

**PHARMACOGNOSTIC, ANALGESIC AND ANTI-INFLAMMATORY
STUDIES ON THE LEAVES OF *MICROTRICHIA PEROTITII* DC
(ASTERACEAE)**

BY

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OCTOBER, 2017

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STUDIES ON LEAVES OF *MICROTRICHIA PEROTITII* DC
(ASTERACEAE)**

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**A THESIS SUBMITTED TO THE SCHOOL OF POST-GRADUATE
STUDIES, AHMADU BELLO UNIVERSITY, ZARIA
IN PARTIAL FULFILMENT FOR THE AWARD OF DOCTOR OF
PHILOSOPHY DEGREE IN PHARMACOGNOSY**

**DEPARTMENT OF PHARMACOGNOSY AND DRUG
DEVELOPMENT FACULTY OF PHARMACEUTICAL SCIENCES
AHMADU BELLO UNIVERSITY
ZARIA – NIGERIA**

OCTOBER, 2017

DECLARATION

I declared that this thesis entitled’’**Pharmacognostic, Analgesic and anti-inflammatory Studies on Leaves of *Microtrichia perotitii* DC (Asteraceae)**’’ has been carried out by me in the Department of Pharmacognosy and Drug Development under the supervision of Professor N.Ilyas, Professor Hajara Ibrahim and Professor Kabir Y. Musa. 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 presented for another degree or diploma at any University.

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CERTIFICATION

This thesis entitled **PHARMACOGNOSTIC, ANALGESIC AND ANTI-INFLAMMATORY STUDIES ON *MICROTRICHIA PEROTITHI* DC (ASTERACEAE)** by Abdullahi Maikudi, NUHU meets the regulations governing the award of the Degree of Doctor of Philosophy in Pharmacognosy of Ahmadu Bello University Zaria and is approved for its contribution to knowledge and literary.

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DEDICATION

This work is dedicated to my late father Mal. Nuhu Abdurrahman and the entire members of his family.

ACKNOWLEDGEMENT

Alhamdulillah, I am most grateful to Allah (SWT) for sparing my life to see to the completion of this Research work.

I wish to sincerely express my profound gratitude to Professor N. Ilyas of the Department of Pharmacognosy and Drug Development Ahmadu Bello University, Zaria for her effective and efficient supervision of this work, reading and correction of the entire manuscript, offering of constructive criticism and advice despite her other official engagements.

My sincere appreciation also goes to Professor Hajara Ibrahim. of the Department of Pharmacognosy and Drug Development for her co-supervision, advices, reading and correction of the entire manuscript and her overall support right from the commencement of this work.

Similarly my appreciation goes to Professor K.Y Musa for his co-supervision, advice, criticism, reading the entire manuscript and his encouragement throughout the cause of this work.

My profound and sincere gratitude goes to my parents Mallam Nuhu(late) and Mallama Hadiza for their parental support and guidance, my immediate family members especially my wives and children for their patience, tolerance during my absence and their encouragement towards my research may Allah (SWT) reward all of them.

My appreciation specially goes to Prof..Garba Ibrahim the HOD of Pharmacognosy ABU Zaria for his words of Advice and encouragement in the cause of this work.

This research work and the study was made possible with the ground support of my employer, Kaduna Polytechnic.

I wish to extend my special appreciation to both academic and non-academic staff of the Department of Pharmacognosy and Drug Development in particular the HOD Professor Garba Ibrahim and the PG Coordinator Dr U. H. Danmallam and Department of Pharmacology and Therapeutics especially Dr. A.H. Yaro and Dr M. G. Magaji, Professor A.O.Oyewale of the Department of Chemistry ABU Zaria the Dean and the entire staff of the Faculty of Pharmaceutical Science, ABU Zaria for their immeasurable support, towards the success of this work. Need of mention are my colleagues in the Department of Applied Science, College of Science and Technology, Kaduna Polytechnic.

Finally, I am so grateful to Malam Musa Muhammad of the Department of Biological Sciences ABU Zaria, my brothers, sisters and my friends. May Almighty Allah (SWT) bless you all.

ABSTRACT

Microtrichia perotitii DC Asteraceae (Compositae) is an herb found in the tropical countries. Ethno-medicinally, it is mainly used for the treatment of pain related diseases such as toothaches, cuts and burns and few ailments like rashes, rheumatism, jaundice, stomachaches and diarrhea in children as well as inflammatory diseases. The aim of this research was to establish its pharmacognostic standards for its identification, purity, isolation and characterisation of its phytoconstituents as well as to evaluate some claims and prove the usage of the herb as an analgesic and anti-inflammatory agent. The macroscopical studies revealed the shape of the leaf as small with acute apex, asymmetric base and pubescent surface. The leaf has long petiole and a serrated margin. Organoleptically, the leaf is green, slightly bitter and pepperish with an unpleasant smell. The microscopical studies showed that the leaf of the herb had epidermal cells with irregularly thickened walls, numerous anomocytic stomata, and multicellular covering trichomes on both upper and lower epidermis. The transverse section of the leaf showed that it was dorsiventral and has vascular bundle. The powdered leaf showed calcium oxalate crystals (prism and rosette), starch (oval), xylem (spiral) and lignified fibres. Quantitative-leaf microscopy revealed the leaf constants as palisade ratio (3.2-3.4); stomatal number (258-285); stomatal index (19.5-24.7); veinlet (6.0-8.0) and veintlet (8.0-11.0). The physico-chemical constants of the leaf showed moisture content (12.67 %), total ash (20.33 %), acid-insoluble ash (27.3 %), water-insoluble ash (81.18 %) water-soluble (40.9 %) and alcohol-soluble extractives (25.45 %). The results of analgesic studies of the leaf for both crude extracts and solvents fractions showed dose-dependent and dose-independent significant ($P < 0.001$; $P < 0.05$) inhibitory activities in

mice. Similarly the crude extracts and solvent fractions significantly ($P < 0.001$; $P < 0.05$) reduced inflammations in the formalin induced paw oedema in rats. The phytochemical investigation on the leaf revealed the presence of tannins, flavonoids, alkaloids, triterpenes which have analgesic and anti-inflammatory activities, saponins, carbohydrates and cardiac glycosides. The overall studies lead to the isolation of an α -amyrin acetate for the first time from the leaves of *Microtrichia perotitii* DC. Pharmacognostic parameters observed in this study will quite be useful for identification, standardization, adulteration, development and preparation of crude drug's formulation and inclusion in various pharmacopoeias for treating ailments. The current observations will help in differentiating the leaf of this species from closely related species of same genus and family.

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ABBREVIATIONS

ANOVA = Analysis of variance

ANS= Anthocyanidin

Bw=body weight

CDER= Centre for Drug Evaluation and Research

COSY= Homonuclear correlation spectroscopy

COX= Cyclooxygenase

COX-1= Cyclooxygenase 1

COX-2= Cyclooxygenase 2

CHS= Chalcone synthase

CNS= Central nervous system

C4H= Cinnamate-4-hydroxylase

¹³C-NMR= Carbon -13 Nuclear magnetic resonance

DEPT= Distortionless enhancement by polarization transfer

DF= Degree of freedom

DFR= Dihydroflavonol-4-reductase

F3H= Flavones-3-hydroxylase

FTIR= Fourier transformed infrared

g = Grammes

GC-MS= Gas chromatography mass spectrometer

GI = Gastro intestine

HHDP= Hexahydroxydiphenyl

HMBC= Heteronuclear multiple bond correlation

HSQC= Heteronuclear single quantum coherence

HPLC= High performance liquid chromatography

$^1\text{H-NMR}$ = Proton nuclear magnetic resonance

I= Spin quantum number

i.p.= Intraperitoneally

L= Leaf

LD₅₀= Lethal dose

MC= Moisture content

ml= Milliliter

Mm= Millimeter

mg/kg= Milligram per kilogram

ml/kg = milliliter per kilogram

M/e= Mass to charge ratio

MPAE= *Microtrichia perotitii* aqueous extract

MPE= Maximum possible effect

MPME= *Microtrichia perotitii* methanolic extract

Nm= Nano metre

NMR= Nuclear magnetic resonance

NOESY= Nuclear overhauser effect spectroscopy

NSAIDs= Non-steroid anti-inflammatory drugs

PAL= Phenylalanine ammonia-lyase

PGG= Penta-galloyl-glucose

PGE= Prostaglandin-E

PGS= Prostaglandins

PGF = Prostaglandin-F

Ph = Hydrogen optimum

Ppm = Parts per million

R_f= Retardation factor

S.E = Standard error

TCM= Traditional chinese medicine

TLC = Thin layer chromatography

TS= Transverse section

TNF^α= Tumour neorosis factor

TXA = Thromboxane

GFGT = Glucose-flavonoid-3-O-glycosyl transferase

UNEP= United Nations Environment Programme

UV = Ultra violet

v/v = Volume by volume

WHO = World health organisation

w/w = Weight by weight

μm = Micrometer

CHAPTER ONE

1.0

INTRODUCTION

The word “pharmacognosy” was coined in the early 19th century to designate the discipline related to the study of medicinal plants (Ganzinger, 1982). The science of pharmacognosy became aligned with botany and plant chemistry, and until the early 20th century, it dealt mostly with physical description and identification of whole and powdered plant drugs including their history, commerce, collection, preparation, and storage. Advances in organic chemistry added a new dimension to the description and quality control of these drugs, and the discipline has since expanded to include discovery of novel chemical therapeutic agents from the natural world (Betz *et al.*, 2011). Pharmacognosy therefore, is the study of the structural, physical, chemical and sensory characters of crude drugs of plants, animals and mineral origin. The search for biologically active compounds from these sources has always been of great interest to researchers looking for new and lead sources of drugs useful in infectious and other diseases. A big quantum of research work in the area of authentication of the correct plant source has always been done to provide for means of differentiation among many sources of plants (Ravi and Ruthinam, 2013; Sharma and Pracheta, 2013). Medicinal plants are the greatest asset to human health and a treasure for discovering new potential compounds with various therapeutic effects. A large number of the world’s population especially the developing countries depend upon the medicinal plants as an alternative and complimentary therapy for various ailments. Some of these common practices include the use of the plants crude extracts directly which may contain a broad diversity of compounds often of unknown biological effects (WHO, 2007). The plants are

therefore being used indiscriminately without recourse to their unhealthy or toxic effects. WHO, 1992 has therefore recommended for a complete scientific investigations of such plants.

The plants produce bioactive compounds which act as a defense against disease while at the same time may be toxic. The advantage of natural drugs from these plants cannot be over emphasized because of their easy availability, economic and less side effects but their disadvantage include adulteration which must be taken care-off. Authentication of such medicinal plants is done through morphological, phytochemical and physico-chemical analysis (Dineshkumar, 2007; Sumitra, 2014).

1.1 The Family Asteraceae

The family Asteraceae (Compositae) is the second largest family of flowering plants in terms of number of species. The family comprises of more than one thousand six hundred genera (1,600) and twenty three thousand (23,000) species. The largest genera are *Senecio* (1,500 species), *Vernonia* (1,000 species), *Cousinia* (600 species), and *Centaurea* (600 species) (Daniel, 1887).

1.2 Medicinal plants

The use of medicinal plants for the treatment of various types of ailments is found in almost all cultures in the world and many of these medicinal plants have been found to be efficacious. Therefore, the use of medicinal plants for curative purpose cannot be over emphasized. For instance, malaria fever has been one of the worst of all human diseases and has claimed and is still claiming the lives of so many people especially children. However, it was not until the 17th century that a native remedy for the treatment of certain

diseases made from an infusion of the bark of *Cinchona* (*Cinchona* species; Rubiaceae) was coincidentally found to control malaria. The provincial substance identified from the infusion was Quinine (David, 2003). The quinine has been used for many years in the treatment of the malarial ailment which is a disease caused by protozoa of which the most troublesome is *Plasmodium falciparum* (Paul, 2002).

Similarly, the infusions of Ephedrine species (Ephedraceae) have been used for thousands of years in China for curative purposes and because of its effectiveness it is often referred to as “ma huang”. From the infusions, ephedrine and a series of other related compounds were isolated which as of today have wide application in modern medicine. The Indians of Ancient South America for long were using coca leaves as stimulants and chewing the leaves mixed with lime in order to reduce hunger and pains whereas the lime free alkaloids were later discovered to have local anaesthetic. Cocaine isolated from the Coca plant was being used in surgery (David, 2003).

The use of *Digitalis purpurea* L. (Scrophularaceae) plant for the treatment of heart disease dated back to thousands of years ago. The plant *Papaver somniferum* L. (Papaveraceae) has long been used to alleviate pains in humans, in the Mediterranean and Near East and its latex was ascertained to be a pain killer. A number of plants of the *Veratrum* species were also used for the treatments of various ailments in certain communities several centuries ago. A typical example is *Rauvolfia serpentina* (L.) Benith ex. Kurz (Apocynaceae) which is used to treat hypertension and some certain mental illnesses it contains alkaloids such as reserpine that are extremely potent hypertensive agents. Another plant from the same family is *Catharanthus roseus* (L) G. Don (Apocynaceae) is locally used for the treatment of leukemia (Paul, 2002).

The *Aloe* species has come to the fore in that, the plant species were noted for their medicinal importance. For instance, the seeds of *Strychnosmix vomica* L.(Loganiaceae) were usually collected wide in Africa and Asia and when eaten they provide remedy for paralysis and also stimulate the central nervous system (David, 2003).

Hypericum perforatum (St John's wort) L.(Guttiferae/Hypericaceae) is a native of Europe and Asia where it occurs as weeds and has been used as an effective antidepressant remedy and for other herbal applications. Its extract in vegetable oil is being used for antiseptic and wound healing and some phenolic compounds were isolated from the plant. On the other hand, extracts from the leaves of *Gingko biloba* L.(Ginigonaceae) a primitive member of the gymnosperms and a native of China is used to improve capillary blood flow and improve memory and some aspects of brain function. The active constituents have been characterized as mixtures of terpenoids and flavonoids(Paul, 2002; David, 2003).

The medicinal plants in general have contributed immensely to the development of modern (orthodox) medicines because of the various constituents present in them as well as their biological activities(Sofowora, 1981; Sharon, 2009). In spite of the diverse research from chemistry and biotechnology in producing synthetic drugs, plants are still the sole healing provider to mankind. Some of the benefits derived from using medicines from plants include their relative safety over synthetic drugs, profound therapeutic benefits as well as affordable treatment (Idu *et al.*, 2007).

1.3

Herbal medicines

1.3.1 The practice of herbal medicine

The practice of herbal medicine has served as an impetus to the progress recorded in the development of modern drugs. For instance, over the last century a number of top selling drugs have been developed from plant products. The Plant medicine has been practiced by native tribes for centuries and these include the ancient Egyptians, the Chinese and the Indians (Sharon, 2009). The herbal medicines include the use of herbs and herbal materials, herbal preparations and finished herbal products that contain parts of plants or other plant materials as active ingredients (Sofowora, 2008; WHO, 2008). The practice has gained international diversity and has been adopted into different cultures and regions without the parallel advance of international standards and methods for evaluation (WHO, 2008). This practice has dominated the provision of healthcare to many communities. For instance, in some Asian, Latin America and African countries, 80% of the population depend on the traditional medicine for healthcare, while in many developed countries, about 70–80% of the population has used some form of alternative or complementary medicine e.g. acupuncture (Kim, 2005; WHO, 2008; Solomon, 2009). Specifically in Ethiopia over 90% primary care provision is in the traditional medicines which are recorded in oral traditional and in early medico–religious manuscript and traditional pharmacopeias that dated back to the 15th century AD, whereas in China putting acupuncture and herbal medicine for provision in primary healthcare has been documented for over 2,500 years. The herbs as medicines may be used directly as teas, extracts or in the production of drugs (Sofowora, 2008; Solomon, 2009). In the United

States of America, herbal medicine generally refers to a system of medicine that uses European or North American plants (Sharon, 2009).

In summary herbal medicine is the use of plants and medicinal herbs to help prevent and treat disease. Herbalism therefore is a traditional medicine or folk medicine practice based on the use of plants and plant extracts. It is also known as botanical medicine, medical herbalism, herbal medicine, herbology and phytotherapy (Sharon, 2009). Therefore, the scope of herbal medicine is sometimes extended to include fungal and bee products as well as minerals, shells and certain animal parts. The art of herbalmedicine is not far different from traditional medicine practices which in essence is the sum total of knowledge, skill and practices based on the theories and experiences indigenous to different cultures that are used to maintain health as well as diagnose, improve or treat physical and mental illnesses (Acharya and Shrivastava, 2008; WHO,2008). For the purpose of traditional medicines, the modern herbalists often make use of plants from many different regions of the world and they do not restrict their practices only to those plants classified as herbs (a seed plant whose stem withers away annually) instead an herb can be a root, a piece of tree bark, a mushroom or anything else which grows naturally and falls into the plant kingdom (Harry, 2002;UNEP 2008).Traditional systems of medicine has continued to be widely used on the account of population rise, inadequate supply of orthodox drugs, prohibitive cost of treatments with orthodox drugs, side effects of synthetic drugs and development of resistance to currently used drugs for treatment of infectious diseases(WHO, 2008) .

Traditional or indigenous medicine is generally believed to be transmitted orally through a community, family and individual until it is collected within a given culture. The World

Health Organization (WHO, 2008) defined traditional medicine as the sum total of knowledge, skills and practices based on the theories, beliefs and experiences indigenous to different cultures that are used to maintain health as well as to diagnose, improve or treat physical and mental illness. Therefore, traditional medicine describes knowledge systems which developed over centuries within various societies before the era of modern medicine. The practices include herbal, Ayurvedic, Unani, ancient Iranian, Siddha, Islamic, traditional Chinese medicine, acupuncture, Muti, Ifa and other medical knowledge and practices all over the globe. The traditional medicine encompasses, the health practices, approaches, knowledge and beliefs incorporating plants, animals and mineral based medicines, spiritual therapies, manual techniques and exercises applied singularly or in combination to treat, diagnose and prevent illnesses or maintain well being (Kay, 1996; WHO, 2008). Some examples of these cultures include:-

1.3.1.1 *African Traditional Medicines*

The history of the evolution of the practice of traditional medicine in Africa was a mystery and a lot of theories were put forward. For instance, it was reported that the early man deliberately selected specific plant materials for the treatment of his ailments based on knowledge gained by accident, knowledge gained by ancient antiquities, knowledge from wizards and witches as professed by some African traditional healers (Sofowora, 2008). Some were of the opinion that, the art of the practice of traditional medicine came from hunters and spirit of an ancestor who practiced herbalism (Sofowora, 2008 ; Sharon, 2009). However, the practice of traditional herbal medicine has emanated from all traditions in Africa. It has provided about 80% of the primary healthcare needs of Africans and because of this some countries like Ethiopia and Nigeria have

institutionalised the practice of traditional medicine (WHO 2008; Sharon, 2009). The reason for this approach was that many traditional African remedies have performed well in initial laboratory tests to ensure they are not toxic in test animals. For example, it was reported that *Faidherbia albida* Del(Gawo) passed the tests for toxicity and was found suitable for the treatment of fevers, diarrhea and inflammation (Acharya and Shrivastava, 2008).

1.3.1.2 *Ayurvedic Medicine*

The Ayurvedic medicine originated in India about 5000 years (600BC) and is a word combined from two Sanskrit words – ayur (life) and veda (science) which simply means Ayurvedic is the science of life (Sofowora, 2008; Acharya and Shrivastava, 2008). In India, the Ayurvedic medicine is also known as Aashtanga Veda (Science with eight branches) because of its classification into eight different branches of treatment, namely; Kaya Chikitsa (general medicine), kaumara Bhritya (pediatrics), Graha Chikitsa (psychology), shalaky Chikitsa (treatment of the diseases of eyes, nose, throat, ear and head) shalya Tantra (technique for surgery), Agada Tantra (toxicology), Rasayana Chikitsa (Rejuvenation treatment) and vajeekarana Chikitsa (aphrodisiac treatment). According to the Ayurveda, diseases affect not only humans but also all living and non-living things and thus only humans can recognize diseases and take appropriate steps to correct the conditions (Sofowora, 2008 ; Acharya and Shrivastava, 2008). The literature of Ayurveda was recorded on palm leaves, copper plates and stones and the knowledge was transferred through generations by word of mouth and now through systematic education (Acharya and Shrivastava, 2008). Ayurvedic medicine has quite complex formulas with 30 or more ingredients, including a sizeable number of them that have undergone

“alchemical processing” chosen to balance “vata”, pitto” or “kapha” (Henrich, *et al*,2005; WHO, 2008).

1.3.1.3 *Chinese Medicine*

The traditional Chinese medicine (TCM) includes a range of traditional medical practices originating in China. The TCM theory originated 1000 of years ago through meticulous observation of nature, the cosmos and the human body. Major theories include those of Yin- yang, the five phases, the human body channel system, Zang Fa organ theory, six confirmations, four layers (Porkert, 1974). The Chinese herbal medicine as of today is amongst the greatest herbal system of the world and history has it that it dates back to the 31st century BC (Vicky, 2005). The first Chinese herbal book, the shennong Bencao Jing contains lists of 365 medicinal plants and their uses including ma–Huang. The Chinese TCM adopts many methods of treatment but the most important one is the acupuncture which is a technique that adopts the practice of inserting fine needles into specific points in the patient’s body. The intended effect is to increase circulation and balance energy within the body (Porkert, 1974; Yasuo, 1985; Dan *et al.*, 2004). The Chinese TCM is so significant that it was introduced to Japan in the 7th – 9th centuries through text like the Neijing Suwen, Lingshu and material medica. It is called Kampo medicine which is also adopted in Taiwan and exported to the Western countries. The Kampo medicine encompasses acupuncture and other compliments of the Chinese medical system. Some compounds with pharmacological activities have been isolated from some Chinese herbal medicines. They include, artemisinin from worm wood (qinghao) which as of today is used to treat multi drug resistant strains of Falciparum malaria as well as an anti cancer agent (Calvin, 1969; Dan *et al.*, 2004; Subhuti, 2006).

