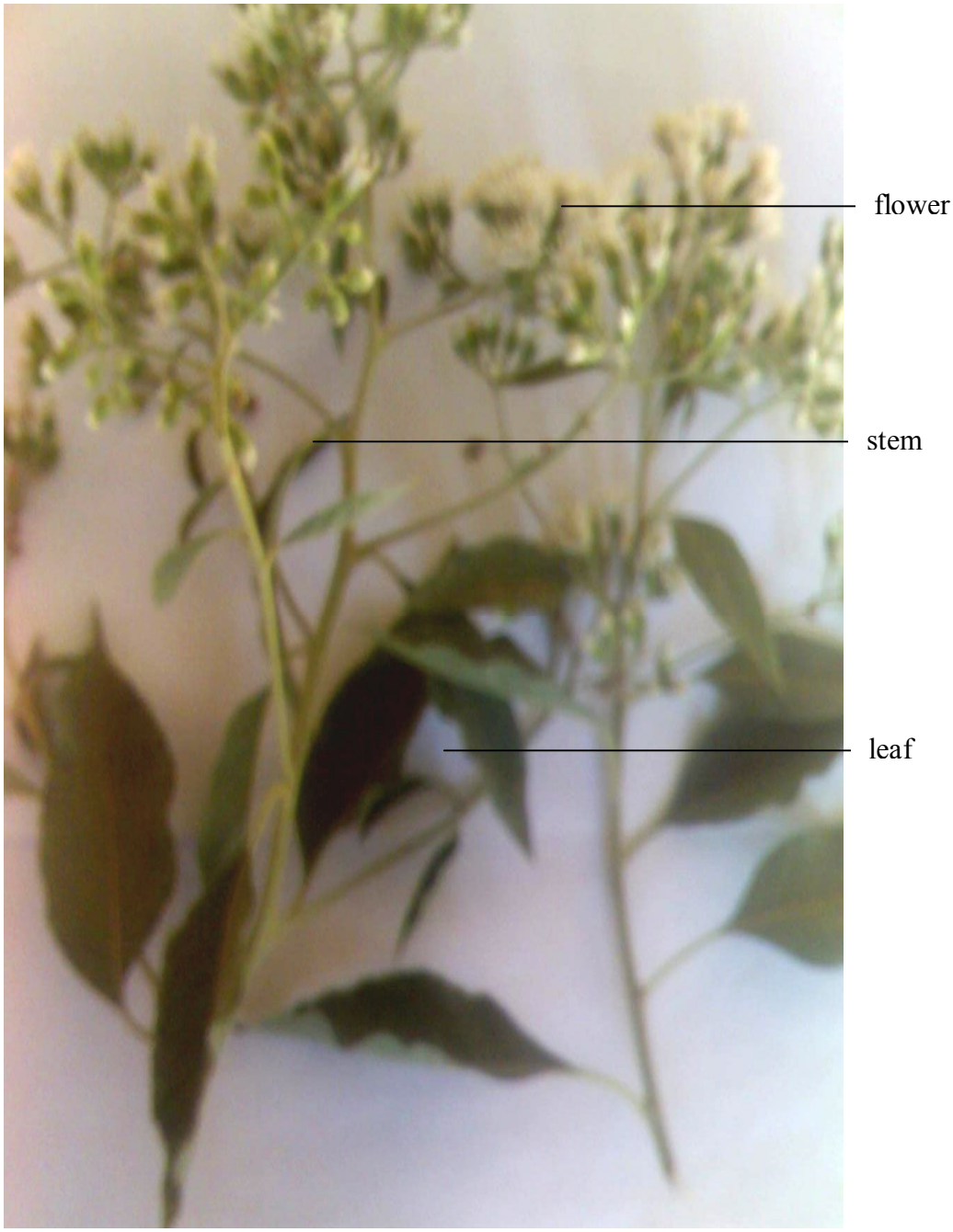


Plate I: Stems, Leaves and Flowers of *Vernonia amygdalina*

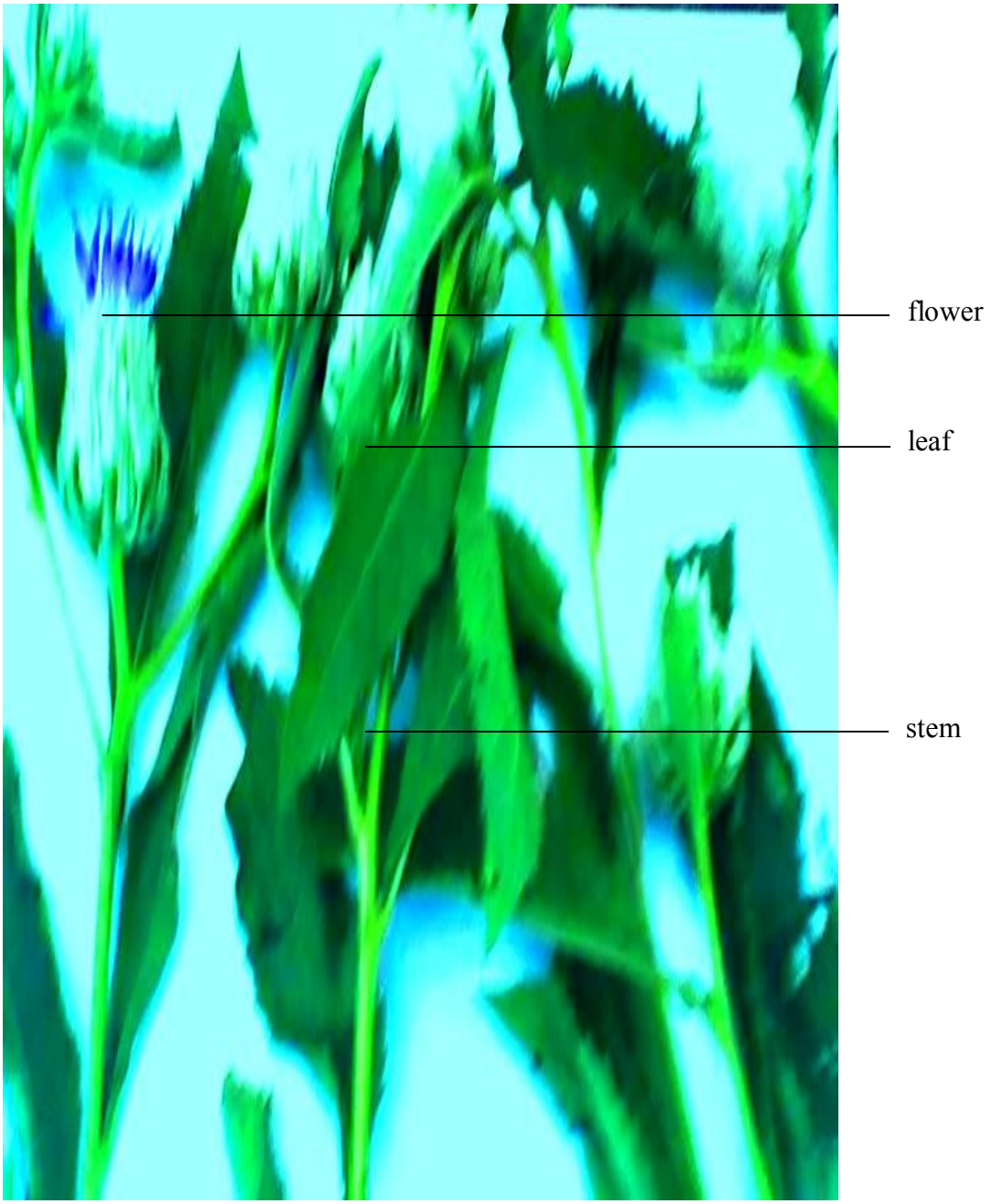


Plate II: Stems, Leaves and Flowers of *Vernonia kotschyana*

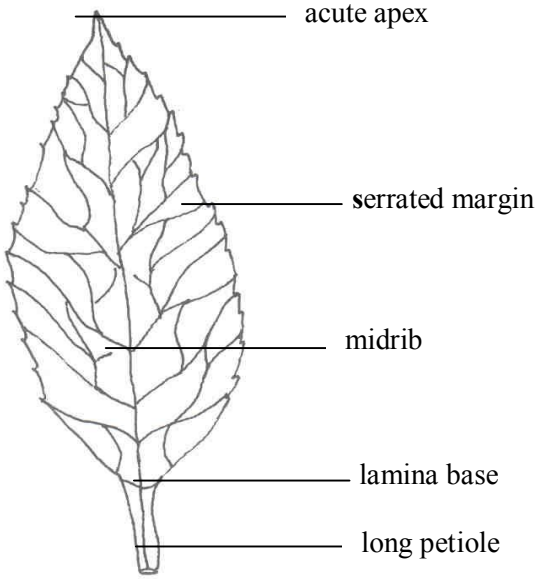
4.3 Preparation of *Vernonia amygdalina* and *V. kotschyana* Leaves

Two (2.0) kg of the leaf powder of each of the two plants were obtained. They were labelled *V. amygdalina* and *V. kotschyana* leaf powders and stored in tightly closed containers. These were subsequently used for (a) quantitative-leaf microscopy, (b) solvent extractive value determinations and (c) Soxhlet extraction.

4.4 Macroscopic Studies on *Vernonia amygdalina* and *V. kotschyana* Leaves

Macroscopically, leaves of the two plant species were found to be alternately arranged, had simple lamina with reticulate venation. They had acute apex, symmetrical base and pubescent surface.

The leaves of *V. amygdalina* (12.0 x 5.7 cm) had long petiole, serrated margin and were lanceolate in shape while those of *V. kotschyana* (17.0 x 6.7 cm) had very short petiole, dented margin and were elliptical in shape with characteristic axillary buds (Figure 4.1 (a & b). Organoleptically, *V. amygdalina* leaves were more greenish in colour and bitter in taste than those of *V. kotschyana*. However, both plants have distinct odour (Table 4.1; Appendix A).



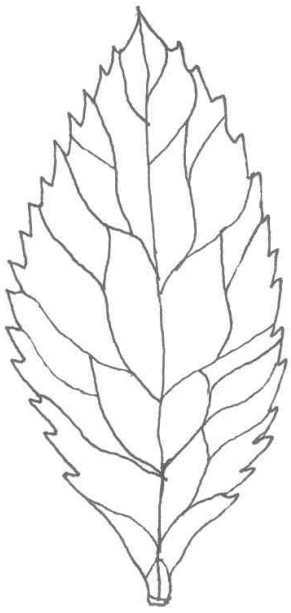
(a) ***Vernonia amygdalina*** **Leaf**

acute apex

dentate margin

lamina base

very short petiole



(b) *Vernonia kotschyana* Leaf

Figure 4.1 (a & b): *Vernonia amygdalina* and *V. kotschyana* Leaves; x1

Note: (a) the serrated and dented margin and (b) the lanceolate and elliptical shape of *V. amygdalina* and *V. kotschyana* leaf respectively.

Table 4.1: Macroscopic Features of *Vernonia amygdalina* and *V. kotschyana* Leaves

FEATURES	DESCRIPTIONS	
	V. amygdalina	***V. kotschyana***
Arrangement	Alternate	Alternate
Petiole	Long petiole	Very short petiole
Lamina: i. Dimension	12.0 x 5.7 cm	17.0 x 6.7 cm

ii. Composition	Simple	Simple
iii Shape	Lanceolate	Elliptical
iv. Venation	Reticulate	Reticulate
v. Margin	Serrated	Dented
vi. Apex	Acute	Acute
vii. Base	Symmetrical	Symmetrical
viii. Surface	Pubescent	Pubescent
ix. Texture	Soft	Papery
Organoleptic properties:		
i. Colour	More greenish	Greenish
ii. Odour	Distinct	Distinct
iii. Taste	More bitter	Bitter

4.5 Calibration of Eyepiece Micrometer

Under x40 objective the 40th small eyepiece micrometer division coincided with the 10th small stage micrometer division. The calibration factor was therefore calculated as:

40 small eyepiece micrometer divisions = 10 small stage micrometer divisions

Since, 1 small stage micrometer division = 10μm

40 small eyepiece = 100 μm (10x10)
micrometer divisions

∴ 1 small eyepiece = 100 μm
micrometer division 40

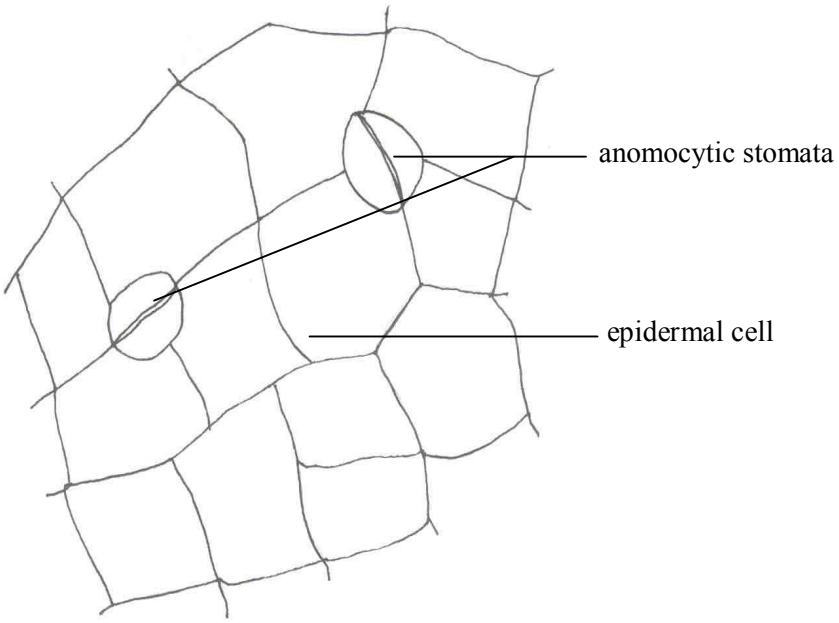
1 small eyepiece = 2.5 μm
micrometer division

∴ Calibration factor = 2.5 μm

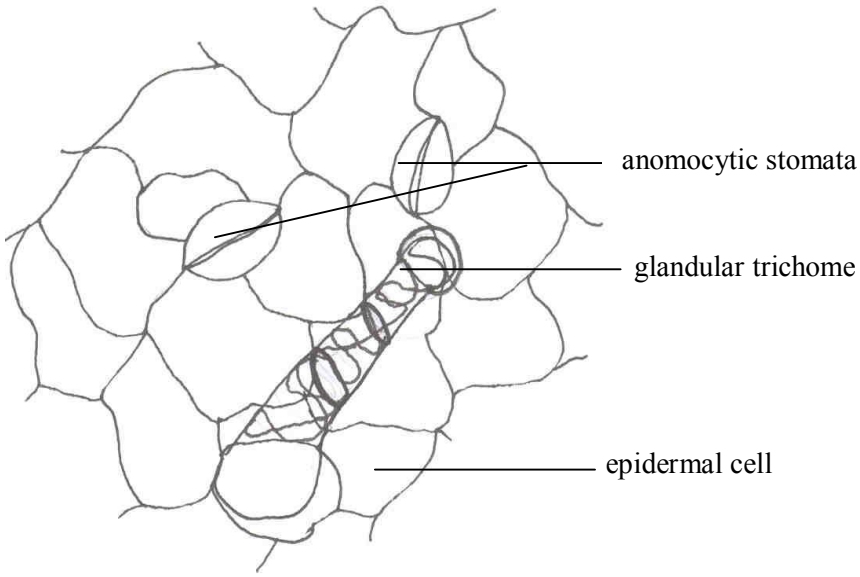
4.6 Microscopic/ Chemo-microscopic/ Quantitative-leaf Microscopic Studies on *Vernonia amygdalina* and *V. kotschyana* Leaves

4.6.1 Anatomical Sections and Powder of Leaves

Microscopic features identified in the leaves of the two plants include: Anomocytic stomata (18.6 - 18.75 - 18.9 μm in *V. amygdalina* and 22.05 - 22.2 - 22.35 μm in *V. kotschyana*). Stomata with 4-6 subsidiary cells surrounding the guard cells occurred on the abaxial and adaxial leaf surfaces in both plants. Epidermal cells with slightly beaded wavy anticlinal walls were found in *V. amygdalina* (29.85 - 30.0 - 30.15 μm) whereas those in *V. kotschyana* (37.35 - 37.5 - 37.65 μm) were deeply wavy in nature. Unicellular and few uniseriate multicellular covering trichomes (62.35 - 62.5 - 62.65 μm) were found on the midrib portion of *V. amygdalina* leaf. However, unicellular and uniseriate multicellular covering trichomes (124.85 - 125.0 - 125.15 μm) and few uniseriate multicellular glandular trichomes with warty surface (87.35 - 87.5 - 87.65 μm) occurred on the lamina and midrib portions of *V. kotschyana* leaf (Figure 4.2 (a & b); Appendix B and Figure 4.3 (a & b); Appendix C).



(a) *Vernonia amygdalina* Upper Epidermis

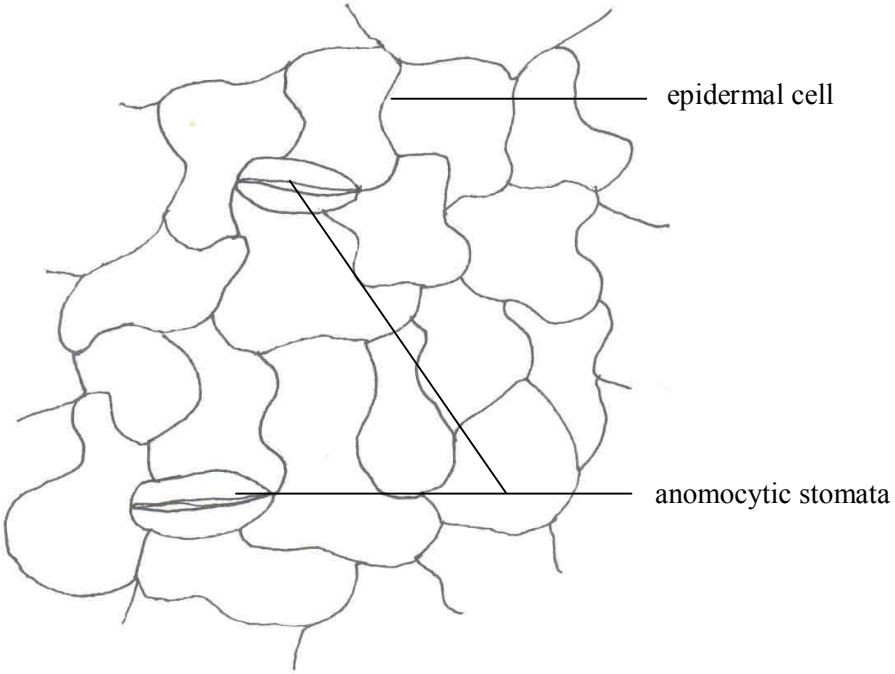


(b) *Vernonia kotschyana* Upper Epidermis

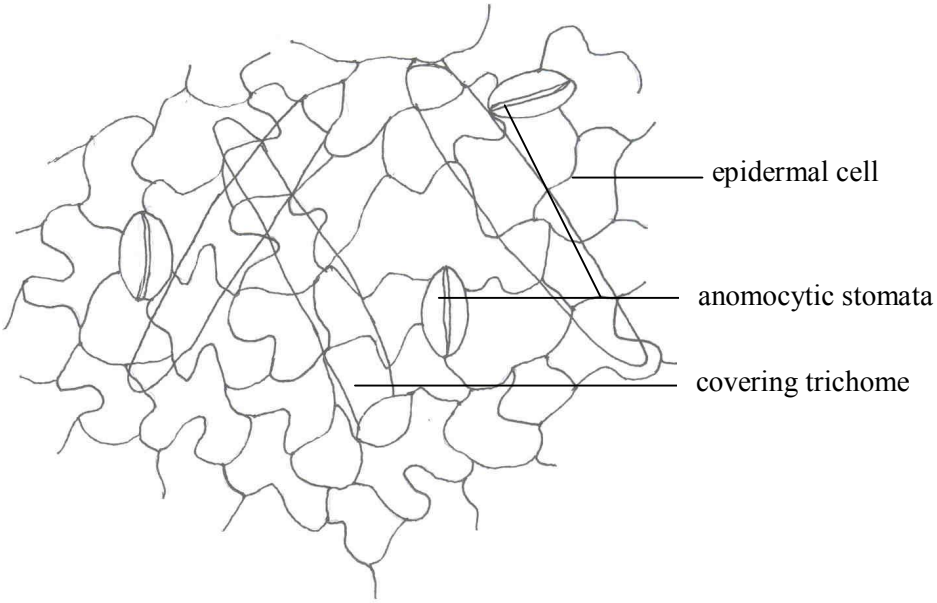
Figure 4.2 (a & b): Surface Preparation from the Upper Epidermis of *Vernonia amygdalina* and *V. kotschyana* Leaves; x400

Note: (a) The anomocytic stomata in both *V. amygdalina* and *V. kotschyana* upper

epidermis and (b) the glandular trichome with warty surfce in *V. kotschyana* only.



