PHARMACOGNOSTIC AND ANTIBACTERIAL STUDIES OF THE LEAVES OF *CHROMOLAENA ODORATA* (L.) KING & ROBINSON (ASTERACEAE)

BY

OHANELE CHUKWUMA CYRINUS

DEPARTMENT OF PHARMACOGNOSY AND DRUG DEVELOPMENT

FACULTY OF PHARMACEUTICAL SCIENCES

AHMADU BELLO UNIVERSITY ZARIA

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BY

OHANELE CHUKWUMA CYRINUS

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DEPARTMENT OF PHARMACOGNOSY AND DRUG DEVELOPMENT

FACULTY OF PHARMACEUTICAL SCIENCES

AHMADU BELLO UNIVERSITY, ZARIA

AUGUST 2011

DECLARATION

I hereby declare that this thesis entitled “Pharmacognostic and Antibacterial Studies of the Leaves of *Chromolaena odorata* (l.) King & Robinson (Asteraceae)” is a record of my own research work under the supervision of Prof. (Mrs.) N. Ilyas and Dr. A. Agunu

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.

Ohanele, Chukwuma Cyrinus	_____	August 2011
Name of Student	Signature	Date

CERTIFICATION

This thesis entitled 'PHARMACOGNOSTIC AND ANTIBACTERIAL STUDIES OF THE LEAVES OF *CHROMOLAENA ODORATA* (L.) KING & ROBINSON (ASTERACEAE)' by **Ohanele**, Chukwuma Cyrinus, meets the regulation governing the award of the degree of Masters of Science of Ahmadu Bello University and it is approved for its contribution to knowledge and literary presentation.

_____ _____

PROF. (MRS.) N. ILYAS; B.Sc., M.Sc., M.Phil., Ph.D. Date

Chairman Supervisory Committee

_____ _____

DR. A. AGUNU; B.Pharm., M.Sc., Ph.D. Date

Member Supervisory Committee

_____ _____

Dr. M. S. ABUBAKAR; B.Pharm., M.Sc., Ph.D. Date

Head of Department

_____ _____

Prof. A.A. JOSHUA; B.A., M.A., Ph.D. Date

Dean Postgraduate School

DEDICATION

This thesis is dedicated to my wife, Mrs. Uchechi Chukwuma Ohanele for her unflinching support.

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ABSTRACT

The plant, *Chromolaena odorata* (L.) King & Robinson is a member of the Asteraceae family. It grows abundantly in Southern Nigeria where its leaves are used as traditional herbal treatment for wounds and fevers. The plant, *C. odorata* was therefore investigated for antibacterial activities. The leaves of *C. odorata* were collected from plants growing in their natural habitat in Kaduna State. Standard pharmacognostic methods were used to conduct macroscopic and microscopic studies of the leaf. Physical constants of the leaf were also determined. Furthermore, phytochemical and biological evaluations of the leaf extracts and fractions were conducted. The matured, fresh leaf of *C. odorata* is green in colour, mildly hairy and deltoid to ovate-lanceolate in shape. The leaf margin is dentate. The leaf measures between 6 – 8 cm in width and 8 – 10 cm in length. The leaf is amphistomatic with anomocytic stomata and non-glandular, uniseriate and multicellular trichomes. The stomata were more in the abaxial surface than in the adaxial with means of 9 ± 1.35 and 3 ± 0.45, respectively. The abaxial surface equally had higher stomatal index of 24.6 % ± 3.69 compared to 7.4 % ± 1.05 for the adaxial surface. Vein islet number and vein termination number were 5.8 ± 0.87 and 4.8 ± 0.72, respectively. The palisade ratio has a mean value of 6.5 ± 0.98. The powdered drug has a moisture content of 9.78 % ± 1.5. The ash value and the acid insoluble ash value were 12.13 % ± 1.82 and 4.11 % ± 0.62 respectively. The alcohol extractive value was 4.87 % ± 0.73, while water extractive value was 3.47 % ± 0.52. The phytochemical study demonstrated the presence of flavonoids, triterpenes, tannins, saponins and anthraquinones in the ethanolic leaf extract. The ethanol

extract was further partitioned into aqueous, I-butanol and chloroform fractions. Flavonoids and triterpenes were present in all the fractions while tannins were found only in the aqueous and butanol fractions. The chromatographic resolution of ethanolic extract with the solvent system Benzene: Methanol (9:1) showed over 10 spots visible in the daylight. Phytochemical tests indicated that most of the bands represented flavonoids. The ethanol extract and the chloroform, butanol and aqueous fractions showed various degrees of antibacterial activities against *Staphylococcus aureus*, *Streptococcus pyogenes*, *Salmonella typi* and *Escherichia coli.* The chloroform fraction however, had the most antibacterial activity with the highest zones of inhibition (*S. aureus* (31 mm), *S. pyogene* (30 mm), *S. typhi* (29 mm) and *E. coli* (29 mm)), lowest minimum inhibitory concentration (MIC) (*S. aureus* (2.5 mg/ml), *S. pyogene* (2.5 mg/ml), *S. typhi* (5 mg/ml) and *E. coli* (5 mg/ml)) and minimum bactericidal concentration (MBC) (*S. aureus* (5 mg/ml), *S. pyogene* (10 mg/ml), *S. typhi* (20 mg/ml) and *E. coli* (10 mg/ml)). This study therefore showed that the leaf extract of *C. odorata* has broad-spectrum antibacterial properties against the common bacteria that infect wounds. This result suggests the scientific reason for the traditional use of the leaf of the plant for wound treatment. Thus, the plant can be an effective and easily accessible herbal drug for the treatment of wounds.

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ABBREVIATIONS, GLOSSARIES AND SYMBOLS

Abaxial	=	Lower
Adaxial	=	Upper
ABUTH	=	Ahmadu Bello University Teaching Hospitals
ABU	=	Ahmadu Bello University
$AlCl_3$	=	Aluminum chloride
ATCC	=	American Type Culture Collection
DMSO	=	Dimethyl sufoxide
et al.	=	And others
A1	=	Aqueous fraction
B1	=	I-Butanol fraction
C1	=	Chloroform fraction
E1	=	70 % Ethanolic extract
P1	=	Petroleum Ether extract
Cm^3	=	Cubic centimetre
Dr	=	Doctor
D/W	=	Distilled Water
$FeCl_3$	=	Ferric chloride

Fig	=	Figure
hrs	=	Hours
HCl	=	Hydrochloric acid
H_2S0_4	=	Sulphuric acid
kg	=	Kilograms
mg	=	Milligram
w/w	=	Weight by weight
mm	=	Millimetre
SE	=	Standard error of mean
±	=	Plus/minus
%	=	Percentage
<	=	probability less than
TLC	=	Thin layer chromatography
R_f	=	Retardation factor
Staph	=	Staphylococcus
MIC	=	Minimum inhibition concentration
MBC	=	Minimum bactericidal concentration
mins	=	minutes

NCTC	=	National Collection of Type Cultures
SE	=	South East
^{0}C	=	Degree Celsius
g	=	Gram
Sq	=	Square
Ppt	=	precipitate
NaOH	=	Sodium hydroxide
Pet ether	=	Petroluem ether (60^oC – 80^oC)
+ve	=	Present
-ve	=	Absent
V/V	=	Volume by volume
RBC	=	Red blood cell (erythrocyte)
Prof	=	Professor
LS	=	Longitudinal section
TS	=	Transverse section

CHAPTER ONE

1.0 INTRODUCTION

1.1 Pharmacognosy

Right from the beginning of recorded history, man has always tried to understand himself and his environment. Man has also strived to use the knowledge gathered from studying his environment to his advantage. He successfully learnt to use materials from his environment to provide food, shelter, clothing and medicine, among others, for himself. The use of plants, animal parts and products, and minerals as medicine by man dates back thousands of years. Information on the medicinal properties of natural products was probably passed from generation to generation orally. However, with the advent of writing, documented records became available. One of the earliest records of the use of plants as medicine is that of species of Hydnocarpus Gaertn. The Chaulmoogra oil from the plant was recorded as a treatment for leprosy in the Pharmocopoeia of the Emperor Shen Nung of China between 2700 and 3000BC (Le Strange, 1977). Other early records of the use of plants as medicine were found in Egypt and India (Evans, 2002).

In modern times, the study and documentation of plants as a source of natural medicine has become more systematic. A complete understanding of medicinal plants involves a number of disciplines such as botany, chemistry, commerce, pharmacology, genetics and quality control. These disciplines, over time, have become unified under one broad subject known as pharmacognosy. The term ‘Pharmacognosy’ was probably first coined by Johann Schimidt (1759 – 1809). It is

derived from the Greek *Pharmakon*, ‘a drug’ and *gignosco*, ‘to acquire knowledge of’. Although pharmacognosy is principally concerned with plant materials, there are small numbers of animal products that are traditionally encompassed within the subject. These animal products include beeswax, gelatin, wool fat, vitamins, antibiotics, etc. Some minerals used by man for their medicinal properties are also of interest to pharmacognosy. Thus, pharmacognosy is the study of the biological effect that chemicals in medicinal plants have on cell cultures, animals and humans. In a broader sense, pharmacognosy can also be described as the study of crude drugs derived from plants, animal and mineral sources (Evans, 2002).

Pharmacognosy has gained impetus in recent years due to the observed shortcomings of modern medicine. Moreover, the realization that plants could hold the answer to many health issues of today such as malaria, cancer and diabetes, among others, has focused attention on pharmacognosy.

1.2 Traditional Medicine

The focus of pharmacognosy is traditional medicine. This is because traditional medicine is the primary source of information for pharmacognosy. Traditional medicine can be described as, ‘the total combination of knowledge and practices, whether explicable or not, used in diagnosing, preventing, or eliminating a physical, mental, or social disease and which may rely exclusively on past experience and observation handed down from generation to generation, verbally or in writing.’ In Africa, traditional medicine is often practiced in such a way that the material world, the sociological environment and the metaphysical forces of the

universe are combined to achieve results. Thus, traditional medicine depending on the situation employs plant, animal and minerals in addition to community participation and invocation of the spirit world through incantations (Sofowora, 1993).

In Nigeria, traditional medicine is sometimes referred to as native medicine, folk medicine or herbal medicine. Practitioners of traditional medicine are officially referred to as Traditional Medicine Practitioners (TMP). Informally however, they are sometimes called 'native doctors' or 'herbalist'. Traditional medicine has remained relevant in Nigeria despite the improvements in modern medicine. For example, 69 % of Yorubas, 47 % of Hausas and 32 % of Igbos admitted to the use of herbs in treating malaria (Idowu *et al*, 2006).

1.3 Medicinal Plants

Most of the drugs studied in pharmacognosy come from plants. A medicinal plant is any plant which in one or more of its organs contains substances that can be used for therapeutic purposes or which are precursors for the synthesis of useful drugs. This definition includes food, spice and perfumery from plant sources such as ginger, used medicinally. Microscopic plants such as fungi used for isolation of drugs (eg antibiotics) are also termed medicinal plants (Sofowora, 1993).

Some medicinal plants have found worldwide application. These include the Cinchona tree and *Artemisia annua* Linn, the sources of the antimalaria drugs, quinine and artemisinin, respectively. Others include *Taxus brevifolia*, the source of promising anticancer drug, taxol and *Rauwolfia serpentine*, the source of

reserpine used for treating hypertension and neuropsychiatric disorders (Evans, 2002). In the USA, from 1959 to 1980, about 25 % of all prescription dispensed in pharmacies contain active ingredients that are extracted from higher plants. Many more medicinal plants products are found in over-the-counter products as food supplements (Farnsworth, 1988).

The therapeutic properties of medicinal plants have been found to be due to the presence of secondary metabolites. The secondary metabolites were thought as waste products. However, it is now believed that secondary metabolites are biosynthesized to aid the producer's survival. Secondary metabolites include a vast array of compounds. Some of which are responsible for the characteristic odours, pungencies and colours of plants. Other secondary metabolites give plants their culinary, poisonous or medicinal properties. However, many secondary metabolites are of obscure value to the plant and to humans. The nature and quantity of secondary metabolites depend on the plant's growth and development. These are affected by environmental conditions such as temperatures, rainfall, length of day and altitude. Thus, the environment under which it grows may affect the medicinal value of a particular medicinal plant (Evans, 2002).

1.4 The Plant: *Chromolaena odorata*

One of the medicinal plants commonly used in some parts of Nigeria is *Chromolaena odorata* King & Robinson (formerly called *Eupatorium odoratum* Linn.). It is commonly known as Siam weed, Christmas bush and common floss flower (Obute and Adubor, 2007). In Igbo language, it is called 'obiara ohuru', 'nsi-

igwulube' or 'achara awolowo'. In Yoruba language, the plant is called 'akintola' or 'awolowo' while in Hausa, it is called 'chiyawa awolowo'. *C. odorata* is a shrub of the sunflower family (Asteraceae). The Asteraceae or Compositae belong to the order Asterales and is the second largest family of flowering plants (Angiosperms), in terms of number of species. Compositae, an older name of the family refers to the characteristic inflorescence. The Asteraceae family comprises of more than 1600 genera and 23,000 species. Asteraceae are cosmopolitan, but most common in temperate regions and tropical mountains. They are commonly referred to as aster, daisy or sunflower family and are native to North America (Scott, 2006). Members of this family including plants such as *Ageratum conyzoides* Linn*, Aspilia africana* C.D. Adams, *Vernonia amygdalina* Del, *Artemisia annua* Linn and *C. odorata,* grow well in wet tropical regions of the world. Some members of the family, including the above-mentioned ones, have medicinal properties and are used in herbal medicine in both Nigeria and some other countries (Obute and Adubor, 2007). The classification of *C. odorata* is as follows (Schmidt and Shilling, 2000):

Kingdom	-Plantae
Subkingdom	-Tracheobionta
Superdivision	- Spermatophyta
Division	- Magnoliophyta
Class	- Magnoliopsida
Subclass	- Asteridae
Order	- Asterales
Family	- Asteraceae

Genus - Chromolaena

Species - *Chromolaena odorata* (L.) King & H. Rob

Chromolaena odorata is a perennial, diffuse, scrambling shrub that is mainly a weed in plantation crops and pastures of Southern Asia and Western Africa. It forms a bush 3 m to 7 m in height when growing in the open. The plant has abundant yellowish, fine lateral roots. Multiple sprouts arise from the root crown and lower stems. Older stems are brown and woody, tips and young stems are green and succulent. The leaves are opposite, deltoid to ovate-lanceolate and usually with dentate margin and a long pointed tip. The inflorescences are corymbs of cylindrical heads located on the terminals of lateral branches. The seeds are brownish gray to black achene that is 4 mm long with pale brown pappus 5 or 6mm long (McFadyen and Skarratt, 1996, and Liogier, 1997). It is a neotropical plant and was spread widely by early navigators in the mid 1800s. In 1900s, it became a weed problem in West, Central and Southern Africa. It was actually accidently introduced into Nigeria in 1937 through contaminated seeds of the forest tree, *Gmelina arborea* . The plant, *C. odorata* is of multiple and sometimes contradictory values to man. It is a nuisance as weed but could be employed as good compost by farmers. The plant also has medicinal purposes such as treatment for fever, toothache, skin infection and dressing of infested wound (Obute and Adubor, 2007).