The practice of herbal medicine is not only limited to African and Asian countries but also to Europe and America. For instance, it was reported that the Kampo medicine of Japan was introduced to America through the literary work of U.S emigrants from Taiwan (Subhuti, 2006). In Europe, the ancient Greeks and Romans were reported to have practiced traditional medicines and their literary works were preserved in the writings of Hippocrates which has provided the patterns for later Western medicines. Therefore, in the early medieval Europe, monasteries became local centres of medicinal knowledge and their gardens provided raw materials for local treatment of diseases. At the same time folk medicines in the homes and villages continue uninterrupted and amongst the herbalists are the “wise-women” who prescribed herbal remedies (Kay, 1996). Medical school known as Dimanstan began to appear from the 19th century in the medieval Islamic world which was generally more advanced than medieval Europe at that time. The Arab travelers had access to plant materials from distant places, therefore Muslim botanists and physicians significantly expanded on the earlier knowledge of material medical such as the works of al-Dinandic which described more than 637 plant drugs in the 19th century as well as Ibn al-Baitar which described more than 1,400 different plants, foods and drugs in the 13th century. This effort was complimented by an Andalusian – Arab botanist Abu al-Abbas al-Nabati who introduced empirical techniques in the testing, description and identification of numerous materials medical where he separated unverified reports from those supported by actual tests and observations. However, this allowed for the study of material medical to evolve into the science of Pharmacology (Fahd, 1996; Diane, 2002; Huff, 2003).

In as much as 3.4 billion people in the developing world are dependent on plant based traditional medicine then the development of herbal medicine would greatly be affected by environmental degradation, poor resource management as well as urbanization (Solomon, 2009). The veracious uncertainty on the safety and usage of the herbal medicines is a point of concern and not just their efficacy. Evidence showed that, there are no standard methods of preparations and whether or not the whole or part of a plant for preparation of a product is more important and lack of sufficient evidence as to the interaction of these herbs with prescribed drugs could be harmful or not (Peter, 2001). To safeguard the continuous usage of herbal medicines, scientific evidence from tests done to evaluate the safe effectiveness of the herbal products and a comparison of the safety, effectiveness and quality of finished herbal medicinal product drugs and the quantity of their source materials is very necessary. On the other hand, the expanding herbal product market could drive over-harvest of plants and threaten biodiversity while poorly managed collection and cultivation practices could lead to extinction of endangered plant species and the destruction of natural resources due to lack of knowledge and sustainability. Similarly, the practice of traditional medicine could be harmful and some instances may result with adverse reactions due to poor quality or taken inappropriately with other medicines (WHO, 2008). In other words, the integration of herbal medicine into modern medical practices would definitely take into account quality and safety, although lack of pharmacological evidence of the majority of herbal medicine products could bring a setback for the inclusion into conventional medical practices. A typical example here is the preference of the use of digitalis leaf for congestive heart failure rather than the pure compound dioxin (digitoxin) isolated from the plant because the

compound is considered dangerous(Harry,2002;Sofowora,2008). Herbal medicine is distinct from pharmaceutical drugs because of the complexity of plant materials, they are prescribed in combination and they only seek to correct internal imbalance rather than to treat symptoms alone (Yasuo, 1985; Bruntone, 1999; Subhuti, 2006).

1.3.2 Standardization of herbal drugs

Standardization of Herbal Medicines or drugs is the process of establishing or prescribing a set of peculiar identities, specific characteristics which are generally unique and of unshared qualities. Whereas pharmacognostic standardization of a drug is a process involving a series of laboratory experiments which will reveal and assemble a set of inherent peculiar characteristics that includes constant parameters, definite qualitative and quantitative value or specific and unique features on the basis of which similar herbal medicines, claimed to be the same, can be compared for the purpose of authenticity, efficacy, genuineness, purity, reproducibility and overall quality assurance(Sumtra, 2014). Specific standards obtained through experimentation may be compiled into a monograph of selected medicinal plant which can be assembled together to constitute an herbal pharmacopoeia. The Pharmacognostic standard ensures plant identity and lays down standardization parameters which will help and prevents adulteration. These parameters include:

*1.3.2.1.Macroscopic Standards:*The macroscopy of a crude drug includes its visual appearance to the naked eyes and its other sensory characteristics such as odour and taste sensations. Macroscopic analysis defines the establishment of morphological characteristics of plant products achieved by organoleptic evaluation. This involves

recording features noted upon observing the specimen with or without the aid of a magnifying lens, as well as evaluation of the specimen using other sensory characters such as smell as well as touch. These tests provide size, colour and odour properties of the specimen being analyzed. Various crude vegetable drugs have been classified or grouped based on their characteristics. This identification and characterization processes can be carried out using various botanical examination techniques (Sumitra, 2014). These physical measurements may often provide the simplest and quickest indication for its identity, purity or quality when compared with the official monograph. Most herbal drugs fall under the following morphological classes: Barks (i.e. tissue in a wooden stem outside the cambium); underground structures (i.e. roots, rhizomes, corms and bulbs); whole herbs (all the above ground parts of the plant); leaves, flowers, fruits and seeds. The shape, size, texture or fracture -in the case of barks, roots or rhizome; leaf arrangement, margins, venation, shape and surface description for leaves and for flowers; the presence or absence, and the type of calyx, corolla, androecium/gynoecium and inflorescence, type of ovary, placentation and seed description; they are all useful parameters for assessing the identity, purity and to a certain extent, quality of the herbal material (Evans, 2009).

1.3.2.2. Microscopical Standards: The microscopical analysis gives the anatomical characteristics of the tissue obtained by transverse, radial and longitudinal sections. This helps to specify the taxonomic position of the crude drug in some cases (Ahmed *et al.*, 2015). Microscopic analysis is certainly the most objective and reliable among the various pharmacognostic techniques of drug identification and standardization. Since it deals with tissue exposition and visualization under the microscope, essentially providing

closer cellular discrimination in their intact natural arrangement as well as for qualitative measurement of the internal structures, no two plant species will possess exactly the same cellular patterns qualitatively and quantitatively in all respects (Ewurum, 2009). In fact, the use of microscopical and micrometric data (known as diagnostic characters) for example; types of cells- (parenchyma, sclerenchyma, collenchyma), size of the cells, trichomes, stomata pore, starch grains, aleuronic grains, fibres, pollen etc. is indispensable for the identification and quality control of herbal drugs especially in powdered forms. The basic layout tissues which is constant in crude drugs of stems, leaves, roots etc can be best ascertained by careful observation of the transverse, longitudinal (radial and tangential) and surface sections. The cells which are most useful for purposes of identification, standardization and quality include fibres, sclereids, tracheids, vessels, cork cells etc and also are least affected by drying process. The cell contents are detectable by using various micro-chemical tests and with greatest importance are starch grains, calcium oxalate crystals, lignins, proteins, and fixed oils (Bauer, 1998).

1.3.2.3. Qualitative Microscopy: Qualitative Microscopy is concerned with the identification of the diagnostic characters present in the soaked, cleared crude drug materials or powdered crude drug with the aid of a microscope. The purity of many underground drugs can be established or confirmed by an examination of calcium oxalate crystals, detailed structure of the trichomes and other features, for the detection of adulterants in powdered drugs. The knowledge of microscopical structures is essential for a given diagnostic character of particular species (Bauer, 1998; Evans, 2009). The quality microscopy involves accurate cellular micrometry of all the issues such as:

i. **Palisade Ratio:** This is the average number of palisade cells beneath one epidermal cell, using four contiguous epidermal cells for the count. This ratio has been shown to be sufficiently constant to serve as a diagnostic character of species belonging to the same genus in certain instances (Evans, 2009).

ii. **Stomata Number:** This is the average number of stomata per square millimeter of epidermis and it varies considerably with the age of the leaf. In recording results, the range as well as the average value should be recorded for each surface of the leaf and the ratio of values for the surface (Evans, 2009).

iii. **Stomata Index:** This is expressed as the percentage of the ultimate divisions of the epidermis of a leaf which has been converted into stomata. It is noted that while the stomata number varies considerably with the age of the leaf, stomata index remains constant irrespective of the age of the leaf (Evans, 2009). Thus the stomata index is expressed as:

$$\% \text{ Stomatal index} = \frac{\text{Total number of stomata in a given area of leaf}}{\text{Total number of epidermal cells in the same area of leaf} + \text{total number of stomata}} \times 100$$

iv. **Vein-islet Number:** This is used to denote the minute area of photosynthetic tissues encircled by the ultimate division of the conducting strands. The number of vein-islets per mm² is calculated from four contiguous square millimeters in the central part of the lamina, midway between the midrib and the margin. When determined on whole leaf, the area examined should be from the central part of the lamina, midway between the margin and midrib. It is independent of the size of the leaf and does not alter with the age of the plant (Evans, 2009).

v. **Veinlet Termination Number:** This refers to the number of veinlet terminations per mm² of the leaf surface. A veinlet termination is the ultimate free termination of a veinlet or branch of a veinlet. It also shares the same advantage as the vein-islet number (Evans, 2009).

1.3.2.4. Ash content: The ash content of a drug may be referred to as analytical Standard which consists of the qualitative and quantitative analysis of the chief chemical constituents of the crude drug. It may also refer to the determination of the various ash values as well as solvent extractive values in accordance with Pharmacognostical Methods (Bauer, 1998; WHO, 1998;WHO, 2007; Evans, 2009). The steps involve include;

(i)**Total ash value:** is a measure of the total amount of material left after burning and includes ash derived from the part of the plant itself and acid insoluble ash (Bauer, 1998). The value also represents the amount of inorganic salts adhering to or occurring naturally in the drug. At times, this may be extended to include inorganic matter added for the purpose of adulteration. Ash determination gives basis for the evaluation of the identity and purity of a crude drug. It also gives an idea of the crude drug's extent of adulteration with inorganic matter (WHO, 1998; Evans, 2009; WHO, 2007).

(ii)**Water soluble ash:** The water soluble ash is subjected to greater reduction than in the total ash. It is thus used in the detection of material exhausted by water and as such is an important indication for the presence of materials substituted for the genuine article (WHO, 1998; Evans, 2009; WHO, 2007).

(iii) **Acid-insoluble ash:** Ash which is insoluble in dilute hydrochloric acid is referred to as acid-insoluble ash. Acid insoluble ash value is often preferred to total ash value. This preference is based on the fact that majority of crude drugs often contain calcium oxalates in large but variable amounts, and as such total ash values may vary within limits for genuine drug specimen (Evans, 2009).

(iv). **Sulphated ash:** This is a process which entails the conversion of all oxides and carbonates to sulphates at higher temperatures. Sulphated ash produces a more constituent ash value (WHO, 1998; WHO, 2007; Evans, 2009).

1.3.2.5. Extractive values: Various methods have been employed in the identification and evaluation of crude vegetable drugs. It is pertinent to highlight the fact that, in most cases, the amount of drug soluble in a given solvent is an index of its polarity. Thus, on this basis, the determination of extractive yields finds its usefulness. The determination of alcohol-soluble extractives and water-soluble extractives are useful means of evaluating drugs whose constituents are not readily estimated by other means (WHO, 1998; WHO, 2007; Evans, 2009).

1.3.2.6. Moisture Content: The most widely accepted method of moisture content determination of plant and food materials is the gravimetric method. Moisture content determination looks very simple in concept, but to practice the accurate determination is complicated by a number of facts which may vary considerably from one sample to another. Among the facts are the relative amounts of water available and the ease with which the moisture can be removed. Air or vacuum oven drying are considered to be reliable methods provided that there is no chemical decomposition of the sample and

water is the only volatile constituent removed. Sample should be dried to a constant weight (WHO, 1998; WHO, 2007; Evans, 2009).

1.4 Concept of toxicity

Toxicity is the degree to which a substance is poisonous or is able to damage an exposed organism. It can also refer to the effect on a whole organism such as animal, bacterium, plant as well as the effect on the structure of the organism such as a cell (cyto-toxicity) an organ (organo-toxicity) or liver (hepato-toxicity). Toxicity is complex with many influencing factors, such as dosage and can be measured by the effects on the organism, tissue or cell and one of such methods (measure) is the lethal dose (LD₅₀). Evaluation of plant crude extract encompasses the study of its possible toxicity and safety (Emily, 2007; Toxicity-Wikipedia, 2009). The study of the plant crude drug involves the following methods:-

- a. Acute Toxicity:- In acute toxicity study, the animal (usually rodents) is administered (exposed) a single dose of the test drug or multiple doses within 24 hours and observation is carried out for a period of atleast 14 days
- b. Repeated dose Toxicity Study:- In a repeated dose toxicity study, the animals(rodents) are exposed to the test drug daily for atleast 14 or 28 days.
- c. Sub chronic Toxicity Study:- In this method , the animals (rodents) are exposed to the test drug (material) for at least 90 days in small doses, which is sufficient in any of the single doses to produce deleterious effects.
- d. Chronic Toxicity Study:- This method arises from repeated and continuous exposure of the substance to small doses of the test drug (material) which may not be eliminated from the body and accumulates to toxic level, that is, it represents

cumulative damage to specific organ systems and it takes many months or years to become a recognizable clinical disease. The basic principles guiding toxicity test in animals are to check the effect of the test substances on laboratory animals and its direct toxic effect on human and exposure of laboratory animals to high doses in order to evaluate its possible hazard on human that are exposed to much lower doses. (Lorke, 1983; CDER, 2006; Emily, 2007; Toxicity-Wikipedia, 2009).

1.5 Analgesics

1.5.1 Concept of analgesics.

An analgesic (pain killer) is any member of the group of drugs used to relieve pain. The drugs act in various ways on the peripheral and central nervous system (CNS). Example of such drugs included paracetamol (paracetylamino-phenol), the non-steroid anti-inflammatory drugs (NSAIDs) such as salicylates and also opioid drugs such as morphine.

1.5.2 Pain

Pain is defined as an unpleasant sensory and emotional experience, associated with actual or potential tissue damage or described in terms of such damage (Merskey, 1986). Pain therefore is a perception similar to vision and hearing and is a symptom that cannot be objectively assessed (Angela-morrow, 2009). Pain is also defined as an unpleasant sensation created by a noxious stimulus mediated along specific nerve pathways to the central nervous system where it becomes interpreted (Norman, *et al*, 1985 Mosby Medical Dictionary, 2009).

1.5.2.1 Pain Feeling and Sensation

The feeling of pain is through special nerve receptors called nociceptors which are nerves designed to detect painful (or noxious) stimuli such as extreme heat, mechanical damage like pinch or irritating agents. Therefore, when the nerve detects a painful stimulus, it will fire an impulse which will travel back along the nerve fibre to the spinal cord. From there the message is conveyed to the brain through a spinal neuron (nerve) travelling up through a part of the brain called the thalamus before ending in many different areas of the brain cortex (BIGPOND Pain, 2010).

1.5.2.2 Pain Mechanism

The gate control theory of pain states that pain signals that reach the nervous system excite a group of small neurons that form a pain pool. In other words non-painful input closes the ‘‘gates’’ to painful input, which prevents pain sensation from travelling to the central nervous system. When the total activity of these nerves reaches a minimum level, a theoretical gate opens up and allows the pain signals to proceed to higher brain centres. The areas in which the gates operate are considered to be in the spinal cord dorsal horn and the brainstem (Mosby, 2009). The sequence of pain sensation includes.

- a. All noxious Stimuli are directed by receptors in pain sensitive tissue
- b. The signal generated by these receptors is transmitted by sensory nerves through the dorsal root ganglia to the dorsal horn of the spinal cord.
- c. The signal received through the peripheral sensory mechanism is processed by the spinal cord segment and transferred via ascending spinal cord path ways to various parts of the brain.

- d. The signal received in the thalamus nuclei, periventricular grey matter and brain stem reticular formations are processed and passed to the sensory cortex giving in the sensation of pain.

The immediate reaction to pain sensation is transmitted over the reflexes by sensory fibres in the dorsal horn of the spinal cord and by synapsing motor neurons in the anterior horn. The sensation of pain is therefore a protective mechanism that warns of danger without giving too much information about the specific nature of the danger. It therefore initiates nociceptive reflexes (McQuay, 1992; Mosby, 2009). The schematic diagram is thus:

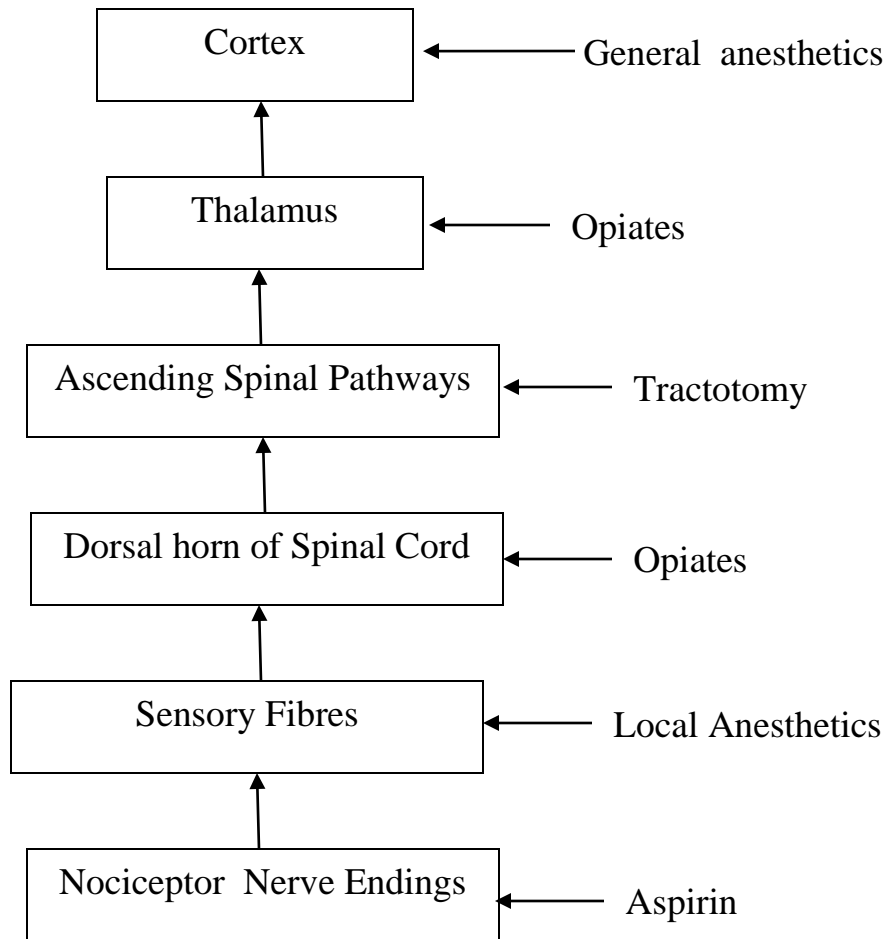


Figure 1.1: Nervous pathways and the site of action of Analgesics (McQuay, 1992)

1.6

Inflammation

1.6.1 Concept of inflammation

Inflammation is a reaction of the body to injury, infections, allergic or chemical irritation. Inflammation arises from all damages resulting from mechanical trauma, chemical, physical and thermal injury, antigen–antibody reactions and infections. The sum total is that inflammation is the complex biological response to vascular tissues to harmful stimuli. Therefore, it is the protective attempt by which the healing process for the tissue without which wounds and infections would never heal and progressive destruction of the tissue would compromise the survival of the organism (George, 2000).

The classic signs of inflammation are redness, swellings, pain (resulting from dilation of the blood vessels in the affected part with loss of plasma and leucocytes in the tissue) and loss of function (dysfunction). Inflammation destroys, dilutes or isolates the injurious agent and the injured tissue (George, 2000). The redness and heat arise from increased blood flow at body core temperature of the inflamed site, swelling by accumulation of fluid and the pain is due to release of chemical that stimulates nerve endings. The nerves that carry signals set up by chemical and mechanical stimulation of sensory receptors that is being perceived as pain also promote an increase in local blood flow through the axon reflex mechanism. The release of prostaglandins and other substances from damaged tissue also increase the sensitivity of sensory nerve endings thereby enhancing pain (George, 2000; Rustan, 2008). Essentially, the inflammatory response allows increase in blood circulation therefore the vessels dilate to increase blood flow to the inflamed area so much that important factors like white blood cell and certain proteins are circulated

around the site (George, 2000; Christine and Alice, 2004). The inflammatory reaction include the followings

- a) Tissue Injury- is the primary source of inflammation
- b) Release of chemical–initiate the inflammatory response (substance like kinins prostaglandins and histamine cause widening of the blood vessels [vasodilation] and their permeability).
- c) Leukocytes migration – White blood cells are transferred to the damaged site so that the neutrophils which are part of the WBC will function by neutralizing harmful bacteria while macrophages will aid the healing process by engulfing bacteria and dead cells so that the injured site is cleared for new cells to grow (Rustan, 2008; Sport injury Clinic, 2010).

The types of inflammation include:

- a) Acute inflammation – This is a short time immune response the body mounts in cases of trauma, infection and allergy. This is done at the initial stage by restricting blood flow to prevent hemorrhage and then increasing blood flow to initiate repair or healing process within seconds and the acute inflammation is mediated therefore by granulocytes and
- b) Chronic inflammation – This arises when the response to the injury is not completely extinguished. Therefore, the inflamed body continues to stimulate pro-inflammatory immune cells even when they are not needed. The presence of these cells in excess might bring about tissue damage such as blood vessels linings,

pancreatic tissue, joint tissue and so on. The chronic inflammation is mediated by mononuclear cells e.g. monocytes and lymphocytes (Christine and Alice, 2004; Micheal and Lynn, 2007).

1.6.2 Mechanism of inflammation

Inflammation basically is a protective mechanism. The leakage of water and protein into the injured area brings humoral factors including antibodies into the injured area and may serve to dilute soluble toxic substance and wash them away. The adherence and migration of leucocytes brings them to the local site to deal with infectious agents (George, 2000; Christie and Alice, 2004). Therefore, as soon as injury or infection is established whether caused by bacteria, trauma, chemicals, heat or by any other phenomenon, the injured tissue releases multiple substances that will cause dramatic changes in the tissue (Chauhan and Jain, 2006). The mechanism of inflammation includes:-

1. An increased blood supply to the inflamed site (vasodilatation of local blood vessels).
2. Increase permeability of capillaries/ to allow leakage of large quantities of fluid.
3. Migration of leukocytes out of the capillaries into surrounding tissues.
4. Swelling of the tissue cells caused by histamine, bradykinins, serotonin and prostaglandins. (Arthur and John, 2006; Chauhan and Jain, 2006).

The mast cells appear to be the key players in the initial stage of inflammation because their cytoplasm is loaded always with granules containing mediators of inflammation (Arthur and John, 2006; Chauhan and Jain, 2006; Michael and Lynn, 2007; Bruce, 2010).

1.7

Analgesics and Anti- Inflammatory Drugs

Analgesic is described as any member of the group of drugs used to relieve pain. The drugs act in various ways on the peripheral and central nervous systems. On the other hand anti-inflammatory refers to the property of a substance or treatment that reduces inflammation. Anti- inflammatory drugs make-up about half of analgesics remedying pain by reducing inflammation as opposed to opioids which affect the central nervous system (Ergelhardt, 1996; Omudhome, 1996;Everts *et al.*, 2000; Praveen and Edward, 2008).