(a) *Vernonia amygdalina* Lower Epidermis

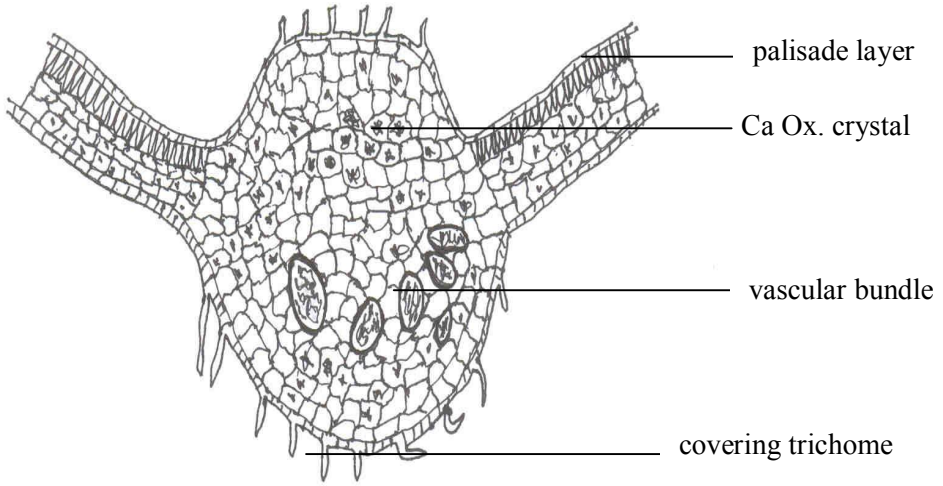


(b) *Vernonia kotschyana* Lower Epidermis

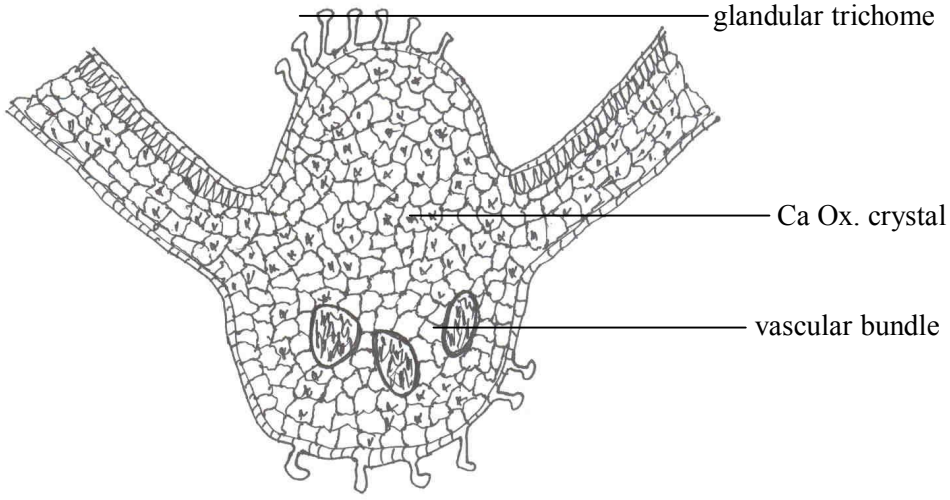
Figure 4.3 (a & b): Surface Preparations from the Lower Epidermis of *Vernonia amygdalina* and *V. kotschyana* Leaves; x400

Note: (a) The slightly and deeply wavy epidermal cells in the lower epidermis of *V. amygdalina* and *V. kotschyana* respectively and (b) the numerous covering trichomes in *V. kotschyana* only.

Transversely, the leaves of *V. amygdalina* and *V. kotschyana* were dorsiventral. The palisade tissues occurred beneath the upper epidermis only and were differentiated from the spongy mesophyll tissues (with much airspace in between) above the lower epidermis. Xylem elements were of the lacunar type which consist of 4-6 strands of the conducting elements (Figure 4.4 (a & b)).



(a) *Vernonia amygdalina* Trasverse Section

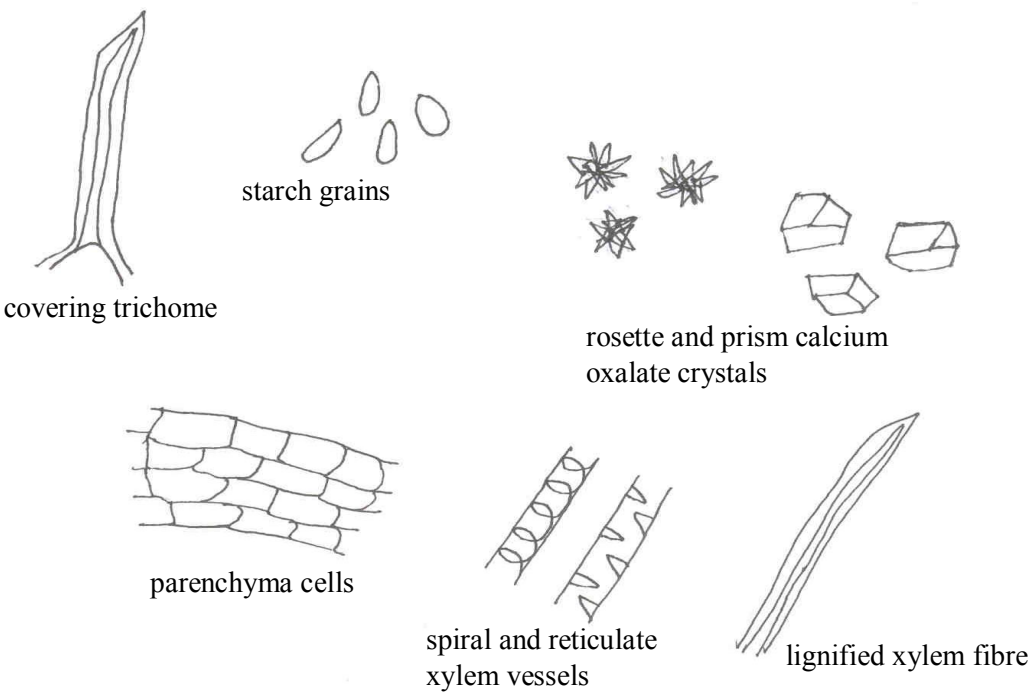


(b) *Vernonia kotschyana* trasverse section

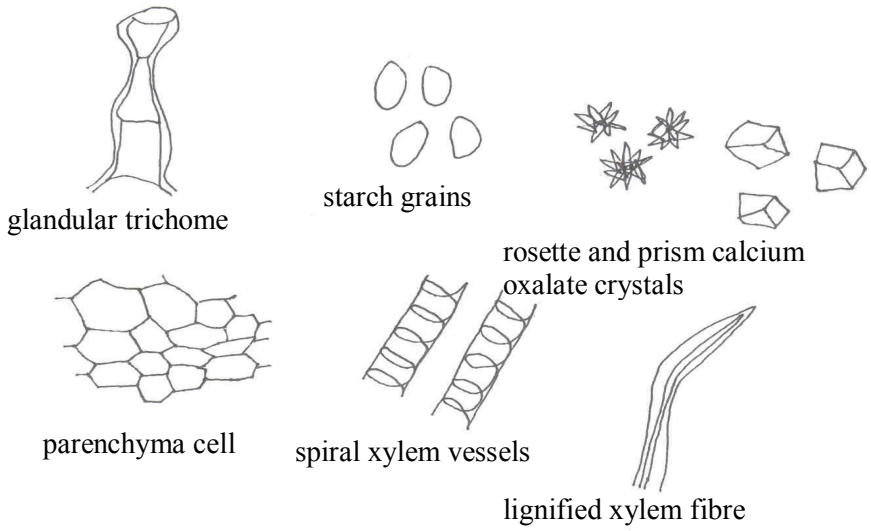
Figure 4.4 (a & b): Transverse Sections across the Midrib of *Vernonia amygdalina* and *V. kotschyana* Leaves; x400

Note: (a) The numerous covering and glandular trichomes in the midrib of *V. amygdalina* and *V. kotschyana* leaf respectively and (b) the numerous calcium oxalate crystals in their collenchymal cells.

Fragments of xylem vessels (reticulate and spiral types), phloem fibres (non-lignified and lignified) were identified in the *V. amygdalina* and *V. kotschyana* leaf powders. Calcium oxalate crystals: prism type (4.85 – 5.0 – 5.15 µm) and rosette form (7.35 – 7.5 – 7.65 µm) were identified in the leaf powders and within the collenchyma cells. Oval-shaped starch grains (9.85 – 10.0 – 10.15 µm) were also identified in the powders and within the chloroplasts (Figure 4.5 (a & b)).



(a) *Vernonia amygdalina* leaf powder



(b) *Vernonia kotschyana* leaf powder

Figure 4.5 (a & b): Some Microscopic Diagnostic Features from Powdered Leaves of *Vernonia amygdalina* and *V. kotschyana*; x400

Note: (a) The covering and glandular trichome and (b) the elongated and polygonal parenchymal cells from the leaf powder of *V. amygdalina* and *V. kotschyana* respectively.

4.6.2 Chemo-microscopic Studies

Different types of cell wall materials and cell inclusions were identified in the leaves of *Vernonia amygdalina* and *V. kotstchyana* as:

(a) Test for Cellulose

Blue colour was obtained on walls of epidermal cells that indicated the presence of cellulose in the two plants.

(b) Test for Lignin

Red stain was obtained on the walls of some lignified collenchyma cell in the two plants that indicated the presence of lignin.

(c) Test for Tannins

Greenish black colour in some parenchyma cells was obtained in both plants, which indicated the presence of tannins.

(d) Test for Starch

Blue-black colour on some grains within the chloroplasts and in leaf powder was obtained in both plants. This was positive for the presence of starch.

(e) Test for Mucilage

No pink colour was obtained in the epidermis and vascular tissues in both plants. This indicated the absence of mucilage-containing cells.

(f) Test for Calcium Oxalate Crystals

Brightly coloured crystals disappeared upon addition of the acid (80% H_2SO_4) in the collenchyma cell of both plants, which indicated the presence of calcium oxalate crystals.

(g) Test for Calcium carbonates ($CaCO_3$)

No effervescence was observed upon disappearance of the crystals in the collenchyma cell of the two plants. This showed the absence of calcium carbonates.

(h) Test for Fixed Oils and Fats

Red colour was observed only in some parenchyma cells in *Vernonia kotchyana.* This was positive for fixed oils and fats.

The chemo-microscopic results from the leaves of both plant species were summarized in Table 4.2.

Table 4.2: Chemo-microscopic Features of the Leaves of *Vernonia amygdalina* and *V. kotschyana*

CONSTITUENTS	TESTS	OBSERVATIONS	
		V. amygdalina	***V. kotschyana***
Cellulose	Chlor-zinc iodine	+	+
Lignin	Phloroglucinol and conc. HCl	+	+
Tannins	5% Ferric chloride solution	+	+
Starch	N/50 iodine	+	+
Mucilage	Ruthenium red	-	-
Ca Ox. Crystals	80% H_2SO_4	+	+
Calcium carbonates	80% H_2SO_4	-	-
Fixed Oils and Fats	Sudan IV Solution	-	+

Keys: + = Present; – = Absent; HCl = Hydrochloric acid;

Ca Ox = Calciumoxalate; H_2SO_4 = Sulphuric acid

4.6.3 Quantitative-Leaf Microscopic Studies

On the average, stomatal numbers (265.2 and 312.1) and indices (19.1 and 38.1) were higher in *Vernonia amygdalina* than in the *V. kotscyana* (118.3 and 227.3) and (17.1 and 28.3) respectively. However, palisade ratio (5.2), vein-islet and veinlet termination nubers (12.7 and 16.5) were higher in the latter plant than in the former plant (4.1, 6.2 and 10.7) respectively (Table 4.3).

Table 4.3: Quantitative-leaf Microscopic Features from *Vernonia amygdalina* and *V. kotschyana*

DETERMINATIONS	*MEAN VALUES	
	V. amygdalina	*V. kotschyana*
Upper Epidermis		
Palisade ratio	3.95 – 4.1 – 4.25	5.05 - 5.2 – 5.35
Stomatal number	265.05 - 265.2 – 265.35	118.15 - 118.3 – 118.45
Stomatal index	18.95 - 19.1 – 19.25	16.95 - 17.1 – 17.25
Lower Epidermis		
Stomatal number	311.95 - 312.1 – 312.25	227.15 - 227.3 – 227.45
Stomatal index	37.95 - 38.1 – 38.25	28.15 - 28.3 – 328.45
Vein-islet number	6.05 – 6.2 – 6.35	12.55 - 12.7 – 12.85
Vein-let termination number	10.55 – 10.7 – 10.85	16.35 - 16.5 – 16.65

* Mean Values of 5 Counts

4.7 Determination of Physical Constants on *Vernonia amygdalina* and *V. kotschyana* Leaves

Physical constants determined from the leaf powder of the two plant species on the average (%w/w) were moisture content, total ash values, acid-insoluble and water-soluble ash values, and alcohol and water extractives (Table 4.4; Appendix D).

Table 4.4: Physical Constants from the Leaf Powders of *Vernonia amygdalina* and *V. kotschyana*

PARAMETER	* MEAN VALUE (% w/w)	
	V. amygdalina	***V. kotschyana***
Moisture content	4.5 ± 2.12	5.0 ± 3.13
Ash	11.0 ± 3.12	8.0 ± 1.11
Acid-insoluble ash	1.5 ± 1.17	1.5 ± 2.15
Water-soluble ash	4.5 ± 2.25	4.2 ± 3.16
Alcohol-soluble extractives	14.0 ± 1.18	13.5 ± 0.97
Water-soluble extractives	22.5 ± 2.33	18.5 ± 3.02

* Mean Values of 5 Counts

4.8 Elemental Analysis on *Vernonia amygdalina* and *V. kotschyana* Leaf Powders

Wide range of mineral elements (essential and trace) were found in the finely ground leaf powder of the two plants. The concentrations in milligram (mg) per 1.0 g of leaf powder of various elements in the plants were expressed as Concentration ± Error. Elements identified included potassium, calcium, iron, manganese, zinc, lead and uranium (Table 4.5).

Table 4.5: Concentrations of Some Elements in *Vernonia amygdalina and V. kotschyana* Leaf Powders

ELEMENT	CONCENTRATION (mg) ± ERROR	
	V. amygdalina	*V. kotschyana*
Chlorine (Cl)	-	4.45±LOD
Silver (Ag)	-	4.35±LOD
Potassium (K)	15.40±1.5	5.40±0.61
Calcium (Ca)	10.10±0.90	8.82±0.44
Iodine (I)	-	0.76±LOD
Uranium (U)	0.007±LDL	-
Titanium (Ti)	-	0.29±LOD
Vanadium (V)	-	0.12±LOD
Manganese (Mn)	0.09±0.03	0.07±0.02
Iron (Fe)	1.50±0.11	0.89±0.03
Cobalt (Co)	-	0.03.0±LOD
Nickel (Ni)	-	0.02±LOD
Cupper (Cu)	0.02±LDL	0.02±LOD
Zinc (Zn)	-	0.02±0.005
Arsenic (As)	-	0.01±0.005
Selenium (Se)	-	0.006±LOD
Lead (Pb)	0.01±LDL	0.02±LOD
Boron (Br)	0.03±0.03	0.01±LOD
Rubidium (Rb)	0.04±0.03	0.02±0.02

Strontium (Sr)	0.08±0.01	0.04±0.002
Ytrium (Y)	0.02±LDL	0.02±LOD
Zirconium (Zr)	0.06±0.01	0.05±0.03
Niobium (Nb)	-	0.004±0.001
Chromium (Cr)	-	0.09±LOD
Molybdenum (Mo)	-	0.004±LOD
Thorium (Th)	0.007±LDL	-

Keys: mg = Milligram; LDL = Least Detectable Limit; LOD = Limit of Detection

4.9 Extraction of *Vernonia amygdalina* and *V. kotschyana* Leaf Powders

The percentage yield of extracts per quantities (800 g of the plant material) obtained in each of the two plant species after exhaustive Soxhlet extractions were appropriately presented in Table 4.6.

Table 4.6: Percentage Yield of the Petroleum Ether and Ethanol Extracts Per 800 g of *Vernonia amygdalina* and *V. kotschyana* Leaf Powders

PLANT	EXTRACT	AMOUNT (g)	% YIELD
V. amygdalina	Petroleum Ether Extract (V.A.P.E.)	92.61	11.58
„	Ethanol Extract (V.A.E.E.)	221.94	27.74
V. kotschyana	Petroleum Ether Extract (V.K.P.E.)	98.72	12.34
„	Ethanol Extract (V.K.E.E.)	195.25	24.41

Keys:

V.A.P.E = *Vernonia amygdalina* Petroleum ether Extract

V.K.P.E = *Vernonia kotschyana* Petroleum ether Extract

V.A.E.E. = *Vernonia amygdalina* Ethanol Extract

V.K.E.E. = *Vernonia kotschyana* Ethanol Extract

4.10 Phytochemical Studies on *Vernonia amygdalina* and *V. kotschyana* Ethanol Leaf Extracts

Various phytochemical constituents were identified from the extracts of each of the two plants as:

(a) Test for Alkaloids

Orange-red precipitates with Dragendorff's, whitish turbidity with Mayer's, brown precipitates with Wagner's and no precipitates with Hager's reagents were obtained in *Vernonia kotschyana* ethanol extract only. Precipitates with the first three reagents are positive chemical tests for alkaloids.

(b) Test for Phenolic Compounds

Blackish-green precipitate was obtained as a positive chemical test for phenolic compounds in the the ethanol extracts of both plants.

(c) Test for Tannins

(i) Lead Acetate Test

Yellow precipitate was obtained as positive chemical test for tannins in the ethanol extracts of both plants.

(ii) Bromine Solution Test

Blue colour was obtained in the ethanol extracts of both plants indicating a positive chemical test for condensed tannins.

(iii) Ammonia Solution Test

No slowly forming green colouration was observed in the ethanol extracts of both plants indicating a negative chemical test for chlorogenic acid.

(d) Test for Carbohydrates

(i) Molisch's Test

Purple colour was obtained in the ethanol extracts of both plants indicating a positive chemical test for carbohydrates.

(ii) Fehling's Test

Brick–red precipitate was obtained in the ethanol extracts of both plants indicating a positive chemical test for reducing compounds.

(iii) Methylene Blue Test

No violet-blue precipitate was obtained in the ethanol extracts of both plants indicating a negative chemical test for mucilage.

(e) Test for Anthracene Derivatives (Borntrager's Reaction)

No cherish-red colour of the alkaline solution was obtained in the ethanol extracts of both plants indicating a negative chemical test for emodols.

(f) Test for Coumarins

No blue or green fluorescence under U.V. light in the alkaline solution was obtained in the ethanol extracts of both species indicating a negative chemical test for coumarins.

(g) Test for Flavonoids

(i) Ferric Chloride Test

Green precipitate was obtained in the ethanol extracts of both species indicating a positive chemical test for phenolic nucleus.

(ii) Lead Acetate Test

Yellow precipitate was obtained in the ethanol extracts of both species indicating a positive chemical test for flavonoids.

(iii) Sodium Hydroxide Test

Yellow colour was obtained in the ethanol extracts of both species indicating a positive chemical test for flavonoids.

(iv) Shinoda Test

Pink colour was obtained in the ethanol extracts of both species indicating a positive chemical test for flavones/flavonols.

(v) Amyl Alcohol Test

Yellow colour was obtained in the ethanol extracts of both species indicating a positive chemical test for flavone/flavonol nucleus.

(h) Test for Saponins (Steroids /triterpenoids)

(i) Frothing Test

Persistent frothing column was observed in the ethanol extracts of both species indicating a positive physical test for saponins.

(ii) Haemolysis Test

Haemolysis of the red blood cells was observed in the ethanol extracts of both species indicating a positive chemical test for saponins.

(iii) Liebermann-Burchard's Reaction

Reddish-brown ring and bluish-green upper layer were obtained in the ethanol extracts of both species indicating a positive chemical test for sterols.

(iv) Salkowski's Test

Reddish-brown colour at the interface was observed in the ethanol extracts of both species indicating a positive chemical test for a steroidal ring.

(i) Test for Carotenoids (Carl Price's Reaction)

No deep blue or bluish green colour was observed in the ethanol extracts of both species indicating a negative chemical test for carotenoids.