1.5 Phytochemicals

Phytochemicals are plant substances synthesized during secondary metabolism. They are responsible for the medicinal properties of medicinal plants. Specific phytochemicals have been identified as responsible for particular pharmacological actions. The isolation and identification of these phytochemicals from a plant help to demonstrate that the reported physiological activity of the plant is real. Detailed pharmacological and other academic studies become possible once a specific biological activity is linked to the presence of a particular chemical in a plant (Sofowora, 1993). Phytochemicals found in most medicinal plants include alkaloids, terpenes, and glycosides (tannins, saponins, anthraquinones and flavonoids, etc.) (Evans, 2002).

1.5.1 Alkaloids

Alkaloids are chemically, heterogeneous group of compounds having a basic character and containing at least one nitrogen in a heterocyclic ring. The term ‘alkaloid’ (or alkali-like) was coined by a German Pharmacist, W. Meissner in 1819 for basic nitrogen-containing components of plant origin. Alkaloids occur in about 15 % of medicinal plants in more than 150 families, 500 genera and over 1,200 species. They are highly potent medicament and possess curative properties no matter how trace they may occur in plants (Osadebe, 2002). Some medicinal uses of alkaloids include analgesic (eg. Morphine), anti-malaria (eg. Quinine), anti-tumors (eg. Vincristine) (Obi and Onuoha, 2000). Qualitative tests for the detection of alkaloids in a drug are mostly based on the formation of

precipitated or microcrystalline complexes. Some reagents used for these tests include, Mayer's, Wagner's, Dragendorff's, and Hager's Reagents (Ashutosh, 2007).

1.5.2 Terpenes

Terpenes or terpenoids are natural products whose structures are composed of several isoprene (C_5H_8) units. Terpenoids are broadly classified based on the number of isoprene units joined in a head to tail manner incorporated into a specific unsaturated hydrocarbon. Classes of terpenoids include monoterpenoids ($C_{10}H_{16}$), sesquiterpenoids ($C_{15}H_{24}$) diterpenoids ($C_{20}H_{32}$), etc. Terpenoids occur in most families of angiosperms, with few exceptions such as the Moraceae family. Terpenoids are of medicinal value to man. They have been found to play roles such as antipyretic, antiseptic, antifungal, etc. (Glasby, 1982). Chemical tests for terpenes include Liebermann Burchard's Reagent for triterpenes (Kokate, 1994).

1.5.3 Glycosides

The term glycoside is a generic named used for natural products chemically bound to one or more sugar. Thus, glycosides consist of two parts – a non-sugar part called aglycone (genin) and a sugar part called glycone. The aglycone portion is soluble in non-polar solvents while the glycone portion is soluble in polar solvents. Based on the chemical nature of the aglycone portion, glycosides are classified into the following types: tannin glycosides, saponin glycosides, anthraquinone glycosides, flavonoid glycosides, cardiac glycosides, etc. Generally, glycosides

play various medicinal roles in man such as cathartic, cardiatonic, analgesic, anti-ulcer, etc. (Ashutosh, 2007).

1.5.3.1 Tannins

Tannins are complex organic, non-nitrogenous derivatives of polyhydroxy benzoic acids. Most tannins are in the form of glycosides. They are non-crystalline substances and form colloidal solution with water. Tannins are precipitated by gelatin and alkaloids. They form complex with proteins, which is resistant to proteolytic enzymes (astringent action). Tannins are widely distributed in plant families such as Leguminosae, Rubiaceae, Asteraceae, Moraceae, etc. They occur in leaves, unripe fruits, barks and roots. Tannins are used by man for tanning of animal hides and skin and precipitation of gelatin and alkaloids in the laboratory. They are also used as astringents, healing of burns, etc. (Evans, 2002). Chemical tests for tannins include, ferric chloride test, Goldbeater's skin test, gelatin test, phenazone test, etc. (Haslan, 1989).

1.5.3.2 Saponins

Saponins are chemically derived from either tetracyclic steroid or the pentacyclic triterpenoid, both with glycosidal linkage at C-3. They are broadly regarded as haemotoxic in nature because they cause haemolysis of erythrocytes. They are soluble in alcohol and water but insoluble in non-polar organic solvents like benzene. Saponins are distributed among several plant families. They are used as demulcents and mild expectorant and for their detergent property

(Ashutosh, 2007). Frothing and haemolysis tests are used to detect saponins (Torsell, 1996).

1.5.3.3 Anthraquinones

Anthraquinones are mostly referred to as anthracene glycosides. They are hydroxyl compounds occurring at different levels of oxidation state in plants. They occur mainly in dicots and the family Liliaceae is the only monocot known to possess them (Glasby, 1982). They are used as laxatives and cathartics. Chemical tests for anthraquinones are Bontrager's test (free anthracene) and Modified Bontrager's test (combined anthracene) (Sofowora, 1993).

1.5.3.4 Flavonoids

Flavonoids constitute one of the largest classes of naturally occurring plant products. They are mostly phenols either in free state or as glycoside. The chemical structure of flavonoids is based on C6-C3-C6 carbon skeleton having a pyran ring with a second benzene ring strategically positioned at C2 and C3 or C4. In nature, they exist as flavones, flavonols, Isoflavones and anthocynidins. They sometimes also occur as biflavonoid. Flavonoids occur in many plant families such as Polygonaceae, Asteraceae, Moraceae, Leguminosae, etc. They are found in stem barks and leaves mostly. They are used as diuretic and antispasmodic agents. Some posses antibacterial and antifungal properties. Some are used to decrease capillary fragility and reduction of capillary bleeding. Tests for flavonoids include ferric chloride ($FeCl_3$), sodium hydroxide (NaOH), lead acetate, zinc

hydrochloric acid test, Wieslen boric acid test and Shinoda's tests (Ashutosh, 2007).

1.6 Extraction and Separation Techniques

Extractives are pharmaceutical preparations obtained from plant and animal tissues with the aid of solvents and standardized extraction procedures. These preparations contain active principles, which have been separated as much as possible from inert constituents. Thus, extraction precedes separation. The major modes of extraction include maceration, percolation, digestion, infusion and decoction (Ashutosh, 2007). In this investigation, extraction by maceration technique was employed.

1.6.1 Maceration Technique

Maceration consists of soaking the properly comminuted drug in the menstrum until the cellular structure is thoroughly penetrated and the soluble portions are softened and dissolved. Powdered drugs are macerated in definite quantities of menstrum that could consist of alcohol of various strengths, ether, ether/alcohol or other specified solvents. After maceration, the extracts are separated using chromatographic techniques (Ashutosh, 2007).

1.6.2 Chromatography

Chromatography is a separation technique based on preferential movements between solvents of two immiscible phases. Various types of chromatographic techniques include column chromatography, Thin Layer Chromatography (TLC),

High Performance Liquid Chromatography (HPLC), etc. The constituent of interest and the nature of work would determine the type of chromatographic technique to be used. However, the most common technique is TLC. TLC usually involves spotting on a silica or alumina plate inserted into a tank containing a solvent mixture. TLC is simple, cheap, and fast and can accommodate many spots. Identification of phytochemicals can also be done with TLC by spraying the developed plate with appropriate detecting reagents. Separated constituents such as alkaloids, flavonoids and steroids are eluted using appropriate solvents (Evans, 2002).

1.7 Bacteria and Antibacterial Assay

Microorganisms or microbes are living organisms that cannot be seen with the naked eyes. They usually grow as single cells or colonies of multicellular filaments (fungi moulds). Many microbes are parasites while some are free living. Examples of microbes include protozoa (Plasmodium), fungi (Candida), viruses (HIV) and bacteria. Bacteria are prokaryotes, which can be classified on morphological basis into ovoid or spheroid coccus (Staphylococcus species), rod or cylindrical bacillus (Bacillus species), spirillium (*Trepanonema palladium*) and vibrio (*Vibrio cholerae*). Bacteria, based on the nature of the cell wall, can also be classified as either Gram positive (Streptococcus species) or Gram negative (*Escherichia coli*). Bacteria could be motile or non-motile; with the motile ones possessing flagella.

Bacteria, like most microbes are mostly harmless to man. Many are in fact of benefit to man. However, some bacteria are harmful to man by causing infection.

An infection that causes noticeable impairment of the body function is called an infectious disease. Disease is a product of the interaction between host and pathogen. The outcome of the relationship depends not only on the pathogenicity or on virulence of the parasite (bacteria), but also on the resistance or susceptibility of the host. A pathogen must first gain access to the host tissues and multiply before producing disease. To do this, the organism must penetrate the surface, such as the skin, mucous membranes or intestinal epithelium, which normally acts as barriers. Thus, injuries like burns and wounds that damage any of these barriers facilitate bacterial infection (Baker *et al*, 1998).

Besides man's natural resistance (immune system) to microbial infections, external agents known as antimicrobials are used to prevent or eliminate infection. Antimicrobials have various mechanisms of action which include the following:

a. Affecting cell wall peptidoglycan biosynthesis.

b. Affecting the cell membrane.

c. Inhibiting DNA synthesis.

d. Inhibiting protein synthesis.

An antimicrobial that acts on bacteria is known as antibacterial. An antibacterial, depending on the mode of action can be bacteriostatic or bactericidal. The effectiveness of an antibacterial agent is determined in the laboratory by susceptibility testing. The two main methods used in antibacterial susceptibility testing are dilution and diffusion methods. Each of these may be carried out by a

variety of techniques like the Stokes Method and Bauer-Kirby technique. For quantitative estimates of antibacterial activity, Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) tests are carried out (Baker *et al*, 1998).

However, many bacteria have developed elaborate strategies for avoiding the host's immune responses and many antibacterial agents. This has necessitated the continuous search for new and different antibacterial agents. Medicinal plants hold enormous potentials in this regard. Some medicinal plants with confirmed antibacterial properties include the fungus, *Penicillium notatum* (penicillin), and species of Juniperus and Pinus (essential oils). Other plants with antibacterial properties are garlic (*Allium sativum*) and ginger (*Zingibir officinale*), among others (Evans, 2002).

1.8 Wound and Wound Healing

Encarta English Dictionaries defined wound as 'injury in which the skin, tissue or an organ is broken by some external force as a blow or surgical incision, with damage to underlying tissue'. The same dictionary defined sore as a painful skin infection or wound. Similarly, the term, wound also includes injury to the skin caused by chemicals, cold, friction, heat pressure and rays; and manifestation in the skin of internal conditions (Roper, 1995).

Wounds arise from tissue damage caused by various agents. These agents could be mechanical, chemical, radiant energy or pathogenic micro-organisms. The severity of the agent determines the extent of injury. Wounds can be classified into

bruises or contusion and open wounds. Contusions are caused mostly by blunt trauma resulting to disruption of the connective tissue with extravasations of blood. The epithelium might remain intact thereby protecting the damaged tissue from microbial insult. On the other hand, open wounds could range from mere loss of the superficial layers of the epithelium to deep penetrating injuries. The former is known as abrasion while the later could be referred to as puncture or lacerated wounds, depending on the causative agent and the size (Badoe *et al,* 2000).

Secondary bacterial invasion is a problem in all open wounds. The likelihood of infection increases with the period of exposure of an open wound. Moreover, with the exception of surgical wounds, all open wounds must be regarded as contaminated. Time is therefore of essence in the management of wounds. Common bacteria implicated in wound infection include *Staphylococcus aureus, Streptococcus pyogenes, Escherichia coli, Pseudomonas aeruginosa* and Bacterioides species, among others (Cheesbrough, 2006).

Treatment of wounds usually involve various procedures including appropriate positioning of the injured part; minimizing blood loss; debridement; prevention/treatment of bacterial infection and wound closure. The body reacts both locally and systemically to commence wound healing, when injured. Essentially, wound healing involves the 3 steps of epithelialization, synthesis of fibrous tissue and wound contraction. Systemically, factors such as age of patient, severe constitutional disease and nutrition affect this process. Locally, oxygen, blood supply and residual infection, among others, affect wound healing. Consequently, it is essential that any treatment of wound must include stopping

excessive loss of blood without damaging blood supply to the wound site. In addition, treatment should include preventing or clearing residual bacterial infection while encouraging formation of fibroblast at the site. Orthodox treatment of wound therefore, usually involves arrest of bleeding using cotton wool and bandages, application of antiseptics (eg. Iodine solution) and administration of anti-inflammatory (eg. ibuprofen) drugs (Badoe *et al,* 2000).

Traditionally, wound treatment in parts of Nigeria ranges from the folkloric application of some herbs as first aid to arrest bleeding, to careful and professional application of herbs to tackle sores and chronic festering wounds. Some medicinal plants used for this purpose include *Aspilia africana* C.D. Adams, *Carica papaya* L. and *Psidium guajava* L., among others (Obute and Adubor, 2007)..

1.9 Statement of Research Problems

Chromolaena odorata is used by folks in many parts of Nigeria as traditional medicine to arrest bleeding and treat infested wounds. While previous studies have shown that styptic and anti-inflammatory properties of the plant could encourage wound healing, broad-spectrum antibacterial activities have not been established. However, a major hindrance to effective wound healing is bacterial infection. There is therefore the need to investigate the plant for antibacterial activities.

1.10 Justification for the Study

Chromolaena odorata is found growing abundantly in many parts of the rain forest belt of Nigeria. The leaf of the plant is widely used as herbal remedy for various

ailments such as fever, skin infections and infested wound by folks in parts of Nigeria. There is therefore the need to evaluate the antibacterial properties of *C. odorata* growing in Kaduna, Northern Nigeria. There is also increasing incidences of resistant strains of bacteria to orthodox antibiotics. The styptic, anti-inflammatory and anti-bacterial properties of the plant coupled with its abundance in many parts of Nigeria promises huge economic potential for rural dwellers.

1.11 Aims and Objectives of the Study

This research will be conducted to achieve the following:

a. To establish the various physical parameters of the leaf of *C. odorata.*

b. To carry out phytochemical screening of the leaf extracts.

c. Conduct antibacterial studies on the leaf extract of *C. odorata*.

1.12 Hypothesis

The leaves of *Chromolaena odorata* have phytochemical constituents with antibacterial activities that make them capable of aiding wound healing.

CHAPTER TWO

2.0 LITERATURE REVIEW

The Asteraceae family to which *C. odorata* belongs to is one of the largest in the plant kingdom. It has thousands of species, some of which are useful to man as ornamental and medicinal plants. Asteraceae family is known to possess many secondary metabolites useful to man such as terpenes, alkaloids and glycosides. Polyphenols like flavonoids and tannins are abundant in the Asteraceae family. Many of these phytochemicals possess styptic, anti-inflammatory, antimicrobial and anti-diabetic properties, among others (Evans, 2002).

Perhaps, the most important member of the Asteraceae family today is the Wormwood, *Artemisia annua* Linn. The plant and its derivatives are the most rapidly acting anti-malarial known and are highly selective against malaria parasites. The drug, artemisinin is a sesquiterpenoid lactone. In Asia, some other species of Artemisia are also used for their medicinal values. *A. capillaries* Thunberg contains essential oils (pinene, capillene, etc) and is used as a diuretic and cholertic. *A. indica* L. has antidiabetic properties (Evans, 2002).