1.8

Statement of Research Problems

Medicinal plants are key players in the world's healthcare systems with about 80% of Africans depending on phytomedicine. The Nigerian medicinal plants are not exceptions in this guise. It could be a common fact that many of these plants especially herbs have not been extensively exploited for their analgesic and or anti-inflammatory agents in general (Elujoba *et al.*, 2005). Accordingly, the few plants that have so far being studied for their efficacies, there are no set pharmacognostic standards for their identities, elemental contents, and industrial base conservation being established. In short, their active principles responsible for their wide ranging analgesic and or anti-inflammatory properties were not identified to a larger extent. *In-vitro* research into individual plant used by the indigenous population whereby the plants have been used as indicators for antibacterial, antiviral, antisickling, analgesic, anti-inflammatory and other biological activities is not enough to ascertain the claim by the traditional medical practitioners or herbalists. Traditionally, a plant used for wound healing, pain relief, fever and headache,

infections, edema, rheumatic disease and many others is not an indication that such a plant should be tested for either analgesic or anti-inflammatory properties. However, *in-vitro* studies or screening have provided an assay of plants for either analgesic or anti-inflammatory properties that have served as prelude to the activity of medicinal plants. On the whole, the analgesic action is on the peripheral and central nervous system (CNS) while for the anti-inflammatory screening the procedures include the inhibition of cyclooxygenase and 5-lipoxygenase which are the two enzymes responsible for producing thromboxanes, prostaglandins and leukotrienes (Norman *et al.*, 1985; George, 2000). By and large, further researches in these activities will reveal more on the potentials of most of these common plants which in the long-run could serve as a source of low cost, affordable, accessible and acceptable and perhaps low toxic analgesic and anti-inflammatory drugs that are of international standards.

The search for phytochemicals from medicinal plants is a worldwide phenomenon because the potentials of the plants have not been properly harnessed. Therefore, continuous search of these components especially those with potent analgesic and anti-inflammatory activities is worthy of consideration in drug discovery as an alternate to orthodox medicines.

1.9

Aim and Objectives Of The Study

- i. Aim: To study the Pharmacognostic standards for the identification of the leaf of *Microtrichia perotitii* (DC) and preparation of monograph as well as to evaluate its traditional usage as an analgesic and anti-inflammatory agent.
- ii. Objectives:
 1. To establish some macroscopic, microscopic, quantitative and chemo-microscopic features of the leaves of the herb.
 2. To determine the median lethal dose (LD₅₀) values of the extracts of the leaf of *Microtrichia perotitii* in laboratory mice.
 3. To evaluate the analgesic and anti-inflammatory activities of both the crude and solvent fractions of the leaf of *Microtrichia perotitii* using animal models (mice and rats).
 4. To identify the phytochemical constituents present in the leaf of *Microtrichia perotitii*.
 5. To isolate and characterize the constituents that might be responsible for the activities of the leaf of *Microtrichia perotitii*

1.10

Statement of Research Hypothesis

The leaves of *Microtrichia perotitii* (DC) contain phytochemical constituents with potential analgesic and anti-inflammatory activities.

CHAPTER TWO

2.0

LITERATURE REVIEW

2.1

The Family Asteraceae (Compositae)

The family Asteraceae Bercht & J. Presl, (Compositae Giseke) also called the aster, daisy or sunflower family is a taxon of dicotyledonous flowering plants and is probably the largest family of flowering plants consisting of over 1,600 genera and over 23,000 species worldwide growing from sea level to the highest mountain peaks. The name of the family is derived from the genus *Aster* and it refers to the star-shaped flower heads of its members, epitomized well by the daisy (Wagner *et al.*, 1990). The old name *compositae* on the other hand was derived from the Latin word *compositus* which means “made up of parts united in one common whole”. This therefore, refers to the collection of different florets arranged together in an inflorescence (Panero and Funk, 2002; Herman, 2004). Generally, the Asteraceae family is divided into decreasing ranking such as subfamilies (Cichorioideae and Asteroideae), Tribes (19), Subtribes, genera and species: The scientific classification of the Asteraceae family is thus:

Kingdom: *Plantae*

Division: *Magnoliophyta*

Class: *Dicotyledons (Magnoliopsida) [Angiosperms]*

Order: *Asterales*

Family: *Asteraceae* Bercht & J. Presl (Alvarenga *et al.*, 2001; Scott, 2006).

Members of the Asteraceae are mostly herbs, rarely small trees, acaulescent, dwarf shrubs and some are scramblers and aquatic. They could be laticiferous or not and without coloured juice. Some do bear essential oils while some members are in between normal plants or switch-plants. In general they could be annual, biennial or perennial plants (Hutchinson, 1973; Elizabeth, 2000; Herman, 2004).

The descriptive features of members of Asteraceae include that, their leaves are arranged alternately and sometimes opposite and when they appear alternate they are usually simple. The annual, biennial and perennial members have basal aggregation of leaves. The most evident characteristic of Asteraceae is their inflorescence (calathid or calathidium or simply flower head) which are aggregated. The stem of members has secretory cavities which could be absent in some species and contain resin or with latex. The fruits of members are non-fleshy, indehiscent, a cypsella or a drupe occasionally. The seeds on the other hand are without endosperm (Hutchinson, 1973; Abulafahh, 1989; Watson and Dattwitz, 1992; McKenzie *et al.*, 2005; Jose and Bonnie, 2008). A comparative study of the leaf epidermis of 12 species of Asteraceae indicated the presence of glandular and non-glandular types of trichomes which ranged from T-shape, K – or – H – shape to amoeboid shape, epidermal cells that are polygonal, irregular to rectangular in shape at either adaxial to abaxial surfaces. Four stomatal types were also recorded and they included; anomocytic, brachyparacytic, anisocytic and diacytic. The presence of starch grains, calcium oxalate crystals and trichomes were also reported from the leaves of members (Jane and Marcia, 2010). Other parameters such as chemo-microscopic, quantitative-leaf microscopy and physical constants were also reported for members of the Asteraceae (Adedeji and Jewoola, 2008; Salahuddin *et al.*, 2010)

2.2

Biological Activities of Some Members

Members of the Asteraceae because of the presence of important compounds in them have been reported to possess medicinal values. Some reported works included their practice in Traditional folkmedicines. Abulafahh,(1989) reported the use of some members of the Compositeae for the treatment of various diseases and sickness such as rheumatism, asthma, diabetes, stomach problems, constipation, eye and ear problems, cold, fever, measles, bladder and urinary disease, toothaches, epilepsy and skin allergy amongst the dissidents of Saudi Arabia.

People of rural Mexico and their Mexican/ Chicano descents in the United States have been reported to be ingesting herbal teas made from some species despite undergoing treatment for ailments with antibiotics or anti-inflammatory drugs because of their belief that the plants they are using contain very important substances that can cure their maladies which afflict them. Therefore, members of the Asteraceae (Compositeae) have contributed the largest number of plants in their Pharmacopeias (Berlin and Berlin, 1996; Heinrich,*et al.*, 1998).

Alotion produced from the species *Artemisia filifolia* was said to be used for the treatment of snake bites, while its herbal tea is good for the treatment of indigestion. The aromatic oil from it also is good in treating colds and coughs amongst the people of southern Brazil (Luz and Anderson, 2001).

Molluscidal activity of some members of the family against adult snail (*Biomphalaria glabrata*) was reported. The snail is a host to *Schistosoma mansoni* which is a parasite that causes schistosoma disease. The plants include *Actinoseris angustifolia*, *Alomia*

myriadenia, *Achyrodine satureiods*, *Verbesina claussemi*, *Pipotocarpha rotundifolia* and *Vanillosmopsis erthropappa* each of which killed 100% of the snail adults after 24hrs with LC₉₀ ranging from 33-43 (Nelymar *et al.*, 1999;Anzar *et al.*, 2007).

Antimicrobial activity of *Pulicaria dysenterica* L. was reported against *Vibrio cholera*, *Staphylococcus aureus* and *Bacillus cereus* that are responsible for infectious diseases. The extract inhibited the growth of the micro-organisms (Bahmanet *et al.*, 2007).

The genus *Artemisia* have been cited in many pharmacopeia indicating the use of species for the treatments of gastric and hepatic(liver) disease, malaria as chologogue, chloleretic, anticonvulsive, antifungal, antihelminthic, bacteriostatic, sedative agent such as sieversiane for thousands of years (Hope , 1975;Van *et al.*, 1997). Similarly, the new genus *Tagetes* have variously been reported for their medicinal values mostly in communities where they are cultivated (Mohammed,*et al.*, 2010)^a.

Medicinal uses of the genus *Eriocephalus* have been reported. Some species such as *E.africana*; *E.recemosus* and *E. punctualtus* have been used for the treatment of respiratory ailments, gastro-intestinal disorders and various skininflammation diseases (Watt and Breyer-B, 1962; Van-Wyk *et al.*, 1997, Van Wyk and Gericke, 2000). Similarly, the essential oils from the species *E. Africana* was found to exhibit antimicrobial activity against *Cryptococusa neoformans* and *Bacillus cereus* thus validating the use of the oils from the genus in Chinese Traditional herbal remedy (Nakatsuet *et al.*, 2000; Candan *et al.*, 2003; Viljoen *et al.*, 2003).

Similarly, Nurhayat *et al.*(2007) reported antifungal activities of the essential oil extracted from *Amica longifolia*, *Aster hesperus* and *Chrysothamus nauseosus* against

some plant pathogens (*Colletotrichum acutatum*, *C. tragariae* and *C. gleosporioides*) with comparable activity of 20mg/ml. In the same vein, Sathishkumar *et al.* (2008) reported the antimicrobial and antitumor activities of *Stevia rebaudiana* against some disease causing microorganisms (*Staphylococcus aureus*; *Salmonella typhi*; *Escherichia coli*; *Bacillus subtilis*; *Aeromonas hydrophila*; *Vibrio cholerae*, *Candida albicans*, *Cryptococcus neoformans*; *Trichophyton mentagrophytes* and *Epidermo* phytospecies. The plants exhibited activity much greater than the standard drug used.

The genus chamomile is also reported for the treatment of human ailments such as hay fever, inflammation, muscle spasms, menstrual disorder, insomnia, ulcers, wounds, gastrointestinal disorders, rheumatic pain and haemorrhoids in some pharmacopias (Hamon, 1989; Fidler *et al.*, 1996; Kyakong *et al.*, 2002).

The medicinal value of the decoctions of *Jasania saxatiles* (Lam) and that of *Matricaria recutita* L. including its infusions were reported to have antidiarrhoeal and antiseptic properties when administered orally (Delors *et al.*, 1997). Similarly, the ethanolic extract of the flowers of *Helichrysum plicatum* was studied for its relaxant activity on isolated rat ileum. The extract showed spontaneous inhibition of the ileum contractions including those induced by acetylcholine, histamine, barium and potassium ions (Dubravka *et al.*, 2010).

In certain communities, *Ageratum conyzoides* L. is used for purgative while the sap of its leaves is used to treat wounds and eye problems. The sap of *Emilia sonchifolia* L. is also used for treating wounds. *Vernonia amygdalina* Del is used to cure measles, small and chicken pox and piles while its roots and epidermis are used to treat diarrhoea (Gbile and

Adesina, 1986). *Inula royleana* DC is also used for the treatment of throat sores, wounds and inflammation, while *Enydra fluctuans*, Lour, *Spilanthe scalva*. DC and *Tagetes erecta* L were reported to be used for the treatment of gastric ulcer, toothache, blood dysentery and wounds using various parts of the plants (Mohammed *et al.*,2010)^b. Another species of the genus *Matricaria* (*Matricaria- chamomilla*) is reported in the tradomedicinal application for centuries some of which included herbalism, relaxation, sedative, soothing, spasinolytic effects, calming, sedative, anti-inflammatory, tenseness, aching muscles, indigestion, acidity, hay fever, asthma, eczema, sore nipples, toothaches, insomnia, aches and pains, flu and allergies in the Hispanic medicine (John and Ted, 2011). It has also been reported that members of Asteraceae contain various important trado-medical applications which led to their citation in many pharmacopeia.

2.3

Phytochemicals

Asteraceae members generally store energy in the form of inulin which is a polysaccharide made up of small polymer units of fructofuranose. Other compounds include polyacetylenes which are characterized by the presence of cyclic, aromatic or heterocyclic end-group, sesquiterpenes lactones, alkaloids, flavonoids, saponins and essential oils (David, 2001). Infact, glycosides, saponins, flavonoids and tannins are their chief constituents. The flavonoids and saponins are sometimes used as taxonomic markers (Alvarenga *et al.*, 2001; Evans, 2009).

Sesquiterpene lactones have been reported to occur in many members of Asteraceae. They are biologically active compounds and are known to poisonlivestock, act as insect feeding deterrents and to cause contact dermatitis in humans (Astreaet *al.*,1990; Neerma,

2003;Frederick, 2007). *Elephantopus scabh.* L. is a plant used for healing wounds and the ethanolic extract of its leaves was found very active against wounds and upon spectroscopic analysis the presence of deoxyelephantopin was revealed which is a sesquiterpene lactone (Singh *et al.*, 2005; Geetha *et al.*, 2011). Christian *et al.*,(2009) reported the isolation of a novel chlorinated sesquiterpene lactone glucoside from *Leontodon palisae*. The isolated compound showed strong cytotoxic activity against all tested microorganisms. It has been reported that two eudemanolides, eight lactucin and five phenolic compounds were isolated from the root of *Cichorium pumilum* for the 1st time. The plant has been used extensively in the folkmedicine of the mediterenean (Bohlman *et al.*, 1982; Goren *et al.*, 1992; Wanda and Klaudia, 2003;Ricardo *et al.*, 2006; Gerard, 2011).

Sesquiterpene lactones have also been isolated from some members of the Asteraceae family such as *Tanacetum praeteritum*Horw from its cytotoxic, antibacterial and antifungal properties. (Goien *et al.*, 1996;Mansilla and Palenzuela, 1999; Neerman, 2003). A sesquiterpene lactonealongside a flavone was isolated from*Tithonia diversifolia* which was screened for germination and growth inhibition activitiese isolated from the root of the plant (Baruah *et al.*, 1996;Rateb *et al.*, 2007; Bianca *et al.*, 2015).

Flavonoids which give colours to flowering plants have been isolated from some members of the Asteaceae family. Forinstance some flavonoids were detected and isolated from the trichomes of *Chromolleaena* species (*C. hirusita* and *C. squalida*) which were associated with antimicrobial activities (Silvia *et al.*,2007). Kaemferol, quercetin and luteolin were isolated from the methanolic extract of *Rhagadioles stellatus* Gaertn a

plant used as food by Cichoreae tribe of the Mediterraneanas well as the methanolic extract of *Aster novibelgii* (Emad *et al.*,2011; (Romana *et al.*, 2011).

The first phytochemical study of *Blackiella bartsiiifolia* SF. Blake revealed the presence of 2 flavonoids (Flavones) from the plants exudates and a labdane diterpene (Maria *et al.*,2010). Silymarin which is a mixture of flavolignans was reported to have been isolated from the seeds of *Silybummananum* Gaertn and was said to passes antihepatotoxic activity (Valenzuela and Garrido, 1994). Some other flavonoids with antioxidant properties were isolated from *Pieris choidus* L a plant thatis used to treat indigestion, intestinal nematodes and their parastities in the mediteranian (Mirjana *et al.*, 2003; Saluk *et al.*, 2010, John and Ted, 2011).

Monika and Tadeuse, (2009) reported the isolation of flavonoids and coumarins from *Hieracium pilosella*, La well known plant in European ethnomedicine and traditionally used to treat inflammations of the urinary tract and skin diseases because of its diuretic, astringent, antiseptic and antiphlogistic activity. Some of the flavonoids included Luteolin, apigenin, glucopyranoside and kaempferol. Similarly, 18 species of the genus *Madia* were phytochemically screened and the results lead to the isolation of different flavonoids and diterpenes which were associated with the wide ranging medicinal utilization of the plants (Eckhard *et al.*, 2003).

Humberto *et al.*, (2009)reported the isolation of a new polyacetylene from *Vernonia scorpioides* Lam. The plant is found useful for the treatment of variety of skin disorders such as chronic wounds (eg ulcers), fungicides and bacteriacdes. The compound isolated was found active against some tumor cells and also has cytotoxic effect.

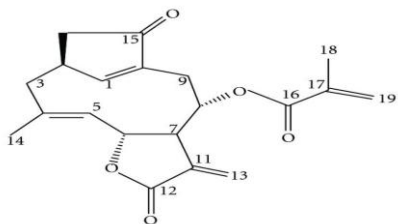
Phytochemical investigation of various parts of *Luchnophora ericoides* Mart indicated the occurrence of steroids, triterpenes, flavonoids, furanoheliangolides and lignans. The furanoheliangolide was found to be cytotoxic against cancer cells (LC₅₀ value of 0.06µg/ml) while various parts of the plant were found useful in analgesic and anti-inflammatory activities (Pierre *et al.*, 2004).

Chromatographic analysis of the essential oil extracted from the flowers of *Chrysanthemum coronarium* L revealed the presence of camphor, α- and β-pinene while the essential oil from *Artemisia* species (*A. absinthium* L, and *A. specigera* C.Koch) indicated the presence of camphor and 1,8 – cineole. Piperitone and piperitenone were also identified from the essential oil of *Tagetes patula* L. (Maria *et al.*, 2007).

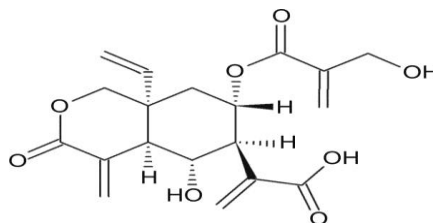
From the crude extract of *Baccharis dracunculifolia* D.C., Isosakuranetic, aromadendrin-4-methylether, baccharis oxide, ferulic acid, cinnamic acid, fredelanol were identified and isolated (Da-Silvia *et al.*, 2008). Similarly, the root extract of *Pluchea indica* has revealed the presence of β-sitosterol and stigmasterol (Gomes *et al.*, 2006) while the active compounds in *Inula helenium* L and *Rudbeckia substantenlosa* Pursh revealed the presence of alantolactone, 10,α-11-α-H-13-dihydroisoalantolactone from the *I. helenium* while alloalanolactone and 3-oxoalloyalantolactone were isolated from *R. substantenlosa* (Cantrell *et al.*, 1999). Oleoic acid and deacylcyanopricirin compounds were isolated from the plant *Cyclolepis genistoides* D.Don. The plant is used widely in folk medicine as diuretic, an antirheumatic and antispasmodic agent in Argentina (Sosa *et al.*, 2011).

A study on the phytotoxins of the genus *Artemisia* and *Chrysanthemum* showed that the species inhibited the growth of *Escherichia coli* and *Saccharomyces cerevisiae* cultures when exposed to a UV-irradiation. Further studies showed that the presence of polyacetylenic and thiophenic metabolites are responsible for the phytotoxicity of the members (Kelsey *et al.*, 1987).

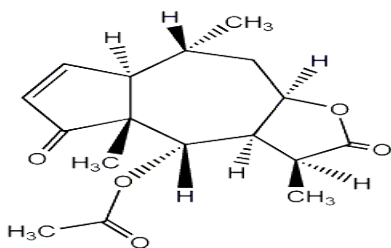
Other important compounds isolated from the Asteraceae family included, chlorogenic acid (*Rhagadioline stellatus*), saponin (*Asternovibelgii*), caffeic acid (*Eupatoriuniperifolialum*), syringaldehyde, caniterinaldehyde and caniferyalcohol (*Cichorium pumilum*), coumarin (*Hieracium pilosella*), biopharizone (*Madia* species) (Eckhard *et al.*, 2003; Mareika *et al.*, 2008; Monika and Tadeusz, 2009; Taha *et al.*, 2010; Emad *et al.*, 2011; Romana *et al.*, 2011).



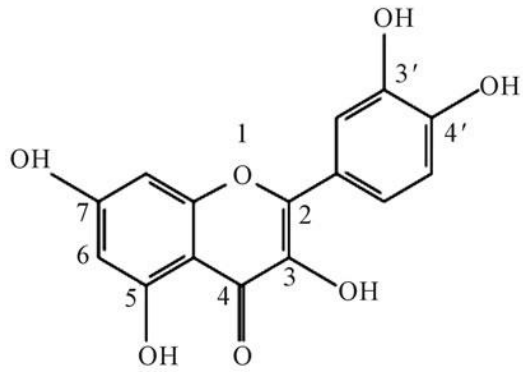
elephantopin.



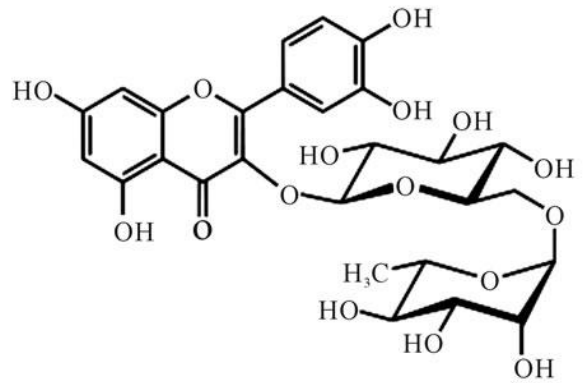
vernodalinol



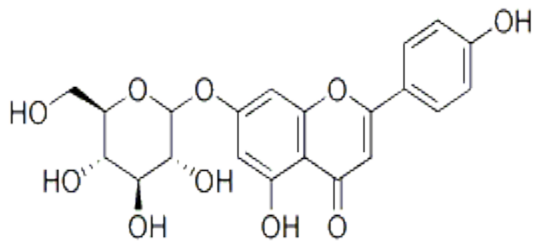
arnicolide



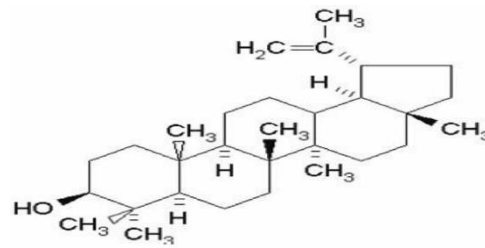
Quercetin



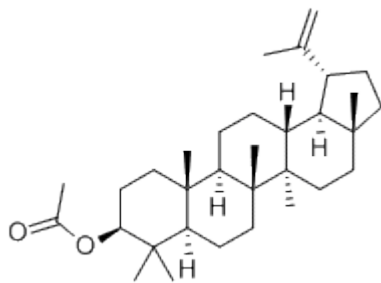
Rutin (Quercetin-3-O-rutinoside)



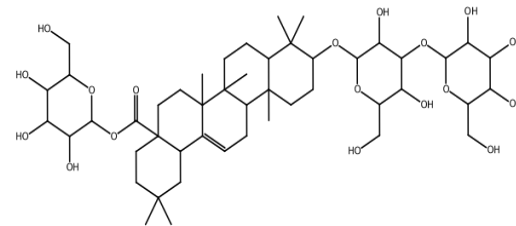
Apigenin



lupeol



Lupeol acetate



Arvensoside

Figure 2.1: Isolated compounds with analgesic and or anti-inflammatory activities from Asteraceae

2.4

The Genus *Microtrichia* DC

The genus has only two species, namely; *Microtrichia perotitii* DC and *Microtrichia dellenis* (Gray 1822) but later another species was added and is named *Microtrichia zavattarii* Lanza (Cufodontis, 2009). The general features of the genus/species include possession of capitula which is hemispherical or broadly campanulate, outer florets, involuoral scales 2-3 seriate, membranous-margined, nearly equal or shorter than the florets. The receptacle is convex and naked. The corolla is tubular with the mouth denticulate. Campanulate is narrowed at the base, shortly 4-5 anthers, the base is obtuse, apex shortly produced, oblong is lanceolate. The style has branches with lanceolate acute tips. The achenes is subterete or slightly compressed; pappus of few cellular subulate, denticulate very caduceous setae. They are usually herbs or under shrub more or less pubescent. Leaves are alternate, dentate. The capitulum is small, many are flowered and yellow. The genus is confined mostly to Tropical Africa (Daniel, 1877).