(j) Test for Fixed Oils and Fats

Clear blue solution was observed only in the *Vernonia kotschyana* ethanol extract indicating a positive chemical test for glycerine.

(k) Test for Resins

No odour of benzaldehyde due to oxidation of benzoic acid was perceived in the two ethanol extracts of both species indicating a negative chemical test for resins.

(l) Test for Cyanophore Glycosides (Guignard's Reaction)

The colour of sodium picrate paper changed from yellow to brick red due to formation of sodium isopurpurate in the ethanol extracts of both species indicating a positive chemical test for cyanogenetic glycosides.

The results of the phytochemical constituents in the ethanol extracts of the two plants were summarized in Table 4.7.

Table 4.7: Some Phytochemical Constituents from the Leaf Extracts of *Vernonia amygdalina* and *V. kotschyana*

CONSTITUENTS	TESTS	OBSERVATIONS	
		V. amygdalina	*V. kotschyana*
Alkaloids	Dragendorff's/Mayer's/ Wagner's/Hager's	-	+
Phenolic compounds	Ferric chloride's	+	+
Tannins (condensed)	Lead acetate/Bromine solution	+	+
Chlorogenic acid	Ammonia Solution	-	-
Carbohydrates	Molisch's/Fehling's	+	+
Mucilage	Methylene blue	-	-
Anthracene derivatives	Borntrager's	-	-
Coumarins	Ammonia/U.V. light	-	-
Flavonoids	Ferric chloride/Lead acetate/NaOH/Shinoda/Amyl alcohol	+	+
Saponins	Frothing/Haemolysis/ Liebermann-Burchard's/ Salkowski's	+	+
Carotenoids	Carl Price's	-	-
Fixed oils and Fats	$CuSO_4$ & NaOH Solution	-	+
Resins	Acetic anhydride/H_2SO_4	-	-
Cyanophore glycosides	Guignard's	+	+

Keys: + = Present; - = Absent; U.V. = Ultra Violet; NaOH = Sodium hydroxide; $CuSO_4$ = Cupper sulphate; H_2SO_4 = Sulphuric acid

4.11 Fractionation of the Ethanol Extracts of *Vernonia amygdalina* and *V. kotschyana* Leaves

The various fractions per quantities obtained from 150.0 g of crude ethanol extracts of *V. amygdalina* and *V. kotschyana* leaves were determined and coded appropriately (Table 4.8).

Table 4.8: Percentage Yield of Crude Flavonoids and Saponins per 150 g of *Vernonia amygdalina* and *V. kotschyana* Ethanol Leaf Extracts

PLANT SPECIES	CONSTITUENT	AMOUNT(g)	%YIELD
V. amygdalina	Crude Flavonoids	2.15	1.43
V. kotschyana	,,	2.02	1.35
V. amygdalina	Crude Saponins	3.32	2.21
V. kotschyana	,,	4.46	2.97

4.12 Chemical Identification of the Crude Flavonoids and Saponins from V*ernonia amygdalina* and *V. kotschyana* Ethanol Leaf Extracts

The results of the various chemical tests on the crude flavonoids and saponins extracts obtained from the two plants are presented in Tables 4.9 and 4.10 respectively.

(a) Identification of Flavonoids

(i) Ferric Chloride Test

Green precipitate was obtained from the crude flavonoid extracts of the two plants, which indicated a positive chemical test for phenolic nucleus.

(ii) Lead Acetate Test

Yellow precipitate was obtained from the crude flavonoid extracts of the two plants, which indicated a positive chemical test for flavonoids.

(iii) Sodium Hydroxide Test

Yellow colour was obtained from the crude flavonoid extracts of the two plants, which indicated a positive chemical test for flavonoids.

(iv) Shinoda Test

Pink colour was obtained from the crude flavonoid extracts of the two plants, which indicated a positive chemical test for flavones/flavonols.

(v) Amyl Alcohol Test

Yellow colour was obtained from the crude flavonoid extracts of the two plants, which indicated a positive chemical test for flavones/flavonols.

(b) Identification of Saponins

(i) Frothing Test

Persistent froth was obtained from the crude saponin extracts of the two plants, which indicated a positive test for saponins.

(ii) Haemolysis Test

Haemolysis of red blood cells was obtained from the crude saponin extracts of the two plants, which indicated a positive chemical test for saponins.

(iii) Liebermann-Burchard's Reaction

Reddish-brown ring and bluish-green supernatant layer were obtained from the crude saponin extracts of the two plants, which indicated a positive chemical test for sterols.

(iv) Salkowski's Test

Reddish-brown colour at the interface was obtained from the crude saponin extracts of the two plants, which indicated a positive chemical test for a steroidal ring.

Table 4.9: Chemical Identification of the Crude Flavonoids from *Vernonia amygdalina* and *V. kotschyana* Ethanol Leaf Extracts

TEST	OBSERVATION	INFERENCE
Ferric Chloride	Green precipitates	Phenolic nucleus present
Lead Acetate	Yellow precipitates	Flavonoids present
Sodium Hydroxide	Yellow colouration	Flavonoids present
Shinoda	Pink colouration	Flavones/flavonols present
Amyl Alcohol	Yellow colouration	Flavones/flavonols present

Table 4.10: Chemical Identification of the Crude Saponins from *Vernonia amygdalina* and *V. kotschyana* Ethanol Leaf Extracts

TEST	OBSERVATION	INFERENCE
Frothing	Persistent copious froth that lasted for more than three hours	Saponins present
Haemolysis	Haemolysis of the red blood cells	Saponins present
Liebermann-Burchard's	Reddish-brown ring with bluish-green supernatant layer	Steroidal nucleus present
Salkowski's	Reddish-brown colour	Steroidal ring present

4.13 Thin Layer Chromatographic Analysis on *Vernonia amygdalina* and *V. kotschyana* Crude Ethanol Extracts, Flavonoids and Saponins

The spots of the crude ethanol extract (flavonoids) of *V. amygdalina* corresponded to the spots of the crude ethanol extract of the plant: p (yellow; 0.55), q (yellow; 0.71), r (brown; 0.76) and a (brown; 0.55), c (brown; 0.71), d (brown; 0.76) whereas, the spots of the crude ethanol extract (flavonoids) of *V. kotschyana* corresponded to the spots of the crude ethanol extract of the plant: u (yellow; 0.67), w (yellow; 0.82), x (brown; 0.95) and j (brown; 0.67), m (brown; 0.82), o (brown; 0.95) respectively (Table 4.11).

However, the spots of the crude ethanol extract (saponins) of *V. amygdalina* corresponded to the spots of the crude ethanol extract of the plant: z (purple; 0.76), aa (blue; 0.82) and d (brown; 0.76), f (brown; 0.82) whereas, the spots of the crude ethanol extract (saponins) of *V. kotschyana* corresponded to the spots of the crude ethanol extract of the plant: ac (brown; 0.58), ad (brown;0.67), af (brown; 0.95) and i (brown; 0.58), j (brown; 0.67), o (brown; 0.95) respectively. There were four spots in *V. amygdalina* crude ethanol extract: b (brown; 0.64), e (brown; 0.78), g (brown; 0.84) and h (brown; 0.95) while there were three spots in *V. kotschyana* crude ethanol extract: k (brown; 0.75), l (brown; 0.78) and n (brown; 0.87) that were neither flavonoids nor saponins. The former plant had one more flavonoid spot than the latter plant (Table 4.11).

The spots of the flavonoid extracts of *V. amygdalina* corresponded to the flavonoid spots of the crude ethanol extract of the plant: ag (yellow; 0.55), ah (yellow; 0.71), ai (brown; 0.76), ak (brown; 0.95) and p (yellow; 0.55), q (yellow; 0.71), r (brown; 0.76), t (brown; 0.95) whereas the spots of the flavonoid extract of *V. kotschyana* corresponded to the flavonoid spots of the crude ethanol extract of the plant: al (yellow; 0.67), am (yellow; 0.76), ao (brown; 0.95) and u (yellow; 0.67), v (yellow; 0.76), x (brown; 0.95) respectively. There was one flavonoid spots each in *V. amygdalina*: aj (yellow; 0.80) and *V. kotschyana*: an (yellow; 0.87) flavonoid extract that did not correspond to any of the flavonoids spots of their crude ethanol extracts (Tables 4.11 & 4.12).

The spots of the saponin extract of *V. amygdalina* corresponded to the saponin spots of the crude ethanol extract of the plant: ap (brown; 0.65), ar (blue; 0.82), as (brown; 0.93) and y (brown; 0.65), aa (blue; 0.82), ab (brown; 0.93) whereas the spots of the saponin extract of *V. kotschyana* corresponded to the saponin spots of the crude ethanol extract of the plant: at (brown; 0.58), au (brown; 0.67), aw (brown; 0.95) and ac (brown; 0.58), ad (brown; 0.67), af (brown; 0.95) respectively. Both plants had shown the same number of saponin spots in their saponin extracts. Even though, one spots each of the *V. amygdalina*: aq (purple; 0.78) and *V. kotschyana*: au (brown; 0.78) did not corresponded to any of the saponin spots of the crude ethanol extracts of the plants (Tables 4.11 & 4.12).

Table 4.11: Crude Ethanol Extracts of *Vernonia amygdalina* and *V. kotschyana* Leaves when chromatographed on Precoated TLC Plates, Exposed to Iodine, Concentrated Ammonia Vapour and Liebermann-Burchard's Reagent Respectively

SPOT COLOURS		R_f VALUES	
V. amygdalina	*V. kotschyayana*	*V. amygdalina*	*V. kotschyayana*
Exposed to Iodine Vapour			
[a]Brown	[i]Brown	0.55	0.58
[b]Brown	[j]Brown	0.64	0.67
[c]Brown	[k]Brown	0.71	0.75
[d]Brown	[l]Brown	0.76	0.78
[e]Brown	[m]Brown	0.78	0.82
[f]Brown	[n]Brown	0.82	0.87
[g]Brown	[o]Brown	0.84	0.95
[h]Brown	-	0.95	-
Exposed to Concentrated Ammonia Vapour			
[p]Yellow	[u]Yellow	0.55	0.67
[q]Yellow	[v]Yellow	0.71	0.76
[r]Brown	[w]Yellow	0.76	0.82
[s]Brown	[x]Brown	0.85	0.95
[t]Brown	-	0.95	-
Sprayed with Liebermann-Burchard's Reagent			
[y]Brown	[ac]Brown	0.65	0.58
[z]Purple	[ad]Brown	0.76	0.67

[aa]Blue	[ae]Brown	0.82	0.76
[ab]Brown	[af]Brown	0.93	0.95

Table 4.12: Crude Flavonoids and Saponins of *Vernonia amygdalina* and *V. kotschyana* when chromatographed on Precoated TLC Plates, exposed to Concentrated Ammonia Vapour and Sprayed with Liebermann-Burchard's Reagent followed by Heating at 105^{o}C Respectively

SPOT COLOURS		**R_f VALUES**	
V. amygdalina	***V. kotschyayana***	***V. amygdalina***	***V. kotschyayana***
	Crude Flavonoids		
[ag]Yellow	[al]Yellow	0.55	0.67
[ah]Yellow	[am]Yellow	0.71	0.76
[ai]Brown	[an]Yellow	0.76	0.87
[aj]Yellow	[ao]Brown	0.80	0.95
[ak]Brown	-	0.95	-
	Crude Saponins		
[ap]Brown	[at]Brown	0.65	0.58
[aq]Purple	[au]Brown	0.78	0.67
[ar]Blue	[av]Brown	0.82	0.78
[as]Brown	[aw]Brown	0.93	0.95

4.14 Acute Toxicity Studies (LD_{50}) on *Vernonia amygdalina* and *V. kotschyana* Ethanol Leaf Extracts

The ethanol leaf extracts of *V. amygdalina* and *V. kotschyana* were found to have LD_{50} values of 288.5 and 471.2 mg/kg body weight intraperitoneally (*i.p.*) in mice respectively (Table 4.13 (a-b) and Table 4.14 (a-b)).

Table 4.13 (a-b): LD_{50} Determination of *Vernonia amygdalina* Ethanol Leaf Extract in Mice

DOSE (mg/kg)	RESULTS
(a) First Phase	
10	0/3
100	0/3
1000	2/3
(b) Second Phase	
140	0/1
225	0/1
370	1/1
600	1/1

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

Table 4.14 (a-b): LD_{50} Determination of *Vernonia kotschyana* Ethanol Leaf Extract in Mice

DOSE (mg/kg)	RESULTS
(a) First Phase	
10	0/3
100	0/3
1000	2/3
(b) Second Phase	
140	0/1
225	0/1
370	0/1
600	1/1

$\therefore$ $LD_{50} = \sqrt{370 \times 600} = 471.2$ mg/kg *i.p.*

4.15 Analgesic Studies on *Vernonia amygdalina* and *V. kotschyana* Crude Ethanol Leaf Extracts, Flavonoids and Saponins

The ethanol extract of *V. amygdalina* (V. A. E. E.) exhibited significant ($p<0.05$) and dose-dependant analgesic effects. The highest inhibition (71.9%) was observed at the 100 mg/kg *i.p.* against acetic acid-induced writhing in mice. The ethanol extract of *V. kotschyana* (V. K. E. E.) produced significant ($p<0.05$) but non dose-dependant analgesic effects. The highest inhibition (74.6%) was observed at the 50 mg/kg *i.p.* against acetic acid-induced writhings in mice. These results were compared with the negative control (normal saline) group. The ketoprofen produced (67.5%) inhibitory effect at 10 mg/kg, which was used as the standard (+ve control) group (Table 4.15; Figure 4.6 and Appendices F-K).

Table 4.15: Analgesic Activities of *Vernonia amygdalina* and *V. kotschyana* Ethanol Extracts (V. A. E. E. and V. K. E. E.) against Acetic Acid-Induced Writhing in Mice

TREATMENT	DOSE (mg/kg)	MEAN NUMBER OF WRITHING ± S.E.M.	%INHIBITION
Normal Saline (-ve Control)		22.8 ±1.77	
V.A.E.E.	25	16.2±1.20*	29.0
V.K.E.E.		11.8±2.73*	48.3
V.A.E.E.	50	13.8±1.59*	39.5
V.K.E.E.		5.8±1.11*	74.6
V.A.E.E.	100	6.4±1.12*	71.9
V.K.E.E.		8.2±1.69*	64.0
Ketoprofen (+ve Control)	10	7.4±1.08*	67.5

Keys: S.E.M. = Standard Error of Mean; V.A.E.E. = *Vernonia amygdalina* Ethanol Extract; V.K.E.E. = *Vernonia kotschyana* Ethanol Extract; * = significant at $p<0.05$

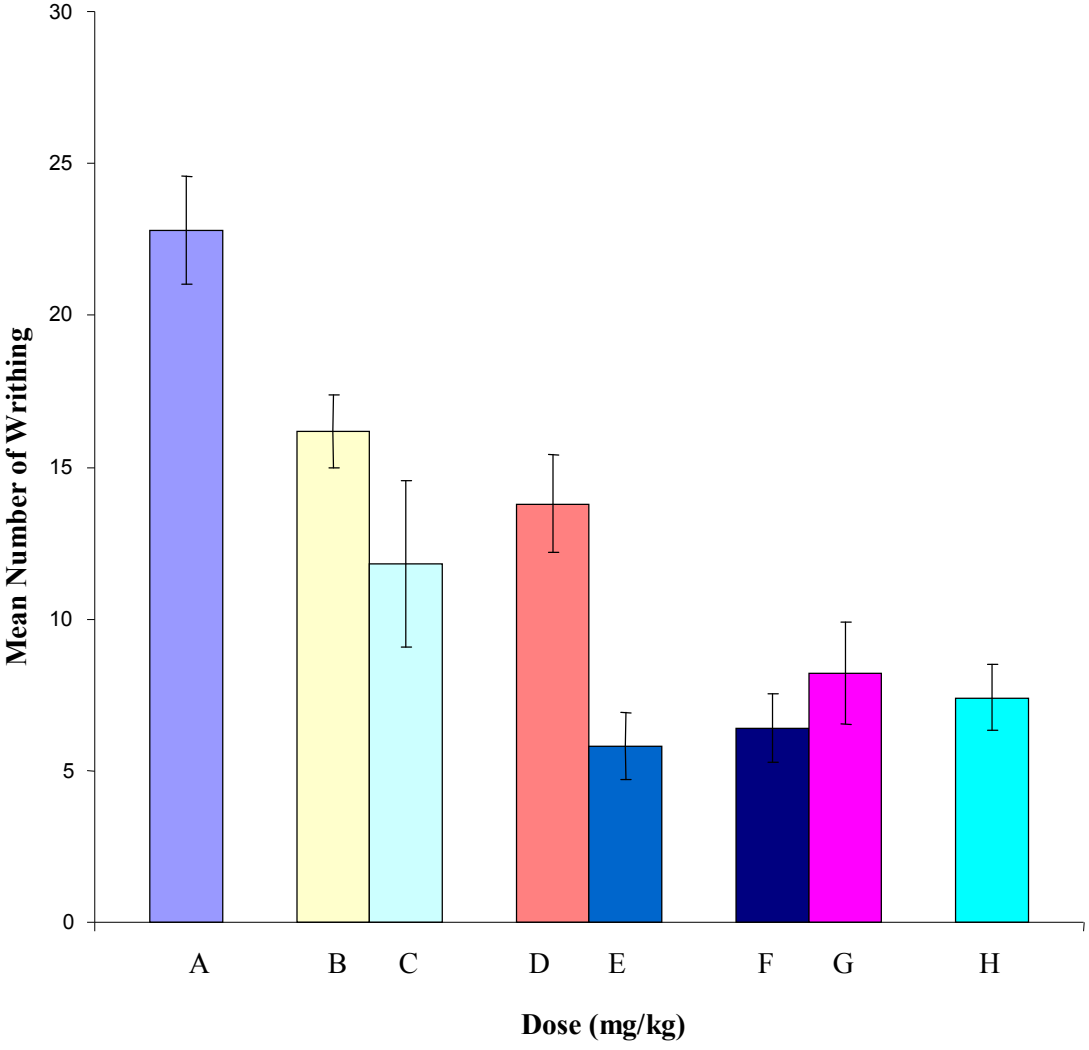

Mean Number of Writhing
30
25
20
15
10
5
0
A
B
C
D
E
F
G
H
Dose (mg/kg)

Figure 4.6: Effects of *Vernonia amygdalina* and *V. kotschyana* Ethanol Extracts (V. A. E. E. and V. K. E. E.) against Acetic Acid-Induced Writhing in Mice

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A – Normal Saline; B – V.A.E.E. (25 mg/kg), C – V.K.E.E. (25 mg/kg); D – V.A.E.E. (50 mg/kg), E – V.K.E.E. (50 mg/kg); F – V.A.E.E. (100 mg/kg), G – V.K.E.E. (100 mg/kg); H – Ketoprofen (10 mg/kg)

The *Vernonia amygdalina* and *V. kotschyana* crude flavonoids (V.AC.F and V.K.C.F.) produced significant ($p<0.05$) analgesic activity, which was dose-dependant. The highest inhibition (97.0% and 99.0%) of the two plant crude flavonoids respectively was observed at the 100 mg/kg *i.p.* against acetic acid induced writhing in mice. However, there wasn't significant inhibition at the 25 mg/kg *i.p* in the case of *V. amygdalina* crude flavonoids even at the same significance level when compared with the negative control group. The ketoprofen produced (58%) inhibitory effect at 10 mg/kg, which served as the standard (+ve control) (Table 4.16; Figure 4.7 and Appendices L-Q).