In Africa, some members of the Asteraceae family used medicinally include *Vernonia amygdalina* Linn. (Bitter leaf, Igbo: *olugbu*, Hausa: *shiwaka*, Yoruba: *ewuro*), *Ageratum conyzoides* Linn (Igbo: *agadi-isi-awo-ocha,* Yoruba: *ako yunyun,* Ijaw*: furu ituka*), *Aspilia africana* C.D. Adams (Igbo: *ura-njila*), *Emilia coccinea* C. Don (Igbo: *ntiene*, Yoruba: *odundun odo.* Ijaw: *kalama toru edede*) and *Chromolaena odorata* Linn. *V. amygdalina* is used as a laxative and to treat

pile and hyperglyceamia, among others. The plant, *V. amygdalina* contains myricetin, a flavonol (Aiyeloja and Bello, 2006). *Ageratum conyzoides* contains luteolin (flavone) and the leaf juice is used to treat eye infection and arrest bleeding. The plant decoction is used to treat fever. *Aspilia africana* contains the flavonol, gossypetin. It is used as a diuretic and to stop bleeding and treat wounds. *C. odorata* has total phenol content of 3.51 mg/g compared to 3.01 mg/g and 1.76 mg/g for *V. amygdalina* and *A. africana*, respectively. Phenolic compounds posses strong anti-oxidant and antimicrobial properties (Obute and Adubor, 2007).

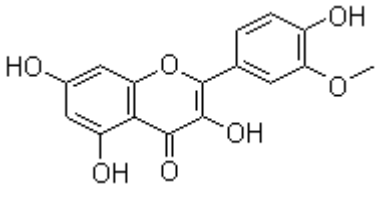
Many members of the family Asteraceae such as *A. conyzoides, A. africana, Emilia coccinea* and *C. odorata* are used for treating wound. Other plants used for wound healing in parts of Nigeria include *Phyllanthus amarus* (family: Euphorbiaceae; Igbo: *nkwu-ana,* Hausa: *geeron tsusaayee,* Yoruba: *eyin olubi*), *Newbouldia laevis* (family: Bignoniaceae; Igbo: *ogilisi,* Hausa: *aduruku,* Yoruba: *akoko*), *Carica papaya* (Caricaceae; Igbo: *okworo bekee,* Hausa: *gwanda,* Yoruba: *ibepe*), *Psidum guajava* (Myrtaceae, Igbo: *ugova*, Hausa: *gwaabaa*, Yoruba: *gurofa*), and *Asclepsis lineolata* (Asclepiadaceae; Igbo: *okazi,*) Aiyeloja and Bello, 2006 and Obute and Adubor, 2007).

In Southern part of Nigeria, the leaves of *C. odorata* are used for wound dressing, skin infection and to stop bleeding. Other uses of the leaf of *C. odorata* include treatment of diabetes (leaf infusion) and skin rashes (crushed leaves) (Metwally and Ekejuba, 1981; Aiyeloja and Bello, 2006 and Obute and Adubar, 2007). In Vietnam and other tropical countries, fresh leaves or decoction of the leaves are

used for the treatment of leech bite, soft tissue wounds, burn wounds, skin infection and dento-alveolitis (Phan *et al*, 2001). A formulation prepared from the aqueous extract of the leaves of *C. odorata* has been licensed for clinical use in Vietnam to treat skin irritations (Ling *et al*, 2005). More support for the medicinal use of the leaves of *C. odorata* include a study which showed that methanol extract of the leaves had anti-inflammatory, antipyretic and antispasmodic properties in mice (Taiwo *et al,* 2000). Aqueous extract of the leaf of *C. odorata* showed anti-inflammatory activity in rat (Owoyele *et al,* 2005). Extracts of leaves of *C. odorata* have been found to encourage proliferation of fibroblasts and delay the visible signs of skin aging such as wrinkles and hyper-pigmentation. This knowledge was used to formulate a cosmetic composition containing extracts of *C. odorata* for the prevention of normal skin conditions due to chrono-ageing or photo ageing (Donovan, 2007). It was also demonstrated that the leaf extract of *C. odorata* has anti-oxidant, anti-inflammatory and anti-staphylococcal properties, but no cytotoxic effect on normal cell lines selected for the study (Ling *et al*, 2006).

The plant, *C. odorata*, besides its medicinal uses is also applied as manure to crops since it decays fast, releasing its high nitrate content to the soil. It also has larvicidal properties and is especially employed against black fly (Matur and Davou, 2007). Although, *C. odorata* is presently not consumed as food in Nigeria, it has been recommended as an edible vegetable. This is because of its comparative high protein content, consumer acceptability test and availability during the dry season when most edible vegetables are scarce (Akpovughaye, 2009).

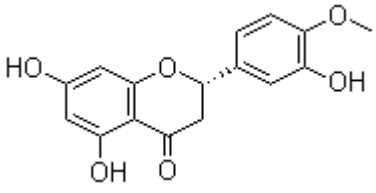
Many phytochemicals have been detected in the leaves of *C. odorata*. The flavonol, isorhamnetin that is known to have therapeutic properties has been isolated from *C. odorata* (Obute and Adubor, 2007).



($C_{16}H_{12}O_7$)

Figure 2.1: Isorhamnetin (3,5,7-Trihydroxy-2-(4-hydroxy-3-methoxyphenyl)-4H-1-benzopyran-4-one)

It has also been shown that the leaves of *C. odorata* have volatile oil in higher percentage from sunset to midnight (Inya-Agha *et al*, 1987). The phytochemicals, tannins, steroids, terpenoids, flavonoids and cardiac glycoside have been detected from both methanolic and aqueous extracts of leaves of *C. odorata*. Alkaloids were only detected in the methanolic extract (Akinmoladun *et al*, 2007). Similarly, six flavonoids were obtained from ethanolic extract of the leaves of *C. odorata*. Three of them were isolated from this plant for the first time including 3,5,4'-trihydroxy-7-methoxyflavanone; 5,7,3'-trihydroxy-5'-methoxyflavanone and 3',5,7-Trihydroxy-4'-methoxyflavanone (Pisutthanan *et al,* 2005).



($C_{16}H_{14}O_6$)

Figure 2.2: Hesperetin (3', 5, 7-Trihydroxy-4'-methoxyflavanone)

Furthermore, 16 compounds consisting of 11 flavonoids and 5 of low molecular weight aromatic compounds have been isolated from the leaves. Moreover,

flavonoids have been identified as the major group of compounds present in the leaves of *C. odorata*. It was also shown that kaempferol-4-methyl ether was the major compound and had high anti-oxidant activities (Ling *et al,* 2006). In general, flavonoids are characteristic of certain plant families including, Asteraceae (Evans, 2002).

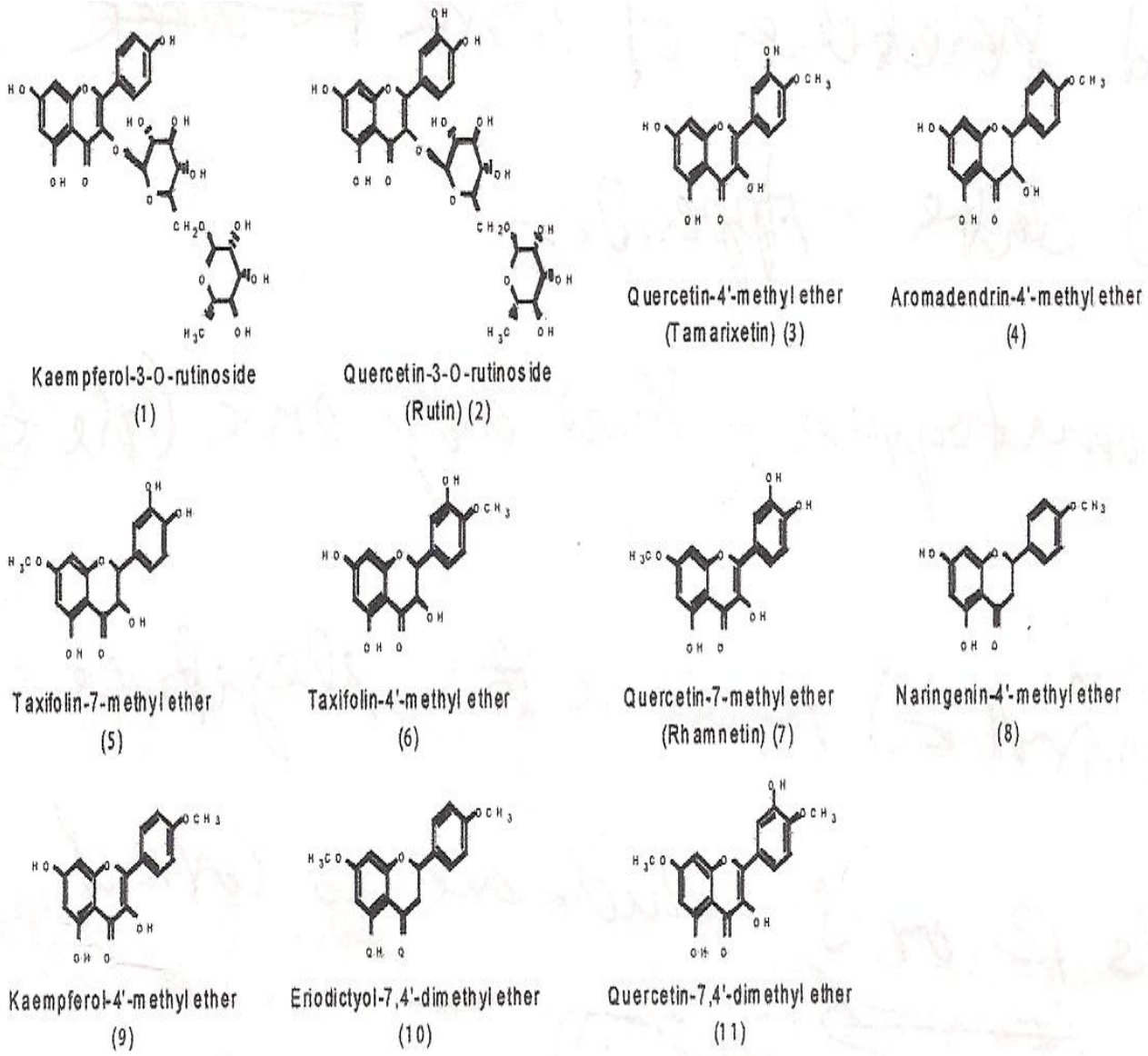


Figure 2.3: Structures of Flavonoids Isolated from C. odorata

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Equipments, Solvents and Reagents

The following equipments, solvents and reagents were used to carry out the experimental procedures of the project:

3.1.1 Equipments

Mettler electronic balance, Fisher Scientific hot air oven (600 series), water bath (HH-S6), Fisher Scientific microscope (S90009 series), Fisher Scientific stage and eyepiece micrometers, Swift-Ives camera Lucida, Pyrex TLC tank, pre-coated TLC plate (made of silica gel on aluminum foil by Merck), Gallenkamp incubator.

3.1.2 Reagents and Solvents

Distilled water (D/W), absolute ethanol, petroleum ether ($60^{o}C$ – $80^{o}C$), chloral hydrate, glycerin water, Mayer's reagent, Wagner's reagent, Dragendoff's reagent, sulphoric acid (H_2SO_4), ferric chloride ($FeCl_3$), phenazone solution, sodium acid phosphate buffer (pH 7.4), benzene, ammonia solution, magnesium chips, zinc chips, hydrochloric acid (HCl), sodium hydroxide (NaOH), l-butanol, chloroform, acetone, methanol, butanone, blood agar (Fluka, Sigma-Aldrich), normal saline, 10 % erythrocyte suspension and 1 % ethanolic solution of aluminum chloride. All of which were of standard grades were used.

3.2 Plant Collection and Identification

The plant was collected fresh, from a forest by the edge of a stream at Sabon Tasha Kaduna, Nigeria. The plant was identified at the herbarium unit of the Department of Biological Sciences, Ahmadu Bello University, Zaria by the University taxonomist, Mr U.S. Gallah where a voucher number of 1128 is deposited for reference.

3.3 Preparation of Plant Material

The leaves were plucked, sorted, washed in clean water and then air-dried for six days. The dried leaves were then powdered using local mortar and pestle into coarse powder.

3.4 Macroscopy

A fresh and matured leaf of *C. odorata* was plucked from the flowering plant and examined macroscopically. The colour, texture, shape and size of the leaf were noted.



Plate I: **Aerial parts of *Chromolaena odorata* Plant**

3.5 Examination of Microscopical Characters

Both qualitative and quantitative microscopy were carried out to determine the quality and quantity of the defining anatomical features of the leaf of *C. odorata* as follows:

3.5.1 Qualitative Microscopy

A fragment of the middle lamina of the leaf was cleared by boiling in chloral hydrate solution. Each surface of the leaf was peeled off, mounted on glycerin water and examined microscopically using X 10 and X 40 objectives. The presence and features of some anatomical characters such as epidermal cells, stomata and trichomes were noted. In addition, TS and LS through the midrib of the leaf were made and examined microscopically (Kokate, 1994).

3.5.2 Determination of Stomatal Number and Stomatal Index

A fragment of leaf from the middle lamina was cleared by boiling in chloral hydrate solution. The upper and lower epidermis of the leaf were peeled using forceps, and mounted separately in glycerin water. A square 2 x 2 mm was drawn using stage micrometer and camera Lucida on drawing paper, and the stage micrometer was replaced by the cleared leaf preparation and focused. The number of the stomata and epidermal cells were counted (two guard cells and ostiole being counted as one) within the square. The stomatal index was calculated using the formula:

$$S.I = \frac{S}{E + S} \times 100$$

Where S.I = Stomatal Index, E = Epidermal Cells, and S = Number of Stomata (Evans, 2002).

3.5.3 Determination of Palisade Ratio

Scrapings from the upper leaf surface were separately cleared by boiling with chloral hydrate solution and mounted in glycerin water and then focused under high power (X 40) magnification. A camera Lucida was attached and the outlines of four continuous epidermal cells were traced and marked using pencil. Palisade cells within each group were counted including those that were more than half covered by the epidermal cells. The values obtained from the counts were divided by four to obtain the palisade ratio (Evans, 2002).

3.5.4 Determination of Vein-Islet and Vein Termination Numbers

A leaf sample was boiled in chloral hydrate solution in a test tube placed in boiling water bath until it was cleared. It was then mounted in glycerin water. A camera Lucida was set up to divide the paper into 2 sq mm by means of the stage micrometer. The stage micrometer was then replaced by the cleared leaf preparation on a slide, and the veins were traced in a square of 2 mm x 2 mm, following the superimposition of image of the leaf portion and paper. The numbers of vein – islets and vein-lets termination present within the square were counted and the total numbers of each were divided by four to get the value in square mm. A total of 4 sets of counts were made (Evans, 2002).

3.6 Determination of Physical Constants

Various physico-chemical parameters of the leaves were conducted using standard methods. These are:

3.6.1 Moisture Content

Three grams of powdered plant was accurately weighed in tarred silica dish. It was then heated for one hour at 105^0C, cooled in desiccators and weighed. The procedure was repeated until there was no further loss in weight (i.e until a constant weight was obtained). The percentage moisture content was calculated with reference to the initial weight of the powdered drug (Kokate, 1994).

$$\text{Moisture content} = \frac{\text{final weight of drug}}{\text{initial weight}} \times 100.$$

The number of replicate (n) is three (3).

3.6.2 Total Ash Value

Two grams of the powdered leaves was weighed accurately into nickel crucibles. The contents were heated gently over Bunsen burner until they were moisture free and completely charred. The heating was continued until all the carbon was removed. The contents were cooled and weighed until constant weight was obtained. The total ash value was then calculated with reference to the initial mass of powdered leaves (2 g) (Kokate, 1994).

$$\text{Ash Value} = \frac{\text{final weight of ash}}{\text{initial weight of drug}} \times 100.$$

The number of replicate (n) is three (3).