2.4.1 The species, *Microtrichia perotitii* DC

Microtrichia perotitii DC is an herb and a diffuse much – branched pubescent or puberulous annual varying to 1ft or more in height (Watson and Dallwitz, 1992). The leaves are obovate, obtuse, cuneately narrowed into petiole, coarsely toothed above, ½- 1½ inch long 1/3-1 inch broad. The petiole of lower leaves is 1 inch or more and the upper is shorter. The capitula is 1/8- 1/6 in diameter, sessile or shortly pedunculate in small terminal or subterminal clusters. Involucral scales are oblanceolate or linear, 2-3 seriate, subequal and rather acute, cellular – dentate, outer loosely pilose. Achenes are

thinly scattered with short spreading minutely glochidiate setae, substerete or slightly compressed (Daniel, 1877; Andrews, 1954).

The plant is widely distributed in West Africa. In Nigeria it is cultivated in Northern Nigeria around Zaria province which extends up to Birnin Gwari (Kaduna State). The plant is called ‘‘maijankai’’ or ‘‘sawun keke’’ because of its colour appearance and its twining around other plants respectively by the Hausa speaking people, ‘‘shaware pepe’’ by Yoruba speaking people and ‘‘osete’’ by the Igbira speaking people. In other parts of West Africa it is common in Senegal, Quassadous, Mali, Port of Guinea, Sieraleone, Ivory coast, Ghana and Dahomey (Hutchinson and Dalziel, 1963).

The plant species is classified as follows:-

Domain: *Eukaryota*
Kingdom: *Plantae*
Division: *Magnoliophyta*
Class: *Magnoliopsida (Dicotyledons)*
Order: *Asteraes*
Sub class: *Asteridae*
Superorder: *Campanulanae*
Family: *Asteraceae (Compositae)*
Genus: *Microtrichia* DC (Grangea Adams)
Species: *Microtrichiaperottitii*. DC

2.4.2 Ethnobotanical uses of *Microtrichia perottitii*

An Hausa medical traditional herbalists at Kasuwar Barci Market at Tudun Wada District of Kaduna and female traditional herbalist (Hawker) at Rigasa Village when consulted reported that the herb (*Microtrichia perottitii*) is very good in treating tooth ache, cuts and burns. Therefore, the wet leaves are chewed continuously until the pepperish taste is exhausted. Sometimes, the cold concoction of the leaves are used as mouth wash or the dried leaves are applied directly to the affected tooth. The fresh flowers are squeezed and can also be placed on the affected tooth. The wet and fresh leaves are squeezed and spread over rashes in children to treat it. Of recent, there are claims of the use of the plant to treat rheumatism in aged persons, diarrhea as well as jaundice when made with concoctions of neem leaves. The herb is also used to treat some inflammatory diseases.

However, Yoruba spiritualists use the plant to dispel evil spirit as explained when they were consulted at Kasuwar Barci Market Kaduna.

2.5 Previous Research on *Microtrichiaperottitii* DC

There were noreported research works for the herb.

CHAPTER THREE

3.0 Materials and Methods

3.1 Equipments, Solvents, Reagents/Solutions and Instruments.

3.1.1 The following equipment were used for the research

Beakers

Beam balance (Denver instrument APX-200)

Bunsen burner

Conical flasks

Desecrator

Developing tanks with covers (Shanden TLC chroma tank)

Droppers

Forceps

Heating mantle

Hot plate (Sharon SUSSEX) MH/4

Hot water bath (baths Macdonald) digital

Hotplate (Gallenkamp, England)

Measuring cylinders

Mechanical shaker (stand scientific flash shaver SF1)

Microscope (fisher scientific Edu, 0602317)

Nickel crucibles.

Oven (fisher scientific midi/6/clad-F)

Pipettes

Pre-coated TLC paper

Sieve (0.6mm)

Slides and cover slips

Soxhlet apparatus (Quickfit)Electrothermal CAT No EM 3000mk1 (3000m1)

Spatula

Stirring rods

Syringes (0.01mm) and needles

Test-tubes

Thistle funnels

Tripod stand

Watch glasses

Weighing balance (mettler P1210)

Whatman filler paper

Wire gauge

3.1.2 Solvents

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

Acetone

Ammonia hydroxide (ammonia solution)

Chloroform

Diethylether

Distilled water

Ethanol

Ethylacetate

Glacial acetic acid

Hydrochloric acid

Methanol

n-Butanol

n-Hexane

Petroleum ether (60-80⁰C)

Tetraoxosulphate vi acid (Sulphuric acid)

3.1.3 Reagents/solution

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

Acetic acid (0.06%)

Ammonia solution (10%)

Chloral hydrate solution

Dragendoff's reagent

Fehlings reagent

Ferric chloride (5%)

Glycerol solutions

Hydrochloric acid (1%)

Hydrochloric acid (10%)

Magnesium chips

Mayer's reagent

Wagner's reagent

Molish reagent

Phloroglucinol

Sudan IV

3.1.4.1 Preparation of fresh reagents

Fresh solutions of the following reagents were prepared for the research by using the process of Ciulei (1994) and WHO (1998) they include;

a. Fehlings' solution (cupric-alkaline solution)

34.66 g of copper (II) sulphate was dissolved in 200ml of distilled water and then diluted to 500 ml to obtain Fehlings solution A, while 17.3 g of sodium and potassium tartrate (Rochelle salt) and 100g of sodium hydroxide were dissolved in 300ml of distilled water and made up to 500ml after cooling to obtain Fehling's solution B. For the use, equal volumes of A and B are usually mixed immediately before use.

b. Dragendoff's reagent

The reagent was prepared by dissolving 0.85g of basic bismuth nitrate in a mixture of 40ml of distilled water and 10ml of Acetic acid. A solution of potassium iodide was prepared by dissolving 8g of its salt in 20ml of distilled water and was then mixed homogeneously.

c. Mayer's reagent

The reagent was prepared by dissolving 1.35g of mercuric chloride in 60ml of distilled water and then mixing it with a solution of potassium iodide which was prepared by dissolving 8g of the salt in 10ml of water. The mixture was diluted with 100ml of distilled water.

d. Molisch's reagent

This reagent was prepared by dissolving 15g of naphthol in 100ml of ethanol. Alternatively it contained not less than 17% v/v of H_2SO_4 and 20% thymol mixed in ethanol.

e. Wagner's reagent

The reagent was prepared by dissolving 2 g of iodine and 6 g of potassium iodide in 100 ml of distilled water.

f. Chloral hydrate

This solution was prepared by dissolving 50g of the salt in 20mls of distilled water.

g. Phloroglucinol

This solution was prepared by dissolving 1g of the salt in 100ml of ethanol

3.1.4 Instrumentation

Double beam microscope (Fischer scientific education 0602317)

Micrometers

Vernier caliper

^1H -NMR spectrophotometer (Bruker 400Mhz)

^{13}C -NMR spectrophotometer (Bruker 400Mhz)

FTIR spectrophotometer (Perkin Elmer Spectrum 100 FT-IR)

Melting point apparatus(Stuart Scientific co Ltd GR)

3.2 Plantcollection

Fresh herbs of *Microtrichia perotitii* DC were collected during the matured and flowering stages of the plant from April 2005. The plant was collected from the swampy areas in Rigasa village a sub-urb of Kaduna metropolis in Kaduna state.

3.3 Identification of *Microtrichia perotitii* DC

The plant was identified and authenticated by Mallam Musa M.of the Herbarium unit of the Department of Biological sciences, Ahmadu Bello University, Zaria. A herbarium specimen number of 998was given for future references.

3.4 Macroscopic examination of the leaf of *Microtrichia perotitii* DC

The macroscopical examinations of the leaves were carried out with the aid of lens and the naked eyes. The observations conducted included, appearance of the leaves such as the surface appearance, dimension, point of attachment, lamina structure which included composition, inclusions, shape, base, venation, margin and apex as well as the organoleptic characters that included, colour, odour, texture and taste (Brain and Turner, 1975; Evans, 2009).

3.5 Microscopic examination of the leaf of *Microtrichia perotitii* DC

The microscopic studies of the leaves of the herb were done using the following methods.

3.5.1 Qualitative microscopic examination

The microscopical features of both fresh and powdered leaves of *Microtrichia perotitii* were carried out. The surface was prepared by gradually peeling off the upper and lower epidermis of the fresh leaves, while the transverse section (T.S) was prepared with the aid of a razor blade. Small quantity of the powdered leaves was also subjected to the microscopic studies and were prepared by clearing them in 70% chloral hydrate solution on a microscope slide after being boiled in a test-tube on a Bunsen flame. The fresh leaves were prepared by boiling on water bath in 70% chloral hydrate. This process dissolved starch grains, plasticid and volatile oils while at the same time expanded collapsed and delicate tissues without causing any under swelling of cell walls or distortion of tissue (Brain and Turner, 1975; WHO, 1998, Evans,2009).

3.5.2 Chemo-microscopic examination

The chemo-microscopy studies were carried out on the powdered leaf material of *Microtrichia perotitii* DC. Small quantity of the powder was cleared of any obscuring materials by boiling in 70% Chloral hydrate solution in a test-tube. The sample was later mounted on clean microscope slide using dilute glycerol. Detecting reagents were applied to detect the presence of some cell inclusions and cell wall materials. (WHO,1998; Evans, 2009).

a. Test for starch:

To a small quantity of the cleared leaf powder N/50 iodine solution was added. Appearance of Blue-black colouration on some grains confirms presence of starch granules.

Similarly, 2 drops of dilute glycerol were added to the cleared leaf. Appearance of brownish colouration confirms positive test for starch(WHO, 1998; Evans, 2009).

b. Test for Lignins

To a small quantity of the cleared leaf powder, phloroglucinol solution was added followed by few drops of concentrated HCl. Appearance of red stain of the lignified walls confirms positive test for lignins(WHO, 1998; Evans, 2009).

c. Test for Cellulose

To a small quantity of the cleared leaf powder, few drops of chloro-zinc iodine solution were added. The occurrence of blue colouration indicates positive test for cellulose on cell walls of the epidermal cells(WHO, 1998; Evans, 2009)

d. Test for Calcium Oxalate crystals

To a small quantity of the cleared leaf powder, few drops of 80% H₂SO₄ were added. Appearance of brightly coloured crystals which disappears upon addition of few drops of concentrated HCl indicates positive test for calcium oxalate crystals(WHO, 1998; Evans, 2009)

e. Test for fixed oils and fats

To a prepared transverse section of the leaf few drops of Sudan IV solution were added and observed for few minutes. The appearance of a reddish colouration which is distinct indicates the presence of fixed oils and fats (Brain and Turner, 1975; Evans, 2009).

f. Test for Calcium carbonates

To a small quantity of the cleared leaf powder few drops of 80% H₂SO₄ were added. Deposition of Calcium carbonate crystal which dissolves upon addition of few drops of HCl with effervescence indicates positive test for CaCO₃(WHO, 1998; Evans, 2009).

g. Test for Mucilages

To a small quantity of the cleared leaf powder, few drops of Rutheniumred were added. Appearance of pink colouration in the mucilage containing cells within the epidermis indicates positive test for mucilaginous walls (WHO, 1998; Evans, 2009).

h. Test for Tannins

To a small quantity of the cleared leaf powder, few drops of 5% Ferric chloride solution were added. Appearance of a greenish black colouration on some parenchyma cells indicates positive test for tannins(WHO, 1998; Evans, 2009)

3.6 Quantitative leaf microscopic determination

Some physical constants were determined on the leaf of *Microtrichia perotitii*DC. These included the results obtained from the microscopic character on the fresh leaf and physical determinations, as described by Evans, 2009.

a. Stomatal number

The average number of stomata per square millimeter of epidermis of both upper and lower sides of the leaves of *Microtrichia perotitii*DC were calculated after the sections of the upper and lower epidermis were cleared with 70% chloral hydrate solution as described earlier. A camera Lucida was set-up with the aid of a stage

micrometer, while a clean paper was divided into squares of 1mm² using a x10 objective (magnification). The stage micrometer was replaced by the cleaved sections (Evans, 2009). The stomata was traced and counted in the fields on a single section of the leaf and thus the average number of the stomata per mm² of epidermis was calculated from:

$$\text{stomatanumber} = \frac{\text{averagenumberofstomata}}{\text{squaremillimeterofepidermis}}$$

b. Stomata index

The percentage proportion of the ultimate divisions of the epidermis of a leaf which has been converted into stomata was determined for the plant. Sections of the upper epidermal portion of the leaf were cleared with the 70% Chloral hydrate solution by boiling over hot water bath. After mounting on a clean microscope slides with dilute glycerol, it was examined with x 40 objective (magnification) (Evans, 2009). The stomata as well as the epidermis were counted and the index calculated.

$$\% \text{ stomatal index} = \frac{\text{numberofstomata}}{\text{numberofepidermalcells} + \text{numberofstomata}} \times 100$$

c. Vein-islet number

The number of vein-islet per mm² of the leaf of *Microtrichia perotitii* DC was calculated in four contiguous squares from the central part of the lamina of the leaf between the midrib and the margin. It was calculated from the cleared leaf which was later treated with Hydrochloric acid (10% v/v) in order to remove any trace of Calcium oxalate crystals for proper visibility.

A set-up camera lucida at x10 objective was used for the determination as described for the stomatal number. Thus each vein was traced and areas which were completely enclosed by the veins were counted except those that were completely enclosed (Evans, 2009).

d. Vein-let termination number

The number of vein-let termination per mm^2 of the leaf of *Microtrichia perotitii* DC at the surface was determined. A set-up camera lucida was prepared as described earlier and thus the Vein-let termination in each square was counted in order to get the vein-let termination number (Evans, 2009).

e. Palisade ratio

The average number of palisade cells beneath the upper epidermal cells for the leaf of *Microtrichia perotitii* DC was determined. The cleared leaf was observed with x40 objective lense (magnification). The camera lucida was arranged to allow for the tracing of both epidermal and palisade cells lying beneath them. Groups of 4 epidermal cells were traced and their outlines linked to them were conspicuous. Therefore, palisade cells lying beneath each group were focused, traced and counted, including the cells that were more than half covered by the epidermal cells. The values obtained were divided by a factor of 4 so as to get the palisade ratio of each group (Brain and Turner, 1975; Evans, 2009).

3.7

Determination of physical constants

The dried powdered leaf material of *Microtrichia perotitii* DC was subjected to the following physical tests.

a. Determination of moisture content (MC)

The moisture content of the leaf of *Microtrichia perititii* DC was determined by using oven drying method (Razavi and Taghadeh, 2005). Therefore, 3g of the powdered leaf material was weighed and transferred into a weighed crucible. The crucible was then heated for 1hr at 105⁰C in drying oven. At the expiration of the period, the crucible and its contents were cooled in a dessicator and the weight loss was determined. The process was repeated five times until a constant weight was obtained (WHO, 1998; Evans, 2009).The % MC was calculated from,

$$\% MC = \frac{wt\ of\ crucible + sample(W_1) - wt\ of\ crucible + ovendryedsample(W_2)}{wt\ of\ crucible + sample(W_1) - wt\ of\ emptycrucible(W_0)} \times 100$$

b. Determination of total ash

3g of the powdered leaf material of *Microtrichia perotitii* DC was weighed accurately into a previously weighed crucible. The powder material was spread evenly. The crucible was charred in a muffle furnace by gradual increase in temperature (400-500⁰) until the content turned white thus indicating the absence of carbon. The crucible was cooled to room temperaturein a desiccator andlaterre-weighed (WHO, 1998; Evans, 2009; Pratima and Mathad, 2011).. The total ash was expressed as a percentage.

$$\% totalash = \frac{wtcrucible + sample(W_1) - wt\ of\ crucible + ash(W_2)}{wt\ of\ crucible + sample(W_1) - wt\ of\ emptycrucible(W_0)}$$

c. Determination of acid-insoluble ash

The crucible containing the total ash from (b) was gradually transferred into a beaker and the content washed with 25 ml of 25 % (v/v) hydrochloric acid. The beaker was covered with watch-glass and boiled gently for 5 minutes. The watch-glass was rinsed with 5ml of hot water into the beaker and then the content was

filtered through a whatman filter paper (No.44) (ashless). There was continuous washing with hot water until the filtrate was neutral. The insoluble ash thus retained on the filter paper was gradually dried gently in an oven. It was ignited in an earlier weighed crucible at 100⁰C, cooled and weighed (WHO, 1998; Evans, 2009; Pratima and Mathad, 2011). The % acid insoluble ash was calculated from;.

$$\% \text{ Acid - insoluble Ash} = \frac{\text{weight of residual ash}}{\text{initial weight of sample}} \times 100$$

d. Determination of water-soluble ash

Experiment on determination of total ash was repeated. Therefore, to the crucible containing the total ash was washed into a beaker with 25ml of water and then boiled for 5 minutes. After rinsing the watch-glass as described in c above, the crucible was ignited for 15 minutes in an oven at a temperature of 400⁰C (WHO, 1998; Evans, 2009; Pratima and Mathad, 2011). The % water-soluble ash was calculated from;

$$\% \text{ water - soluble ash} = \frac{\text{initial wt of ash} - \text{wt of res. ash}}{\text{initial weight of sample}} \times 100$$

e. Determination of extractable matter

(i) Water-soluble extractive value

10g of the powdered leaf material of *Microtrichia perotitii* DC was macerated for 24hr in a stoppered conical flask containing 100ml chloroform-water mixture (2.5 ml of chloroform in 100 ml of water). The mixture was intermittently shaken during the first 6hrs using a shaker. After the 24hr the mixture was filtered and 20ml of the filtrate was transferred into a beaker and evaporated to dryness on water bath. The

drying was completed when constant weight was obtained.(WHO, 1998; Evans, 2009; Pratima and Mathad, 2011). The percentage water-soluble extractive value was obtained from;

$$\% \text{ Water – soluble extractive value} = \frac{wtofresidue}{wtof sample} \times 100$$

(ii) Alcohol – soluble extractive value

10g of the powdered leaf of *Microtrichia perotitii* DC was macerated for 24hr in a stoppered conical flask containing 100ml of methanol. The mixture was intermittently shaken during the first 6hrs using a shaker. The mixture was filtered and 20ml of the filtrate was transferred into a beaker and evaporated to dryness on water bath. The drying was completed when constant weight was obtained(WHO, 1998; Evans, 2009; Pratima and Mathad, 2011). The percentage alcohol-soluble extractive value was obtained;

$$\% \text{ Alcohol – soluble extractive value} = \frac{wtpfresidue}{wtof sample} \times 100$$

3.8 Acute Toxicity Study.

3.8.1 Determination of median lethal dose (LD₅₀) of the extract of the leaves of *Microtrichia perotitii* DC

The LD₅₀ determination was conducted using the method of Lorke (1983). Therefore, in the initial phase (phase 1) 9 mice were divided into 3 groups of three mice each and treated with extracts at doses of 10, 100 and 1000mg/kg body weight intraperitoneally (*i.p*) and observed for 24hr for signs of toxicity including mortality and general

behaviour. In the second phase or final phase (phase 2), the mice were divided into 4 groups of one mouse each and were given specific doses of 200,400,800 and 1,600mg/kg of body weight (*i.p*) that arose from the results of phase 1 and monitored for 24hr. The geometric mean of the lower dose that killed the animals and the highest dose that did not kill was taken as the median lethal dose and was calculated as shown in the formula below (Akhila *et al.*, 2007).

$LD_{50} = \sqrt{(\text{highest lethal that gave no mortality} \times \text{lower non-lethal dose that produced mortality})}$

3.9 Experimental animals

Adult Swiss albino mice (18 – 25 gm) and Wistar rats (180 – 200 gm) of both sexes and body weight were obtained from the animal house of the Department of Pharmacology and therapeutics, Ahmadu Bello University Zaria and were used for this research work. They animals were kept in well-ventilated room and fed with standard excel feeds Plc (Kaduna) grower mash. The animals were allowed access to food and water *ad libitum*. The animals were handled well in compliance with the National Regulations for Animal Research which is in conformity with the regulations of Ahmadu Bello University Animal Research and ethics guidelines.