Table 4.16: Analgesic Activities of *Vernonia amygdalina* and *V. kotschyana* Crude Flavonoids (V. A. C. F. and V. K. C. F.) against Acetic Acid-Induced Writhing in Mice

TREATMENT	DOSE (mg/kg)	MEAN NUMBER OF WRITHING ± S.E.M.	%INHIBITION
Normal Saline (-ve Control)		20.0±2.59	
V.A.C.F.	25	13.8±3.69	32.0
V.K. C.F.		8.0±2.97*	60.0
V.A. C.F.	50	5.4±1.36*	73.0
V.K. C.F.		4.4±1.44*	78.0
V.A. C.F.	100	0.6±0.40*	97.0
V.K. C.F.		0.2±0.20*	99.0
Ketoprofen (+ve Control)	10	8.4±1.86*	58.0

Keys: S.E.M. = Standard Error of Mean; V.A.C.F. = *Vernonia amygdalina* Crude Flavonoids; V.K.C.F. = *Vernonia kotschyana* crude Flavonoids; * = significant at $p<0.05$

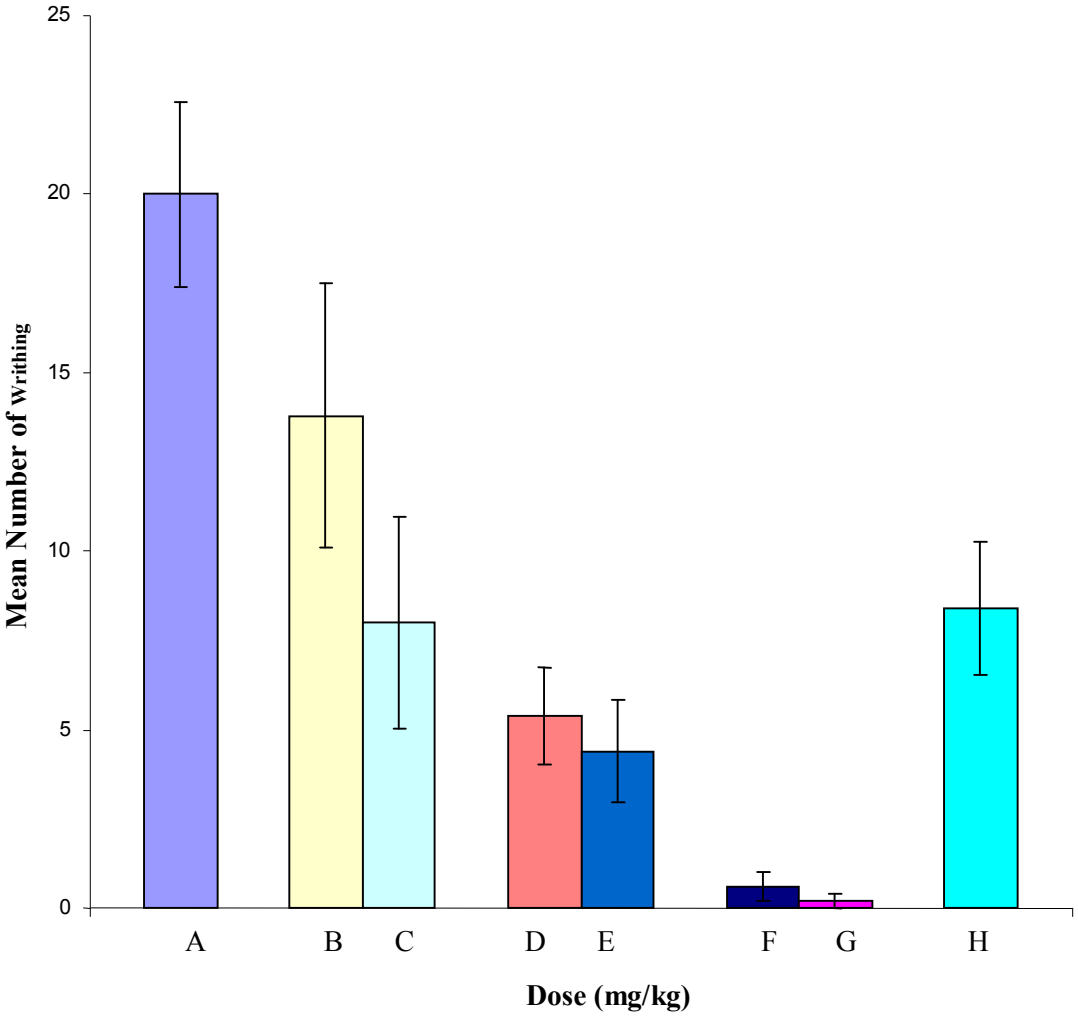


Figure 4.7: Effects of *Vernonia amygdalina* and *V. kotschyana* Crude Flavonoids (V. A. C. F. and V. K. C. F.) against Acetic Acid-Induced Writhing in Mice

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A – Normal Saline; B – V.A.C.F. (25 mg/kg), C – V.K.C.F. (25 mg/kg); D – V.A.C.F. (50 mg/kg), E – V.K.C.F. (50 mg/kg); F – V.A.C.F. (100 mg/kg), G – V.K.C.F. (100 mg/kg); H – Ketoprofen (10 mg/kg)

The crude saponins from *V. amygdalina* and *V. kotschyana* (V. A. C. S. and V. K. C. S.) had significant ($p<0.05$) dose-dependant analgesic activity. The highest inhibition of the crude saponins of the two plant species (78.0% and 97.0%) was respectively observed at the 100 mg/kg *i.p.* The crude saponins were used against acetic acid induced writhing in mice. However, there was no significant inhibition by the crude saponins extracts of both plants at the 25 mg/kg *i.p.* when compared with the negative control group. The ketoprofen produced (58%) inhibitory effect at 10 mg/kg, which served as the standard (+ve control) (Table 4.17; Figure 4.8 and Appendices R-W).

Table 4.17: Analgesic Activities of *Vernonia amygdalina* and *V. kotschyana* Crude Saponins (V. A. C. S. and V. K. C. S.) against Acetic Acid-Induced Writhing in Mice

TREATMENT	DOSE (mg/kg)	MEAN NUMBER OF WRITHING ± S.E.M.	%INHIBITION
Normal Saline (-ve Control)		20.0±2.59	
V.A.C.S.	25	13.6±2.66	32.0
V.K.C.S.		12.6±2.32	37.0
V.A. C.S.	50	9.6±2.27*	52.0
V.K.C.S.		8.2±2.94*	59.0
V.A.C.S.	100	4.4±2.11*	78.0
V.K.C.S.		0.6±0.40*	97.0
Ketoprofen (+ve Control)	10	8.4±1.86*	58.0

Keys: S.E.M. = Standard Error of Mean; V.A.C.S. = *Vernonia amygdalina* Crude Saponins; V.K.C.S. = *Vernonia kotschyana* crude Saponins; * = significant at $p<0.05$

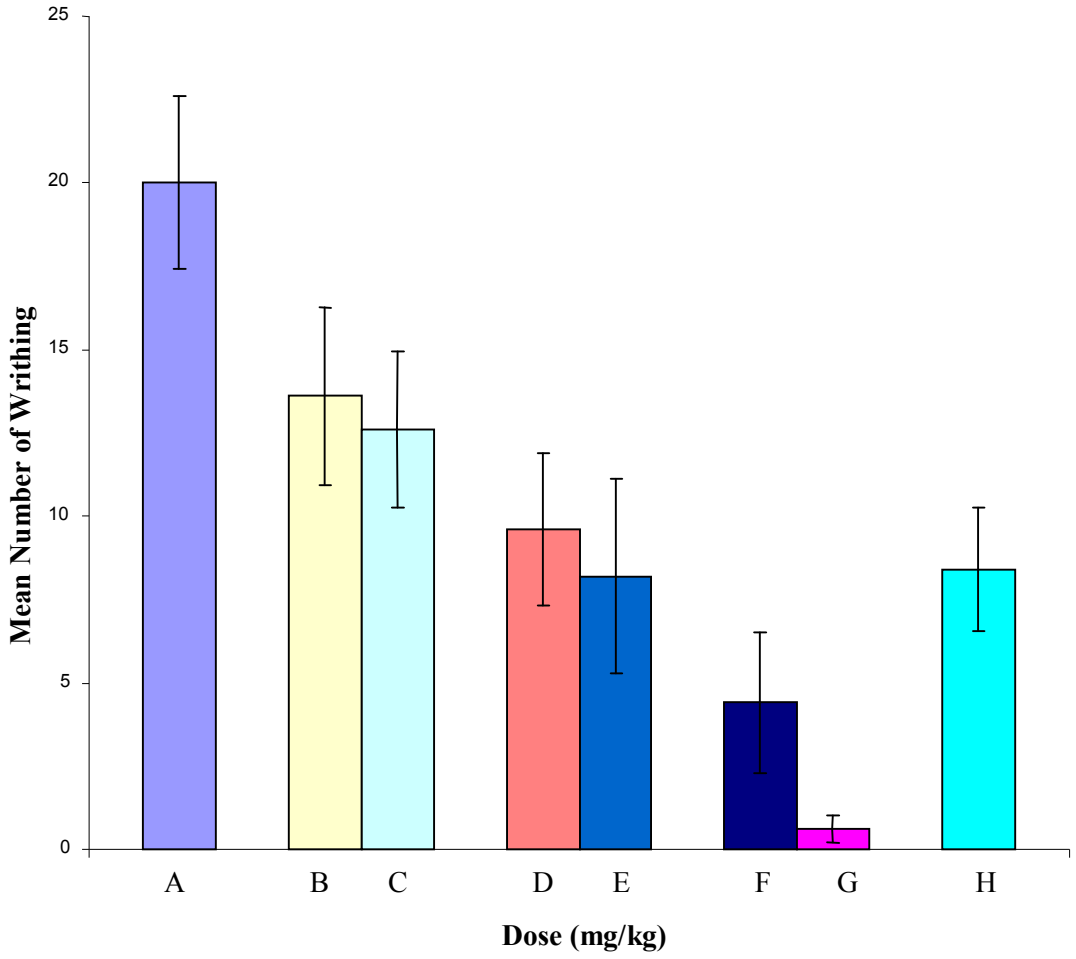


Figure 4.8: Effects of *Vernonia amygdalina* and *V. kotschyana* Crude Saponins (V. A. C. S. and V. K. C. S.) against Acetic Acid-Induced Writhing in Mice

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A – Normal Saline; B – V.A.C.S. (25 mg/kg), C – V.K.C.S. (25 mg/kg); D – V.A.C.S. (50 mg/kg), E – V.K.C.S. (50 mg/kg); F – V.A.C.S. (100 mg/kg), G – V.K.C.S. (100 mg/kg); H – Ketoprofen (10 mg/kg)

4.16 Anti-inflammatory Studies on *Vernonia amygdalina* and *V. kotschyana* Crude Ethanol Leaf Extracts, Flavonoids and Saponins

The *Vernonia amygdalina* ethanol extract (V. A. E. E.) exhibited significant ($p<0.05$) and dose-dependant anti-inflammatory activity with the highest (45.5%) inhibition observed at the 100 mg/kg *i.p.* Similarly, the ethanol extract of *V. kotschyana* (V. K. E. E.) produced significant ($p<0.05$) but non dose-dependant anti-inflammatory activity with the highest (45.5%) inhibition observed at the 50 mg/kg *i.p.* that was used against carragenan-induced oedema in rats. The activities were compared with the negative control (normal saline) group. The ketoprofen produced (63.6%) inhibitory effect at 10 mg/kg, which served as the positive control group (Table 4.18; Figure 4.9 and Appendices X-AC).

Table 4.18: Anti-inflammatory Activities of *Vernonia amygdalina* and *V. kotschyana* Ethanol Extracts (V. A. E. E. and V. K. E. E.) against Carrageenan-Induced Oedema in Rats

TREATMENT	DOSE (mg/kg)	MEAN PAW OEDEMA (cm) ± S.E.M. 1	2	3	4	5	%INHIBITION
Normal saline (-ve Control)		0.19 ± 0.01	0.25 ± 0.02	0.23 ± 0.02	0.21 ± 0.02	0.20 ± 0.02	
V.A.E.E.	25	0.11 ± 0.01*	0.15 ± 0.02*	0.16 ± 0.02*	0.14 ± 0.01*	0.16 ± 0.02*	36.4
V.K.E.E.		0.12 ± 0.01*	0.15 ± 0.01*	0.14 ± 0.01*	0.12 ± 0.01*	0.13 ± 0.01*	40.9
V.A.E.E.	50	0.10 ± 0.01*	0.16 ± 0.01*	0.15 ± 0.01*	0.12 ± 0.01*	0.12 ± 0.01*	40.9
V.K.E.E.		0.09 ± 0.01*	0.14 ± 0.01*	0.14 ± 0.01*	0.14 ± 0.02*	0.11 ± 0.01*	45.5
V.A.E.E.	100	0.13 ± 0.01*	0.14 ± 0.02*	0.11 ± 0.01*	0.11 ± 0.02*	0.10 ± 0.01*	45.5

V.K.E.E.		0.11 ± 0.01*	0.15 ± 0.01*	0.16 ± 0.02*	0.12 ± 0.01*	0.09 ± 0.01*	40.9
Ketoprofen (+ve Control)	10	0.08 ± 0.01*	0.07 ± 0.03*	0.09 ± 0.03*	0.07 ± 0.03*	0.09 ± 0.03*	63.6

Keys: S.E.M. = Standard Error of Mean; V.A.E.E. = *Vernonia amygdalina* Ethanol Extract; V.K.E.E. = *Vernonia kotschyana* Ethanol Extract; * = significant at $p<0.05$

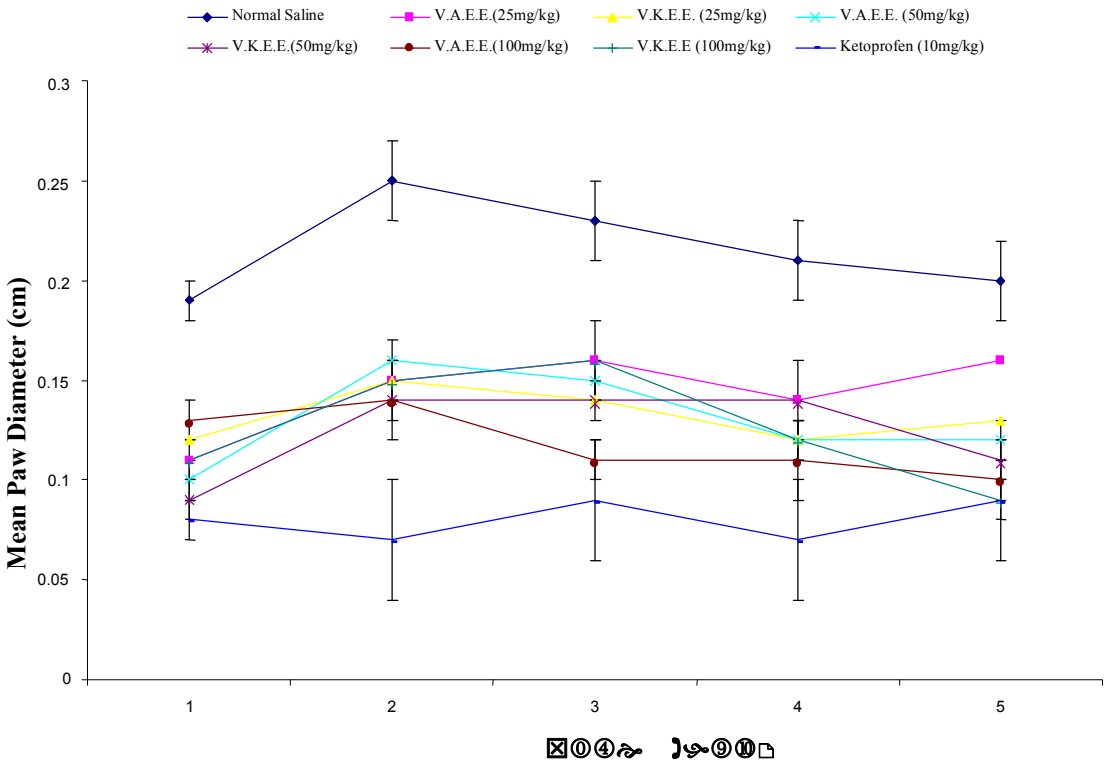


Figure 4.9: Effects of *Vernonia amygdalina* and *V. kotschyana* Ethanol Extracts (V. A. E.E. and V. K. E. E.) against Carrageenan-Induced Oedema in Rats

The crude flavonoids of *V. amygdalina* (V. A. C. F) exhibited significant ($p<0.05$) and dose-dependant anti-inflammatory activity. The highest (45.5%) inhibition was observed at the 100 mg/kg *i.p.* However, the crude flavonoids of *V. kotschyana* (V.K.C.F.) produced significant ($p<0.05$) but non dose-dependant anti-inflammatory activity. The highest (36.7%) inhibition was observed at the 50 mg/kg *i.p.* The crude flavonoids were used against carrageenan-induced oedema in rats. In both cases, there was no significant inhibition at the 25 mg/kg *i.p.* at the same significance level when compared with the negative control group. The ketoprofen produced (50%) inhibitory effect at 10 mg/kg, which served as the standard (+ve control) group (Table 4.19; Figure 4.10 and Appendices AD-AI).

Table 4.19: Anti-inflammatory Activities of *Vernonia amygdalina* and *V. kotschyana* Crude Flavonoids (V. A. C. F. and V. K. C. F.) against Carrageenan-Induced Oedema in Rats

TREATMENT	DOSE (mg/kg)	MEAN PAW OEDEMA (cm) ± S.E.M. 1	2	3	4	5	%INHIBITION
Normal saline (-ve Control)		0.19 ± 0.01	0.25 ± 0.02	0.23 ± 0.02	0.21 ± 0.02	0.20 ± 0.02	
V.A.C.F.	25	0.22 ± 0.01	0.22 ± 0.01	0.18 ± 0.01	0.18 ± 0.02	0.15 ± 0.01	13.6
V.K.C.F.		0.21 ± 0.01	0.22 ± 0.01	0.19 ± 0.02	0.19 ± 0.01	0.16 ± 0.01	13.6
V.A.C.F.	50	0.16 ± 0.01*	0.15 ± 0.01*	0.15 ± 0.01*	0.11 ± 0.02*	0.11 ± 0.01*	36.4
V.K.C.F.		0.15 ± 0.01*	0.16 ± 0.02*	0.13 ± 0.01*	0.13 ± 0.01*	0.13 ± 0.01*	36.7
V.A.C.F.	100	0.15 ± 0.02*	0.13 ± 0.01*	0.11 ± 0.01*	0.08 ± 0.01*	0.11 ± 0.01*	45.5
V.K.C.F.		0.18 ± 0.02*	0.22 ± 0.02*	0.18 ± 0.01*	0.14 ± 0.02*	0.14 ± 0.02*	22.7
Ketoprofen	10	0.11	0.12	0.12	0.10	0.07	50.0

(+ve Control)	± 0.01*	± 0.02*	± 0.01*	± 0.02*	± 0.01*

Keys: S.E.M. = Standard Error of Mean; V.A.C.F. = *Vernonia amygdalina* Crude Flavonoids; V.K.C.F. = *Vernonia kotschyana* Crude Flavonoids; * = significant at $p<0.05$

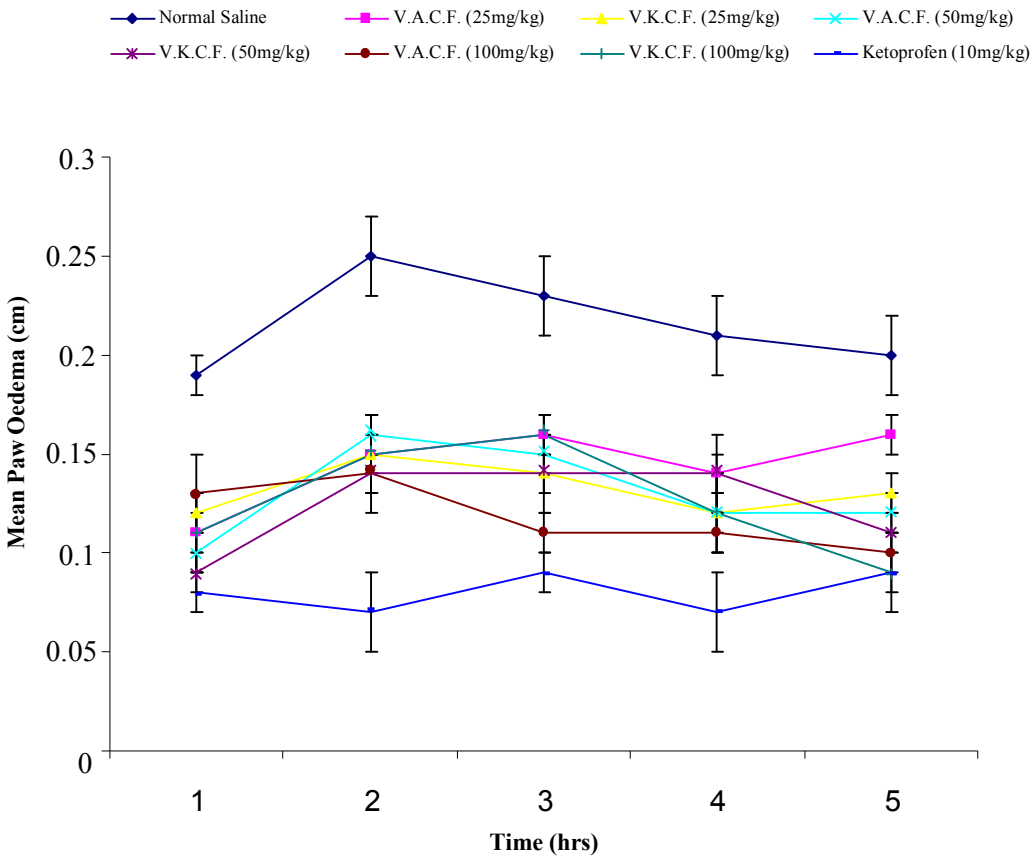


Figure 4.10: Effects of *Vernonia amygdalina* and *V. kotschyana* Crude Flavonoids (V. A. C. F. and V. K. C. F.) against Carrageenan-Induced Oedema in Rats

The crude saponins of *V. amygdalina* and *V. kotschyana* (V. A. C. S. and V. K. C. S.) produced significant ($p<0.05$) and dose-dependant anti-inflammatory effects. The highest (50.0% and 40.9%) inhibition respectively was observed at the 100 mg/kg *i.p.* that was used against carrageenan-induced oedema in rats. This effect was compared with the negative control group. The ketoprofen produced (50%) inhibitory effect at 10 mg/kg, which served as the standard (+ve control) (Table 4.20; Figure 4.11 and Appendices AJ-AO).