3.6.3 Acid Insoluble Ash Value

The total ash produced from the above (3.6.2) was transferred to a beaker containing 25ml of dilute HCl and boiled for five minutes. The insoluble matters were then filtered through an ash-less filter paper. The contents in the beakers were washed in hot water, and the washings were passed through the filter paper. The washings were continued until the beaker was free from acid. The residue and the filter paper were dried gently and ignited in tarred crucibles. The crucibles were cooled and weighed. Acid insoluble ash values were then calculated with reference to the initial weight of the drugs (Kokate, 1994).

$$\text{Acid insoluble ash value} = \frac{\text{weight of residue}}{\text{initial weight of drug}} \times 100.$$

The number of replicate (n) is three (3).

3.6.4 Alcohol Soluble Extractive Value

Five grams of powered drugs were shaken with 95 % ethanol in 250 ml Stoppard flasks for 6 hrs using mechanical shaker and allowed to stand for 18 hrs. The mixture was filtered immediately. Then 20 ml of the filtrates were transferred into tarred evaporating dish whose weight was already known. Using hot plate, the filtrate was evaporated to dryness and dried to constant weight in an oven and the final weight known. The alcohol extractive values were calculated with reference to the initial weight of powdered drugs (Kokate, 1994).

$$\text{Alcohol Soluble extractive} = \frac{\text{final weight of extract}}{\text{initial weight of drug}} \times 100.$$

The number of replicate (n) is three (3).

3.6.5 Water Soluble Extractivpe Value

Five grams of powered drugs were shaken with chloroform water (0.25 % v/v chloroform in pure water) in 250 ml stoppered flasks for 6 hrs using mechanical shaker and allowed to stand for 18 hrs. It was then filtered and 20 ml of the filtrates were transferred into tarred evaporating dish. Using hot plate, they were evaporated to dryness and dried to constant weight in an oven and the final weight known. The water-soluble extractive values were calculated with reference to the initial weight of powdered drugs (Kokate, 1994).

$$\text{Water soluble extractive} = \frac{\text{final weight of extract}}{\text{initial weight of drug}} \times 100.$$

The number of replicate (n) is three (3).

3.7 Extraction and Fractionation of Plant Materials

Dried and coarsely powdered leaves of *C. odorata* (500 g) were mixed with 1000 ml petroleum ether (60 – 80^{o}C) in a conical flask covered and left to stand, with frequent shaking, for 2 days at room temperature. The extract was decanted off and fresh quantity of petroleum ether was added, covered and left to stand for 2 days (also with frequent shaking). The combined petroleum ether extracts (14.68 g or 4.9 %) were concentrated and designated P1. The defatted leaves were completely dried and extracted with 70% ethanol in water. The

combined ethanol extracts were concentrated on water bath whereby 72.65 g (14.53 %) brownish mass was obtained and designated E1. The percentage yield was then calculated using the following formula:

$$\text{Percentage yield} = \frac{\text{weight of extract}}{\text{Weight of powdered drug}} \times 100$$

Thereafter, 30 g of E1 was then treated with hot water and filtered. The water-soluble fraction was then partitioned with chloroform and l-butanol, each of the fractions were dried under reduced pressure and marked C1 and B1 respectively. The aqueous residue was concentrated on water bath and marked A1.

3.8 Phytochemical Screening of Ethanolic and Pet Ether Extracts and Fractions

Phytochemical screenings of the leave extracts and fractions (P1, E1, C1, B1 and A1) of *C. odorata* were carried out. The extracts (P1 and E1) and fractions (C1, B1 and A1) were screened for the presence of alkaloids, triterpenes and glycosides (tannins, saponins, anthraquinones, flavonoids, cardiac and steroidal glycosides).

3.8.1 Test for Alkaloids

About 0.5 g of each sample and fraction was stirred with 5 ml of 1 % aq. hydrochloric acid on a steam bath. One milliliter of the filtrate was treated with few drops of the following reagents:

Dragendorff's reagent: When Dragendorff's reagent was added to 1 ml of the filtrate, an orange ppt. was taken to indicate the presence of alkaloids (Evans, 2002).

***Mayer's reagent*:** When Mayer's reagent was added to 1 ml of the filtrate, turbidity or cream ppt. was taken as evidence of the presence of alkaloids (Evans, 2002).

***Wagner's reagent*:** When Wagner's reagent was added to 1 ml of the filtrate, a reddish-brown ppt. was taken as evidence of the presence of alkaloids (Evans, 2002).

3.8.2 Test for Triterpenes

***Lieberman Burchad's test*:** About 0.5 g of the sample was dissolved in 2 ml of acetic anhydride and cooled well in ice. Concentrated sulphuric acid was then carefully added. Development of reddish colour at the interphase indicates the presence of triterpenes (Evans, 2002).

3.8.3 Test for Tannins

About 0.5 g of the sample was stirred with 10 ml of distilled water and then filtered and used for the following tests:

***Ferric chloride test*:** Two drops of 10 to 15 % $FeCl_3$ was added to the filtrate, a green precipitate was taken for the presence of phenolic compounds which could be condensed tannin (Evans, 2002).

***Phenazone test*:** To about, 5 ml of the extract dissolved in water, 0.5 g of sodium acid phosphate was added, warmed, cooled and filtered. To the filtrate, 25 % solution of phanazone was added, a bulky, coloured ppt indicates the presence of tannins (Evans, 2002).

3.8.4 Test for Saponins

***Frothing test*:** About 0.5 g of plant sample was stirred in a test tube containing 5 ml of water. Foaming which persisted on warming was taken as preliminary evidence for the presence of saponins (Kokate, 1994).

***Haemolysis test*:** About 0.5g of the sample was boiled briefly with 50 ml of phosphate buffer (pH 7.4) and then allowed to cool and filtered. A few drops of the filtrate was added to 5 ml of blood, mixed and allowed to stand for one hour. Complete haemolysis of the blood cells was taken as evidence of presence of saponins (Kokate, 1994).

3.8.5 Test for Anthraquinones

Borntrager's test: About 0.5 g of the extracts was shook with 10 ml of benzene and filtered. Thereafter, 5 ml of 10 % ammonia solution was added to the filtrate and stirred. The presence of pink-red colour was taken for the presence of free anthraquinones (Sofowora, 1993).

***Modified Borntrager's test*:** A small quantity of the extract was boiled with 10 ml aq. sulphuric acid and filtered while hot. The filtrate was shaken with 5 ml of benzene. The benzene layer was separated and half its volume of 10 % ammonia solution was added. A pink, red or violet colouration in the ammonia phase (lower layer) indicated the presence of combined anthraquinones (Trease and Evans, 1989).

3.8.6 Test for Flavonoids

***Sodium hydroxide test*:** Two drops of aqueous NaOH solution were added to 0.5 g of the extract dissolved in ethanol, appearance of yellow colouration indicated the presence of flavonoids (Evans, 2002).

***Shinoda's test*:** To 2 - 3 ml of the extract dissolved in ethanol, a pinch of magnesium chips was added. Concentrated hydrochloric acid was then added; a bright violet colour confirmed the presence of flavonoids (flavones and flavonols) (Krishnaswamy, 1996).

***Zinc hydrochloric acid test*:** A small portion of the sample in was dissolved in ethanol and a few chips of zinc metal added. To the mixture, a few drops of HCl was added and heat. Pink colour develops showing the presence of flavonones and flavonols (Ashutosh, 2007)

***Wilson boric acid test*:** A small portion of the sample was dissolved in ethanol and to it, a 1:1 mixture of acetone and saturated boric acid (v/v) was added. To the solution add 10 % citric acid in acetone. A yellow colour develops indicating the presence of flavonoids (flavonones and flavonols) (Ashutosh, 2007).

3.8.7 Test for Cardiac Glycosides

Salkowski's test: About 0.5 g of the sample was dissolved in 2 ml of chloroform. Sulphuric acid was carefully added to form a lower layer. A reddish-brown colour at the interphase indicated the presence of a steroidal ring (aglycone portion of cardiac glycoside) (Sofowora, 1993).

Kellar-Kilani test: About 0.5 g of the sample was dissolved in 2 ml of glacial acetic acid containing one drop of ferric chloride solution. This was then under-layed with 1 ml concentrated sulphuric acid. A brown ring formed at the interphase indicated the presence of deoxy sugar characteristic of cardenolides (Sofowora, 1993).

3.9 Chromatographic Analysis of the Extracts and Soluble Fractions of the Leaves of C*. odorata* Using TLC Techniques

Thin Layer Chromatography plates 20 cm by 20 cm silica gel on aluminium foil (prepared by the manufacturer, Merck GMBH) were used for the chromatographic resolution of the 70 % aq. ethanol and pet ether extracts. Subsequently, the same materials were also used to chromatograph the chloroform, aqueous and I-butanol fractions. The crude extracts were dissolved in methanol, while the soluble fractions were dissolved in their respective solvents (ie. Chloroform, I-butanol and distilled water). The choiceof the solvent systems was by various trials following the suggestions advanced for the chromatographic resolutions of flavonoids and other glycosides (Stahl, 1969). The following solvent systems were tried:

- Butanol:Chloroform (5:5)
- Benzene:Methanol (9:1)
- Benzene:Methanol (8:2)
- Benzene:Acetone (9:1)
- Methanol:Chloroform (8:2)
- Water:Butanone:Methanol (4:3:3)

The solvent systems – benzene:methanol (9:1) and methanol:chloroform (8:2) presented the best separation of spots for the crude extracts and the fractions, respectively. The the spots were sprayed with appropriate reagents to determine the class of compounds they represent, thereafter, their R_f values were calculated.

3.10 Biological Evaluation of the Extracts and Soluble Fractions of the Leaves of *C. odorata*

3.10.1 Organisms

Clinical isolates of *Staphylococcus aureus*, *Strptococcus pyogenes, Corynebacterium ulceran, Salmonella typhi*, *Escherichia coli* and *Pseudomonas aeruginosa;* and Standard isolates of *S. aureus* (ATCC 13709), *S. typhi* (ATCC 9184), *E. coli* (NCTC 10418) and *P. aeruginosa* (NCTC 6750)

3.10.2 Antibacterial Screening of the Extracts of *C. odorata*

The antibacterial activities of crude extracts (E1 and P1) of the leave of *C. odorata* was determined using six pathogenic micro-organisms. The microbes were clinical isolates obtained from the Department of Medical Microbiology, Ahmadu Bello University Teaching Hospital (ABUTH) Shika, Zaria. All samples were checked for purity and maintained in slants of nutrient agar. Agar Well diffusion method as adapted from Cos *et al* (2006) was used to determine the antibacterial activities of the extracts. Half gram of each extract was emulsified in 2 ml of DMSO and then dissolved in 8 ml of distilled water to give a concentration of 50 mg/ml.

Blood agar (Fluka, Sigma-Aldrich) was used as the growth medium. It was prepared according to the manufacturer's instruction. The agar was dissolved and

boiled in conical flask capped with cotton wool. It was then sterilized at 121°C for 15 mins. The medium was cooled to 45°C before being poured into sterile petri dishes. The plates were allowed to cool and the agar solidify. The prepared medium was then seeded (prepared bacterial suspension dropped over the medium) with standard inoculum of *S. aureus* test micro-organism. Using sterile swab, the inoculum was spread evenly over the surface of the medium. The seeded plates were then allowed to dry by incubating at 37°C for 30 mins. A sterile cork borer of 6 mm in diameter was used to cut a well at the centre of each seeded plate. The solution of each extract of concentration 50 mg/ml was then inoculated into each well on the medium. Thereafter, 10 mins was allowed for diffusion of the extracts into the agar gel. The plates were then incubated at 37°C for 24 hrs, after which they were observed for zones of inhibition of growth. The zones were measured with transparent ruler and the result recorded in mm. The procedure was repeated for all the test bacteria used in this study. All tests were carried out in duplicate along with positive (ciprofloxacin and erythromycin) and negative (70 % ethanol and pet ether) controls.

3.10.3 Estimation of Minimum Inhibitory Concentration of Extracts of *C. odorata*

The Minimum Inhibitory Concentration (MIC) of the extracts was carried out on the test organisms. This was done by broth dilution method. Nutrient broth was prepared according to the manufacturer's instruction (Fluka, Sigma-Aldrich). Ten milliliters of the broth was dispensed into each test tube and sterilized at 121°C for 15 mins. The broth was allowed to cool. McFarland's turbidity

standard scale of 0.5 was prepared to give a turbid solution. Normal saline was used to make turbid suspension of the micro-organisms. This was incubated for 6 hrs at 37°C. Dilution of the micro-organisms with normal saline was done continuously until the turbidity marched that of the McFarland's by visual comparison. Two-fold serial dilution of the extract in the broth was made to obtain concentrations of 50 mg/ml, 25 mg/ml, 12.5 mg/ml, 6.25 mg/ml and 3.125 mg/ml. The initial concentration was obtained by dissolving 0.5 g of the extract in 10 ml of DMSO. Having obtained the different concentrations of the extracts in the broth, 0.1 ml of the standard inoculum of the test micro-organism was then inoculated into the different concentrations of the extracts in the test tubes. The test tubes were incubated at 37°C for 24 hrs. The lowest concentration of the extract in the test tube which shows no turbidity was recorded as the MIC of that extract (Cheesbrough, 2006).

3.10.4 Estimation of Minimum Bactericidal Concentration of the Extracts of *C. odorata*

Minimum Bactericidal Concentration (MBC) is the lowest concentration of antimicrobial required to kill a bacteria. MBC of the extracts was carried out in order to determine whether the test micro-organisms were killed or only their growth was inhibited by the samples. Blood agar was prepared according to the manufacturer's instruction. The agar was boiled to dissolve in conical flask, capped with cotton wool and was sterilized at 121°C for 15 mins, cooled to 45°C and then poured into sterile Petri dishes. The medium was allowed to cool and solidify. The contents of the tubes used in the serial dilution to obtain the MIC were

then sub-cultured onto the prepared medium, incubated at 27°C for 24 hrs. After, the plates were visually observed for bacterial colonies growth. Positive (known *S. aureus* suspension with no antibiotic) and negative (D/W) control tubes were also set up. The MBC was the plate with the lowest concentration without colony growth (Cheesbrough, 2006).

3.10.5 Antibacterial Screening of the Ethanolic Extract and Soluble Fractions of the Leaves of C*. odorata*

The screening for antibacterial activities of crude extract (E1) and fractionated portions (C1, B1 and A1) of the leaf of *C. odorata* was determined using standard strains (*S. aureus* (ATCC 13709), *S. typhi* (ATCC 9184), *E. coli* (NCTC 10418) and *P. aeruginosa* (NCTC 6750)) and clinical isolate (*S. pyogenes*). All samples were checked for purity and maintained in nutrient agar slants. Agar diffusion method of Kirby-Bauer (Cheesbrough, 2006) was used to determine the antibacterial activities of the samples. 0.8 g of the ethanol extract and 0.2 g of the each of the fractions were weighed and dissolved in 10 ml of DMSO to obtain concentrations of 80 mg/ml and 20 mg/ml respectively.