3.10 Analgesic studies on *Microtrichia perotitii* DC

3.10.1 Acetic acid induced writhing test in mice

The acetic acid induced writhing test was carried out based on the method outlined by Koster *et al.*, 1959; Hokanson, 1978). Thus, the crude aqueous and methanol leaf extracts as well as the solvent fractions were used. Swiss albino mice (18 – 25 gm) were divided

into 5 groups of 5 mice each. The first group received 10 mg/kg of normal saline solution (0.9 % w/v NaCl) to serve as negative control, while groups 2,3 and 4 received 25, 50, and 100mg of extract per kg body weight (*ip*) respectively. Group 5 received proxicam at a dose of 10mg/kg body weight (*i.p*) as positive control. Thirty minutes later, all the groups were treated with acetic acid (0.6% v/v *i.p*). The mice were placed individually back in to their plastic transparent cages. After 5mins of treatment with the acetic acid, the number of abdominal constrictions was counted for a period of 10mins using a tally counter. The percentage inhibition of writhing was calculated and recorded using the formula. $\% inhibition = \frac{meannumberofwrithing(control) - meannumberofwrithing(treated)}{meannumberofwrithing(control)} \times 100$

3.10.2 Hot plate test

This test was carried out based on the methods described by Eddy and Leimbach,(1953) and Williamson, *et al.*,(1996) The experimental animals (Swiss albino rats weighing 20 - 25 gm) of either sexes were randomly selected and divided into five groups consisting of five mice each to serve as control and test groups respectively. The first group (negative control) received 10 mg/kg of normal saline solution (0.9% w/v NaCl), while groups 2,3 and 4 received 25,50 and 100mg of extract/kg body weight *p.o* respectively. Group 5 (positive control) received morphine sulphate at a dose of 4 mg/kg body weight *p.o*. The animals were positioned on a hot plate (Gallenkamp thermostat) kept at a temperature of $55 \pm 0.5^{\circ}\text{C}$. A cut off period of 15 seconds was observed to avoid damage to the paw. Reaction time was recorded when animals licked their fore or hind paws or jumped prior to and at 30, 60 and 90 min after oral administration of the samples(Kulkarni, 1999;Toma *et al.*, 2003; Heidari *et al.*, 2007). The percentage inhibition is calculated from

$$\% inhibition = \frac{\text{post-treatment latency} - \text{pretreatment latency}}{\text{cut off time} - \text{pretreatment latency at zero seconds}} \times 100$$

Post-treatment= latency after drug treatment;

Pre-treatment= latency before drug treatment (zero time)

3.11 Anti-inflammatory studies on *Microtrichia perotitii* DC

3.11.1 Formalin induced paw Oedema

The anti-inflammatory activities of both aqueous and methanolic extracts as well as the methanol fractions were evaluated by formalin induced paw oedema method. Wistar rats weighing between 180-200g were used for this work. The rats were divided into 5 groups of 5 rats each and 30mins before injection of formalin (formaldehyde 2.5% v/v) that is 50µl volume in the subplanter region of the right hind paw of the rat, the groups were treated intraperitoneally (*i.p*) as follows: group 1 received 1ml of normal saline solution (10 mg/kg /0.9% w/v NaCl) as negative while groups 2 3 and 4 received doses of extracts and fractions at 25, 50 and 100mg per kg body weight respectively. Group 5 received 10mg proxicam per kg as positive control. The paw Oedema caused by the inflammogen (formalin) was measured at 0, 1, 2, 3, 4 and 5 hrs. Thus, the increase in paw diameter was measured using vernier caliper by subtracting the diameter of the inflamed hind limb from non-inflamed hind limb (Winter and Nuss, 1963 ; Dubuisson and Dennis, 1977; Hunskar and Hole, 1987; Tjøisen *et al.*, 1992). The percentage inhibition of the oedema by both aqueous, methanolic extracts and fractions was calculated as follows:

$$\% \text{ inhibition} = \frac{\text{meanpawdiamether}(-\text{vecontrol}) - \text{meanpawdiamter}(\text{treated})}{\text{meanpawdiameter}(-\text{vecontrol})} \times 100$$

3.12 Statistical analyses

The results obtained in course of this research work were expressed as mean \pm standard error of mean (mean \pm S.E) using SigmaStat version 8.0, SPSS Science, USA and the data were analysed using student's *t*-test and the data were statistically evaluated by use of one-way ANOVA, followed by post hoc Scheffe's test using version 13 of SPSS software (SPSS Science, USA) and Microsoft office excel 2010. The difference between the control and treated groups (i.e DF = 4) at probability level of $P < 0.05$ and $P < 0.001$ were considered significant (Crossland, 1980).

3.13 Preparation of Sample

The fresh herb *Microtrichia perotitii* DC after collection was washed with running tap water, followed with rinsing with distilled water and then allowed to dry under room temperature for three weeks to ensure proper drying. The leaves were ground into coarse powder using pestle and mortar. (Agarwal, 2008).

3.14 Extraction of Plant Materials

i. Maceration

300 g of the coarse powdered leaves of *Microtrichia perotitii* was weighed and soaked in 450 ml of distilled water in a conical flask. The flask was shaken, corked and left to stand for 72 hours at room temperature. At the end of the 72 hours, the mixture was filtered and the extract was collected and concentrated by

evaporation to dryness in an evaporating dish on boiling hot waterbath at low temperature (regulated). The extractable was allowed to cool and placed in a clean container and kept sealed in a desiccator until use and was marked MPAE (WHO, 1998; Evans, 2009).

ii. Soxhlet apparatus

300 g of the powdered leaves of *Microtrichia perotitii* (300 g) were refluxed with 3L of petroleum ether (60-80⁰C) for 8 hours in a soxhlet apparatus. The extract was decanted off and fresh quantity of petroleum ether (3L) was added again and then refluxed for another 5 hr. The defatted leaves (marc) were completely dried and later extracted with 3L of methanol in an Soxhlet apparatus for 8 hours. Thereafter, the round bottom flask was removed and distilled gradually and the crude evaporated to dryness on boiling hot water bath. The extractable was allowed to cool and placed in a clean container and kept sealed in a desiccator until use and was marked MPME (Vogel, 1989; Brain and Turner, 1975; Ciulei, 1994; Wang and Weller, 2006; Anees *et al.*, 2009).

3.15 Phytochemical Studies on *Microtrichia perotitii* DC

Each of the crude extracts of the leaves of *Microtrichia perotitii* was subjected to preliminary phytochemical screening using standard procedure (Agarwal, 2008).

(a) Test for Carbohydrates

(i) Molisch's test

0.2g of each crude extract was dissolved in 10mls of distilled water. To this solution in a test-tube few drops of Molisch's reagent were added. This was followed by the addition of 1ml of concentrated H₂SO₄ gradually down the side of the test-tube so that the acid forms a layer beneath the aqueous layer. The mixture was allowed to stand for 2min and then diluted with 5ml of water. Formation of a red to dull, violet colour or brown ring at the interface of the two layers indicates positive test for general carbohydrates (Sofowora, 2008; Evans, 2009).

(ii) Fehling's test (Reducing Sugar)

About 0.5 g of each of the plant extracts was hydrolysed by boiling with 5ml dilute hydrochloric acid and the resulting solution was neutralized with sodium hydroxide solution. To each of the solutions few drops of Fehling's A and B solutions were added and then heated on boiling water bath for 2mins. Formation of brick red or reddish brown precipitate of cuprous oxide indicates the presence of carbohydrate (Evans, 2009).

(iii) Benedict's test

About 1 g of each of the plant extracts was dissolved in 5 ml of distilled water. To this was added 2 mls of Benedict's solution and then boiled gently for 2 min.' Appearance of green, yellow or orange-red precipitate indicates the presence of carbohydrates (Sofowora, 2008;Evans, 2009).

(b) Test for Tannins

(i)Lead Acetate Test

To 2ml each of the aqueous solution of the extracts, 2ml of water was added in a test -tube followed by addition of 5 drops of lead acetate solution and then the test-tube was shaken intermittently. Appearance of red precipitate indicates the presence of tannins. (Kokate *et al.*, 2005; Evans, 2009).

(ii)Ammonium hydroxide solution

0.2 g of the aqueous solution of the extract was transferred into a test-tube. To this was added 3 mls of water, shaken vigorously and then filtered. To the filtrate 3 ml of 25 % ammonia solution was added. Appearance of a slowly forming green colouration or emulsion indicates the presence of tannins (Sofowora,2008)

(iii)Ferric chloride Test

A small quantity of each extract was boiled with 5 ml of ethanol for 5 mins. After filtration and cooling, few drops of 5% Ferric chloride solution were added to the filtrate. Appearance of a blue-black, green or blue-green precipitate indicates the presence of tannins (kokate, 2005; Evans, 2009).

(c) Test for Phlobatannins

10ml of aqueous extract of each sample was boiled with 1% HCl acid in a test-tube after filtration. Appearance or deposition of a red precipitate indicates the presence of phlobatannins (Sofowora, 2008; Evans, 2009).

(d) Test for Flavonoids

(i) Shinoda's test

To few mls of the alcoholic extract, few pieces of Magnesium turnings were added followed by two drops of concentrated hydrochloric acid and warming. Occurrence of effervescence with formation of dark brown solution which gradually changes to a deep red solution or appearance of pink colouration indicates the presence of flavonoids (Sofowora,2008;Evans, 2009)

(ii) Ferric chloride test

5ml of distilled water was added to the powdered leaves and boiled for 2min in a test-tube. The content was filtered and to 2ml of the filtrate, few drops of 10% ferric chloride solution were added. Occurrence of green-blue to violet indicates the presence of a phenolics nucleus (Evans, 2009).

(iii)Lead acetate test

0.2g of each crude extract was dissolved in water and then filtered. In a test-tube few drops of 10% lead acetate was added to 5mls of the filtrate. Appearance of buff coloured precipitate indicates the presence of flavonoids (Brain and Turner, 1975;Harborne, 1998; Evans,2009).

(iv)Sodium hydroxide test

To 2ml of each extract in a test-tube was added 10% of sodium hydroxide solution. Appearance of yellow colouration indicates the presence of flavonoids (Evans, 2009).

(e) Test for Alkaloids

3ml of each extract was stirred with 5ml of 1% aqueous hydrochloric acid on hot water bath. The content was filtered and divided into 3 portions. The filtrates were treated as follows:

(i) Dragendoff's reagent

To the first portion was added few drops of Dragendoff's reagent (Potassium bismuth iodide solution) and occurrence of orange red precipitate indicates the presence of alkaloids.

(ii) Wagner's reagent

To the second portion was added few drops of Wagner's reagent (solution of iodine in potassium iodide) and occurrence of reddish brown colour indicates the presence of alkaloids.

(iii) Mayer's reagent

To the third portion few drops of Mayer reagent (Potassium mercuric iodide solution) were added and appearance of cream colour precipitate indicates the presence of alkaloids (Evans, 2009).

(f) Test for cardiac glycosides

(i) Keller-Kiliani test

0.5g of each crude extract was dissolved in 2ml of 3.5% Ferric chloride solution in glacial acetic acid in a test-tube. To this was added 2mls of concentrated

H₂SO₄. Occurrence of reddish-brown ring at the interphase indicates the presence of cardiac glycoside(de-oxy sugar) (Kokate, 2005; Evans, 2009).

(ii)Baljet's test

Few drops of sodium picrate reagent were sprayed to a piece of lamina or thick section of the leaf. Appearance of yellow to orange colouration indicates the presence of glycoside(Ajazuddin, 2010).

(iii)Legal's test

0.2 g of the extract was dissolved in 3 mls of pyridine. Few drops of 2 % NaOH were added. A deep red colouration which fades to brownish yellow indicates the presence of cardenolides (5-membered lactone ring)(Evans, 2009).

(iv)Kedde's test

1 ml of an 8 % solution of the extract in methanol was mixed with 1 ml of a 2 % solution of 3,5-dinitrobenzoic acid in methanol and 1 ml of a 5.7 % aqueous NaOH. An immediate violet colour indicates the presence of cardenolides (5-membered lactone ring) (Evans, 2009).

(v)Raymond's test:

To 0.2g of extract of the leaves was added a few ml of 50 % ethanol and 0.1 ml solution of m-dinitrobenzene in ethanol. To the solution was added 2-3 drops of 20 % sodium hydroxide solution. Appearance of violet colouration confirms the presence of active methylene group at C-21 in the lactone ring(Ajazuddin, 2010).

(g) Tests for Steroids and Terpenoids

(i)Salkowski's Test

0.2g of each crude extract was dissolved in 2ml of chloroform in a test-tube. To this was added carefully from the side sulphuric acid to form a lower layer. Occurrence of reddish-brown colour at the interface indicates the presence of steroidal nucleus (aglycone portion of the cardiac glycoside) (Sofowora, 2008; Harborne, 1998).

(ii) Libermann-Burchard Test

0.2g of each crude extract was dissolved in 2mls of chloroform in a test-tube. Few drops of acetic anhydride were added to the test-tube and was boiled and cooled. Concentrated H₂SO₄ was added from the side of the test-tube using a pipette. Formation of a brown ring at the junction of the two layers and the turning of the upper layer to green shows the presence of steroids while formation of deep red colour indicates the presence of triterpenoids (Culei, 1994; Harborne, 1998; Mosa *et al.*, 2013).

(h) Test for Saponins

Frothing Test

0.5 g of the crude powder leaves was shaken vigorously with 10ml of distilled water in a test-tube. The test-tube was warmed for 5mins and later shaken for observance of frothing. The persistence of the froth indicates the presence of saponins. The frothing was mixed with 3 drops of olive oil and observed for the formation of emulsion which confirms the presence of saponins (Kapoor *et al.*, 1969; Harbone, 1973; Sofowora, 2008).

(i) Test for Anthraquinones

(a) Borntrager's Test (free anthraquinones)

2g of each powdered leaves was boiled with 4ml of 10% HCl for 3min. The mixture was filtered while still hot and the filtrate was allowed to cool. The cooled filtrate was then shaken with equal volume of chloroform to extract the anthraquinone. The chloroform layer was then transferred into a clean test-tube and treated with equal volume of 10% ammonia. The mixture was then shaken well and the colour of the upper layer noted. Appearance of bright pink, red or violet colouration in the aqueous layer indicates the presence of anthraquinone (free hydroxylanthraquinones) (Evans, 2009).

(b) Modified Borntrager's test for anthraquinones

5g of plant material were boiled in 10 ml of 10 % HCl for an hour and then filtered while hot. This will hydrolyse the glycosides to yield aglycones which are soluble in hot water only. The filtrate was cooled and extracted with 5ml of benzene. The benzene layer was filtered off and shaken gently with half its volume of 10% ammonia solution. Appearance of a rose-pink or cherry red colour indicates the presence of anthraquinone derivatives (Evans, 2009).

(j) Test for Resins:

(i) Potassium permanganent solution

To 0.5g of the extract, 5ml of 10% KMnO_4 solution was added and then heated gently in a test-tube over Bunsen flame. Perception of odour of benzaldehyde

due to oxidation of benzoic acid indicates the presence of resins (Brain and Turner, 1975; Evans, 2009).

(ii) Copper acetate solution

To the extracts, 3-4 ml of copper acetate solution was added separately and the tubes were shaken vigorously for 1-2 mins and the resulting solution was allowed to separate. Formation of green coloured precipitate indicates the presence of of resins (Evans, 2009;Kokate *et al.*, 2002).

3.16 Fractionation of theCrude extracts of theLeaves of *Microtrichia perotitii* DC

The aqueous and methanol crude extracts of the leaves of *Microtrichia perotitii* (MPAE and MPME) were fractionated using the scheme as proposed by Woo *et al.*,1980(Figure 3.1).

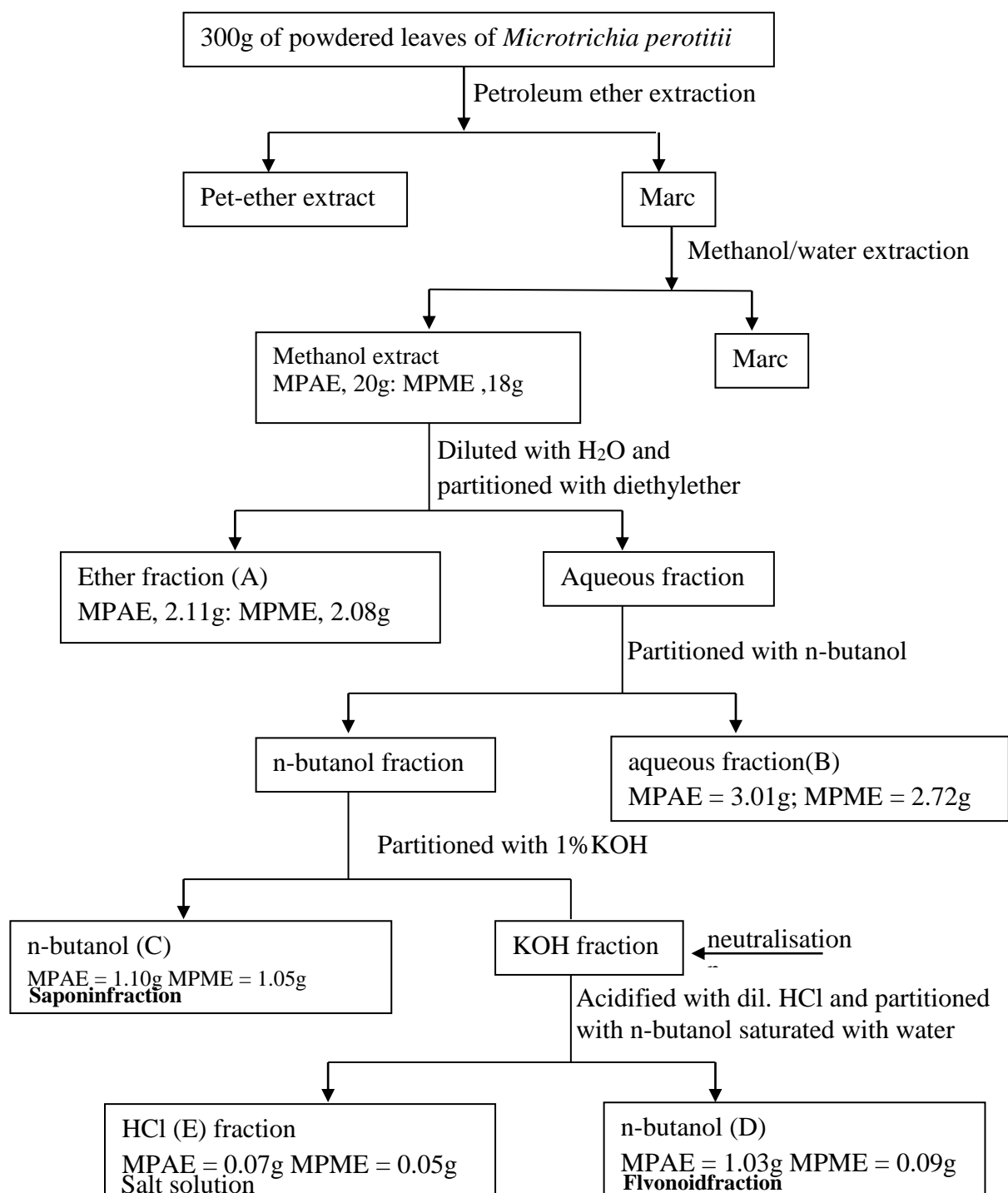


Figure 3.1: Schematic chart for the extraction (Woo *et al.*, 1980)

Each of the extract was evaporated gently over hot water bath for about 30min with intermittent removal from the bath. Thereafter each was transferred into sterilized bottle, labeled and kept in dessicator until use. Accordingly, the residues were identified as; A(ether soluble Fraction), B(aqueous fraction), C(n-Butanol fraction), D(n-Butanol fraction) and E (aqueous hydrochloric fraction). Each fraction was weighed and recorded.

3.17 Isolation and structural elucidation of compounds

Soluble fraction C of the crude methanol extract of the leaves of *Microtriachia perotitii* (MPME) was the most active in the analgesic and anti-inflammatory studies and was therefore subjected to column chromatography in order to isolate the active component(s).

3.17.1 Thin layer chromatography

3.17.2 Preparation of TLC Plates

Glass plates (10cm x 7cm) were coated with silica gel (Kieselgel, 60, Merck) using spreader, the thickness was within 0.006mm and deionised water was used as a binder for the adsorbent. The prepared plates were left for about 20 minutes in the air and then heated in an oven at 120⁰C for 30 minutes in order to activate the plates(Rajauria and Abu-Ghannam,2013).

3.17.3 Spotting of soluble fraction C of the methanol crude extract on TLC plates

In this analysis, 0.2g of n-butanolsoluble fractions C of the crude methanol extract of the powdered leaves of *Microtriachia perotitii* (MPME) was dissolved in methanol and applied on pre-coated plates with the aid of capillary tubes about 1 cm from the bottom in

a straight line and 0.5 cm away from the sides. The spots were allowed to dry at room temperature before placing them into a chromatographic tank which contained the solvent mixtures (solvent systems) prepared about 30 minutes earlier. The mobile phase moved across the plate in an ascending order carrying with it separated components of the fraction (extract). As soon as the mobile phase reached towards the top end of the plate, it was then removed and the solvent front marked with a pencil. The plates were allowed to dry and then sprayed with p-anisaldehyde (Sharma, 2000; Rajauria and Abu-Ghannam, 2013). The retardation factor (R_f) was therefore calculated from the measurements of distances as in the equation below.

$$R_f = \frac{\text{distance travelled by substance}}{\text{distance travelled by solvent (solvent front)}}$$

3.17.4 Development of Thin layer Plate

Flat bottom chamber with cover containing mixture of Butanol:Acetic acid:Water(8:1:1) and Hexane:Ethylacetate(7:3) were used to develop the various chromatograms (Sharma, 2000).

3.18 Column chromatography

This was done in order to isolate and purify the constituents in the n-butanol soluble fraction C of the methanol crude extract of the leaves of *Microtrichia perotitii*. (MPME).

3.18.1 Packing of column

A clean dried glass column of 3.0 cm diameter and 60 cm in length was held in place by retort stand and was sealed with a glass-wool at the tapered end. The column was packed

with hexane and silica gel as adsorbent. The column was tapped in order to remove air-bubbles. 4 gm of the sample was dissolved in methanol and then transferred gradually on to the uppermost part of the column. The column was then covered at the top with aluminium foil to avoid contamination (Zygmunt and Namiesnik, 2003; Franzet *al.*, 2013).

3.18.2 Elution of column

The organic component was placed on top of the column containing the stationary phase was eluted. The eluents slowly passed through the stationary phase to advance the component. The different components travelled down at different speed thus made easy to collect each fraction as coloured bands. The column was first eluted with the least polar solvent and the polarity was increased with addition of more polar solvents in different ratios as mobile phase. The mobile phases used were:

1. Hexane (100 %)
2. Hexane-Ethylacetate (2:1)
3. Hexane-Ethylacetate (1:1)
4. Hexane-Ethylacetate (1:2)
5. Ethylacetate (100 %)
6. Ethylacetate-Methanol (2:1)
7. Ethylacetate-Methanol (1:1)
8. Ethylacetate-Methanol (1:2)
9. Methanol (100 %).

Beakers of 100 cm³ capacity were used to take fractions of 40 ml at a time and a total of 76 fractions were collected. Fractions with similar TLC profiles were grouped or pooled together as A(C₁),B(C₂) and C(C₃) at the end of the elution (Zygmunt and Namiesnik, 2003; Franz *et al.*,2013). The compounds C₁,C₂ and C₃were subjected to Spectrophotometric analyses with FTIR (8400S HIMADZU), ¹H-NMR spectrophotometer (Bruker400Mhz), and ¹³C-NMR spectrophotometer(Bruker400Mhz) spectroscopic analysis.