Table 4.20: Anti-inflammatory Activities of *Vernonia amygdalina* and *V. kotschyana* Crude Saponins (V. A. C. S. and V. K. C. S.) against Carrageenan-Induced Oedema in Rats

TREATMENT	DOSE (mg/kg)	MEAN PAW OEDEMA (cm) ± S.E.M. 1	2	3	4	5	%INHIBITION
Normal saline (-ve Control)		0.19 ± 0.01	0.25 ± 0.02	0.23 ± 0.02	0.21 ± 0.02	0.20 ± 0.02	
V.A.C.S.	25	0.18 ± 0.01*	0.21 ± 0.01*	0.18 ± 0.01*	0.17 ± 0.01*	0.16 ± 0.02*	18.2
V.K.C.S.		0.16 ± 0.01*	0.20 ± 0.01*	0.16 ± 0.02*	0.17 ± 0.01*	0.14 ± 0.01*	22.7
V.A.C.S.	50	0.20 ± 0.02*	0.21 ± 0.02*	0.15 ± 0.02*	0.11 ± 0.02*	0.11 ± 0.01*	27.3
V.K.C.S.		0.17 ± 0.02*	0.20 ± 0.01*	0.15 ± 0.01*	0.18 ± 0.01*	0.12 ± 0.01*	27.3
V.A.C.S.	100	0.15	0.16	0.09	0.08	0.09	50.0

		± 0.01*	± 0.02*	± 0.02*	± 0.02*	± 0.02*	
V.K.C.S.		0.13 ± 0.02*	0.19 ± 0.02*	0.13 ± 0.02*	0.12 ± 0.01*	0.09 ± 0.02*	40.9
Ketoprofen (+ve Control)	10	0.11 ± 0.01*	0.12 ± 0.02*	0.12 ± 0.01*	0.10 ± 0.02*	0.07 ± 0.01*	50.0

Keys: S.E.M. = Standard Error of Mean; V.A.C.S. = *Vernonia amygdalina* Crude Saponins; V.K.C.S. = *Vernonia kotschyana* Crude Saponins; * = significant at $p<0.05$

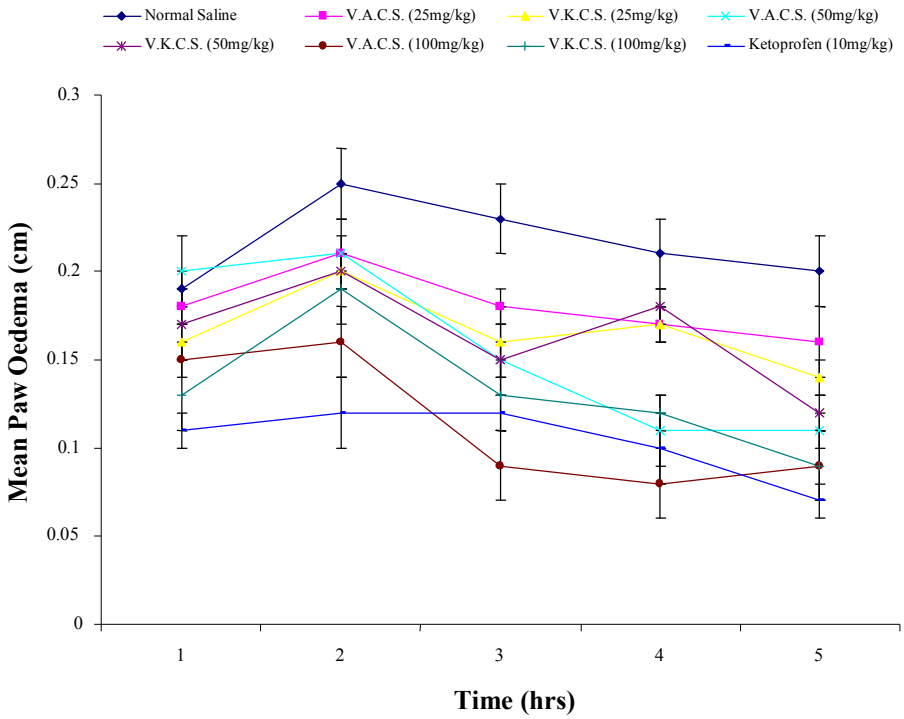


Figure 4.11: Effects of *Vernonia amygdalina* and *V. kotschyana* Crude Saponins (V.A.C. S. and V.K.C.S.) against Carrageenan-Induced Oedema in Rats

CHAPTER FIVE

5.0 DISCUSSION

Macroscopically, the study revealed that, the leaves of the two plants were alternately arranged; they had acute apices, symmetrical bases and pubescent surfaces. Their lamina was simple with reticulate venation.

This study revealed that the leaves differ with respect to petioles, margins and shapes. The leaves of *Vernonia amygdalina* (12.0 x 5.7 cm) had long petioles, serrated margins and lanceolate shapes; the leaves of *V. kotschyana* (17.0 x 6.7 cm) had very short petioles, dented margins and elliptical shapes. Organoleptically, *Vernonia amygdalina* leaf was more greenish in colour and bitter in taste than the *V. kotschyana* leaf, although both plants had distinct odour (Figure 4.1 (a & b; Appendix A; Table 4.1).

The macroscopic features of the two plants were in conformity with the characteristic macroscopic features of the leaves of some members of the family Asteraceae. Cronquist (1981) reported that, leaves of some members of Asteraceae are petiolate; lamina is simple with acute apex and reticulate venation.

Microscopically, the leaves of *V. amygdalina* and *V. kotschyana* were found to have some diagnostic features in common. The diagnostic features identified in their leaves were:

(a) Anomocytic stomata that were surrounded by 4-6 subsidiary cells. These stomata were found abaxially (more numerous) and adaxially.

(b) Numerous unicellular and few uniseriate multicellular covering trichomes. These were found on the midrib portion of *V. amygdalina* and the lower epidermis of *V. kotschyana.*

(c) Few unicellular covering and numerous uniseriate multicellular glandular trichomes on respectively the upper epidermis and midrib of *V. kotschyana* leaves.
(d) Few unicellular and uniseriate multicelluar covering trichomes on the epidermis of *V. amygdalina* leaves.

However, the two plant species differed in the nature of their epidermal cells. The epidermal cells of the *V. amygdalina* had slightly wavy while those of *V. kotschyana* had deeply wavy anticlinal walls (Figure 4.2 (a & b); Appendix B and Figure 4.3 (a & b); Appendix C).

The occurrence of the above mentioned characteristic features in the two plants were supported by Metcalfe and Chalk (1965); Cronquist (1981) and Nwosu (1999) who reported that epidermis of young stems and leaves of some members of Asteraceae are characterized by numerous trichomes, anomocytic (ranunculaceous) or occasionally anisocytic (cruciferous) stomata, wavy, oblong to slightly acicular epidermal cells, peripheral phloem and xylem segments with simple perforation (scalariform or reticulate).

Transverse sections of the leaves of *V. amygdalina* and *V. kotschyana* across the midrib portions show that the leaves are dorsiventral. Their xylem elements were multilacunar and calcium oxalate crystals occur as prisms and rosettes (Figure 4.4 (a & b)). Chemo-

microscopic study on the leaves of the two plants also show the presence of starch grains (oval-shaped), fragments of xylem vessels (reticulate and spiral) and cellulose and fixed oils (*V. kotschyana* only) (Figure 4.5 (a& b) Table 4.2).

The microscopic features identified in *V. amygdalina* and *V. kotschyana* leaves were similar to earlier findings by David (1971) and Cronquist (1981) who reported that the most common type of nodal vascular structures among Dicotyledons, in the primitive and advanced families such as Rosaccae, Asteraceae (Compositae), Fagaceae and Olacaceae are the multilacunar types.

The presence of calcium oxalate crystals was consistent with earlier findings by Hall (1976) who reported that plant species such as some members of Asteraceae (Compositae) that contain calcium element in trace soluble amounts in their cells, it is precipitated in form of crystalline calcium salt of oxalic acid.

The quantitative-leaf microscopic features of the leaves of the two plants show that *V. kotschyana* has higher palisade ratio (5.2), vein-islet (12.7) and vein-let termination number (16.5) than those of *V. amygdalina* that had 4.1, 6.2 and 10.7 respectively. However, stomatal numbers and indices: 265.2 and 19.1 (upper epidermis) and 312.1 and 38.1 (lower epidermis) were higher in *V. amygdalina* than in *V. kotschyana,* which had 118.3 and 17.1 and 227.3 and 28.3 respectively (Table 4.3). Some of these quantitative parameters are very significant in crude drug evaluations. This was supported by Evans (1996), who reported that, stomatal numbers are usually useless in distinguishing closely

allied species but other parameters such as stomatal indices, vein-islet numbers and vein-let terminations are very useful in distinguishing leaves of co-generic species.

The physical constants that were determined (% w/w) from the leaf powder of the two plants show that *V. kotschyana* had higher (5.0%) moisture content than *V. amygdalina* (4.5%). However, the latter plant had higher total ash value (11.0%) than the former (8.0%), although, both plants had similar acid-insoluble ash value (1.5%). The water-soluble ash (4.5%), alcohol and water extractive values (14.0 and 22.5%) were higher in *V. amygdalina* than in the *V. kotschyana* (4.2, 13.5, and 18.5% respectively) (Table 4.4).

Moisture is significant in crude drug evaluation. The moisture content (4.5 and 5.0% w/w) in the leaf powder of the *V. amygdalina* and *V. kotschyana* respectively, was found to be relatively low to provide suitable conditions for the proliferation of micro-organisms that will eventually lead to deterioration. According to A. P. (1986), the general moisture requirement for microbial (bacteria, fungi and/or yeast) growth should not be less than 14%.

On the other hand, the total and acid-insoluble ash values respectively show that the two plants were in good physiological state and that they contain low extraneous matters (such as silica, soil and sand). These findings agree with the A. P. (1986) report which states that the total ash represents both the physiological ash (from the plant tissues) and non-physiological one (from extraneous matter such as sand and soil adhering to the surface of crude drugs) (Appendix D).

The mineral element concentrations in milligram (mg) per 1.0 g of the leaf powder of the two plants show that there was significant difference between them. Generally, *V. kotschyana* was found to contain more mineral elements than *V. amygdalina*. Uranium (0.006±LDL mg) was present only in the latter plant. However, chlorine (4.45±LOD mg), silver (4.4±LOD mg), iodine (0.76±LOD mg), titanium (0.29±LOD mg), vanadium (0.11±LOD mg), cobalt (0.03.0±LOD mg), nickel (0.02±LOD mg), zinc (0.02±0.004), arsenic (0.01±0.004), selenium (0.006±LOD) and niobium (0.004±0.001) were only present in *V. kotschyana* but were absent in the other plant. Although, potassium, calcium, manganese and iron among others were found to be common in both plant species, however, their concentrations varied (Table 4.5).

The respective concentrations of iron (1.50±0.11 and 0.89±0.03) mg and copper (0.02±LDL and 0.02±LOD) mg per 1.0 gm leaf powders of *V. amygdalina* and *V. kotschyana* (Table 4.5) could provide some of the daily need for individuals. Wohl and Goodhart (1964) reported that the daily intake of iron and copper in human are 10–30 mg and 2.5-5.0 mg/day respectively. Worwood (1977) reported that iron is known to play a vital role in amelioration of anaemia.

Manganese is essential for growth and reproduction in animals. It has been shown to be related to some enzyme systems namely metallo enzymes and metal enzyme complexes such as hydrolases, kinases, decarboxylases and transferases (Vallee and Coleman, 1964). The normal daily requirement of manganese for an adult man is 12–20 mg. Therefore, the presence of this element (0.09±0.03 and 0.07±0.02) mg in the leaves of *V. amygdalina* and

V. kotschyana respectively (Table 4.5), could provide some of its daily need for individuals, since both plants are used as vegetables.

Calcium plays a fundamental role in the constitution of biological systems, such as bone formation, which provide organisms with the required rigidity and support (Rubin, 1974). The recommended daily intake of calcium is estimated at 450 mg /day (Davidson, *et al.,* 1972). *Vernonia amygdalina* and *V. kotschyana* leaf powders were found to contain (10.10±0.90 and 8.82±0.44) mg of calcium respectively (Table 4.5).

Maynard *et al.* (1979) said that deficiency of potassium results in non-specific gross symptoms, heart lesions (myocardial degeneration) and tubular degeneration of the kidneys. The estimated requirement of this element is 0.2–0.6 per cent of the dry ration of the animals. As such the concentration of potassium (15.40±1.45 mg) in the leaves of *V. amygdalina* and *V. kotschyana* (Table 4.5) could provide some required amount to the body.

More importantly, iron that was found to be in minimal concentration (1.50±0.11 and 0.89±0.03) mg in the leaves of *V. amygdalina* and *V. kotschyana* respectively, it plays a vital role in inflammatory processes. This is evidently demonstrated in the fact that during infection, host body becomes metabolically adjusted to make iron unavailable to microorganisms which results in some biochemical changes as highlighted by Kennedy (2001) in the following sequences:

(a) Decreased intestinal absorption of iron from the diet.

(b) Decrease of iron in the plasma and an increase in iron in storage as ferritin.

(c) Increased synthesis of the human iron-binding proteins (iron chelators) lactoferrin and transferrin **(**which trap iron for use by human cells while making it unavailable to most microbes) coupled with the febrile response.

(d) Decreased ability of bacteria to synthesize their own iron chelators called siderophores.

(e) Prior stationing of lactoferrin at common sites of microbial invasion such as in the mucous membranes.

(f) Denied entry of transferrin into the tissue during inflammation.

These processes ensure lack of iron which is needed for the bacterial electron transport chain, and therefore inhibit the growth of many bacteria and some inflammatory processes (Kennedy, 2001).

The potentially toxic elements, namely lead (0.01±LDL and 0.02±LOD) mg in *Vernonia amygdalina* and *V. kotschyana* respectively, uranium (0.007±LDL mg) in the former plant and selenium (0.006±LOD mg) in the latter plant were all in their least detectable limit. Lackey and Vegunopal (1977) reported that, chronic lead, uranium or selenium toxicity results in renal malfunction, anaemia, brain and liver damage, cancer, hyperactivity and general psychological impairment.

The percentage yield of extracts obtained out of 800 g of leaf powder (Table 4.6) from the two plants show that the yield of petroleum ether extract was more in *V. kotschyana* (12.34%) than in *V. amygdalina* (11.58%). However, the ethanol extractive yield was more in the latter plant (27.74%) than in the former one (24.41%). The results of phytochemical

studies on the extracts showed that the two plants differ in some chemical constituents. It was found that alkaloids, fixed oils and resins were detected only in *V. kotschyana*. However, tannins, reducing sugars, saponins, flavonoids and steroids were common to both plants. On the other hand carotenoids, anthracenosides, coumarins and polyuronides were absent in the two plants (Table 4.7).

The presence of saponins and flavonosides in the two plants was in accordance with Ohigashi *et al.* (1991) and Jisaka *et al.* (1993). They reported that, vernoniosides A_1, A_2 A_3, and B_1 (steroidal glycosides) were identified in *V. amygdalina* and *V. kotschyana.* Three flavones namely luteolin, luteolin 7–0-β-glucuronoside and luteolin 7–0-β-glucoside were identified in the former plant (Igile, *et al.,* 1994).

The amount of the crude flavonoids and saponins from the ethanol leaf extracts of the two plants show that in general, the crude saponins content in the ethanol leaf extract of the two plants was higher than the crude flavonoids. *Vernonia amygdalina* had higher crude flavonoids (1.43%) than *V. kotschyana* (1.35%). However, the latter plant had higher crude saponins (2.97%) than the former one (2.21%) as in Table 4.8. The crude flavonoids and saponins were identified by using various chemical tests (Tables 4.9 and 4.10) respectively. The presence of the flavonoids and saponins in the two plants was supported by earlier findings by Evans (1999) and Sanogo (1998), that members of the Asteraceae are characterized by the presence of flavonoids, saponins and tannins.

The Thin Layer Chromatographic (TLC) analysis on the crude ethanol extracts, flavonoids and saponins of *V. amygdalina* and *V. kotschyana* show the presence of different types of these constituents in the two plants. This was because, range of colours (yellow, purple, brown and green) and R_f values (0.55 - 0.95) of the spots in the crude extracts and flavonoids were identified. Furthermore, range of colours (brown, purple, blue, yellow and green) and R_f values (0.58 – 0.95) in the crude extracts and saponins were identified. The chromatograms of the crude flavonoids and saponins were visualized by exposure to concentrated ammonia vapour and Liebermann-Burchard's reagent followed by heating at 105^{o}C respectively. However, the chromatograms of the extracts were visualized by exposure to Iodine vapour, concentrated ammonia vapour and Liebermann-Burchard's reagent (Tables 4.11 – 4.12). The various colours and range of R_f values were within the range of colours and R_f values that are characteristic of different types of flavonoids and saponins among medicinal plants.

Harborne (1973) reported that, various classes of flavonoids namely flavonols (Quercetin), flavones (Luteolin), glycosylflavones (Vitexin) and biflavonone (Kayaflavone) show different colours when their chromatograms are visualized under UV light or by exposure to ammonia vapour. These colours ranged from yellow, black, yellow-green, blue, green and brown and R_f values (0.45 – 0.95) (Bladt and Wagner, 1996).

Saponins in forms of glycosides of triterpenes or sterols give wide range of colours when TLC chromatograms were sprayed with a number of detecting reagents including Liebermann-Burchard's reagent followed by heating at 85 – 90^{o}C for 15 minutes. Even the

simplest laboratory solvents, namely water can be used as a spray for detecting steroids on TLC plates (Harborne, 1973). Steroid and triterpenoid saponins were found to exhibit different colours ranging from brown, violet, blue, yellow, red and the like. Their TLC chromatograms were visualized by using different detecting reagents, Carl Price and Liebermann-Burchard's reagents inclusive. Their R_f values ranged from 0.2 – 0.75, depending on the developing solvents used (Bladt and Wagner, 1996).

The acute toxicity study on the ethanol leaf extracts from the two plants show that *V. amygdalina* is relatively more toxic (288.5 mg/kg) than *V. kotschyana* (471.2 mg/kg) body weight *i.p.* to the experimental mice (Tables 4.13 (a-b) and Table 4.14 (a-b). The present findings agree with Lorke (1983) who said that, substances toxic at less than 1 mg / kg are said to be highly toxic. It is therefore, important to establish the LD_{50} exactly. Substaces that are toxic at 1 mg to 5000 mg are said to be moderately toxic. On the other hand, those substances that are toxic at more than 5000 mg / kg are said to be relatively safe. As such, to establish their LD_{50} values are of no practical interest since, the LD_{50} value is usually used as an adequate index to estimate the risk of acute intoxication.

There was significant ($p < 0.05$) inhibitory effect produced by the ethanol leaf extracts of the two plants against acetic acid-induced writhings in mice. The ethanol extract of *V. kotschyana* (V. K. E. E.) produced higher inhibition (74.6%) at 50 mg/kg than that of *V. amygdalina* (V. A. E. E.) that had a dose dependant inhibition (71.9%) at 100 mg/kg when compared with the negative control (normal saline) group. These activities were comparable to that produced by

ketoprofen (67.5%) at 10 mg/kg that served as the positive control (Table 4.15; Figure 4.6 and Appendices F-K).

There was significant ($p<0.05$) and dose-dependant analgesic activities in *V. amygdalina* and *V. kotschyana* crude flavonoids (V.A.C.F and V.K.C.F.). These show that the latter plant has higher inhibition (99.0%) than that produced by the former one (97.0%) both at 100 mg/kg against acetic acid-induced writhings in mice. These activities were compared with the negative control group. The activities were comparable to that produced by ketoprofen (58.0%) at 10 mg/kg that was used as the standard (+ve control) (Table 4.16; Figure 4.7 and Appendices L-Q).