Blood agar (Fluka, Sigma-Aldrich) was used as the growth medium. It was prepared according to the manufacturer's instruction. The agar was dissolved in water and boiled in conical flask capped with cotton wool. It was then sterilized at 121°C for 15 minutes. The medium was cooled to 45°C and 20 ml portions were then poured into sterile Petri dishes. The plates were allowed to cool and agar solidify. The prepared medium was then seeded with 0.1 ml of the standard inoculum of *S. aureus*. Using sterile swab, the inoculum was spread evenly over

the surface of the agar. The seeded plates were then allowed to dry at 37°C for 30 minutes. A sterile cork borer of 6 mm in diameter was used to cut wells on each seeded plate. The extracts and controls were then inoculated into each well on the medium. The plates were then incubated at 37°C for 24 hrs, after which they were visually observed for zones of inhibition of growth. The zones were measured with transparent ruler and the result recorded in mm (Cheesbrough, 2006). The procedure was repeated for all the bacteria used in this study.

3.10.6 Estimation of the Minimum Inhibitory Concentration of the Ethanolic Extract and the Soluble Fractions of the Leaves of *C. odorata*

MIC of the extract and fractions was carried out on the test micro-organisms. This was done by broth dilution method. Nutrient broth (Fluka, Sigma-Aldrich) was prepared according to the manufacturer's instruction. Ten ml of the broth was dispensed into each test tube and sterilized at 121°C for 15 minutes. The broth was allowed to cool. McFarland's turbidity standard scale number 0.5 was prepared to give a turbid solution. Normal saline was used to make turbid suspension of the micro-organisms. This was incubated for 6 hrs at 37°C. Dilution of the micro-organisms in normal saline was done continuously until the turbidity marched that of the McFarland's by visual comparison. Two-fold serial dilution of the extract in the broth was made to obtain the concentrations of 80 mg/ml, 40 mg/ml, 20 mg/ml, 10 mg/ml and 5 mg/ml for the ethanol extract, and 20 mg/ml, 10 mg/ml, 5 mg/ml, 2.5 mg/ml and 1.25 mg/ml for the chloroform, aqueous and l-butanol extracts. The initial concentration of the ethanol extract was

obtained by dissolving 0.8 g of the extract in 10 ml of DMSO while 0.2 g of the soluble fractions were dissolved in 10 ml of DMSO. Having obtained the different concentrations of the extracts in the broth, 0.1 ml of the inoculum of the test micro-organism in normal saline was then inoculated into the different concentrations of the extracts in the test tubes. The test tubes were incubated at 37°C for 24 hrs. The lowest concentration of the extract in the test tube which shows no turbidity was recorded as the MIC of that extract.

3.10.7 Estimation of Minimum Bactericidal Concentration of the Ethanolic Extract and the Soluble Fractions of the Leaf of *C. odorata*

MBC of the extract and fractions was carried out in order to determine whether the test micro-organisms were killed or only their growth was inhibited. Blood agar (made by Fluka, Sigma-Aldrich) was prepared according to the manufacturer's instruction. The agar was boiled to dissolve in conical flask, capped with cotton wool and was sterilized at 121°C for 15 mins, cooled to 45°C and then poured into sterile Petri dishes. The medium was allowed to cool and solidify. The contents of the tubes used in the serial dilution to obtain the MIC were then sub-cultured onto the prepared medium, incubated at 27°C for 24 hrs. After, the plates were observed for bacterial colonies growth. The MBC was the plate with the lowest concentration without colony growth.

CHAPTER FOUR

4.0 RESULT

4.1 Macroscopy

The matured fresh leaf of *C. odorata* is green in colour. The arrangement of the leaves on the stem is opposite and decussate. The leaves are petiolated and deltoid to ovate-lanceolate in shape. The leaf has acute base and reticulate venation, with major veins camptodromous. The leaf has dentate margin and acuminate tip. The leaf surface is puberulent. The leaf measures between 6 – 8cm in width and 8 – 10 cm in length (Plate II).



Plate II: Upper Surface of Leaf of *Chromolaena odorata (X 1)*

4.2 Determination of Microscopical Characteristics of the Leaves of *Chromolaena odorata*

Qualitative microscopy of the leaf showed the leaf surface is amphistomatic and without cuticular striations. Both surfaces of the leaf have many non-glandular, uniseriate and multicellular trichomes (usually 4 to 6 cells), and starch granules (Plate III). The epidermal cells are polygonal to irregular to rectangular in shape. The stomata are anomocytic and circular to elliptic in shape on both surfaces (Figure 4.1).

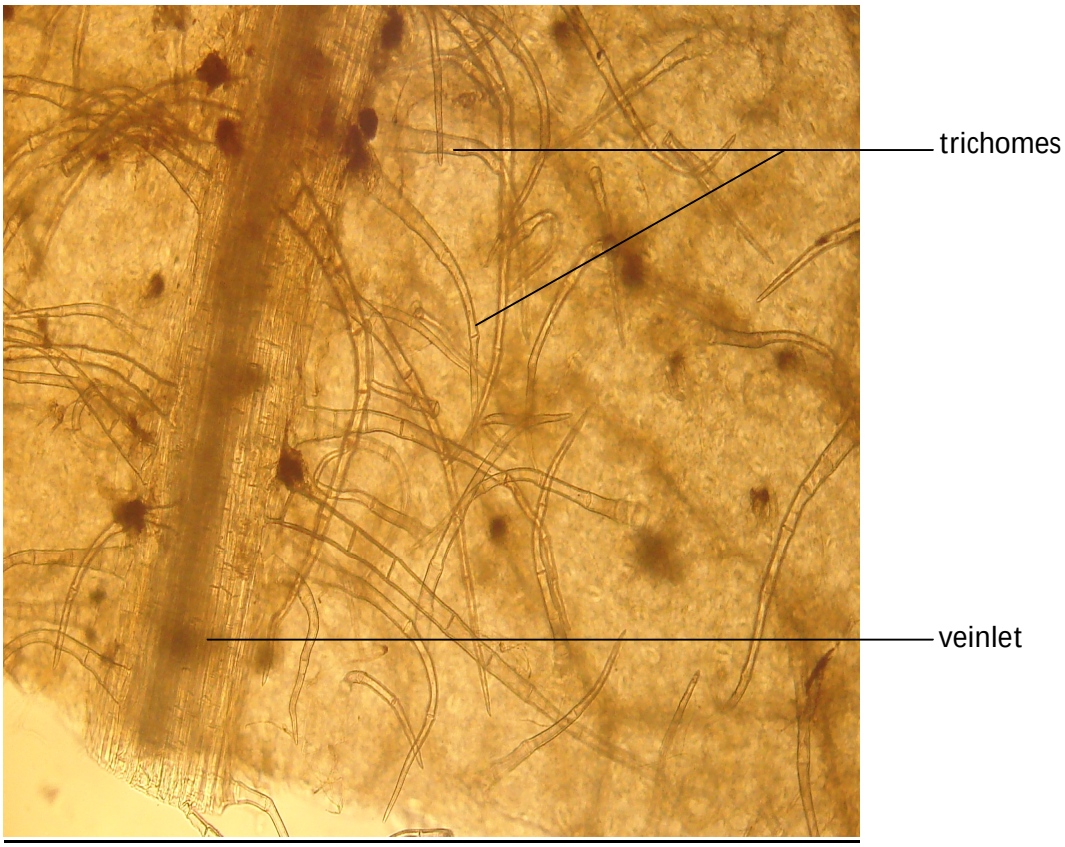


Plate III: Some Microscopic Features of the Leaf of *Chromolaena odorata* (X 400)

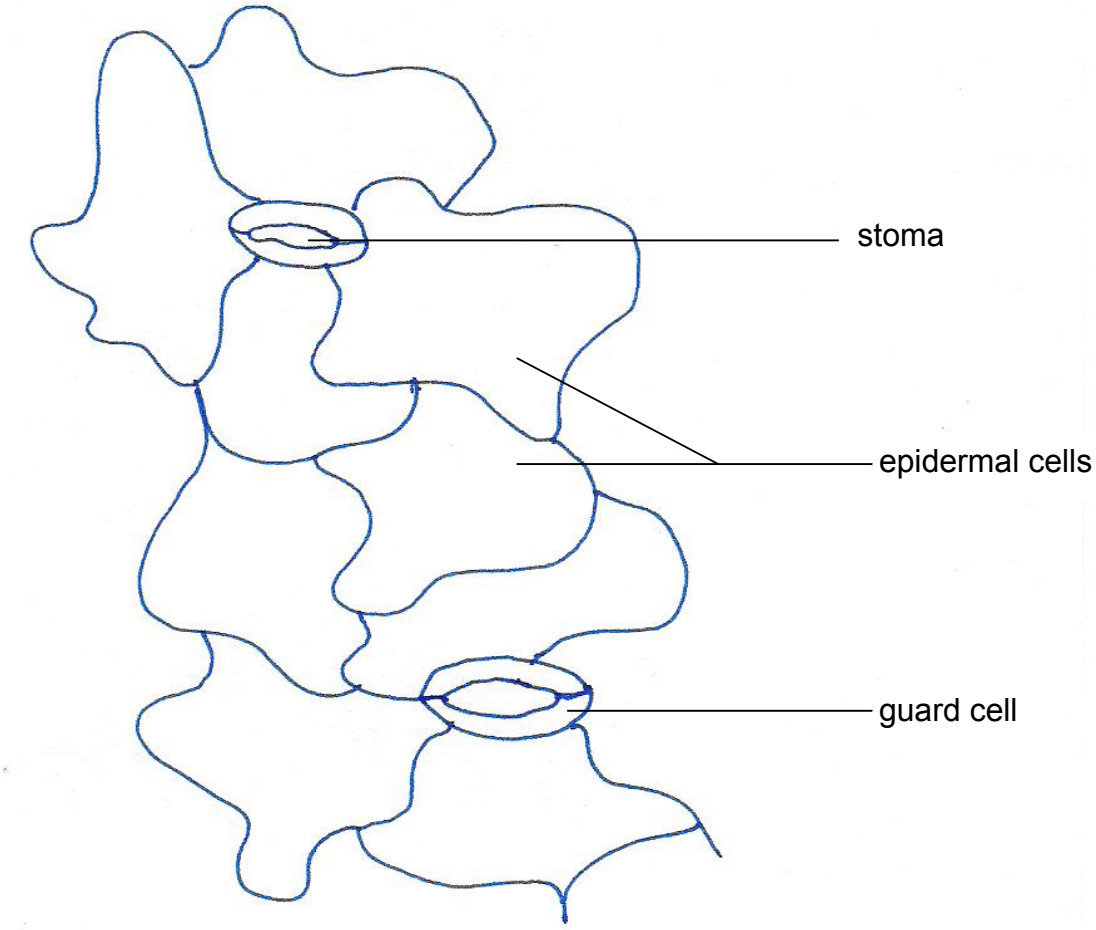


Figure 4.1: *Chromolaena odorata* Showing Anomocytic Stomata

Transverse and longitudinal sections cut through the midrib of the leaf showed epidermal cells on both surfaces covered with thin cuticle. Below the upper epidermal cells are 1 to 2 layers of vertically oriented palisade cells. The central midrib contains the main vascular bundle made up of the xylem (upper part) and the phloem. The fundamental conducting element of the xylem is the spiral vessel. Surrounding the vascular bundle tissue is the parenchyma cells, which help to support the leaf by their turgidity. Below the parenchyma cells are large cells of the spongy mesophyll (Figures 4.2 and 4.3).

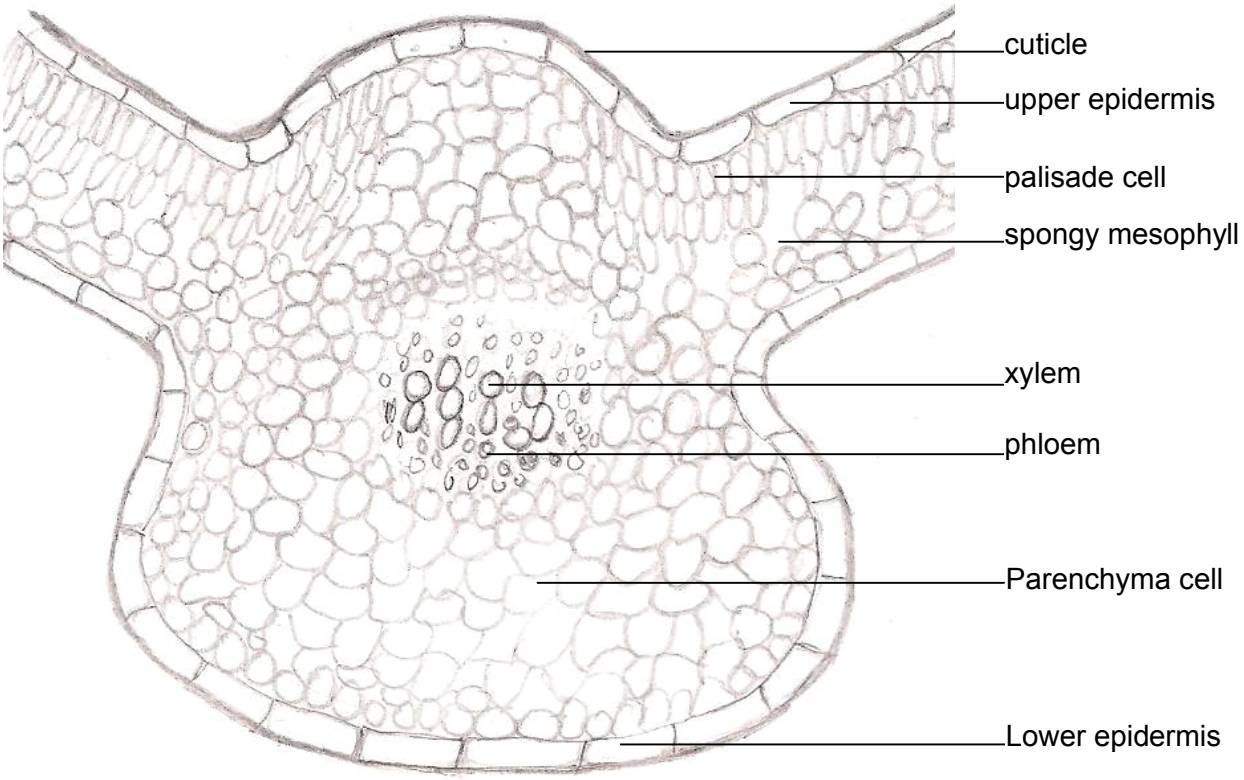


Figure 4.2: TS of the Leaf of *C. odorata* taken through the Midrib of the Leaf (X 400)