CHAPTER FOUR

4.0

RESULTS

4.1 Plant Collection and Identification of *Microtrichia perotitii* DC

Microtrichia perotitii DC collected at Rigasa village, Kaduna was identified and authenticated by Malam Musa Muhammad of the Department of Biological Sciences, Ahmadu Bello University, Zaria. Herbarium specimen was prepared, preserved (with methylated spirit and mercury chloride), stored and deposited at the Herbarium of the Department of Biological Science ABU. The specimen was given a voucher number, 998 for further reference.



Plate 4.1: Single stripe of *Microtrichia perotitii* plant



Plate 4.2: Bunchappearance of *Microtrichia perotitii* herb

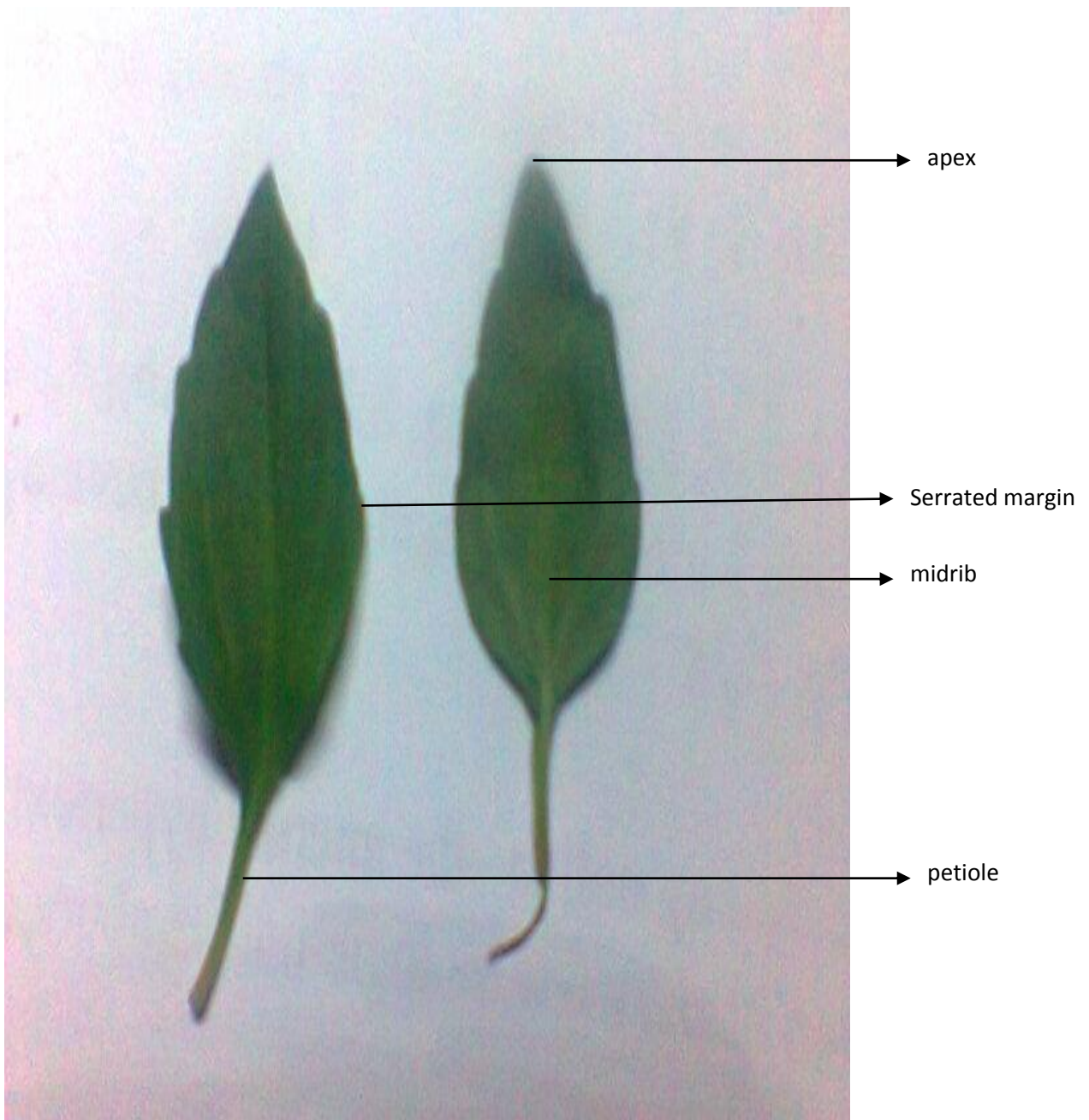


Plate 4.3: Adaxial and Abaxial surfaces of the leaves of *Microtrichia perotitii* herb

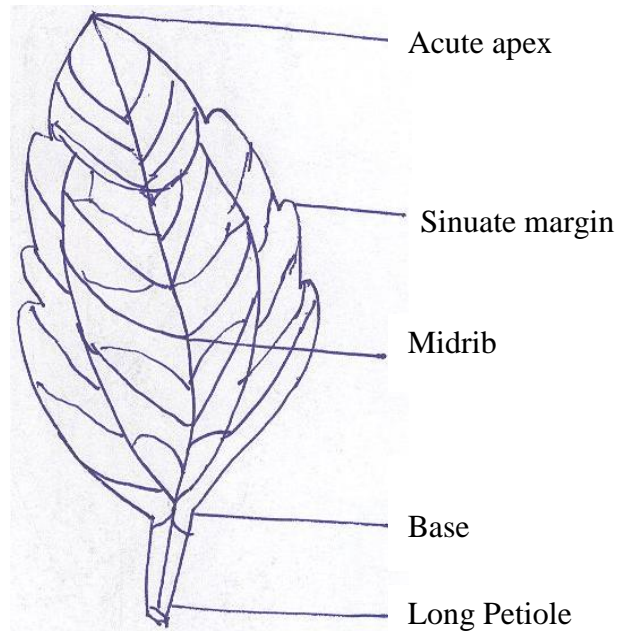


Figure 4.1: Structure of the leaf of *Microtrichia perotitii*

4.2 Preparation of the Leaves of *Microtrichia perotitii* DC for Studies

Both fresh and powdered leaves of the herb including its crude extracts were subjected to macroscopy, quantitative leaf microscopy and phytochemical studies. The results were as presented as follows:

4.3 Macroscopic Features of the Leaves of *Microtrichia perotitii*

The result of macroscopic observations of the features of the leaves were presented in plates 4.1, 4.2 and 4.3 and the organoleptic observations of the leaf indicated that it is green in colour, posses bitter and pepperish tastes and has an unpleasant aromatic odour (Table 4.1).

Table 4.1: Macroscopic features of the leaves of *Microtrichia perotiti*

Features	Descriptions
Shape	Small
Dimension (length x breadth)	(3.6 – 3.7mm) x (1.6 – 2.1mm)
Arrangement	Alternate
Petiole	Long
Lamina:	
i. composition	Simple
ii. venation	Pinnate
iii. margin	Serrated
iv. apex	Acute
v. base	Unequal
vi. texture	Brittle
vii. surface	Pubescent
Organoleptic properties:	
i. colour	Greenish
ii. odour	Unpleasant
iii. tastes	Slightly bitter and pepperish

4.4 Microscopic/Chemo-microscopic and Quantitative Leaf Microscopic Studies of *Microtrichia perotitii*

4.4.1 Microscopic Examination

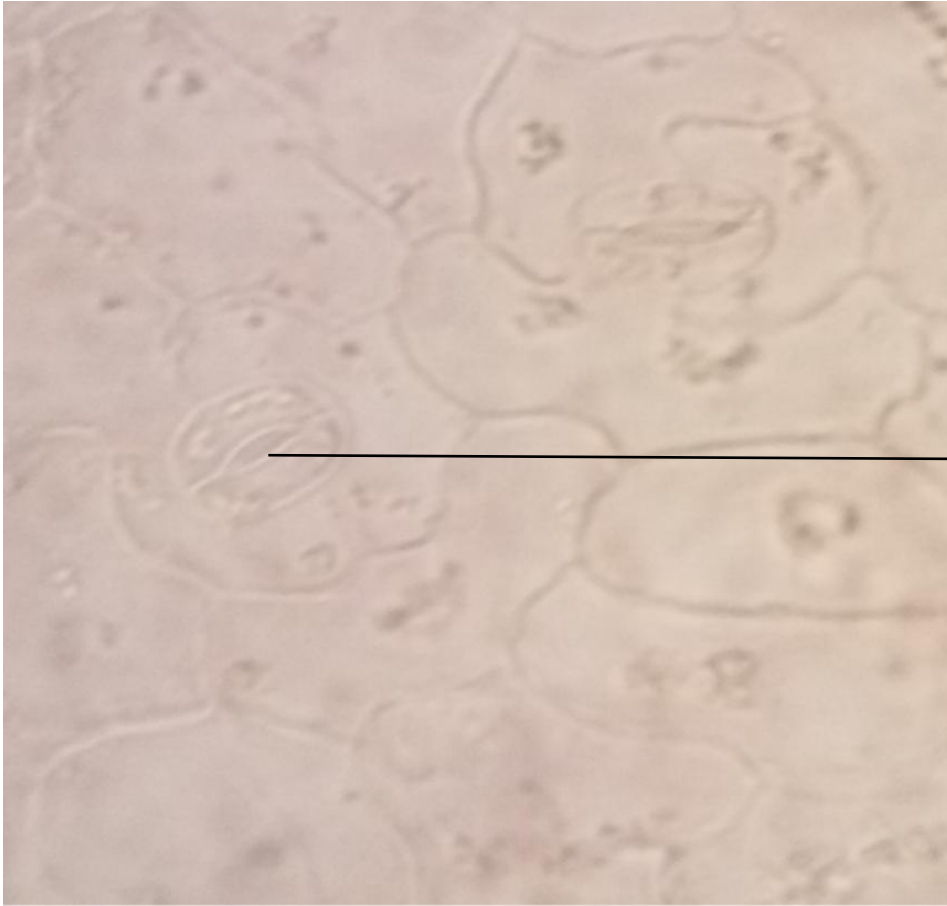
4.4.1.1 Anatomical sections and powder of Leaf

The leaf of *Microtrichia perotitii* is dorsiventral with the palisade tissues occurring beneath the upper epidermis. The various epidermal characters of the leaves and their descriptions are given in table 4.2 and their photomicrographs on plates 4.4-4.7 (Figures 4.8).

Table 4.2: Epidermal characters and their descriptions

Features	Characters	
	lower epidermis	upper epidermis
i.Cells: Shapes	elongated	polygonal
Anticlinal wall	Wavy	straight
Thickening	smooth & cellulose	smooth & cellulose
Papillae	Absent	absent
Cuticle	Present	present
ii.Stomata: Type	Anomocytic	anomocytic
Frequency	numerous	frequent
Size (l,b,µm)	27x16.2	27x13.5
iii.Trichomes:Type	uniseriate multicellular	uniseriate multicellular
Frequency	frequent	rare
Size(h,bµm)	135x21.6	132x21.6

Key: l = length; base= base; h = height



→ Anomocytic stomata

Plate 4.4: Surface preparation of upper epidermis of *Microtrichia perotitii* x 100

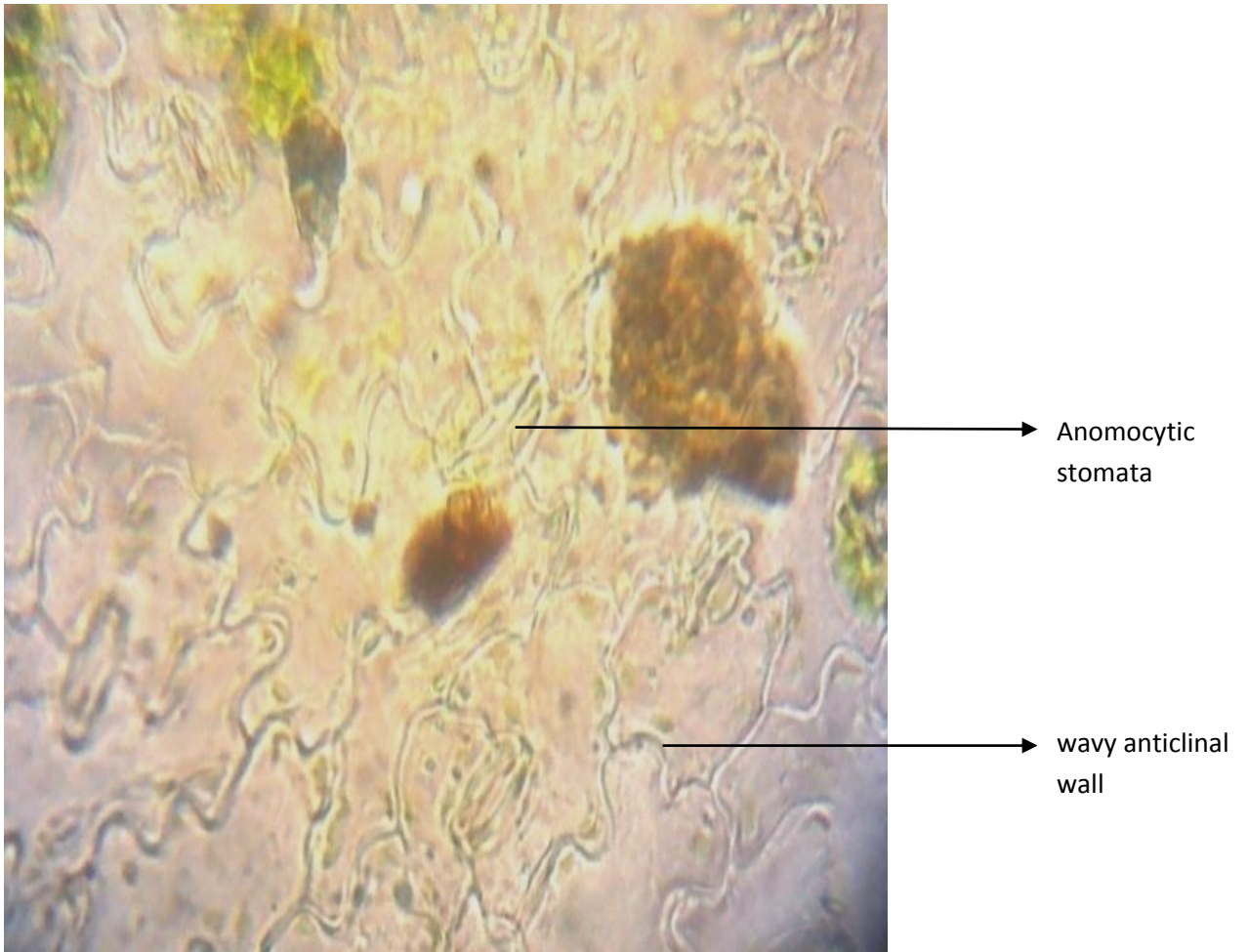


Plate 4.5: Surface preparation of lower epidermis of *Microtrichia perotitii* x 100

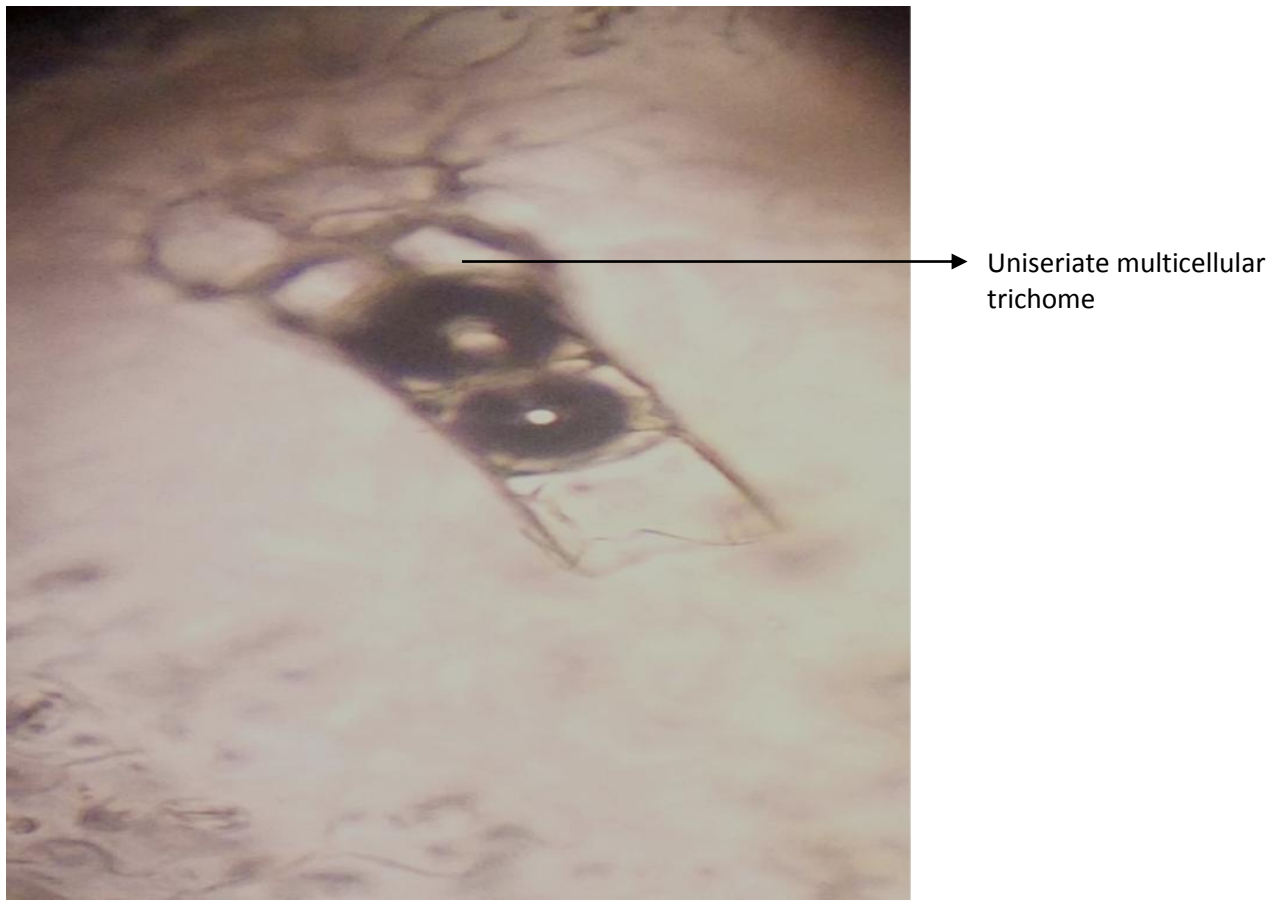


Figure 4.6: uniseriate multicellular trichomes at upper epidermis of the leaves of
Microtrichia perotitii x 100

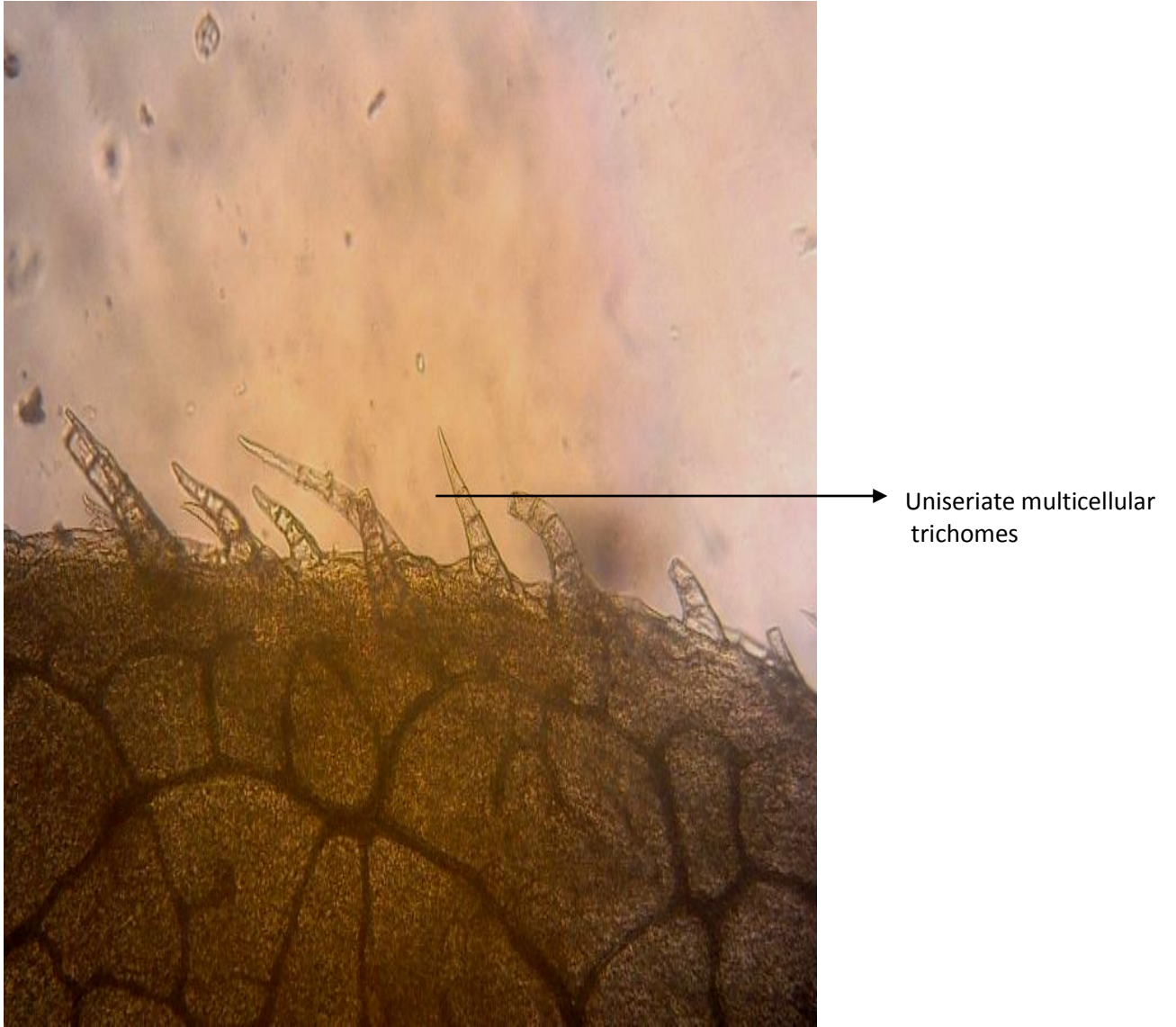


Figure 4.7: uniseriate multicellular trichomes of at lower epidermis of the leaves
of *Microtrichia perotitii* x 100