There was significant ($p<0.05$) dose-dependant analgesic activities produced by the crude saponins from the two plants. It shows that the crude saponins of *V. kotschyana* (V. K. C. S.) had higher inhibitory effects (97.0%) than that (78.0%) of *V. amygdalina* (V. A. C. S.) both at 100 mg/kg against acetic acid-induced writhings in mice. The effect was compared with the negative control group. The ketoprofen that served as the positive control produced comparably less effect (58.0%) at 10 mg/kg than those produced by the crude saponins (Table 4.17; Figure 4.8 and Appendices R-W).

The analgesic activities of the crude ethanol leaf extracts, flavonoids and saponins of *V. amygdalina* and *V. kotschyana* were worthy of consideration. The method used for the study also known as the abdominal constriction response, is a very sensitive one. It is able to detect antinociceptive (analgesic) effects of compound at a dose that could not be detected by other methods such as the tail-flick test (Collier *et al.*, 1968). The abdominal

constriction responses were postulated to partly involve local peritoneal receptors (Bentley *et al.*, 1981; 1983).

The antinociceptive activities of the extracts could be due to some of the phytochemical constituents namely tannins, flavonoids or saponins that were identified in the plants. Analgesic effect has been reported in plant species containing tannins and/or flavonoids (Ahmadiani *et al.*, 1998). The terpenic saponin kalopanaxsaponin A isolated from *Kalopanax pictus* Nakai (Araliaceae) prevented the formation of TNFα by RAW 264.7 macrophages stimulated with LPS at concentrations up to 5 μM. The compound was found to be a useful substance in pain and inflammation (Kim *et al.,* 2002).

Pain induction in form of abdominal constrictions by acetic acid was shown to involve peripheral mechanism of nociception that chiefly involve local peritoneal receptors by promoting the synthesis of prostaglandins and some cyclo-oxygenases (Valber *et al.* 2007). The possible mechanism of action for the significant analgesic activities displayed by the crude ethanol extracts, flavonoids and saponins of *V. amygdalina* and *V. kotschyana* might be similar to that of ketoprofen. It might therefore, inhibit the synthesis of prostaglandins and some cyclo-oxygenases since; acetic acid induced-writhing was known to involve local peritoneal receptors.

This was further supported by (Jerussi *et al.*, 1998) that, ketoprofen (2-arylpropionic acid derivative) which is a racemic mixture of the two enantiomeric forms: R- and S-enantiomers, exerts its analgesic effect by inhibiting cyclooxygenase synthesis. The main

part of the analgesic activity of ketoprofen may be attributed to its R-isomer whereas one of its main side effects, gastric damage, could be attributed to its S-isomer.

There was significant ($p<0.05$) dose-dependant and/or non dose-dependant anti-inflammatory activities against carragenan-induced oedema in rats by the *V. amygdalina* ethanol extract (V. A. E. E.) and that of *V. kotschyana* (V. K. E. E.). It shows that, the ethanol extracts of the two plants had similar inhibition (45.5%) at 100 and 50 mg/kg respectively when compared with the negative control (normal saline) group. The activity was lower than that produced by ketoprofen (63.6%) at 10 mg/kg that served as the positive control (Table 4.18; Figure 4.9 and Appendices X-AC).

There was significant ($p<0.05$) dose-dependant/ non dose-dependant anti-inflammatory activity of the crude flavonoids of *V. amygdalina* (V.AC.F) and that of *V. kotschyana* (V.K.C.F.). This shows that the former plant had dose-dependant higher inhibitory effect (45.5%) at 100 mg/kg than the non dose-dependant one (36.7%) produced by the latter plant at 50 mg/kg against carrageenan-induced oedema in rats. The effect was compared with the negative control group. This activity was lower than that produced by ketoprofen (50.0%) at 10 mg/kg that was used as the positive control (Table 4.19; Figure 4.10 and Appendices AD-AI).

There was significant ($p<0.05$) and dose-dependant anti-inflammatory effect produced by the crude saponins of *V. amygdalina* and *V. kotschyana* (V. A. C. S. and V. K. C. S.). It was found that the former plant had higher inhibition (50.0%) than the latter one (40.9%) both at 100 mg/kg against carrageenan-induced oedema in rats. This effect was compared

with the negative control group. However, the effect was similar in one of the plants (*V. amygdalina*) to that produced by ketoprofen (50.0%) at 10 mg/kg that served as the positive control (Table 4.20; Figure 4.11 and Appendices AJ-AO).

The significant anti-inflammatory activities of the crude ethanol leaf extracts, flavonoids and saponins of the two plants could be due to some of the phytochemical constituents such as tannins or flavonoids or saponins that were identified in the plants. This was supported by earlier finding by Ahmadiani *et al.*, (2000) that, anti-inflammatory effects have been reported in plant species containing tannins and/or flavonoids. It was reported by An *et al.* (2002) that, catalposide, the major glycoside obtained from the stem bark of *Catalpa ovata* G. Don (Bignoniaceae) was found to be useful in inflammatory conditions. This constituent was effective in preventing the production of TNFα, IL-1β and IL-6 in LPS-activated macrophages, possibly via NF-κB inhibition.

Aqueous and ethanol stigma extracts, as well as ethanol petal extract of *Crocus sativus* L. (saffron) exerted chronic anti-inflammatory effect. Phytochemical screening of the extracts indicated the presence of flavonoids, tannins and anthocyanins in the petal aqueous and ethanol extracts. Alkaloids and saponins were found in the stigma aqueous and ethanol extracts (Hosseinzadeh and Younesi, 2002).

The mechanism of induction of oedema by carrageenan has been established to involve two phases. The first phase involves the release of histamine and serotonin while the second phase is due to the release of bradykinin, protease, prostaglandin and lysosome

(Crunckhon and Meacock, 1971). The time course of mediators involved in carrageenan-induced oedema showed that histamine and hydroxytryptamine are released within the $1^1/_2$ hours post induction. Bradykinins and prostaglandins are involved during the 1–2 hours and 2–6 hours post induction respectively (Di Rossa *et al.,* 1971).

The significant anti-inflammatory activity exhibited by the crude ethanol extracts, flavonoids and saponins of *V. amygdalina* and *V. kotschyana* which was comparable to that displayed by a standard non-steroidal anti-inflammatory drug (ketoprofen) suggests that the two plants might have exerted their anti-inflammatory effect by inhibiting the release of bradykinins and protaglandins, since, they showed more activities during the late hours post oedema induction.

Carrageenan-induced edema is a standard prostaglandin-dependent model of inflammation in which the S-isomer of ketoprofen shows a very good inhibitory action. The anti-inflammatory effect of this isomeric compound usually correlates with its potency as a peripheral prostaglandin inhibitor (Brune *et al.*, 1992). However, the R-isomer of ketoprofen has a weak cyclooxygenase inhibitory effect and could reduce paw swelling only at high doses (Mauleon *et al.*, 1996).

Terpenic saponin, kalopanaxsaponin A isolated from *Kalopanax pictus* Nakai (Araliaceae) prevented the formation of TNFα by RAW 264.7 macrophages stimulated with LPS (Kim *et al.,* 2002). The compound was found to be a useful substance in pain and inflammation.

CHAPTER SIX

6.0 SUMMARY, CONCLUSION AND RECOMMENDATIONS

6.1 Summary

The macroscopic studies showed that the leaves of the two plant species possess certain diagnostic features in common and differed in some others. Their leaves were alternately arranged with acute apices, symmetrical bases and pubescent surfaces. *Vernonia amygdalina* leaves are relatively smaller (12.0 x 5.7 cm) than those of *V. kotschyana* (17.0 x 6.7 cm). The *V. amygdalina* leaves have long petiole while those in *V. kotschyana* have very short one. The leaf margin was serrated and dented while the shape was lanceolate and elliptical for *V. amygdalina* and *V. kotschyana* respectively (Figure 4.1 (a & b); Appendix A). Organoleptically, *V. amygdalina* leaves are more greenish in colour and bitter in taste than those of *V. kotschyana* eventhough, the two plants have distinct odour (Table 4.1).

Microscopically, the leaves of both plants had anomocytic stomata and were found abaxially (more numerous) and adaxially. There were numerous unicellular and few uniseriate multicellular covering trichomes in both plants. Few uniseriate multicellular glandular trichomes were found in *V. kotschyana*. However, epidermal cells were slightly and deeply wavy in *V. amygdalina* and *V. kotschyana* respectively (Figure 4.2 (a & b); Appendix B and Figure 4.3 (a & b); Appendix C)

Transversely, leaves of both plants were dorsiventral with multilacunar vascular bundles. Calcium oxalate crystals (prism and rosette), starch grains (oval-shaped) and xylem vessels (reticulate and spiral) were also identified (Figure 4.4 (a & b) and Figure 4.5 (a & b).

Quantitative-leaf microscopy showed that *V. kotschyana* had higher palisade ratio (5.2), vein-islet (12.7) and vein-let termination number (16.5) than that of *V. amygdalina* that had 4.1, 6.2 and 10.7 respectively. Stomatal numbers and indices: 265.2 and 19.1 (upper epidermis) and 312.1 and 38.1 (lower epidermis) were higher in the latter plant than in the former one that had 118.3 and 17.1 and 227.3 and 28.3 respectively (Table 4.3).

The physical constants (%w/w) had shown that *V. kotschyana* had higher moisture content (5.0%) than *V. amygdalina* (4.5%). The latter plant had higher total ash value (11.0%) than the former one (8.0%). Both of them had similar acid-insoluble ash value (1.5%). Water-soluble ash (4.5%), alcohol and water extractive values (14.0 and 22.5%) were higher in *V. amygdalina* than in *V. kotschyana* that had 4.2, 13.5, and 18.5% respectively (Table 4.4 and Appendix D).

The mineral element concentrations in milligram (mg) per 1.0 g of the leaf powder of the two plants vary considerably. The *V. kotschyana* was found to contain more mineral elements than *V. amygdalina.* It was found that uranium (0.006±LDL mg) was only present in the latter plant. Potassium, calcium, manganese, and iron among others were common to both plants but with varied concentrations (Table 4.5).

The yield of extracts out of 800 g of leaf powder (Table 4.6) from the two plants showed that the petroleum ether extract was more in *V. kotschyana* (12.34%) than in *V. amygdalina* (11.58%). The ethanol extract was more in the latter (27.74%) than in the former (24.41%). Phytochemical investigation on the extracts showed that the two plants varied in respect of the presence of chemical constituents (Table 4.7).

The crude flavonoids and saponins from the ethanol leaf extracts of the two plants show that *V. amygdalina* have higher content of crude flavonoids (1.43%) than *V. kotschyana* (1.35%). However, the latter plant had higher crude saponins (2.97%) than the former one (2.21%) as in Table 4.8. Their chemical identities were given in Tables 4.9 and 4.10.

The crude ethanol extracts of *V. amygdalina* and *V. kotschyana* showed the presence of different flavonoids and saponins. The various spots were found to have different colours and R_f values that are characteristic of both flavonoids and saponins when visualized by exposure to iodine, concentrated ammonia vapour and spraying with Liebermann-Burchard's reagent (Tables 4.11; 4.12).

The crude flavonoids of *V. amygdalina* and *V. kotschyana* showed five and four different spots respectively when chromatographed and exposed to concentrated ammonia vapour. However, their R_f values slightly varied, with only one spot from both plant species having the same value (Table 4.12).

The crude saponins of *V. amygdalina* and *V. kotschyana* showed the presence of four different spots in both cases when chromatographed on pre-coated TLC plates and sprayed with Liebermann-Burchard's reagent, followed by heating at 105^{o}C. Only one spot from both plant species had the same R_f value but the remaining ones had their R_f values either slightly and/or grossly different (Table 4.12).

The acute toxicity study on the ethanol leaf extracts of plants show that *V. amygdalina* was relatively more toxic (288.5 mg/kg) than *V. kotschyana* (471.2 mg/kg) body weight *i.p.* to the experimental mice (Tables 4.13 (a-b) and Table 4.14 (a-b).

The analgesic studies on the ethanol leaf extracts of the two plants show that the *V. kotschyana* ethanol extract (V. K. E. E.) had significantly ($p< 0.05$) higher inhibition (74.6%) at 50 mg/kg than that (71.9%) of *V. amygdalina* (V. A. E. E.) at 100 mg/kg against acetic acid-induced writhing in mice when compared with the negative control (normal saline) group. The activity was comparable to that produced by ketoprofen at (67.5%) that served as the positive control (Table 4.15; Figure 4.6 and Appendices F-K).

The significant ($p<0.05$) and dose-dependant analgesic activities of the crude flavonoids show that the *V. kotschyana* crude flavonoids (V.K.C.F.) had higher inhibition (99.0%) than that (97.0%) of the *V. amygdalina* (V.AC.F) both at 100 mg/kg against acetic acid-induced writhing in mice when compared with the negative control group. This inhibitory

effect was comparable to that produced by ketoprofen (58.0%) at 10 mg/kg that was used as the standard (+ve control) (Table 4.16; Figure 4.7 and Appendices L-Q).

The significant ($p<0.05$) and dose-dependant analgesic activities of the crude saponins show that the crude saponins of *V. kotschyana* (V. K. C. S.) had higher inhibitory effect (97.0%) than that (78.0%) of *V. amygdalina* (V. A. C. S.) both at 100 mg/kg against acetic acid-induced writhing in mice when compared with the negative control group. The ketoprofen that served as the positive control had comparably less inhibitory effect (58.0%) at 10 mg/kg than those produced by the crude saponins (Table 4.17; Figure 4.8 and Appendices R-W).

The anti-inflammatory studies on the two plants show that the ethanol extracts of *V. amygdalina* (V. A. E. E.) and that of *V. kotschyana* (V. K. E. E.) had significant ($p<0.05$) and similar inhibition (45.5%) at 100 and 50 mg/kg respectively against carragenan-induced oedema in rats. This effect was compared with the negative control (normal saline) group. The effect was lower than that of ketoprofen (63.6%) at 10 mg/kg that served as the positive control (Table 4.18; Figure 4.9 and Appendices X-AC).

The significant ($p<0.05$) anti-inflammatory activities of the crude flavonoids of *V. amygdalina* (V.AC.F) and that of *V. kotschyana* (V.K.C.F.) show that the former plant had higher inhibitory effect (45.5%) at 100 mg/kg than that (36.7%) of the latter plant at 50 mg/kg against carrageenan-induced oedema in rats. The effect was compared with the negative control group. It was found that this effect was lower than that of ketoprofen

(50.0%) at 10 mg/kg that was used as positive control (Table 4.19; Figure 4.10 and Appendices AD-AI).

The significant ($p<0.05$) and dose-dependant anti-inflammatory effect of the crude saponins of both plants show that the crude saponins of *V. amygdalina* (V. A. C. S.) had higher inhibition (50.0%) than that (40.9%) of *V. kotschyana* (V. K. C. S.) both at 100 mg/kg against carrageenan-induced oedema in rats when compared with the negative control group. The effect was similar to that of ketoprofen (50.0%) at 10 mg/kg that served as the positive control (Table 4.20; Figure 4.11 and Appendices AJ-AO).

Generally, the crude ethanol extracts and flavonoids of both plants had higher analgesic and anti-inflammatory activities than the crude saponins. The crude ethanol extract, flavonoids and saponins of *V. kotschyana* had higher analgesic activities than those of *V. amygdalina* but the ethanol extracts of both plants had similar anti-inflammatory activity. However, the crude flavonoids and saponins of the *V. amygdalina* had higher anti-inflammatory activities than those of the *V. kotschyana*.

6.2 Conclusion

Macroscopic, microscopic and quantitative (including the elemental analyses) studies were carried out on *V. amygdalina* and *V. kotschyana* leaves (fresh and powdered forms). These studies revealed that the two plants had some diagnostic features in common such as anomocytic stomata on the adaxial and abaxial epidermal surfaces and numerous

unicellular covering trichomes on the midribs. The two plants differed with respect to the presence of numerous unicellular covering trichomes abaxially and few uniseriate multicellular glandular trichomes adaxially on the leaf epidermal surface of *V. kotschyana* only; the leaves of *V. amygdalina* had higher stomatal numbers and indices than those of *V. kotschyana.* However, palisade ratio, vein-islet and vein-let termination numbers, and mineral elements were more in the latter plant than those in the former one.

The analgesic and anti-inflammatory studies carried out on the crude ethanol extracts, crude flavonoids and saponins of the two plants showed that both of them possess strong analgesic and anti-inflammatory activities.

The results of the macroscopic, microscopic, elemental, quantitative and extractive studies on *V. amygdalina* and *V. kotschyana* could therefore, serve usefully and form bases for their easy identification and differentiation. They could consequently be of great assistance to those individuals that confuse the two plants.

The results of the analgesic and anti-inflammatory studies carried out on *V. amygdalina* and *V. kotschyana* support the traditional claims of using the plants in the management of tooth ache, stomach ache and some inflammatory diseases such as pain of the joints and gingivitis. The results show that the two plants could serve as potential sources of both analgesic and anti-inflammatory agents of medicinal plant origin.

6.3 Recommendations

It is therefore recommended that more research work should be conducted in order to:

(a) Prepare monographs for the two plants for their possible inclusion into the Nigerian/African Pharmacopoeias.

(b) Further evaluate biologically, the flavonoids and saponins of the two plants which were found to have potent analgesic and anti-inflammatory activities. Since, they can serve as potential sources of modern drugs.

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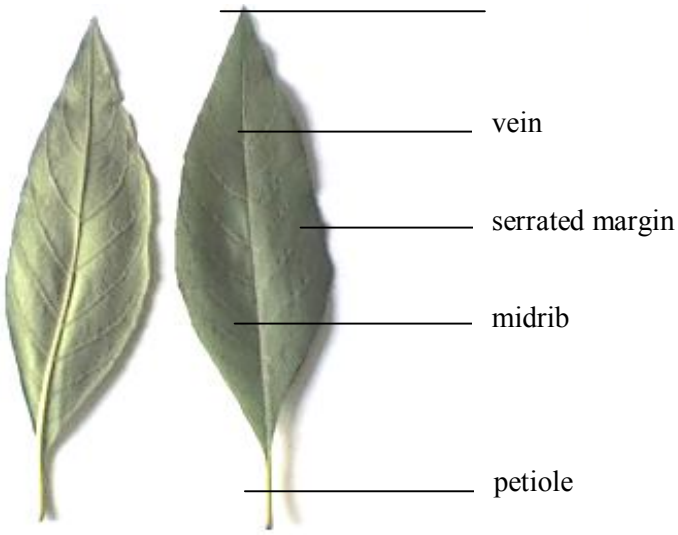
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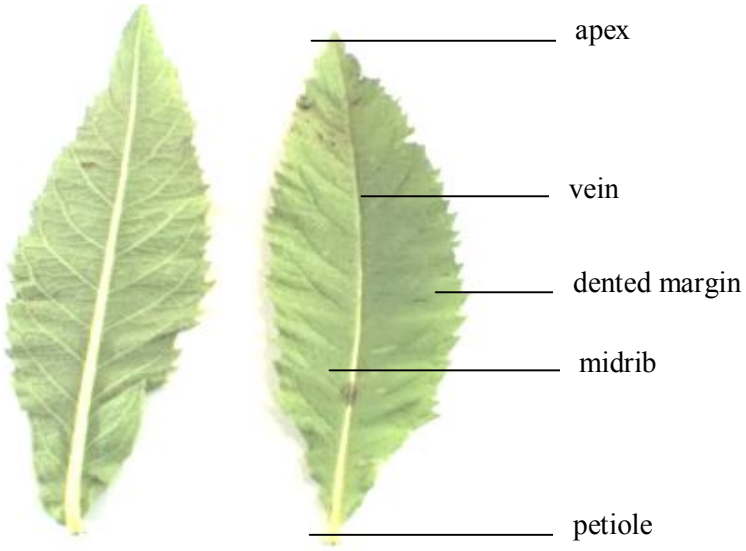
APPENDICES