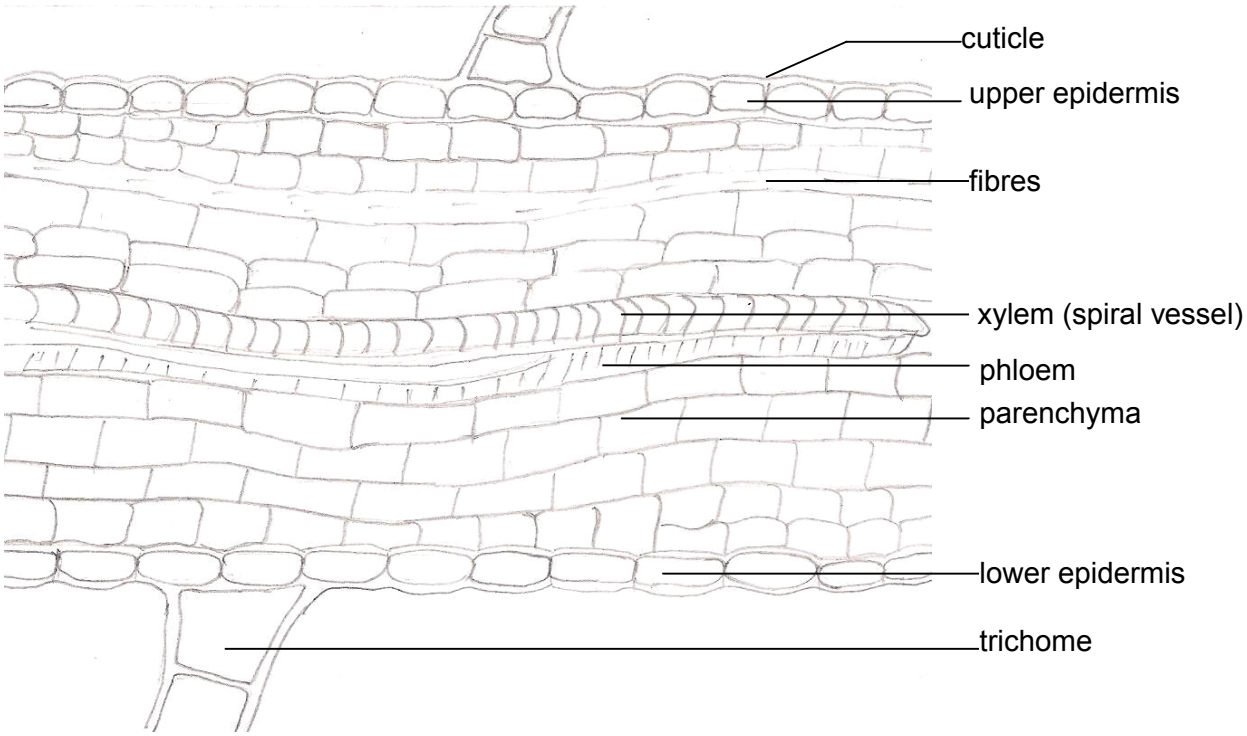


Figure 4.3: LS of the Leaf of *C. odorata* taken through the Midrib of the Leaf (X 400)

Quantitative microscopy of the leaf showed that the anomocytic stomata were more in the lower surface (abaxial) than the upper (adaxial) with means of 9 ± 1.35 and 3 ± 0.45, respectively. The abaxial surface equally had higher stomatal index of 24.6 % ± 3.69 compared to 7.4 % ± 1.05 for the adaxial surface (Table 4.3.1). Vein islet number and vein termination number were 5.8 ± 0.87 and 4.8 ± 0.72, respectively (Table 4.3.1). The palisade ratio was 5 – 7 and a mean of 6.5 ± 0.98 (Table 4.1).

TABLE 4.1: Quantitative Microscopy of the Leaves of *Chromolaena odorata*

Parameter	Upper Surface	Lower Surface
Stomatal number	2 - **3** - 4	8 - **9** - 10
Stomatal index (%)	5.95 - **7.4** - 8.05	20.91 - **24.6** -28.29
Palisade ratio	5 - **6.5** - 7	**
Vein islet number	5 - **5.8** - 7	-
Vein termination number	4 - **4.8** - 6	-

Key: ****** = Not applicable, **-** = not carried out.

4.3 Physical Constants of the Leaf of *C. odorata*

The powdered drug showed moisture content of 9.78 % ± 0.15. The ash value and the acid insoluble ash value were 12.13 % ± 1.82 and 4.11 % ± 0.62 respectively. The alcohol extractive value was 4.87 % ± 0.73 while that of water extractive value was 3.47 % ± 0.52 (Table 4.2).

Table 4.2: Physical Constants for the Leaf of *Chromolaena odorata*

Parameter	Value (%)
Moisture content	8.29 - **9.78** - 11.28
Ash value	10.31 - **12.13** - 13.95
Acid insoluble ash	3.49 - **4.11** - 4.73
Alcohol extractive value	4.14 - **4.87** - 5.6
Water extractive value	2-95 - **3.47** - 3.99

n = 3

4.4 Extraction of the Powdered Drug and Partitioning

The pet ether (P1) extract yielded 14.68 g (4.9 % w/w) of crude drug while the 70 % aq. ethanol (E1) extract yielded 72.65 g (14.53 % w/w) of crude drug Partitioning of 30 g of E1 yielded 0.52 g (1.73 % w/w) of C1, 4.14 g (13.8 %) of B1 and 12.42 g (41.4 %) of A1 fractions.

4.5 Phytochemical Screening of the Crude Extracts and Soluble Fractions of Leaves of *C. odorata*

Phytochemical screening of the ethanolic and pet. ether extracts showed the presence of flavonoids, tannins and cardiac glycosides in both. However, saponins, triterpenes, and anthraquinones were found only in the ethanolic extract (Table 4.3). Thus, more phytochemicals were recovered in the ethanolic extract than the pet ether extract.

Following the partitioning of the ethanolic extract, the aqueous, butanol and chloroform fractions all contained flavonoids and triterpenes. Tannins were present in the aqueous and butanol fractions but not in chloroform fraction. Alkaloid was absent in all fractions. (Table 4.4)

Table 4.3 Phytochemical Screening of the Crude Extracts of the Leaf of *Chromolaena odorata*

Constituent	Test	Observation		Inference	
		Ethanol	Pet ether	Ethanol	Pet ether
Alkaloids	Mayer's	No ppt.	no ppt.	-	-
	Wagner's	No ppt.	no ppt.	-	-
	Dragendoff's	No ppt.	no ppt.	-	-
Triterpenes	Lieberman Burchard's	reddish colour	bluish colour	+	-
Tannins	Ferric chloride	Green ppt.	green ppt.	+	+
	Phenazone	Thick coloured ppt.	thick coloured ppt.	+	+
Saponins	Frothing	foaming which persisted after warming	no foaming	+	-
	Haemolysis	complete haemolysis of RBCs	no haemolysis	+	-
Anthraquinone	Bontrager's	pink to reddish colour	colourless	+	-
	Modified Bontrager's	Pink to reddish colour	colourless	+	-
Flavonoid	Sodium hydroxde	yellow colouration	yellow colouration	+	+
	Shinoda	pink to reddish colour	pink to reddish colour	+	+
	Wilson boric	violet colour	violet colour		

	acid			+	+
	Zinc hydrochloric acid	wine red colour	wine red colour	+	+
Cardiac glycosides	Salkwoski's	reddish interphase	greenish interphase	+	-
	Kellar-Killani	brown interphase	brown interphase	+	+

Key: **+** = present; **-** = absent; ppt = precipitation; RBC = red blood cells

Table 4.4 Summary of Phytochemical Screening of the Fractions of the Crude Extract of the Leaves of *Chromolaena odorata*

Constituent	Test	Soluble fractions		
		Aqueous	**I-Butanol**	**Chloroform**
Alkaloids	Mayer's	-	-	-
	Wagner's	-	-	-
	Dragendoff's	-	-	-
Triterpenes	Lieberman Burchard's	+	+	+
Tannins	Ferric chloride	+	+	-
	Phenazone	+	+	-
Flavonoid	Sodium hydroxde	+	+	+
	Shinoda	+	+	+
	Wilson boric acid	+	+	+
	Zinc hydrochloric acid	+	+	+

Key: **+** = present; **-** = absent;

4.6 TLC of the Crude Extracts and Soluble Fractions of the Leaves of *C. odorata*

The chromatographic resolution of E1 with the solvent system Benzene/ Methanol (9:1) showed about 8 bands visible in the daylight (Plate IV). Colour reaction tests using $FeCl_3$ and NaOH indicated that most of the bands represented flavonoids with various R_f values as shown in Table 4.5. Thereafter, 10 % sulphuric acid, ferric chloride and 1 % ethanolic solution of aluminum chloride (visualized with UV light – 366nm) were used as detecting agents (Plates V).

TLC of the fractions revealed a couple of bands in A1 and B1 portions and a single band in the C1 portion. These were observed also with different solvent systems. However, the solvent system Methanol/Chloroform (8:2) gave the best resolution. The spots were positive for flavonoids and tannins in A1 and B1 fractions. The spot in C1 was identified as flavonoids (yellowish under daylight) using 1 % ethanolic solution of aluminum chloride as detecting agent.

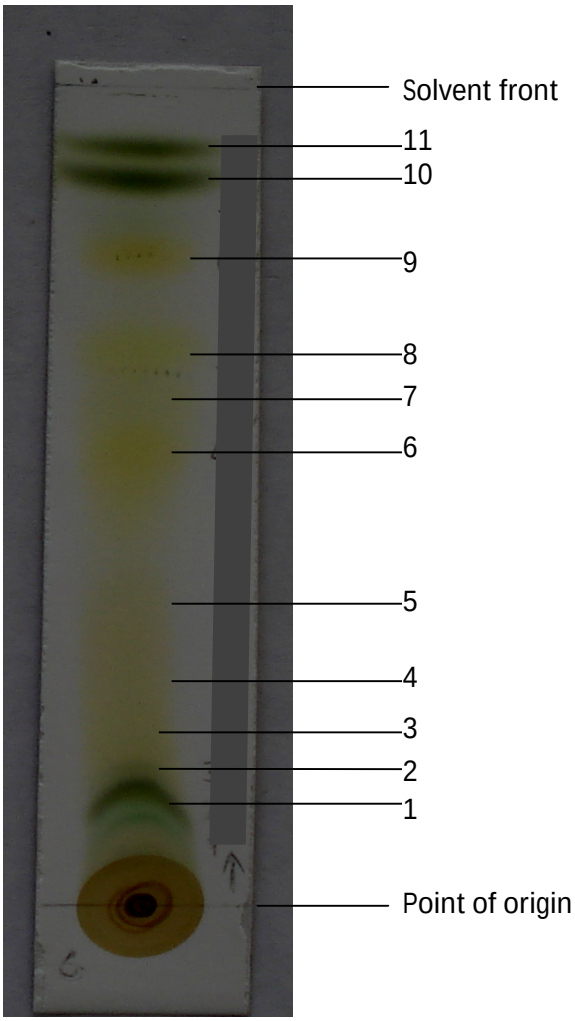


Plate IV: Chromatographic Resolution of Ethanolic Extract of leaves of *Chromolaena odorata* in the daylight

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Chromatographic resolution of the ethanolic extract of leaves of *C. odorata* using Benzene/Methanol (9:1) as the solvent system produced at least 10 distinct bands observed under daylight.

Table 4.5: Chemical Classes of Some of the Spots and their R_f Values Following TLC of E1 Using Ferric Chloride and Aluminum Chloride as Detecting Agents

Phytochemical	Band	Colour	R_f Value
Flavonoids	2	yellow	0.26
	3	yellow	0.28
	4	yellow	0.42
	5	yellow	0.43
	6	yellow	0.55
	8	yellow	0.71
	9	yellow	0.73
Tannins	7	Greenish brown	0.61

Solvent system: Benzene/Methanol (9:1)

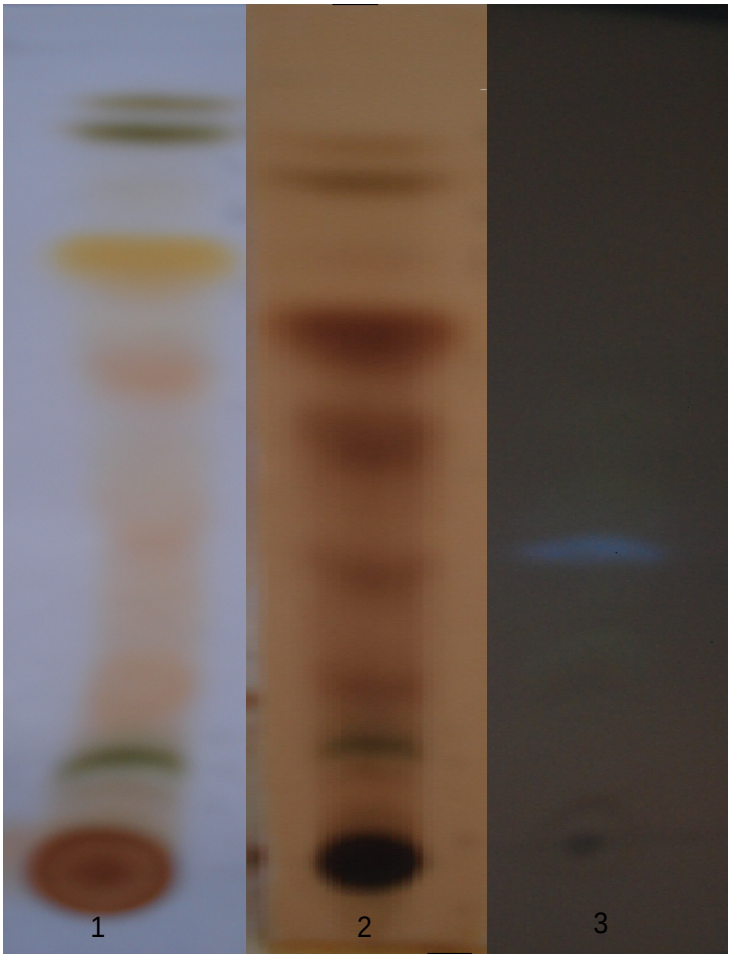


Plate V: Chromatographic Resolution of Ethanolic Extract of Leaves of *Chromolaena odorata* after Spraying with Detecting Agents

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Chromatographic resolution of the ethanolic extract of leaves of *C. odorata* using Benzene/methanol (9:1) as the solvent system. Various detecting agents were used to visualize the plates as follows:

Plate 1: Sprayed with 10 % sulphuric acid (H_2SO_4).

Plate 2: Sprayed with ferric chloride ($FeCl_3$). The resultant colour reaction revealed the presence of phenolic compounds.

Plate 3: Sprayed with 1 % ethanolic solution of aluminum chloride ($AlCl_3$) and visualized under ultra violet light (366 nm). Yellowish flourescence confirmed the presence of flavonoids.

4.7 Antibacterial Screening of the Crude Extracts of *C. odorata*

Evaluation of the antibacterial activities of the crude extracts against the clinical isolates showed that ethanolic extract (50 mg/ml) was more active against the test organisms. *S. pyogenes*, *C. ulcerans* and *P. aeruginosa* were sensitive to both extracts while *E. coli* was only sensitive to ethanolic extract. *S. aureus* and *S. typhi* were resistant to both ethanolic extract (50 mg/ml) and pet. ether extract (50 mg/ml). Negative control using D/W showed no activity against the test bacteria. On the other hand, positive controls using ciprofloxacin and erythromycin showed that the test bacteria were not multi-drug resistant strains. The zones of inhibition for ciprofloxacin on the bacteria were *C. ulcerans* (32 mm), *S. typhi* (37 mm) and *E. coli* (30 mm). For erythromycin, the zones of inhibition were *S. aureus* (30 mm), *S. pyogenes* (37 mm), *C. ulcerans* (35 mm), *S. typhi* (27 mm), *E. coli* (29) and *P. aeruginosa* (24 mm). The positive controls generally, showed higher zones of inhibition than the extracts, where applicable (Table 4.6)

Zones of inhibition by ethanolic extract on the bacteria were *S. pyogenes* (24 mm), *C. ulcerans* (20 mm), *E. coli* (17 mm) and *P. aeruginosa* (20 mm). For pet. ether extract, the zones of inhibition against *S. pyogenes*, *C. ulcerans* and *P. aeruginosa* were 15 mm, 16 mm and 17 mm, respectively.