The transverse section (TS) of the midrib showed parenchyma, collenchymas, phloem, and xylem. The midrib showed epidermal cells with rectangular to round in shape as shown in plate 4.8 and figure 4.3

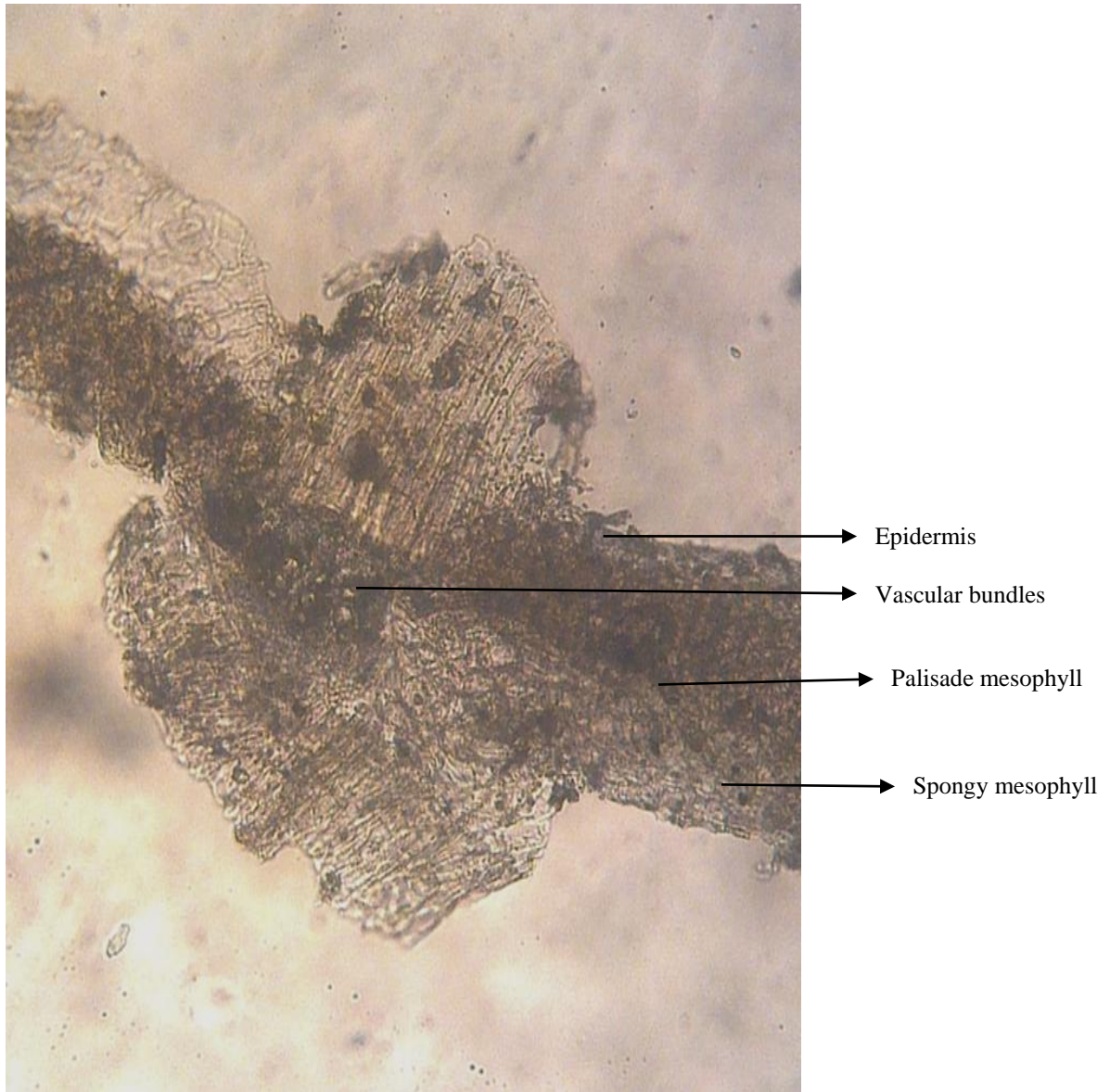
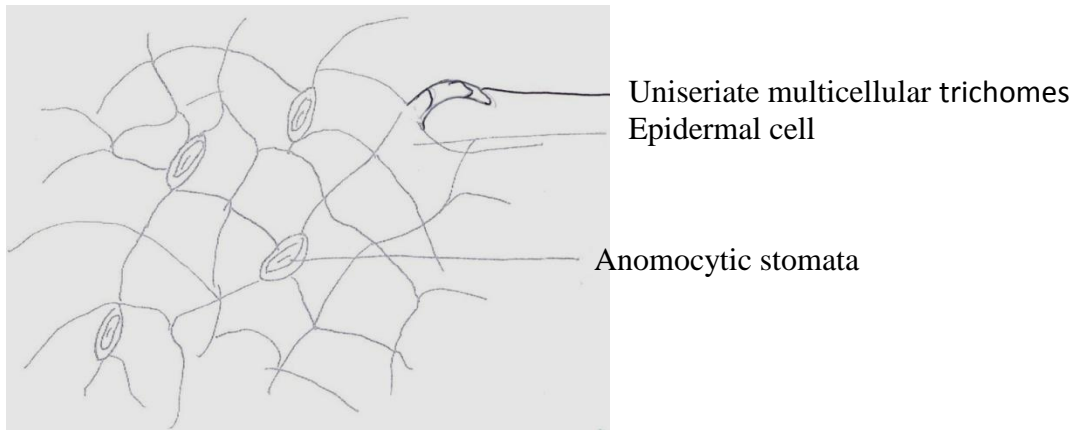
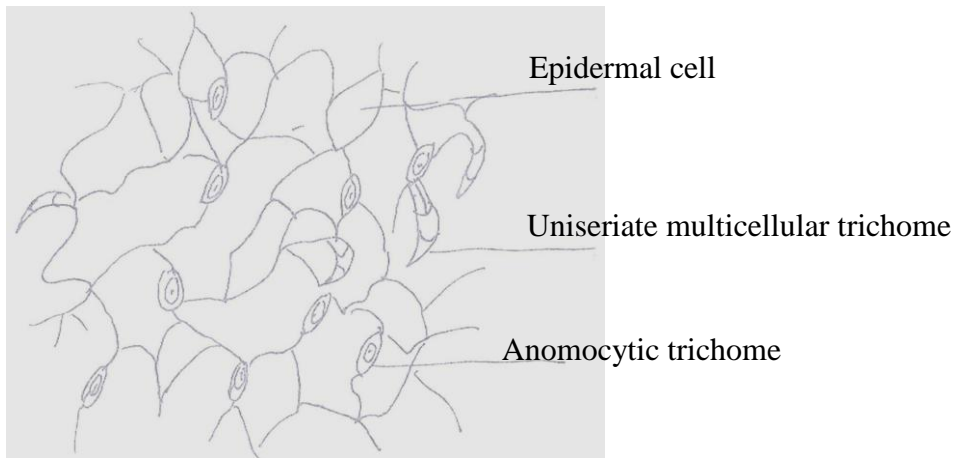


Plate 4.8: Transverse section of the midrib of the leaf of *Microtrichia perotitii* x 100



Upper epidermis



Lower epidermis

Figure 4.2: Surface preparations from the upper and lower epidermis of the leaves of *Microtrichia perotitii* x 400

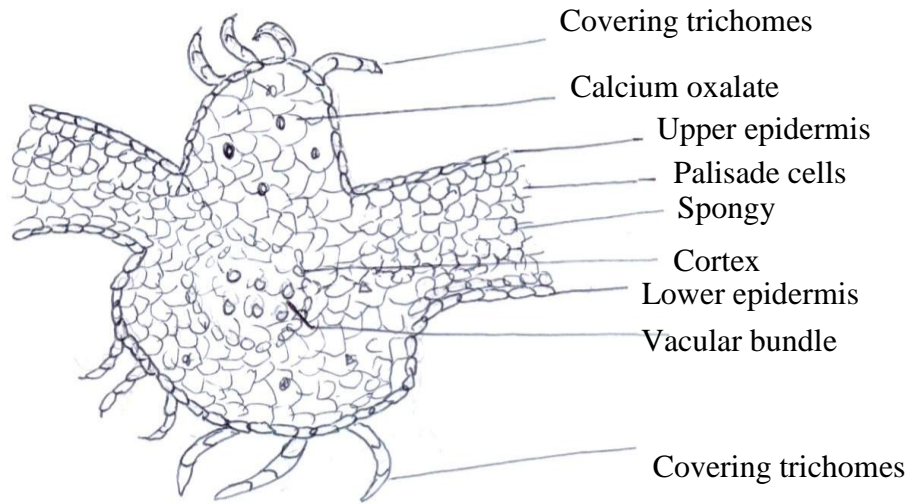


Figure 4.3: Transverse sections of midrib of the leaf and lamina of the leaf of *M. perotitiat*
x 400

From the powdered leaves, fragments of spiral types of xylem vessels, calcium oxalate crystals and starch grains were also identified (figure 4.4).

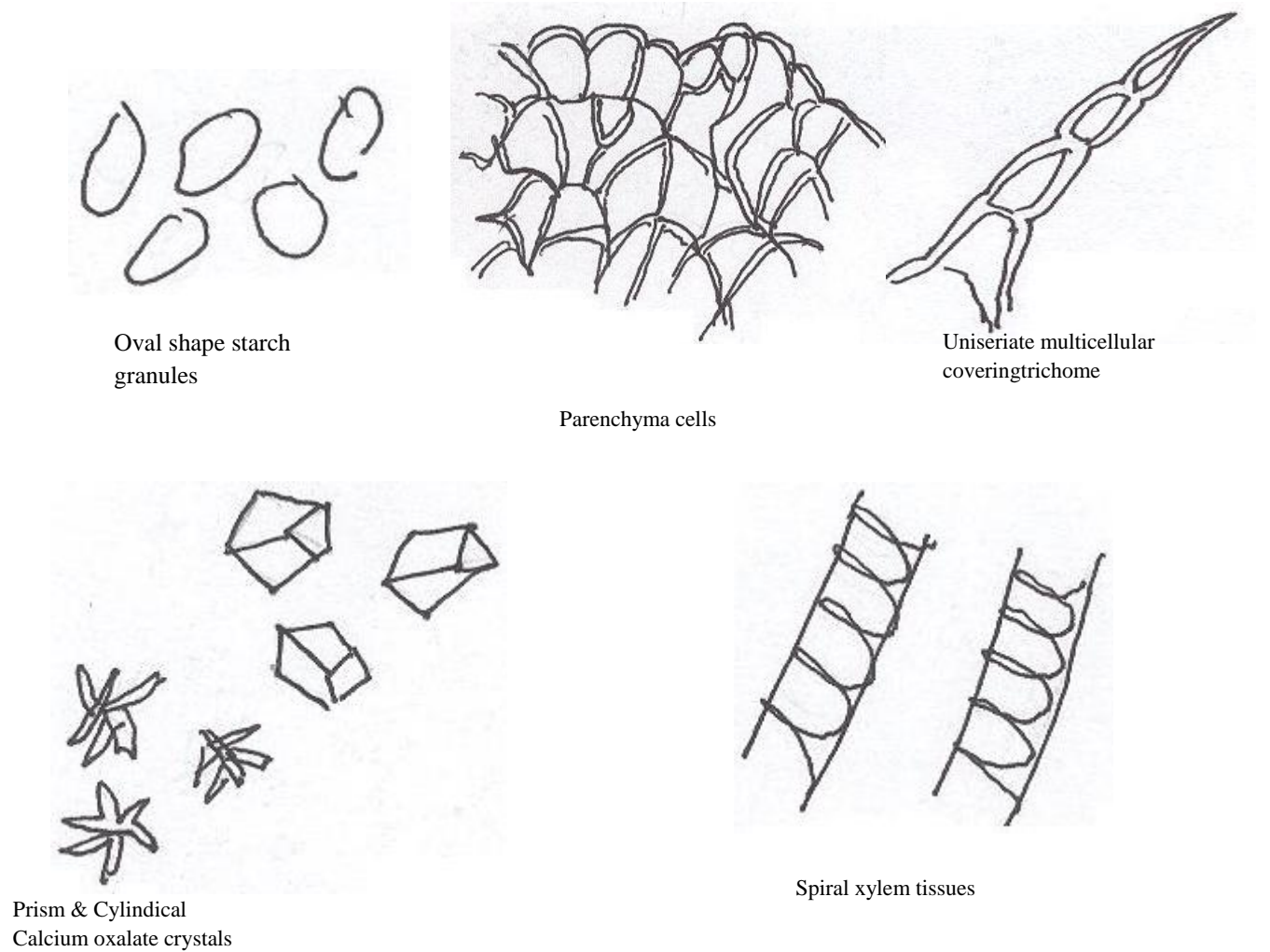


Figure 4.4: Some identified microscopic diagnostic features from the powdered leaves of *Microtrichia perotitii* x 400

4.4.1.2 Quantitative-leaf microscopic studies.

The leaf constants of *Microtrichia perotitii* are relatively constant for the herb and can be used to differentiate it from closely related species. The results such as palisade ratio, stomatal number, stomatal index, vein-islet number and veinlet termination were determined and tabulated in Table 4.3. The result of the quantitative microscopy of the leaf has given valuable information regarding leaf constants of the herb.

Table 4.3: Quantitative leaf microscopic features of *Microtrichia perotitii*

FEATURES	CHARACTERISTICS
i. Palisade ratio	3.2 -3.3* - 3.4
ii. Stomata number	258-271.5* -285(upper epidermis) 289-299.5*-310(lower epidermis)
iii. Stomatal index (%)	19.5- 22.1*-24.7 (upper epidermis)
iv. Vein-islet number	6.0 - 7.2* - 8.0
v. Veinlet termination number	8.0- 9.6* -11.0

* mean values of 5 counts

*4.4.1.3 Chemo-microscopic studies of the leaves of *Microtrichia perotitii**

Different types of cell-wall materials and inclusions were identified from the powdered leaves of the herb. This included presence of starch, calcium oxalates, tannins, cellulose cell wall, lignified cell wall and oil & fats as presented in Table 4.4. The result of the chemo-microscopy of the leaf has revealed the presence of tannins which have been reported to have some pharmacological actions.

Table 4.4: Chemo-microscopy of the leaf of *Microtrichia perotitii*

Constituents	Mounting reagent	Result/observation	Inference
Starch	Iodine solution(N/50)	Blue-black colouration appeared on some grains within the chloroplasts and powdered leaves	+
Lignin & conc. HCl	Phloroglucinol	No red colouration observed on the walls of some lignified collenchymas.	+
Cellulose Solution	Chloro-zinc iodine	Blue colouration observed on walls of epidermal cells	+
Calciumoxalate	Chloralhydrate solution	brightly coloured crystals observed which later dissolved in conc. HCl and disappeared in the collenchymas cells	+
Oils and fats	Sudan iv solution /warming	Reddish colouration was observed in some parenchyma cells and was distinct	+
Mucilages	Ruthenium red solution	Dark solution observed at epidermis and vascular tissues	-
Tannins	5% ferric chloride solution	Greenish black colouration was observed in some parenchyma cells	+

Key: += present, -= absent, Conc HCl = concentrated hydrochloric acid solution

4.5 Determination of physical constants of powdered leaves of *M. perotiti*

The physical constants (%w/w) determined from the powdered leaves were presented in Table 4.5. The moisture content is below 14 % meaning that the leaves would not easily get spoiled by microbes or fungi. The total ash is particularly important in the evaluation of purity of drugs that is for the presence or absence of foreign inorganic matter such as metallic salts and/or silica.

Table 4.5: Physicochemical constants from the leaves powder of *Microtrichia perotitii*

Parameters	Mean values (%)
Moisture content	12.67±0.62
Total Ash	20.33±0.20
Acid-insoluble Ash	2.73±0.13
Water-soluble Ash	8.18±0.30
Water-soluble extractives	40.9±1.73
Alcohol- soluble extractives	25.45±1.24

4.6 Median Lethal Dose (LD₅₀) of *Microtrichia perotitii* Leaves Extract

The result of the LD₅₀ using the method of Lorke (1983) is presented in Table 4.6 below.

Table 4.6: Median lethal (LD₅₀) for aqueous extract of the leaves of *Microtrichia perotitii* in mice

Dose (mg/kg) <i>i.p</i>	Results
Phase one	
10	0/3
100	0/3
1000	2/3
Phase two	
200	0/1
400	0/1
800	0/1
1600	1/1

$$\therefore LD_{50} = \sqrt{800 \times 1600} = 1131.4 \text{ mg/kg } i.p$$

The LD₅₀ value of 1131.4 mg/kg *i.p* means that the plant is not toxic (Kenned *et al*, 1986).

4.7 Analgesic studies of the leaves extracts of *Microtrichia perotitii*

4.7.1 Effects of aqueous and methanol crude extracts of *Microtrichia perotitii* on acetic acid-induced writhing test in mice

The aqueous extract (MPAE) of the leaves exhibited dose-dependent analgesic activity while the methanol (MPME) extracts of the leaves exhibited dose-independent analgesic activity in mice. The MPAE showed maximum inhibition of 86.96 % at the dose of 100 mg/kg while MPME showed maximum inhibition of 100 % at the dose of 50 mg/kg. The values were significant at $p < 0.05$ (Table 4.7; Figure, 4.5, Appendix A).

Table 4.7: Analgesic activities of aqueous (MPAE) and methanol (MPME) crude leaves extracts of *Microtrichia perotitii* on acetic acid induced writhing in mice

Treatment	Dose (mg/kg)	Mean number of writhes \pm S.E.M	% inhibition
Normal saline	10(ml/kg)	23.0 \pm 2.85	-
M.P.A.E	25	9.50 \pm 1.56*	58.70
M.P.M.E	25	11.50 \pm 2.09*	50.00
M.P.A.E	50	6.33 \pm 0.72*	72.48
M.P.M.E	50	0.00 \pm 0.00*	100.00
M.P.A.E	100	3.00 \pm 0.37*	86.96
M.P.M.E	100	2.17 \pm 2.17*	90.57
Proxicam	10	8.00 \pm 0.93*	65.22

Data was presented as mean \pm SEM and were analysed by ANOVA followed by the student's *t*-test. *significant at $p < 0.05$

Keys: M.P.A.E = *Microtrichia perotitii* aqueous extracts; M.P.M.E = *Microtrichia perotitii* methanol extract; S.E.M = standard error of mean.

4.7.2 Effects of soluble fractions A, B, C and D of methanol crude extract of *Microtrichia perotitii* on acetic acid induced writhes test in mice

The soluble fractions A, B, C and D of the methanol crude extract(MPME) of the leaves of *Microtrichia perotitii* exhibited differing analgesic activities in mice. Fractions A,B and D showed dose-independent activity while C showed dose-dependent analgesic activity in mice. Fraction B(aqueous) showed maximum inhibition of 53.96 % at a dose of 25 mg/kg, fraction C(BuOH) of 65.51 % at a dose of 100 mg/kg and D(BuOH) of 69.20 % at a dose of 25 mg/kg when compared to Proxicam of 45.14 % used as a standard. The values were significant at $p < 0.05$ (Table 4.8; Figure 4.6, Appendix A).

Table 4.8: Effects of solvent fractions (A, B, C, and D) of methanol crude extract of the *Microtrichia perotitii* (MPME) against acetic-acid induced writhings in mice

Treatment	Dose (mg/kg)	Number of writhes mean±SEM	% Inhibition
Normal saline	10 (ml/kg)	18.83 ± 2.07	-
A (ether)	25	20.33 ± 3.30	7.97
	50	15.83 ± 2.07	15.93
	100	18.50 ± 2.27	1.75
B (aqueous)	25	8.67 ± 1.54	53.96
	50	11.00 ± 1.84	41.58
	100	14.67 ± 3.04	22.09
C (butanol)	25	8.33 ± 1.15*	55.76
	50	7.33 ± 1.28*	61.20
	100	6.50 ± 0.91*	65.51
D (butanol)	25	5.83 ± 1.49*	69.20
	50	12.50 ± 2.28	33.91
	100	7.67 ± 2.21*	59.27
Proxicam	10	10.33 ± 2.43	45.14

Data was presented as mean ± SEM and were analysed by ANOVA followed by the student's *t*-test N/S = Normal Saline; *significant at $p < 0.05$.

Key: S.E.M = Standard Error of Mean.

4.7.3. Effect of *Microtrichia perotitti* on thermally induced pain in mice

The central nervous analgesia of the leaves of the herb were tested using thermal stimuli in Mice.

*4.7.3.1 Effect of Aqueous Leaf Extract of *Microtrichia perotitii* on Hotplate Test*

The aqueous extract at tested doses of 25, 50 and 100 mg/kg *b.w* significantly ($p < 0.05$; $p < 0.001$) increased the reaction time of mice to thermal induced heat at 60 and 90 minutes exposure (Table, 4.9; Figure, 4.7, Appendix A)

Table 4.9: Effect of aqueous leaves extract (MPAE) of *Microtrichia perotitii* on hot plate induced pain in mice

Groups	Dose (mg/kg)	Time intervals (mins)	Mean latency \pm S.D	% Inhibition
Normal saline	10(ml/kg)	0	1.44 \pm 0.16	-
		30	1.35 \pm 0.21	-
		60	1.51 \pm 0.19	-
		90	1.23 \pm 0.52	-
1	25	0	1.67 \pm 0.21	1.69
		30	1.50 \pm 0.22	1.09
		60	1.97 \pm 0.23*	3.41
		90	1.68 \pm 0.19*	3.37
2	50	0	1.35 \pm 0.21	0.66
		30	1.11 \pm 0.23**	1.76
		60	1.27 \pm 0.18**	1.78
		90	1.35 \pm 0.11	0.87
3	100	0	1.50 \pm 0.16	0.44
		30	1.00 \pm 0.23*	3.24
		60	1.09 \pm 0.15*	3.11
		90	1.16 \pm 0.17	0.51
Morphine sulphate 4		0	1.33 \pm 0.15	0.81
		30	1.44 \pm 0.06**	1.54
		60	1.29 \pm 0.09**	1.63
		90	1.35 \pm 0.12**	0.87

Data was presented as mean \pm SEM and were analysed by ANOVA followed by the student's *t*-test. Key: *significant at $p < 0.05$, ** significant at $p < 0.001$. S. E, M = Standard error of mean

4.7.3.2 Effect of Methanol Crude Extract of Microtrichia perotitii on Hot plate Test in Mice

The methanol extract showed significant inhibition ($p < 0.05$) in a dose-dependent manner with the highest inhibition at a dose of 100 mg/kg at 60mins. The extract showed ($p < 0.001$) inhibition at at all doses (Table, 4.10; Figure, 4.8, Appendix A).