Appendix A (a & b): *Vernonia amygdalina* and *V. kotschyana* Leaves; x1

apex

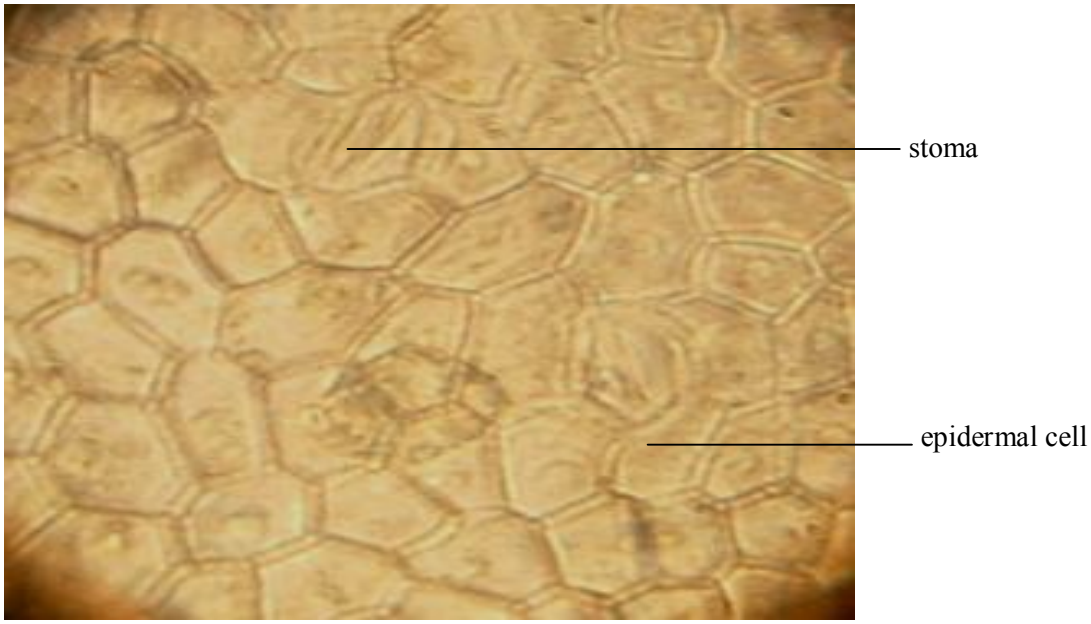


(a) *Vernonia amygdalina* Leaves

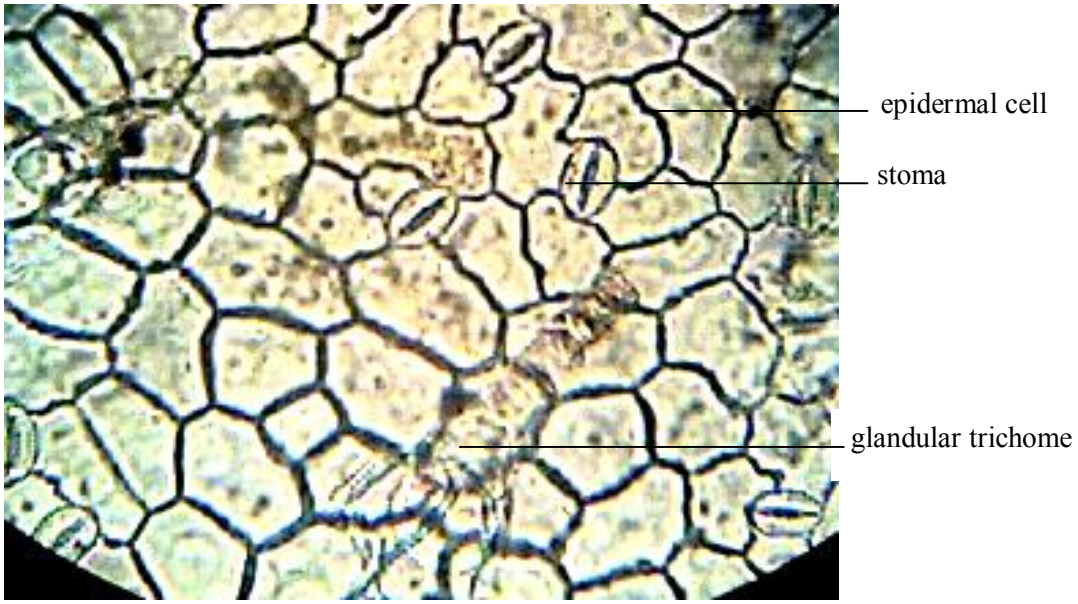


(b) *Vernonia kotschyana* Leaves

Appendix B (a & b): Surface Preparation from the Upper Epidermis of *Vernonia amygdalina* and *V. kotschyana* Leaves; x400

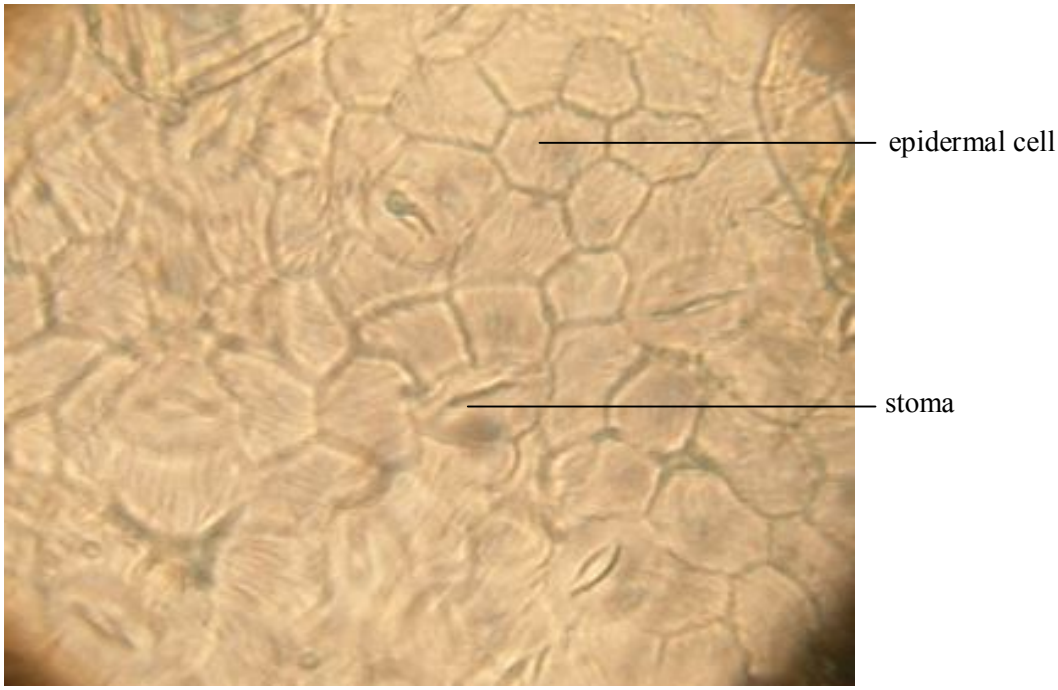


(a) Upper Epidermis of *Vernonia amygdalina*



(b) Upper Epidermis of *Vernonia kotschyana*

Appendix C (a & b): Surface Preparations from the Lower Epidermis of *Vernonia amygdalina* and *V. kotschyana* Leaves; x400



(a) Lower Epidermis of *Vernonia amygdalina*



(b) Lower Epidermis of *Vernonia kotschyana*

Appendix D: Details of the Calculation Procedures of Physical Constants Determination

(i) Moisture Content

Weight of drug = a

Constant wt. of empty crucible = b

Weight of crucible + drug = c

Constant wt. of crucible + drug after drying = d

$\therefore$ Weight of moisture = c – d = e

$$\% \text{ Moisture} = \frac{e}{a} x \frac{100}{1}$$

(ii) Ash Value

Weight of drug = a

Constant wt. of crucible = b

Weight of crucible + drug = c

Constant weight of crucible + residual ash = d

$\therefore$ Weight of ash = c – d = e

$$\% \text{ Ash} = \frac{e}{a} x \frac{100}{1}$$

(iii) Acid-insoluble ash value

Initial weight of drug = a

Constant weight of crucible = b

Weight of crucible + residual ash = c

∴ Weight of acid-insoluble ash = c – b = d

$$\% \text{ Acid-insoluble ash} = \frac{d}{a} x \frac{100}{1}$$

(iv) Water-soluble ash value

Weight of drug = a

Constant weight of crucible = b

Initial weight of crucible + ash = c

Weight of crucible + residual ash = d

∴ Weight of water-soluble ash = c – d = e

$$\% \text{ Water- soluble ash} = \frac{e}{a} x \frac{100}{1}$$

(v) Alcohol-soluble extractive value

Weight of drug = a

Constant weight of empty crucible = b

Constant wt. of crucible + 20 ml residue = c

∴ Weight of residue from 20 ml filtrate = c–b= d

Weight of residue in 100 ml = d x 5 = e

$$\therefore \% \text{ Alcohol-soluble extractives} = \frac{e}{a} x \frac{100}{1}$$

(vi) Water-soluble extractive value

Weight of drug = a

Constant weight of empty crucible = b

Weight of crucible + 20 ml residue = c

∴ Wt. of residue from 20 ml filtrate = c – b = d

∴ Weight of 100 ml residue = d x 5 = e

% Water-soluble extractives = $\frac{e}{a} x \frac{100}{1}$

Appendix E: Procedures for Statistical Analyses

(i) Arithmetic Mean

$$\overline{X} = \frac{\Sigma x}{N}$$

Where Σx denotes the summation of the observed values

N denotes the total number of the observations

(ii) Standard Deviation

$$S = \pm \sqrt{\Sigma \frac{(x - \overline{x})^2}{n - 1}}$$

Where X denotes the individual observations

$\overline{X}$ denotes the arithmetic Mean

n-1 denotes the degree of freedom

$\sqrt{}$ denotes square root

(iii) Variance

$$S^2 = \Sigma \frac{(x - \overline{x})^2}{n - 1}$$

(This is just the square of the standard derivation).

(iv) Co- efficient of Variation

$$\text{C.V} = \frac{SD}{\overline{x}} x100\%$$

Where SD denotes the standard deviation

$\overline{X}$ denotes the arithmetic mean

100% denotes hundred per cent

(v) Standard Error

$$\text{S.E} = \frac{SD}{\sqrt{n}}$$

Where SD denotes the standard deviation

$\sqrt{n}$ denotes the square root of the total observation.

(vi) Dependant Student's *t*-test

$$t = \frac{\overline{xj} - \overline{xi}}{S.Ej + S.Ei}$$

Where:

j denotes the control group

i denotes the treated group

The analyses were carried out with the aid of Graphpad Prism software by using student *t*-test to determine the significance level at $p<0.05$.

Appendix F: **Statistical Analysis of the Analgesic Study on *Vernonia amygdalina* Ethanol Extract (VAEE) at 25 mg/kg in Mice *i.p.* versus Normal Saline (negative control)**

ANALYSIS	RESULTS
Column A	N/saline
vs	vs
Column B	25(VAEE)
Unpaired t test	
P value	0.0150
P value summary	*
Are means signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=3.084 df=8
How big is the difference?	
Mean ± SEM of column A	22.80 ± 1.772 N=5
Mean ± SEM of column B	16.20 ± 1.200 N=5
Difference between means	6.600 ± 2.140
95% confidence interval	1.665 to 11.54
R squared	0.5431
F test to compare variances	
F, DFn, Dfd	2.181, 4, 4
P value	0.2344
P value summary	ns
Are variances significantly different?	No

Appendix G: **Statistical Analysis of the Analgesic Study on *Vernonia amygdalina* Ethanol Extract (VAEE) at 50 mg/kg in Mice *i.p.* versus Normal Saline (negative control)**

ANALYSIS	RESULTS
Column A	N/saline
vs	vs
Column B	50(VAEE)
Unpaired t test	
P value	0.0054
P value summary	**
Are means signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=3.776 df=8
How big is the difference?	
Mean ± SEM of column A	22.80 ± 1.772 N=5
Mean ± SEM of column B	13.80 ± 1.594 N=5
Difference between means	9.000 ± 2.383
95% confidence interval	3.504 to 14.50
R squared	0.6406
F test to compare variances	
F, DFn, Dfd	1.236, 4, 4
P value	0.4211
P value summary	ns
Are variances significantly different?	No

Appendix H: **Statistical Analysis of the Analgesic Study on *Vernonia amygdalina* Ethanol Extract (VAEE) at 100 mg/kg in Mice *i.p.* versus Normal Saline (negative control)**

ANALYSIS	RESULTS
Column A	N/saline
vs	vs
Column B	100(VAEE)
Unpaired t test	
P value	P<0.0001
P value summary	***
Are means signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=7.818 df=8
How big is the difference?	
Mean ± SEM of column A	22.80 ± 1.772 N=5
Mean ± SEM of column B	6.400 ± 1.122 N=5
Difference between means	16.40 ± 2.098
95% confidence interval	11.56 to 21.24
R squared	0.8843
F test to compare variances	
F, DFn, Dfd	2.492, 4, 4

P value	0.1990
P value summary	ns
Are variances significantly different?	No

Appendix I: **Statistical Analysis of the Analgesic study on *Vernonia kotschyana* Ethanol Extract (VKEE) at 25 mg/kg in mice *i.p.* versus Normal Saline (negative control)**

ANALYSIS	**RESULTS**
Column A	N/saline
vs	vs
Column B	25(VKEE)
Unpaired t test	
P value	0.0096
P value summary	**
Are means signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=3.382 df=8
How big is the difference?	
Mean ± SEM of column A	22.80 ± 1.772 N=5
Mean ± SEM of column B	11.80 ± 2.728 N=5
Difference between means	11.00 ± 3.253
95% confidence interval	3.499 to 18.50
R squared	0.5884

F test to compare variances	
F, DFn, Dfd	2.369, 4, 4
P value	0.2120
P value summary	ns
Are variances significantly different?	No

Appendix J: **Statistical Analysis of the Analgesic Study on *Vernonia kotschyana* Ethanol Extract (VKEE) at 50 mg/kg in Mice *i.p.* versus Normal Saline (negative control)**

ANALYSIS	**RESULTS**
Column A	N/saline
vs	vs
Column B	50(VKEE)
Unpaired t test	
P value	P<0.0001
P value summary	***
Are means signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=8.123 df=8
How big is the difference?	
Mean ± SEM of column A	22.80 ± 1.772 N=5
Mean ± SEM of column B	5.800 ± 1.114 N=5

Difference between means	17.00 ± 2.093
95% confidence interval	12.17 to 21.83
R squared	0.8919
F test to compare variances	
F, DFn, Dfd	2.532, 4, 4
P value	0.1951
P value summary	ns
Are variances significantly different?	No

Appendix K: **Statistical Analysis of the Analgesic Study on *Vernonia kotschyana* Ethanol Extract (VKEE) at 100 mg/kg in Mice *i.p.* versus Normal Saline (negative control)**

ANALYSIS	**RESULTS**
Column A	N/saline
vs	vs
Column B	100(VKEE)
Unpaired t test	
P value	0.0003
P value summary	***
Are means signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=5.970 df=8

How big is the difference?	
Mean ± SEM of column A	22.80 ± 1.772 N=5
Mean ± SEM of column B	8.200 ± 1.685 N=5
Difference between means	14.60 ± 2.445
95% confidence interval	8.961 to 20.24
R squared	0.8167
F test to compare variances	
F, DFn, Dfd	1.106, 4, 4
P value	0.4624
P value summary	ns
Are variances significantly different?	No

Appendix L: **Statistical Analysis of the Analgesic Study on *Vernonia amygdalina* Crude Flavonoids (VACF) at 25 mg/kg in Mice *i.p.* versus Normal Saline (negative control)**

ANALYSIS	**RESULTS**
Column A	N/saline
vs	vs
Column B	25 (VACF)
Unpaired t test	
P value	0.2055
P value summary	ns
Are means signif. different? (P < 0.05)	No

One- or two-tailed P value?	Two-tailed
t, df	t=1.378 df=8
How big is the difference?	
Mean ± SEM of column A	20.00 ± 2.588 N=5
Mean ± SEM of column B	13.80 ± 3.680 N=5
Difference between means	6.200 ± 4.499
95% confidence interval	-4.174 to 16.57
R squared	0.1919
F test to compare variances	
F, DFn, Dfd	2.021, 4, 4
P value	0.2562
P value summary	ns
Are variances significantly different?	No

Appendix M: **Statistical Analysis of the Analgesic Study on *Vernonia amygdalina* Crude Flavonoids (VACF) at 50 mg/kg in Mice *i.p.* versus Normal Saline (negative control)**

ANALYSIS	**RESULTS**
Column A	N/saline
vs	vs
Column B	50 (VACF)
Unpaired t test	

P value	0.0011
P value summary	**
Are means signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=4.990 df=8
How big is the difference?	
Mean ± SEM of column A	20.00 ± 2.588 N=5
Mean ± SEM of column B	5.400 ± 1.364 N=5
Difference between means	14.60 ± 2.926
95% confidence interval	7.853 to 21.35
R squared	0.7569
F test to compare variances	
F, DFn, Dfd	3.602, 4, 4
P value	0.1211
P value summary	ns
Are variances significantly different?	No

Appendix N: **Statistical Analysis of the Analgesic Study on *Vernonia amygdalina* Crude Flavonoids (VACF) at 100 mg/kg in Mice *i.p.* versus Normal Saline (negative control)**

ANALYSIS	**RESULTS**
Column A	N/saline
vs	vs

Column B	100 (VACF)
Unpaired t test	
P value	P<0.0001
P value summary	***
Are means signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=7.407 df=8
How big is the difference?	
Mean ± SEM of column A	20.00 ± 2.588 N=5
Mean ± SEM of column B	0.6000 ± 0.4000 N=5
Difference between means	19.40 ± 2.619
95% confidence interval	13.36 to 25.44
R squared	0.8727
F test to compare variances	
F, DFn, Dfd	41.88, 4, 4
P value	0.0016
P value summary	**
Are variances significantly different?	Yes

Appendix O: **Statistical Analysis of the Analgesic Study on *Vernonia kotschyana* Crude Flavonoids (VKCF) at 25 mg/kg in Mice *i.p.* versus Normal Saline (negative control)**

ANALYSIS	RESULTS

Column A	N/saline
vs	vs
Column B	25 (VKCF)
Unpaired t test	
P value	0.0159
P value summary	*
Are means signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=3.048 df=8
How big is the difference?	
Mean ± SEM of column A	20.00 ± 2.588 N=5
Mean ± SEM of column B	8.000 ± 2.966 N=5
Difference between means	12.00 ± 3.937
95% confidence interval	2.921 to 21.08
R squared	0.5373
F test to compare variances	
F, DFn, Dfd	1.313, 4, 4
P value	0.3990
P value summary	ns
Are variances significantly different?	No

Appendix P: **Statistical Analysis of the Analgesic Study on *Vernonia kotschyana* Crude Flavonoids (VKCF) at 50 mg/kg in Mice *i.p.* versus Normal Saline (negative control)**

ANALYSIS	RESULTS
Column A	N/saline
vs	vs
Column B	50 (VKCF)
Unpaired t test	
P value	0.0008
P value summary	***
Are means signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=5.271 df=8
How big is the difference?	
Mean ± SEM of column A	20.00 ± 2.588 N=5
Mean ± SEM of column B	4.400 ± 1.435 N=5
Difference between means	15.60 ± 2.960
95% confidence interval	8.775 to 22.43
R squared	0.7764
F test to compare variances	
F, DFn, Dfd	3.252, 4, 4
P value	0.1399
P value summary	ns
Are variances significantly different?	No

Appendix Q: **Statistical Analysis of the Analgesic Study on *Vernonia kotschyana***

Crude Flavonoids (VKCF) at 100 mg/kg in Mice *i.p.* versus Normal Saline (negative control)

ANALYSIS	RESULTS
Column A	N/saline
vs	vs
Column B	100 (VKCF)
Unpaired t test	
P value	P<0.0001
P value summary	***
Are means signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=7.627 df=8
How big is the difference?	
Mean ± SEM of column A	20.00 ± 2.588 N=5
Mean ± SEM of column B	0.2000 ± 0.2000 N=5
Difference between means	19.80 ± 2.596
95% confidence interval	13.81 to 25.79
R squared 0.8791	
F test to compare variances	
F, DFn, Dfd	167.5, 4, 4
P value	0.0001
P value summary	***
Are variances significantly different?	Yes

Appendix R: **Statistical Analysis of the Analgesic Study on *Vernonia amygdalina* Crude Saponins (VACS) at 25 mg/kg in Mice *i.p.* versus Normal Saline (negative control)**

ANALYSIS	RESULTS
Column A	N/saline
vs	vs
Column B	25(VACS)
Unpaired t test	
P value	0.1228
P value summary	ns
Are means signif. different? (P < 0.05)	No
One- or two-tailed P value?	Two-tailed
t, df	t=1.725 df=8
How big is the difference?	
Mean ± SEM of column A	20.00 ± 2.588 N=5
Mean ± SEM of column B	13.60 ± 2.657 N=5
Difference between means	6.400 ± 3.709
95% confidence interval	-2.154 to 14.95
R squared	0.2712
F test to compare variances	
F, DFn, Dfd	1.054, 4, 4
P value	0.4804
P value summary	ns
Are variances significantly different?	No

Appendix S: **Statistical Analysis of the Analgesic Study on *Vernonia amygdalina* Crude Saponins (VACS) at 50 mg/kg in Mice *i.p.* versus Normal Saline (negative control)**