Table 4.6: Zone of Inhibition of the Extracts of the Leaves of *Chromolaena odorata* Against the Test Bacteria (mm)

Test Organism	Extracts		Negative Control	Positive Control	
	E1	P1	D/W	Ciprofloxacin	Erythromycin
Staphylococcus aureus	0	0	0	0	30
Streptococcus pyogenes	24	15	0	0	37
Corynebacterium ulcerans	20	16	0	32	35
Salmonella typhi	0	0	0	37	27
Escherichia coli	17	0	0	30	29
Pseudomonas aeruginosa	20	17	0	0	24

Concentration of Drugs:

Ciprofloxacin = 5 mg/ml

Erythromycin = 5 mg/ml

MIC test showed that the ethanolic extract still have better activities against the test bacteria with lower MIC than pet. ether extract. The ethanolic extract had a MIC of 6.25 mg/ml compared to 12.5 mg/ml of pet. ether extract against *S. pyogenes*, *C. ulcerans* and *P. aeruginosa*. MIC of ethanolic extract against *E. coli* was 12.5mg/ml (Table 4.7).

The crude extracts were not only bacteriostatic but also bactericidal as shown by the MBC. At concentration of 25 mg/ml, ethanolic extract was bactericidal to *C. ulcerans*, *E. coli* and *P. aeruginosa*. At the same concentration, pet. ether extract was bactericidal to *S. pyogenes* and *P. aeruginosa*. This further confirms that the ethanolic extract had more active phytochemicals against the test bacteria (Table 4.8).

Table 4.7: Minimum Inhibitory Concentration of the Extracts of the Leaves of *Chromolaena odorata* Against the Test Bacteria

Test Organism	Ethanolic Extract					Pet. Ether Extract				
	50mg/ml	25mg/ml	12.5mg/ml	6.25mg/ml	3.125mg/ml	50mg/ml	25mg/ml	12.5mg/ml	6.25mg/ml	3.125mh/ml
Staphylococcus aureus	0	0	0	0	0	0	0	0	0	0
Streptococcus pyogenes	-	-	-	x	+	-	-	x	+	++
Corynebacterium ulcerans	-	-	-	x	+	-	-	x	+	++
Salmonella typhi	0	0	0	0	0	0	0	0	0	0
Escherichia coli	-	-	x	+	++	0	0	0	0	0
Pseudomonas aeruginosa	-	-	-	x	+	-	-	x	+	++

KEY: **0** = Resistant; **-** = No turbidity (no growth); **x** = MIC;

+ = Turbid (light growth); **++** = Moderate turbidity

Table 4.8 Mininmum Bactericidal Concentration of Extracts of the Leaves of *Chromolaena odorata* Against the Test Bacteria

Test Organism	Ethanolic Extract					Pet. Ether Extract				
	50mg/ml	25mg/ml	12.5mg/ml	6.25mg/ml	3.125mh/ml	50mg/ml	25mg/ml	12.5mg/ml	6.25mg/ml	3.125mh/ml
Staphylococcus aureus	0	0	0	0	0	0	0	0	0	0
Streptococcus pyogenes	-	-	x	+	++	-	x	+	++	+++
Corynebacterium ulcerans	-	x	+	++	+++	x	+	++	+++	++++
Salmonella typhi	0	0	0	0	0	0	0	0	0	0
Escherichia coli	-	x	+	++	+++	0	0	0	0	0
Pseudomonas aeruginosa	-	x	+	++	+++	-	x	+	++	+++

KEY: **o** = Resistant; **-** = No colony growth; **x** = MBC; **+** = Scanty colonies growth; **++** = Moderate colonies growth; **+++** = Heavy colonies growth

4.8 Antibacterial Screening of the Ethanolic Extract and the Soluble Fractions of the Leaves of *C. odorata*

The ethanolic extract, chloroform and aqueous fractions were active against *S. aureus* (ATCC13709), *S. pyogenes* (clinical), *S. typhi* (ATCC 9184) and *E. coli* (NCTC 10418). Their zones of inhibition are as shown in Table 4.9. The butanol fraction was only active against *S*. *typh*i and *E. coli*. The crude extract and fractions did not show any activity against *P. aeruginosa* (NCTC 6750).

The activity of chloroform and aqueous fractions were better than that of ethanolic extract. The chloroform fraction had the most activity against the test bacteria with the lowest MIC (Table 4.10).

Table 4.9 Zone of Inhibition of the Extracts of the Leaves of *Chromolaena odorata* Against the Test Bacteria (mm)

Test Organism	Extract/Fractions (mm)			
	Ethanol	**Chloroform**	**Aqueous**	**Butanol**
Staphylococcus aureus (ATCC 13709)	27	31	27	0
Streptococcus pyogenes (Clinical)	24	30	26	0
Salmonella typhi (ATCC 9184)	28	29	24	20
Escherichia coli (NCTC 10418)	20	29	26	21
Pseudomonas aeruginosa (NCTC 6750)	0	0	0	0

Key: **0** = Resistant

Table 4.10: Minimum Inhibitory Concentration of the Extracts of the Leaves of *Chromolaena odorata* Against the Test bacteria

Test Organism	Extract/Fractions																			
	Ethanol					Chloroform					Aqueous				Butanol					
	80mg/ml	40mg/ml	20mg/ml	10mg/ml	5mg/ml	20mg/ml	10mg/ml	5mg/ml	2.5mg/ml	1.25mg/ml	20mg/ml	10,g/ml	5mg/ml	2.5mg/ml	1.25mg/ml	20mg/ml	10,g/ml	5mg/ml	2.5mg/ml	1.25mg/ml
Staphylococcus aureus (ATCC 13709)	-	-	X	+	++	-	-	-	X	+	-	-	X	+	++	O	O	O	O	O
Streptococcus pyogenes (Clinical)	-	-	X	+	++	-	-	-	X	+	-	-	X	+	++	O	O	O	O	O
Salmonella typhi (ATCC 9184)	-	-	X	+	++	-	-	X	+	++	-	-	X	+	++	-	-	X	+	++
Escherichia coli (NCTC 10418)	-	X	+	++	+++	-	-	X	+	++	-	-	X	+	++	-	-	X	+	++
Pseudomonas aeruginosa (NCTC 6750)	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O

Key: **-** = No turbidity (no growth); X = MIC; **+** = Turbid (light growth); **++** = Moderate turbidity

CHAPTER FIVE

5.0 DISCUSSION

The leaves of *C. odorata* used for this study were collected from plants growing by the banks of River Kaduna in Kaduna. The plant is found abundantly in humid Southern Nigeria, it rarely grows in the drier North. However, it could be found near streams/rivers in parts of Kaduna State when there is enough humidity. This agrees with other works, which stated that *C. odorata* grows in humid tropical and temperate parts of Africa, America and Asia. (Muniappen *et al*, 2005).

The leaves of *C. odorata* are arranged alternately. The leaf is simple, ovate-lanceolate with dentate margin and mildly hairy. The leaf is broad and has reticulate venation. These macroscopic features are characteristic of the Asteraceae family (Obute and Adubor, 2007)

The leaf of the plant has anomocytic stomata that were distributed more on the lower epidermis of the leaf. This arrangement helps the plant to minimise the rate of transpiration through the leaves. Moreover, the relatively low stomatal numbers of 3 ± 0.45 and 9 ± 1.35 for upper and lower epidermis respectively could be another adaptation to withstand harsh weather during dry seasons. This also helps the plant to remain evergreen all year round (Akpovughaye, 2009). The non-glandular, multicellular trichome, shape of the epidermal cells, the anomocytic stomata and the stomatal index are identical to those reported by Adedeji and Jewoola (2008). The spiral vessel present in the xylem of the leaf of *C. odorata* constitutes the fundamental conducting element in most xylem of the leaves of

angiosperms (Evans, 2002). Moreover, the microscopic features of the dorsiventral leaf such as the stomatal index, and the vein islet and vein termination numbers are within the range expected of the Asteraceae family (Ajithadoss, 2006). The percentage moisture content of 9.78 % is just below 10 % w/w which is the upper limit recommended for crude drugs (Edward and Tyler, 1970). Excess moisture content of drugs could suggest the buyer may be paying a high price for unwanted water but also that the drug has been prepared incorrectly. This could also be due to poor storage following preparation. High moisture content could lead to breakdown of important constituents due to enzyme activity or microbial attacks. However, the relatively high moisture content of the leaf here could indicate another ability of the plant to retain moisture when exposed to harsh, dry condition. The other physical constants such as ash value (12.13 %), acid insoluble ash value (4.11 %), alcohol extractive value (4.87 %) and water extractive value (3.47 %) are within acceptable ranges. This showed that the leaves were well prepared and preserved with little or no contamination by other plant materials. The values for the physical constants obtained in this study could therefore be used as reference for future studies.

Extraction of the crude drugs showed that 70 % aq. ethanol gave more yield than pet ether. Ethanolic extraction yielded 14.53 % while pet ether yielded 4.9 % of the drug. Pet ether recovered fats/oils in the leaves and could be of advantage if the objects of interest included the fixed and volatile oils. However, this was not the case in this study. Hence, the use of 70 % aq. ethanol as solvent was appropriate for the yield of most of the phytochemicals. The use of 70 % ethanol as solvent of

extraction was with the view to combine the extractive powers of alcohol and water. Most studies on *C. odorata* used aqueous (Owoyele, *et al*, 2005) or alcohol (Taiwo *et al*, 2000) extraction methods. Moreover, it has been stated that water extracts more polar constituents than alcohol but most plant active constituents are extracted by ethanol (Obi and Onuoha, 2000).

Phytochemical screening of the ethanolic extract showed the presence of triterpenes, tannins, saponins, anthraquinones and flavonoids. This agrees with similar works (Akinmoladun *et al,* 2007). On the other hand, only flavonoids and tannins were detected in the pet. ether extract. Phenolics and terpenes detected in the extracts are compounds that have been documented to possess medicinal properties and health-promoting effects (Okwu, 2004 and Liu, 2004). Phenolics are also known to be the largest group of phytochemicals and have been said to account for most of the antioxidant activity of plant extracts (Thabrew *et al*., 1998). Moreover, terpenes and flavonoids are taxonomic markers of the Asteraceae family (Evans, 2002). Chromatographic resolution of both extracts also led credence to the fact that the ethanolic extract had more phytochemicals. Furthermore, preliminary antibacterial screening of both extracts still showed that the ethanolic extract was more active against the test bacteria with higher zones of inhibition and lower MIC and MBC. Therefore, phytochemically and biologically speaking, 70 % aq. ethanol extract was better than pet. ether extract in antibacterial activity of the leaf of *C. odorata*. Based on the above reasons, the pet. ether extract was dropped and further studies were carried out using ethanolic extract.

The chemical constituents found in the extract contribute to the biological activities of the plant. Tannins have been reported to prevent the development of microorganisms by precipitating microbial proteins and making nutritional proteins unavailable for them (Fluck, 1973 and Sodipo *et al*, 1991). Moreover, tannins are responsible for the astringent properties of the leaf of *C. odorata*. This has made the leaf popular in prevention of blood loss from wounds (Iwu, 1993).

The most dominant phytochemical class detected in the extract was flavonoids. It was detected in the ethanolic and pet. ether extracts and the subsequent chloroform, butanol and aqueous fractions. This is in conformity with the fact that phenolic compound such as flavonoids, constitute the largest group of naturally occurring plant products (Evans, 2002). Moreover, other researchers using ethanolic, methanolic or aqueous extracts of *C. odorata* reported the presence of many flavonoids (Metwally and Ekejuba, 1981; Pisutthanan *et al*, 2005; Ling *et al*, 2006 and Akinmoladun *et al*, 2007). Colour reactions indicated that the flavonoids present included flavonols and flavonones. Many flavonols and flavonones such as kaempferol-4´- methyl ether, quercetin-7, 4´- dimethyl ether, naringenin-4´- methyl ether, etc. have been found in leaf extract of *C. odorata* (Ling *et al*, 2006). The chromatographic resolution of the ethanolic crude extract showed at least seven types of flavonoids. This is within the range of 6 flavonoids obtained by Pisuthanan *et al* (2005) and 11 isolated by Ling *et al* (2006) from the leaf of *C. odorata*. This is not surprising since flavonoids are characteristic of Asteraceae family (Evans, 2002). It has also been shown that leaf extracts of *C. odorata* encourage proliferation of fibroblast and delay the visible signs of aging

such as wrinkles, hyper-pigmentation, etc (Donovan, 2007) . It has been further demonstrated that the leaf extracts of *C. odorata* have anti-staphyloccocal properties. Most of the documented medicinal properties of the leaf extracts of *C. odorata* could therefore be attributed to the dominant presence of flavonoids in them (Ling *et al,* 2006). However, it is pertinent to note that phytochemicals in herbal drugs interact with each other in different ways to produce medicinal effect. The phytochemicals could be antagonistic to each other thereby reducing the overall effect of such crude drug. In such case, there would be the need to purify the drug. Conversely, the chemicals could act synergistically to produce better effect than each acting individually. This synergistic effect common in herbal medicine prompted Evans (2002) to state that, 'one of the tenets of herbal medicine is that the maximum effectiveness of drug is derived from whole drug or its crude extract rather than from isolated compounds'.

Following fractionation of the ethanolic extract, the presence of flavonoids was detected in all the fractions. Traces of triterpenes also occurred in all the fractions. Tannins were detected only in the aqueous and butanol fractions. Chromatographic resolution showed that the chloroform fraction was the most homogenous, composing almost entirely of flavonoids.

The evaluation of the antibacterial properties of the leaf extracts of *C. odorata* in this study showed multi-spectrum activity against both Gram positive and negative bacteria. It was bactericidal to microorganisms such as *S. aureus*, *E. coli*, *C. ulcerans* and *P. aeruginosa* that normally infect wounds and *S. typhi*, which causes enteric fever (Cheesbrough, 2006). This, in addition to its astringent

properties justifies its use in treating wounds. Furthermore, its activity against *S. typhi* justifies its use by some folks to stop fever and or treat typhoid and malaria fever.

Comparison of antibacterial activities of the fractions and the ethanolic crude extract revealed that the butanol fraction was weaker than the crude extract. The aqueous and chloroform fractions had better activities than the ethanolic extract. The chloroform fraction was however, the most active against standard strains of *S. aureus*, *S. typhi*, *E. coli* and clinical isolates of *S. pyogenes*. It exhibited higher zones of inhibition than other fractions and the crude extract and had lowest MIC. The methylated flavonoids are soluble in chloroform (Evans, 2002). They account for most of the bioactivity of flavonoids (Ling *et al*, 2006)

The varying degrees of antibacterial activities of the fractions could be due to the relative abundance of flavonoids in each. Although phytochemical tests indicated the presence of the same classes of compounds in aqueous and butanol fractions, it could be assumed that the aqueous fraction had more flavonoids. The dominant roles flavonoids play in medicinal properties of *C. odorata* have been confirmed by many researchers (Suksamrarn et al, 2004; Ling et al, 2006 and Akinmoladun et al, 2007).

One major application of *C. odorata* in traditional medicine is in the treatment of wounds. It is essential that any treatment of wound must include the following (Badoe *et al,* 2000):

►Stopping excessive loss of blood without damaging blood supply to the wound site.

►Preventing or clearing residual bacterial infection.

►Encouraging formation of fibroblasts at the wound site.

Iwu (1993) showed that with its astringent properties, the leaf of *C. odorata* is able to arrest bleeding at wound sites thereby fulfilling the first condition above. Furthermore, this study has shown that the leaf of *C. odorata* is capable of preventing the growth and even causing the death of bacteria that could infect wounds. Thus, the second condition has been met. Moreover, Donovan (2007) showed that the leaf extracts of *C. odorata* do encourage proliferation of fibroblasts. This takes care of the third condition given for treatment of wounds (Badoe *et al,* 2000). Consequently, the leaf of *C. odorata* has all the properties required for effective treatment of wound. Its uses in traditional medicine to treat wound is therefore justified scientifically.

CHAPTER SIX

6.0 SUMMARY, CONCLUSIONS AND RECOMMENDATIONS

6.1 Summary

The plant *C. odorata* is normally found growing by the banks of some streams/rivers in Kaduna town. This is unlike in Southern Nigeria, where they are commonly found as weeds in forests and cultivated farmlands. The quantitative microscopy and physical constants of the leaf were mostly within the ranges expected of the family Asteraceae. However, the percentage moisture content was relatively high at 9.78 %. This probably enables the plant to remain evergreen even during the hot, dry season when most shrubs wither out.

Phytochemical tests revealed the presence of flavonoids, tannins, triterpenes and saponins. Furthermore, chromatographic resolution of the crude extract indicated the presence of at least seven flavonoids. The ethanolic extract were bactericidal to standard strains of *S. aureus*, *S. typhi*, *E. coli* and clinical isolates of *S. pyogenes* and *P. aeruginosa*. The chloroform fraction, composed mostly of flavonoids, showed the most antibacterial activity.

6.2 Conclusion

Flavonoids were the major group of phytochemical in the leaf extract of *C. odorata*. The flavonoids are largely responsible for the antibacterial properties of the plant since more activity was recorded in its presence and less of other phytochemicals. This study showed that the leaf extract of *C. odorata* has broad-spectrum

antibacterial activities against the common bacteria that infect wounds. This together with the other documented medicinal properties of the leaf of *C. odorata* makes it an effective and easily accessible drug for the treatment of wounds.

6.3 Recommendations

The following recommendations are made:

a. More studies should be carried out to find out if a single active compound is responsible for the antibacterial activities of *C. odorata*. There is the need to isolate and purify such a compound if it exists considering the enormous potentials.

b. The bactericidal activity of the leaf extract of *C. odorata* against the enteric fever pathogen, *S. typhi* (as shown in this study) needs to be investigated. This would help elucidate the basis for the use of *C. odorata* in traditional medicine as a treatment for typhoid fever.

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APPENDICES

1.0 SUMMARY OF CALCULATIONS

1.1 Quantitative Microscopy of the Leaves of *Chromolaena odorata*

Parameter		Observations					Total	n	□	S	SEM	Result
		1st	2nd	3rd	4th	5th						
Stomatal no.	upper	2	3	4	3	2	14	5	3	0.78	0.35	2 – **3** - 4
	lower	11	8	9	10	9	47	5	9	1.1	0.49	8 – **9** - 10
Stomatal index	upper	4.1	7.0	11.1	-	-	22.2	3	7.4	2.87	1.66	5.95 – **7.4** – 8.05
	lower	23.4	26.7	23.7	-	-	73.8	3	24.6	1.49	0.86	20.91 – **24.6** – 28.29
Palisade ratio		3, 1,	4, 1,	5, 3,	2, 1,	3, 2	25	10	2.5	1.29	0.41	5 – **6.5** - 7
Vein islet no.		4	7	8	4	-	23	4	5.75	1.79	0.89	5 – **5.8** - 7
Vein termination no.		5	6	4	4	-	19	4	4.75	0.83	0.42	4 – **4.8** - 6

1.2 Physical Constants of the Leaves of *Chromolaena odorata*

Parameter	Observations (%)			Total	n	□	S	SEM	Result (%)
	1st	2nd	3rd						
Moisture content	9.67	9.33	10.33	29.33	3	9.78	0.5	0.29	8.29 – 9.78 – 11.28
Total ash value	12	11.7	12.7	36.4	3	12.13	0.42	0.24	10.31 – 12.13 – 13.95
Acid insoluble ash value	4.33	3.67	4.33	12.33	3	4.11	0.31	0.18	3.49 – 4.11 - 4.73
Alcohol soluble extractive value	4.8	4.7	5.1	14.6	3	4.87	0.17	0.098	4.14 – 4.87 – 5.6
Water soluble extractive value	3.4	3.4	3.6	10.4	3	3.47	0.13	0.075	2.95 – 3.47 – 3.99

Key: n = Number of observations.

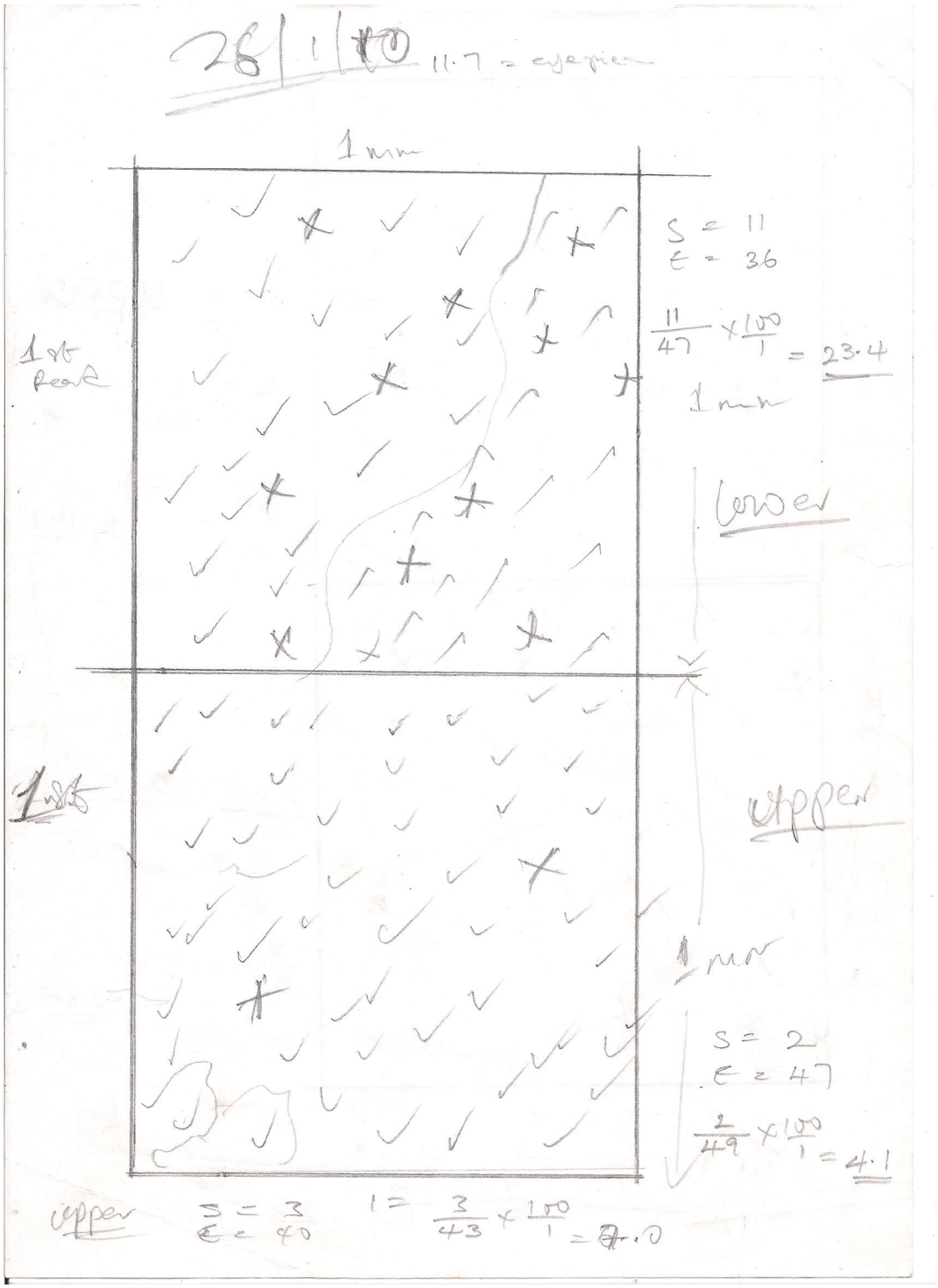
□ = Mean = Total/n

S = Standard deviation of the population = $\sqrt{\frac{\square(x - \square)^2}{n}}$

SEM = Standard error of the mean = $\frac{S}{\sqrt{n}}$

2.0 **Quantitative Microscopy of the Leaves of *Chromolaena odorata***

2.1 Sketch Used to Aid Determination of Stomatal Number and Stomatal Index



2.2 Sketch Used to Aid the Determination of Vein Islet and Vein Termination Numbers of the Leaves of *Chromolaena odorata*



3.0 Sections of the Leaf of *C. Odorata*

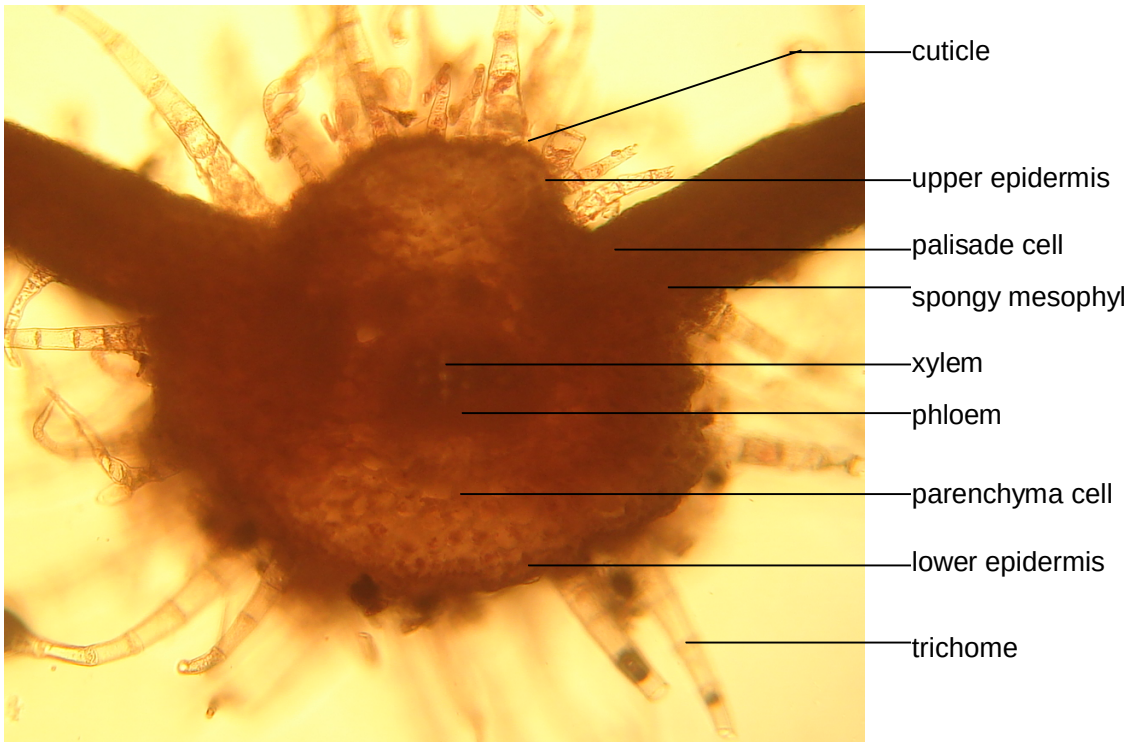


Plate 3.1: TS of the Leaf of *C. odorata* taken through the Midrib of the Leaf (X 400)

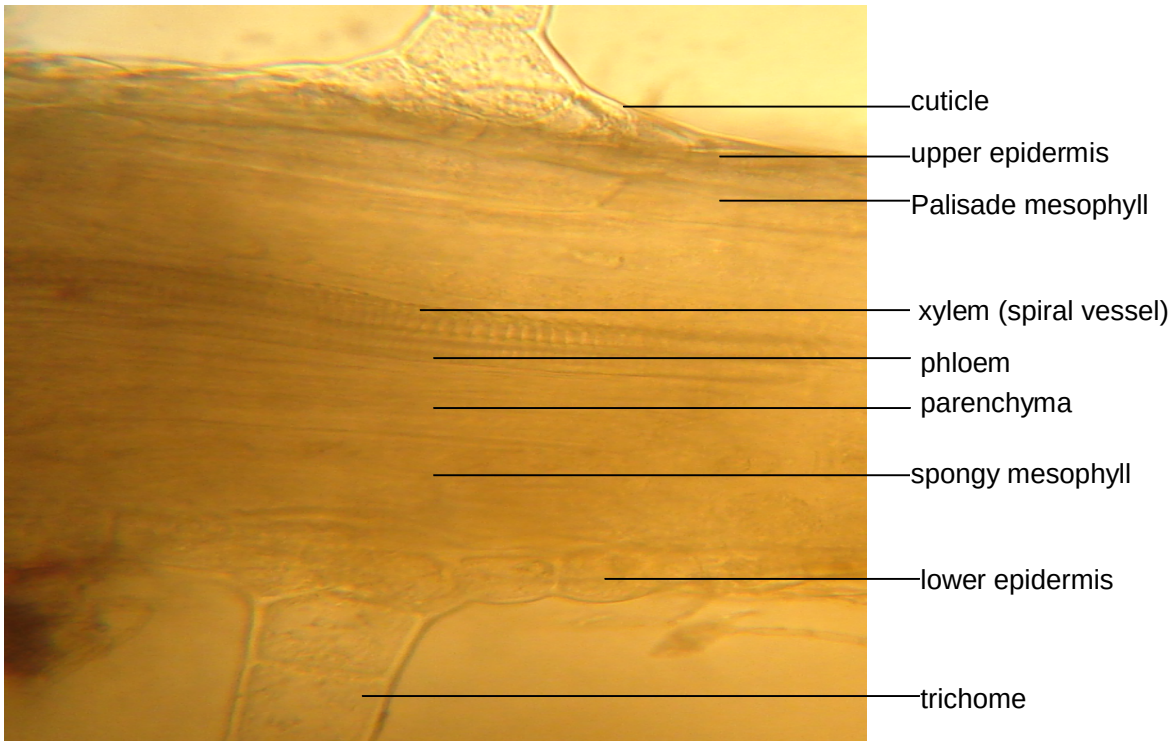


Plate 3.2: LS of the Leaf of *C. odorata* taken through the Midrib of the Leaf (X 400)

4.0 Composition of Reagents Used to Test for Alkaloids

4.1 Dragendorff's Reagent:

Solution (a): Dissolve 0.85g basic bismuth nitrate in 10ml glacial acetic acid and 40 ml water under heating. If necessary, filter.

Solution (b): Dissolve 8 g potassium iodide in 30 ml water.

Stock solution: (a) + (b) are mixed 1:1

4.2 Mayer's reagent:

Dissolve 1.358g of merciuc chloride HgCl2 in 60ml of water and pour into a solution of 5g of potassium iodide (KI) in 10ml of H2O Add sufficient water to make 100 ml.

4.3 Wagner's reagent *(*Iodo-potasium iodide*)*:

Dissolve 2g of iodine and 6g of KI in 100ml of water.