ANALYSIS	RESULTS
Column A	N/saline
vs	vs
Column B	50 (VACS)
Unpaired t test	
P value	0.0166
P value summary	*
Are means signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=3.020 df=8
How big is the difference?	
Mean ± SEM of column A	20.00 ± 2.588 N=5
Mean ± SEM of column B	9.600 ± 2.272 N=5
Difference between means	10.40 ± 3.444
95% confidence interval	2.459 to 18.34
R squared	0.5327
F test to compare variances	
F, DFn, Dfd	1.298, 4, 4
P value	0.4032
P value summary	ns
Are variances significantly different?	No

Appendix T: **Statistical Analysis of the Analgesic Study on *Vernonia amygdalina* Crude Saponins (VACS) at 100 mg/kg in Mice *i.p.* versus Normal Saline (negative control)**

ANALYSIS	RESULTS
Column A	N/saline
vs	vs
Column B	100 (VACS)
Unpaired t test	
P value	0.0016
P value summary	**
Are means signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=4.670 df=8
How big is the difference?	
Mean ± SEM of column A	20.00 ± 2.588 N=5
Mean ± SEM of column B	4.400 ± 2.112 N=5
Difference between means	15.60 ± 3.341
95% confidence interval	7.896 to 23.30
R squared	0.7316
F test to compare variances	
F, DFn, Dfd	1.502, 4, 4
P value	0.3515
P value summary	ns
Are variances significantly different?	No

Appendix U: **Statistical Analysis of the Analgesic Study on *Vernonia kotschyana* Crude Saponins (VKCS) at 25 mg/kg in Mice *i.p.* versus Normal Saline (negative control)**

ANALYSIS	RESULTS
Column A	N/saline
vs	vs
Column B	25(VKCS)
Unpaired t test	
P value	0.0657
P value summary	ns
Are means signif. different? (P < 0.05)	No
One- or two-tailed P value?	Two-tailed
t, df	t=2.131 df=8
How big is the difference?	
Mean ± SEM of column A	20.00 ± 2.588 N=5
Mean ± SEM of column B	12.60 ± 2.315 N=5
Difference between means	7.400 ± 3.473
95% confidence interval	-0.6082 to 15.41
R squared	0.3621
F test to compare variances	
F, DFn, Dfd	1.250, 4, 4
P value	0.4170
P value summary	ns
Are variances significantly different?	No

Appendix V: **Statistical Analysis of the Analgesic Study on *Vernonia kotschyana* Crude saponins (VKCS) at 50 mg/kg in Mice *i.p.* versus Normal Saline (negative control)**

ANALYSIS	RESULTS
Column A	N/saline
vs	vs
Column B	50(VKCS)
Unpaired t test	
P value	0.0167
P value summary	*
Are means signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=3.013 df=8
How big is the difference?	
Mean ± SEM of column A	20.00 ± 2.588 N=5
Mean ± SEM of column B	8.200 ± 2.939 N=5
Difference between means	11.80 ± 3.917
95% confidence interval	2.768 to 20.83
R squared	0.5315
F test to compare variances	
F, DFn, Dfd	1.290, 4, 4
P value	0.4057
P value summary	ns
Are variances significantly different?	No

Appendix W: **Statistical Analysis of the Analgesic Study on *Vernonia kotschyana* Crude Saponins (VKCS) at 100 mg/kg in Mice *i.p.* versus Normal Saline (negative control)**

ANALYSIS	RESULTS
Column A	N/saline
vs	vs
Column B	100(VKCS)
Unpaired t test	
P value	P<0.0001
P value summary	***
Are means signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=7.407 df=8
How big is the difference?	
Mean ± SEM of column A	20.00 ± 2.588 N=5
Mean ± SEM of column B	0.6000 ± 0.4000 N=5
Difference between means	19.40 ± 2.619
95% confidence interval	13.36 to 25.44
R squared	0.8727
F test to compare variances	
F, DFn, Dfd	41.88, 4, 4
P value	0.0016

P value summary	**
Are variances significantly different?	Yes

Appendix X: **Statistical Analysis of the Anti-inflammatory Study on *Vernonia amygdalina* Ethanol Extract (VAEE) at 25 mg/kg in Rats *i.p.* versus Normal Saline (negative control)**

ANALYSIS	**RESULTS**
Column A	N/saline
vs	vs
Column B	25 (VAEE)
Unpaired t test	
P value	0.0010
P value summary	***
Are means signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=5.066 df=8
How big is the difference?	
Mean ± SEM of column A	0.2160 ± 0.01077 N=5
Mean ± SEM of column B	0.1440 ± 0.009274 N=5
Difference between means	0.0720 ± 0.01421
95% confidence interval	0.03923 to 0.1048
R squared	0.7624

F test to compare variances	
F, DFn, Dfd	1.349, 4, 4
P value	0.3894
P value summary	ns
Are variances significantly different?	No

Appendix Y: **Statistical Analysis of the Anti-inflammatory Study on *Vernonia amygdalina* Ethanol Extract (VAEE) at 50 mg/kg in Rats *i.p.* versus Normal Saline (negative control)**

ANALYSIS	**RESULTS**
Column A	N/saline
vs	vs
Column B	50 (VAEE)
Unpaired t test	
P value	0.0005
P value summary	***
Are means signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=5.598 df=8
How big is the difference?	
Mean ± SEM of column A	0.2160 ± 0.01077 N=5
Mean ± SEM of column B	0.1300 ± 0.01095 N=5
Difference between means	0.08600 ± 0.01536

95% confidence interval	0.05057 to 0.1214
R squared	0.7966
F test to compare variances	
F, DFn, Dfd	1.034, 4, 4
P value	0.4873
P value summary	ns
Are variances significantly different?	No

Appendix Z: **Statistical Analysis of the Anti-inflammatory Study on *Vernonia amygdalina* Ethanol Extract (VAEE) at 100 mg/kg in Rats *i.p.* versus Normal saline (negative control)**

ANALYSIS	**RESULTS**
Column A	N/saline
vs	vs
Column B	100 (VAEE)
Unpaired t test	
P value	P<0.0001
P value summary	***
Are means signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=7.516 df=8

How big is the difference?

Mean ± SEM of column A	0.2160 ± 0.01077 N=5
Mean ± SEM of column B	0.1180 ± 0.007348 N=5
Difference between means	0.0980 ± 0.01304
95% confidence interval	0.06793 to 0.1281
R squared	0.8760

F test to compare variances

F, DFn, Dfd	2.148, 4, 4
P value	0.2386
P value summary	ns
Are variances significantly different?	No

Appendix AA: **Statistical Analysis of the Anti-inflammatory Study on *Vernonia kotschyana* Ethanol Extract (VKEE) at 25 mg/kg in Rats *i.p.* versus Normal Saline (negative control)**

ANALYSIS	**RESULTS**
Column A	N/saline
vs	vs
Column B	25 (VKEE)

Unpaired t test

P value	0.0001
P value summary	***
Are means signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed

t, df	t=6.859 df=8

How big is the difference?

Mean ± SEM of column A	0.2160 ± 0.01077 N=5
Mean ± SEM of column B	0.1320 ± 0.005831 N=5
Difference between means	0.08400 ± 0.01225
95% confidence interval	0.05576 to 0.1122
R squared	0.8547

F test to compare variances

F, DFn, Dfd	3.412, 4, 4
P value	0.1308
P value summary	ns
Are variances significantly different?	No

Appendix AB: **Statistical Analysis of the Anti-inflammatory Study on *Vernonia kotschyana* Ethanol Extract (VKEE) at 50 mg/kg in Rats *i.p.* versus Normal Saline (negative control)**

ANALYSIS	**RESULTS**
Column A	N/saline
vs	vs
Column B	50 (VKEE)

Unpaired t test

P value	0.0003

P value summary	***
Are means signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=6.175 df=8
How big is the difference?	
Mean ± SEM of column A	0.2160 ± 0.01077 N=5
Mean ± SEM of column B	0.1240 ± 0.01030 N=5
Difference between means	0.09200 ± 0.01490
95% confidence interval	0.05764 to 0.1264
R squared	0.8266
F test to compare variances	
F, DFn, Dfd	1.094, 4, 4
P value	0.4662
P value summary	ns
Are variances significantly different?	No

Appendix AC: **Statistical Analysis of the Anti-inflammatory Study on *Vernonia kotschyana* Ethanol Extract (VKEE) at 100 mg/kg in Rats *i.p.* versus Normal Saline (negative control)**

ANALYSIS	**RESULTS**
Column A	N/saline
vs	vs
Column B	100 (VKEE)

Unpaired t test	
P value	0.0007
P value summary	***
Are means signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=5.359 df=8
How big is the difference?	
Mean ± SEM of column A	0.2160 ± 0.01077 N=5
Mean ± SEM of column B	0.1260 ± 0.01288 N=5
Difference between means	0.0900 ± 0.01679
95% confidence interval	0.05128 to 0.1287
R squared	0.7822
F test to compare variances	
F, DFn, Dfd	1.431, 4, 4
P value	0.3684
P value summary	ns
Are variances significantly different?	No

Appendix AD: **Statistical Analysis of the Anti-inflammatory Study on *Vernonia amygdalina* Crude Flavonoids (VACF) at 25 mg/kg in Rats *i.p.* versus Normal Saline (negative control)**

ANALYSIS	**RESULTS**

Column A	N/saline
vs	vs
Column B	25 (VACF)
Unpaired t test	
P value	0.1692
P value summary	ns
Are means signif. different? (P < 0.05)	No
One- or two-tailed P value?	Two-tailed
t, df	t=1.511 df=8
How big is the difference?	
Mean ± SEM of column A	0.2160 ± 0.01077 N=5
Mean ± SEM of column B	0.1900 ± 0.01342 N=5
Difference between means	0.02600 ± 0.01720
95% confidence interval	-0.01367 to 0.06567
R squared	0.2221
F test to compare variances	
F, DFn, Dfd	1.552, 4, 4
P value	0.3404
P value summary	ns
Are variances significantly different?	No

Appendix AE: **Statistical Analysis of the Anti-inflammatory Study on *Vernonia amygdalina* Crude Flavonoids (VACF) at 50 mg/kg in Rats *i.p.* versus Normal Saline (negative control)**

ANALYSIS	RESULTS
Column A	N/saline
vs	vs
Column B	50(VACF)
Unpaired t test	
P value	0.0008
P value summary	***
Are means signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=5.252 df=8
How big is the difference?	
Mean ± SEM of column A	0.2160 ± 0.01077 N=5
Mean ± SEM of column B	0.1360 ± 0.01077 N=5
Difference between means	0.0800 ± 0.01523
95% confidence interval	0.04488 to 0.1151
R squared	0.7752
F test to compare variances	
F, DFn, Dfd	1.000, 4, 4
P value	0.5000
P value summary	ns
Are variances significantly different?	No

Appendix AF: **Statistical Analysis of the Anti-inflammatory Study on *Vernonia amygdalina* Crude Flavonoids (VACF) at 100 mg/kg in Rats *i.p.***

versus Normal Saline (negative control)

ANALYSIS	RESULTS
Column A	N/saline
vs	vs
Column B	100(VACF)
Unpaired t test	
P value	0.0002
P value summary	***
Are means signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=6.299 df=8
How big is the difference?	
Mean ± SEM of column A	0.2160 ± 0.01077 N=5
Mean ± SEM of column B	0.1160 ± 0.01166 N=5
Difference between means	0.1000 ± 0.01587
95% confidence interval	0.06339 to 0.1366
R squared	0.8322
F test to compare variances	
F, DFn, Dfd	1.172, 4, 4
P value	0.4406
P value summary	ns
Are variances significantly different?	No

Appendix AG: **Statistical Analysis of the Anti-inflammatory Study on *Vernonia kotschyana* Crude Flavonoids (VKCF) at 25 mg/kg in Rats *i.p.* versus Normal Saline (negative control)**

ANALYSIS	RESULTS
Column A	N/saline
vs	vs
Column B	25(VKCF)
Unpaired t test	
P value	0.1780
P value summary	ns
Are means signif. different? (P < 0.05)	No
One- or two-tailed P value?	Two-tailed
t, df	t=1.477 df=8
How big is the difference?	
Mean ± SEM of column A	0.2160 ± 0.01077 N=5
Mean ± SEM of column B	0.1940 ± 0.01030 N=5
Difference between means	0.0220 ± 0.01490
95% confidence interval	-0.01236 to 0.05636
R squared	0.2142
F test to compare variances	
F, DFn, Dfd	1.094, 4, 4
P value	0.4662
P value summary	ns
Are variances significantly different?	No

Appendix AH: **Statistical Analysis of the Anti-inflammatory Study on *Vernonia kotschyana* Crude Flavonoids (VKCF) at 50 mg/kg in Rats *i.p.* versus Normal Saline (negative control)**

ANALYSIS	RESULTS
Column A	N/saline
vs	vs
Column B	50 (VKCF)
Unpaired t test	
P value	0.0003
P value summary	***
Are means signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=6.085 df=8
How big is the difference?	
Mean ± SEM of column A	0.2160 ± 0.01077 N=5
Mean ± SEM of column B	0.1400 ± 0.006325 N=5
Difference between means	0.07600 ± 0.01249
95% confidence interval	0.04720 to 0.1048
R squared	0.8223
F test to compare variances	
F, DFn, Dfd	2.900, 4, 4
P value	0.1635
P value summary	ns
Are variances significantly different?	No

Appendix AI: Statistical Analysis of the Anti-inflammatory Study on *Vernonia kotschyana* Crude Flavonoids (VKCF) at 100 mg/kg in Rats *i.p.* versus Normal Saline (negative control)

ANALYSIS	RESULTS
Column A	N/saline
vs	vs
Column B	100(VKCF)
Unpaired t test	
P value	0.0441
P value summary	*
Are means signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=2.386 df=8
How big is the difference?	
Mean ± SEM of column A	0.2160 ± 0.01077 N=5
Mean ± SEM of column B	0.1720 ± 0.01497 N=5
Difference between means	0.0440 ± 0.01844
95% confidence interval	0.001479 to 0.08652
R squared	0.4158
F test to compare variances	
F, DFn, Dfd	1.931, 4, 4
P value	0.2698
P value summary	ns
Are variances significantly different?	No

Appendix AJ: Statistical Analysis of the anti-inflammatory Study on *Vernonia amygdalina* Crude Saponins (VACS) at 25 mg/kg in Rats *i.p.* versus Normal Saline (negative control)

ANALYSIS	RESULTS
Column A	N/saline
vs	vs
Column B	25 (VACS)
Unpaired t test	
P value	0.0297
P value summary	*
Are means signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=2.640 df=8
How big is the difference?	
Mean ± SEM of column A	0.2160 ± 0.01077 N=5
Mean ± SEM of column B	0.1800 ± 0.008367 N=5
Difference between means	0.03600 ± 0.01364
95% confidence interval	0.004550 to 0.06745
R squared	0.4655
F test to compare variances	
F, DFn, Dfd	1.657, 4, 4
P value	0.3183
P value summary	ns
Are variances significantly different?	No

Appendix AK: **Statistical Analysis of the Anti-inflammatory Study on *Vernonia amygdalina* Crude Saponins (VACS) at 50 mg/kg in Rats *i.p.* versus Normal Saline (negative control)**

ANALYSIS	RESULTS
Column A	N/saline
vs	vs
Column B	50(VACS)
Unpaired t test	
P value	0.0364
P value summary	*
Are means signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=2.509 df=8
How big is the difference?	
Mean ± SEM of column A	0.2160 ± 0.01077 N=5
Mean ± SEM of column B	0.1560 ± 0.02135 N=5
Difference between means	0.0600 ± 0.02392
95% confidence interval	0.004849 to 0.1152
R squared	0.4403
F test to compare variances	
F, DFn, Dfd	3.931, 4, 4
P value	0.1067
P value summary	ns
Are variances significantly different?	No

Appendix AL: **Statistical Analysis of the Anti-inflammatory Study on *Vernonia amygdalina* Crude Saponins (VACS) at 100 mg/kg in Rats *i.p.* versus Normal Saline (negative control)**

ANALYSIS	RESULTS
Column A	N/saline
vs	vs
Column B	100(VACS)
Unpaired t test	
P value	0.0009
P value summary	***
Are means signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=5.087 df=8
How big is the difference?	
Mean ± SEM of column A	0.2160 ± 0.01077 N=5
Mean ± SEM of column B	0.1140 ± 0.01691 N=5
Difference between means	0.1020 ± 0.02005
95% confidence interval	0.05576 to 0.1482
R squared	0.7639
F test to compare variances	
F, DFn, Dfd	2.466, 4, 4
P value	0.2017
P value summary	ns

Are variances significantly different?	No

Appendix AM: **Statistical Analysis of the Anti-inflammatory Study on *Vernonia kotschyana* Crude Saponins (VKCS) at 25 mg/kg in Rats *i.p.* versus Normal Saline (negative control)**

ANALYSIS	**RESULTS**
Column A	N/saline
vs	vs
Column B	25(VKCS)
Unpaired t test	
P value	0.0089
P value summary	**
Are means signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=3.434 df=8
How big is the difference?	
Mean ± SEM of column A	0.2160 ± 0.01077 N=5
Mean ± SEM of column B	0.1660 ± 0.009798 N=5
Difference between means	0.05000 ± 0.01456
95% confidence interval	0.01642 to 0.08358
R squared	0.5958
F test to compare variances	

F, DFn, Dfd	1.208, 4, 4
P value	0.4295
P value summary	ns
Are variances significantly different?	No

Appendix AN: **Statistical Analysis of the Anti-inflammatory Study on *Vernonia kotschyana* Crude Saponins (VKCS) at 50 mg/kg in Rats *i.p.* versus Normal Saline (negative control)**

ANALYSIS	**RESULTS**
Column A	N/saline
vs	vs
Column B	50(VKCS)
Unpaired t test	
P value	0.0173
P value summary	*
Are means signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=2.992 df=8
How big is the difference?	
Mean ± SEM of column A	0.2160 ± 0.01077 N=5
Mean ± SEM of column B	0.1640 ± 0.01364 N=5
Difference between means	0.0520 ± 0.01738
95% confidence interval	0.01193 to 0.09207

R squared	0.5281
F test to compare variances	
F, DFn, Dfd	1.603, 4, 4
P value	0.3293
P value summary	ns
Are variances significantly different?	No

Appendix AO: **Statistical Analysis of the Anti-inflammatory Study on *Vernonia kotschyana* Crude Saponins (VKCS) at 100 mg/kg in Rats *i.p.* versus Normal Saline (negative control)**

ANALYSIS	**RESULTS**
Column A	N/saline
vs	vs
Column B	100(VKCS)
Unpaired t test	
P value	0.0026
P value summary	**
Are means signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=4.309 df=8
How big is the difference?	
Mean ± SEM of column A	0.2160 ± 0.01077 N=5

Mean ± SEM of column B	0.1320 ± 0.01625 N=5
Difference between means	0.08400 ± 0.01949
95% confidence interval	0.03905 to 0.1290
R squared	0.6989
F test to compare variances	
F, DFn, Dfd	2.276, 4, 4
P value	0.2227
P value summary	ns
Are variances significantly different?	No

Appendix AP: Statistical Analysis of Ketoprofen (10 mg/kg) used as a Standard Analgesic in Mice *i.p.* versus Normal Saline (negative control) when Evaluating Crude Extracts of the two Plants

ANALYSIS	**RESULTS**
Column A	N/saline
vs	vs
Column B	10 (Ketoprofen)
Unpaired t test	
P value	P<0.0001
P value summary	***
Are means signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=7.427 df=8

How big is the difference?	
Mean ± SEM of column A	22.80 ± 1.772 N=5
Mean ± SEM of column B	7.400 ± 1.077 N=5
Difference between means	15.40 ± 2.074
95% confidence interval	0.62 to 20.18
R squared	0.8733
F test to compare variances	
F, DFn, Dfd	2.707, 4, 4
P value	0.1791
P value summary	ns
Are variances significantly different?	No

Appendix AQ: Statistical Analysis of Ketoprofen (10 mg/kg) used as a Standard Analgesic in Mice *i.p.* versus Normal Saline (negative control) when Evaluating Crude Flavonoids and Saponins of the two plants

ANALYSIS	**RESULTS**
Column A	N/saline
vs	vs
Column B	10 (Ketoprofen)
Unpaired t test	
P value	0.0066
P value summary	**

Are means signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=3.639 df=8

How big is the difference?

Mean ± SEM of column A	20.00 ± 2.588 N=5
Mean ± SEM of column B	8.400 ± 1.860 N=5
Difference between means	11.60 ± 3.187
95% confidence interval	4.250 to 18.95
R squared	0.6234

F test to compare variances

F, DFn, Dfd	1.936, 4, 4
P value	0.2689
P value summary	ns
Are variances significantly different?	No