Table 4.10:Effect of methanol crude leaves extract of *Microtrichia perotitii* (MPME) on hot plate test induced pain in mice

Groups	Dose (mg/kg)	Time intervals (mins)	Mean latency \pm S.D	% Inhibition	
1	10	0	1.44 \pm 0.16	-	
		30	1.35 \pm 0.21	-	
		60	1.51 \pm 0.19	-	
		90	1.22 \pm 0.53	-	
	25	0	1.50 \pm 0.16	0.44	
		30	1.33 \pm 0.15	0.81	
		60	1.32 \pm 0.13**	1.41	
		90	1.37 \pm 0.13	1.02	
	2	50	0	1.27 \pm 0.54	1.25
			30	1.20 \pm 0.05	1.09
			60	1.26 \pm 0.43**	1.85
			90	1.35 \pm 0.09	0.87
3	100	0	1.33 \pm 0.15	0.81	
		30	1.00 \pm 0.03*	2.56	
		60	1.07 \pm 0.06*	3.19	
		90	1.14 \pm 0.05**	1.90	
Morphine sulphate 4		0	1.33 \pm 0.15	0.81	
		30	1.14 \pm 0.06**	1.54	
		60	1.29 \pm 0.09**	1.63	
		90	1.35 \pm 0.12	0.87	

Data was presented as mean \pm SEM and were analysed by ANOVA followed by the student's *t*-test. Key: *significant at $p < 0.05$, ** significant at $p < 0.001$; S. E, M = Standard error of mean.

4.8 Anti-inflammatory study of crude leaves extract of *Microtrichia perotiti* Formalin Induced Oedema in Rats

4.8.1 Effects of Aqueous and Methanol crude extracts of *Microtrichia perotiti* on formalin induced paw Oedema in rats.

The injection of formalin (formaldehyde, 2.5 % v/v) in the paws of rats produced local oedema which gradually increased in diameter. The aqueous crude extract (MPAE) exhibited dose-independent inhibition while the methanol crude extract (MPME) exhibited a dose-dependent inhibition. The aqueous extract showed maximum inhibition of 60.80 % at a dose of 100 mg/kg while the methanol extract showed 97.60 mg/kg at a dose of 100 mg/kg. Both results were significant at $p < 0.05$ (Table 4.11; Figure 4.9, Appendix A).

Table 4.11: Effect of crude aqueous(MPAE) and methanol(MPME)extracts of leaves of *Microtrichia perotitii* against formalin induced paw Oedema in rats.

Treatment	Dose mg/kg	Mean volume of Oedema (cm)±S.E.M	% Inhibition
Normal saline	10(ml/kg)	1.25±0.04	-
M.P.A.E	25	0.50±0.03*	60.00
M.P.M.E		0.33±0.01*	73.60
M.P.A.E	50	0.09±0.02*	48.80
M.P.M.E		0.28±0.05*	92.80
M.P.A.E	100	0.49±0.03*	60.80
M.P.M.E		0.28±0.05*	97.60
Proxicam	10	0.66±0.07*	47.20

Data was presented as mean ± SEM and were analysed by ANOVA followed by the student's *t*-test.

Key: *significant at $p < 0.05$.M.P.A.E = *Microtrichia perotitii* aqueous extract; M.P.M.E = *Microtrichia perotitii* methanolic extract; S.E.M = Standard error of mean.

4.8.2 Effects of Solvent fractions A, B,C and D of Crude Methanol Extract of *Microtrichia perotitii* (MPME) on Formalin Induced Paw Oedema in Rats

Solvent fractions C and D exhibited significant ($p < 0.05$ and $p < 0.01$) inhibitions as the time increased. The peak of inflammation was reached in the fifth (5th) hour in normal saline while Proxicam (10 mg/kg) exhibited peak of inflammation ($p < 0.01$) at the fourth (4th) hour. Both soluble fraction C and D reduced the inflammation ($p < 0.05$) at the third (3rd) hour (Table, 4.12).

Table 4.12: Anti-inflammatory activities of solvent fractions A,B,C and D of methanol crude extracts(MPME) of powdered leaves of *Microtrichia perotitii* against formalin induced oedema in rats.

Fraction	Treatment mg/kg	Mean paw diameter (cm) in hours					
		0	1	2	3	4	5
Normal saline	-	-	0.20±0.02	0.18±0.13	0.14±0.01	0.14±0.02	0.15±0.02
A (ether)	25	0.15±0.02	0.16±0.01	0.16±0.01	0.14±0.01	0.14±0.03	0.15±0.1
	50	0.18±0.01	0.19±0.02	0.18±0.01	0.15±0.01	0.15±0.01	0.16±0.01
	100	0.20±0.03	0.23±0.02	0.20±0.02	0.18±0.03	0.16±0.02	0.16±0.02
B (aqueous)	25	0.17±0.01	0.19±0.02	0.15±0.01	0.19±0.01	0.19±0.01	0.21±0.01
	50	0.17±0.01	0.20±0.01	0.17±0.01	0.20±0.01	0.20±0.01	0.18±0.01
	100	0.15±0.02	0.17±0.01	0.16±0.01	0.16±0.01	0.17±0.01	0.20±0.02
C (Butanol)	25	0.16±0.02	0.17±0.01	0.14±0.01	0.13±0.01	0.12±0.01*	0.11±0.01
	50	0.13±0.03	0.15±0.03	0.14±0.02	0.13±0.03*	0.11±0.02**	0.09±0.03
	100	0.12±0.02	0.13±0.01	0.13±0.02	0.12±0.02*	0.11±0.02**	0.10±0.02
D (Butanol)	25	0.13±0.02	0.15±0.01	0.14±0.01	0.11±0.01	0.10±0.01**	0.13±0.01*
	50	0.15±0.02	0.18±0.02	0.19±0.02	0.12±0.01*	0.13±0.01*	0.14±0.01**
	100	0.22±0.01	0.23±0.03	0.18±0.03	0.14±0.01	0.14±0.02	0.15±0.02
Proxicam	10	0.15±0.01	0.16±0.02	0.17±0.02	0.12±0.01*	0.16±0.01**	0.14±0.01**

Data was presented as mean ± SEM and were analysed by ANOVA followed by the student's *t*-test.

Key: * significant at $p < 0.05$, ** significant at $p < 0.01$. S. E, M = Standard error of mean.

4.9 Extractive values of powdered leaves of *Microtrichia perotitii*

The % yield from the extraction of 300 g of the powdered leaves by maceration (water) and soxhlet apparatus (methanol) are presented in Table 4.13. This result indicates that the leaf of the herb contains a lot of polar compounds since water is more polar than methanol.

Table 4.13: Extraction of Powdered Leaves of *Microtrichia perotitii* with Water (aqueous) and Methanol

Plant	Solvent	Amount(g)	% Yield
<i>M.perotitii</i>	Aqueous extract (MPAE)	20	6.67
<i>M.perotitii</i>	Methanol extract (MPME)	18	6.00

key:M.P.A.E =*Microtrichia perotitii* aqueous extract

M.P.M.E =*Microtrichia perotitii* methanol extract.

4.9.1 Extractive values from solvent fractions of water (aqueous) and methanol crude extracts of the powdered leaves of *Microtrichia perotitii*

The amount of materials from partitioned fractions of aqueous (MPAE) and methanol (MPME) crude extracts were as presented in table Table 4.14. The % yield from the aqueous soluble fraction (B) was the highest in both the crude aqueous (MPAE) and methanol (MPME) extracts being more polar than the other solvents.

Table 4.14: Percentage yield from partitioned fractions of crude extracts of powdered leaves of *Microtrichia perotitiin* in water and methanol

Soluble Fraction	Aqueous (g)	% Yield	Methanol (g)	% Yield
Diethylether (A)	2.11	21.1	2.08	20.8
Aqueous (B)	3.01	30.1	2.72	27.2
n-butanol(C)	1.10	11.0	1.05	10.5
n-butanol (D)	1.03	10.3	0.09	0.9
Hydrochloric acid (E)	0.07	0.7	0.05	0.5

4.10 Phytochemical screening

The phytochemical screening of aqueous(MPAE) crude extract and methanol(MPME) crude extracts with its soluble fractions A,B, C and D of the powdered leaves of *Microtrichia perotitii* revealed the following and the results were summerised Tables 4.15 and 4.16.

4.10.1 Test for carbohydrates

a) Molish test.

The aqueous crude extract, methanol crude extract and soluble fractions were tested with Molish's reagents. A violet colouration was formed which indicated the presence of carbohydrates.

b) Fehling's test

The aqueous crude extract, methanol crude extract and soluble fractions were tested with Fehling's solution. A brownish precipitate was formed which indicated the presence of carbohydrates.

c) Benedict's test

The aqueous crude extract, methanol crude extract and soluble fractions were tested with Benedict's solution. An orange-red precipitate appeared which indicated the presence of carbohydrates.

4.10.2 Test for tannins

a) Lead acetate test

The aqueous crude extract, methanol crude extract and soluble fractions were tested with lead acetate solution. A red precipitate appeared in the crude extracts and soluble fraction D which indicated the presence of tannins.

b) Ammonia test

The aqueous crude extract, methanol crude extract and soluble fractions were tested with ammonia solution. Green colouration was observed in the crude extracts and soluble fraction D which indicated the presence of tannins.

c) Ferric chloride test

The aqueous crude extract, methanol crude extract and soluble fractions were tested with ferric chloride reagent. A blue-green precipitate appeared in the crude extracts and soluble fraction D which indicated the presence of tannins.

4.10.3 Test for Phlobatannins

a) Hydrochloric acid test

The aqueous crude extract, methanol crude extract and soluble fractions were tested with hydrochloric acid solution. No red precipitate was deposited which indicated absence of phlobatannins.

4.10.4 Test for flavonoids

a) Shinoda's test

The aqueous crude extract, methanol crude extract and soluble fractions were tested with shinoda's reagent. Pink colouration appeared in the crude extracts and soluble fraction D which indicated the presence of flavonoids.

b) Ferric chloride test

The aqueous crude extract, methanol crude extract and soluble fractions were tested with ferric chloride solution. Blue-green coloration appeared in the crude extracts and soluble fraction D which indicated the presence of flavonoids.

c) Lead acetate test

The aqueous crude extract, methanol crude extract and soluble fractions were tested with lead acetate solution. Buff coloured precipitate appeared in the crude extracts and soluble fraction D which indicated the presence of flavonoids.

d) Sodium hydroxide test

The aqueous crude extract, methanol crude extract and soluble fractions were treated with sodium hydroxide solution. Yellow colouration appeared in the crude extracts and soluble fraction D which indicated the presence of flavonoids.

4.10.5 Test for alkaloids

a) Dragendoff's test

The aqueous crude extract, methanol crude extract and soluble fractions were tested with Dragendoff's reagent. An orange-red precipitate was observed which indicated the presence of alkaloids.

b) Wagner's test

The aqueous crude extract, methanol crude extract and soluble fractions were tested with Molish's reagents. reddish-brown colouration appeared which indicated the presence of alkaloids.

c) Mayer's test

The aqueous crude extract, methanol crude extract and soluble fractions were tested with Mayer's reagent. Creamed coloured precipitate appeared which indicated the presence of alkaloids.

4.10.6 Test for cardiac glycosides

a) Keller-killiani test

The aqueous crude extract, methanol crude extract and soluble fractions were tested with Keller-killiania reagent. A reddish brown ring was observed at the interphase in the crude extracts and soluble fractions C and D which indicated the presence of cardiac glycosides,

b) Baljet's test

Yellow colouration appeared on the thick section of the leaf which indicated the presence of cardiac glycosides.

c) Legal's test

The aqueous crude extract, methanol crude extract and soluble fractions were tested with Legal's reagent. No deep colouration was observed which indicated the absence of cardinolides.

d) Kedde's test

The aqueous crude extract, methanol crude extract and soluble fractions were tested with Kedde's reagent. No immediate violet colour was observed which indicated the absence of cardinolides.

e) Raymond's test

The aqueous crude extract, methanol crude extract and soluble fractions were tested with Raymon's reagent. No violet colouration was observed which indicated the absence of active methylene group at C-21 in a lactone ring.

4.10.7 Test for saponins

a) Frothing test

The aqueous crude extract, methanol crude extract and soluble fractions were shaken with distilled water. Frothing persisted which indicated the presence of saponins in the crude extracts and soluble fractions B and D.

4.10.8 Test for anthraquinones

a) Borntrager's test

No appearance of colouration in the aqueous extract of the leaf which indicated the absence of free anthraquinone

b) Modified Borntrager's test

No appearance of colouration in the ammonium solution which indicated the absence of anthraquinone derivative.

4.10.9 Test for steroids and terpenoids

a) Salkowski's test

The soluble fractions were treated with sulphuric acid solution. No redish-brown colouration was observed at the interphase which indicated the absence of steroidal nucleus.

b) Libermann-Burchard test

The soluble fractions were treated with concentrated sulphuric acid. Brown ring was formed at the interphase and upper layer turned deep-red which indicated the presence of terpenoids in soluble fractions C and D.

4.10.10 Test for resins

a) Potassium permanganate test

The aqueous crude extract, methanol crude extract and soluble fractions were treated with potassium permanganate solution. No odour of benzaldehyde was perceived after boiling which indicated absence of resins

b) Copper acetate test

The aqueous crude extract, methanol crude extract and soluble fractions were treated with copper acetate solution. Green coloured precipitate was not observed which indicated absence of resins

Table 4.15: Summary of phytochemical constituents from the crude extracts of the powdered leaves of *Microtrichia perotitii*.

Constituent	Tests	Aqueous	Methanol
Tannins	Lead acetate	+	+
	Ammonia Solution	+	+
	Ferric chloride test	+	+
Phlobatannins	Hydrochloric acid	-	-
Flavonoids	Shinoda's test	+	+
	Ferric chloride test	+	+
	Lead acetate test	+	+
	Sodium hydroxide	+	+
Alkaloids	Dragendoff's reagent	+	+
	Wagner's reagent	+	+
	Mayer's reagent	+	+
Carbohydrate	Molisch's test	+	+
	Fehlings' solution	+	-
	Benedicts's test	+	-
Cardiac glycosides	Keller-kiliani	+	+
	Baljet's test	+	+
	Legal's test	-	-
	Kedde's test	-	-
	Raymond's test	-	-
Saponins	Frothing test	+	+
Anthraquinones	Bontrager's test	-	-
	Modified Borntrager's test	-	-
Resins	10% KMnO ₄	-	-
	copper acetate	-	-

Keys: + = Present; - = absent; HCl = Hydrochloric acid; KmnO₄ = Potassium tetraoxomanganate vii, MPAE = *Microtrichia perotitii* aqueous extract, MPME = *Microtrichia perotitii* methanol extract.

Table 4.16: Summary of phytochemical constituents from fractions A,B,C and D of crude methanol extract of *Microtrichia perotitii*(MPME)

The result revealed the presence of triterpenes, saponins, flavonoids and tannins which could be responsible for the analgesic and ant-inflammatory activities of the fractions.

Constituents	Test	Fraction		Fraction	
		A	B	C	D
Carbohydrates	Molich test	+	+	+	+
	Fehlingss solution	+	+	+	+
	Tollens' reagent	+	+	+	+
	Benedict's reagent	+	+	+	+
Alkaloids	Dragendoff's reagent	-	-	+	+
Steroids/ Triterpenes	Salkowski Tests	-	-	-	-
	Lieberman-Burchard test	-	-	+	+
Cardiac glycosides	Keller-killani	-	-	-	-
	Baljet's test	-	-	+	+
	Legal's test	-	-	+	+
	Kedde's test	-	-	+	-
Saponins	Raymond's test	-	-	-	+
Flavonoids	Frothing test	-	+	-	+
	Shinoda	-	-	-	+
	Ferric chloride	-	-	-	-
Phlobatannins	Lead acetate	-	-	-	+
Tannins	Hydrochloric acid	-	-	-	+
	Sodium hydroxide	-	-	-	+
	Lead acetate	+	+	+	+
	Ammonia solution	+	+	+	+
	Ferric chloride	+	+	+	+

Key: (+) positive; (-) negative

4.11 Isolation of active components from solvent fraction C(BuOH) of the crude methanol extract of *Microtrichia perotitii* (MPME)

Isolation and characterisation of the active constituents in the leaves of *Microtrichia perotitii* was achieved through the following processes.

4.11.1 Thin layer chromatography (TLC)

The TLC profile of soluble fraction C (BuOH) gave 5 distinct spots; A,B,C,D and E in Butanol: Acetic acid:Water(BAW)[8:1:1] while it gave 4 spots A,B,C and D in Hexane: EtoAc(7:3) solvent mixture(Plates 4.9 and 4.10).

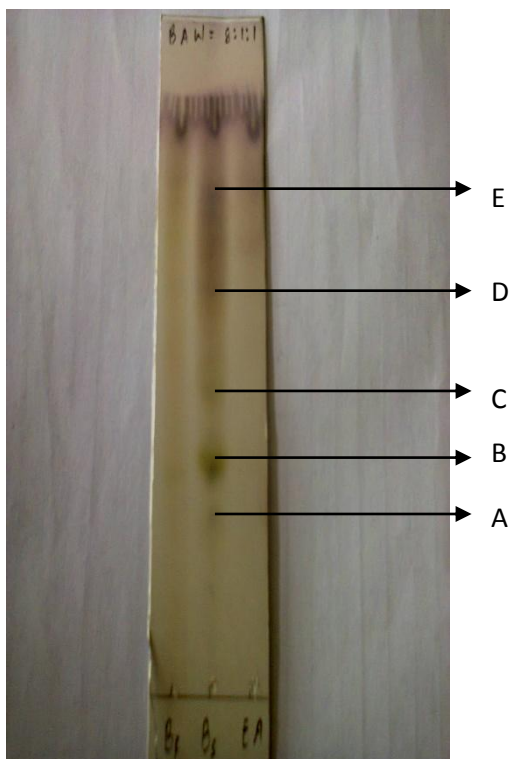


Plate 4.9: TLC plate of n-Butanol fraction in B:A:W: (8:1:1) solvent mixture.

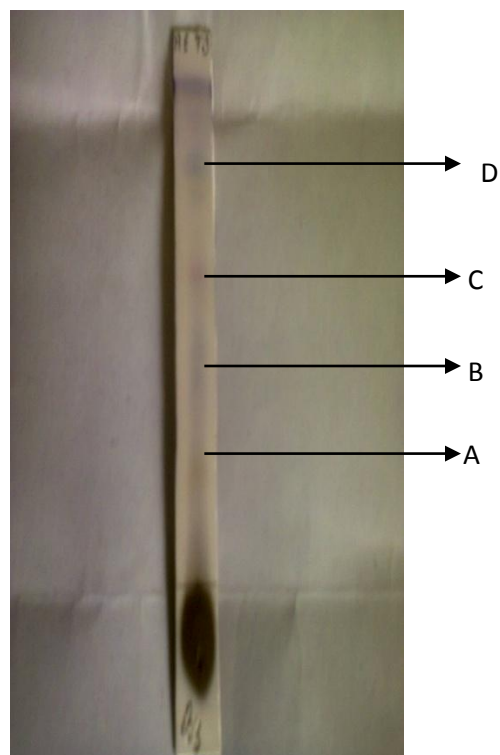


Plate 4.10: TLC plate of n-Butanol in Hex:EtoAc(7:3)solvent mixture.

The R_f values of soluble fraction C in different solvent systems were presented in Tables 4.17.1 and 4.17.2

Table 4.17.1: R_f values of spots of solvent fraction C in Butanol:Acetic acid:Water (8:1:1) solvent mixture

Spots	R_f
A	0.26
B	0.35
C	0.46
D	0.68
E	0.83

Solvent front= 7.35cm

Table 4.17.2: R_f values of spots of soluble fraction C in Hexane:Ethylacetate (7:3) solventmixture

Spots	R_f
A	0.25
B	0.61
C	0.82
D	0.89

Solvent front=2.80 cm

4.11.2 Column chromatography

4.11.2.1 Thin layer chromatography profile of A,B and C.

The eluents from the column were pooled as compound A(36-50),B(51-65) and C (66-76) and their TLC profiles were presented below:

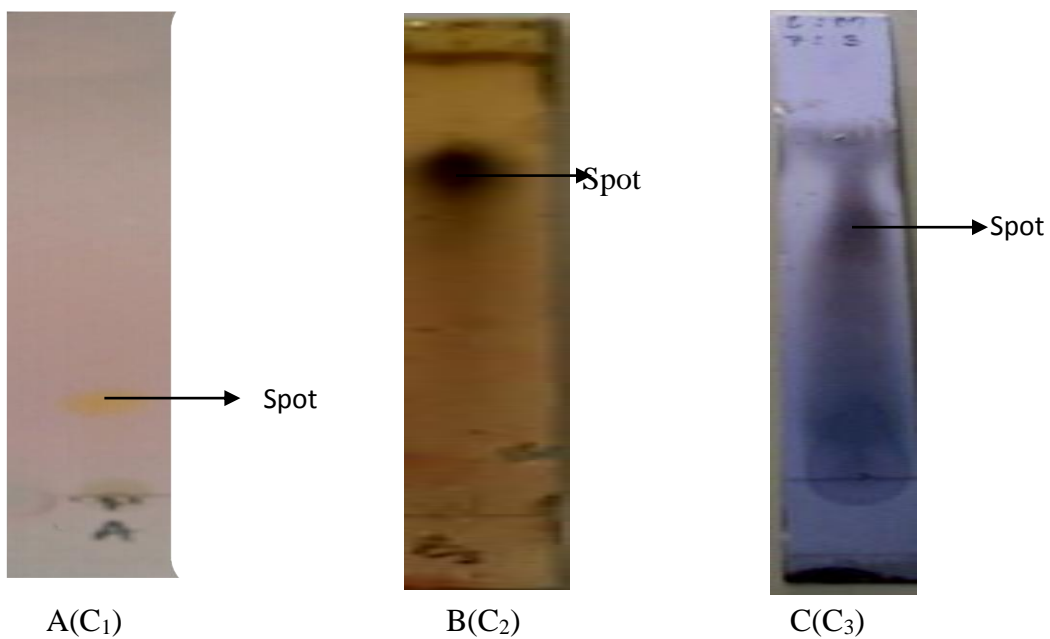


Plate4.11:TLC profiles of A(C₁),B(C₂) and C(C₃) in Hex:EA(7:3)

4.11.2.2 *Melting points of A(C₁),B(C₂) and C(C₃) of soluble fraction C of methanol crude extract of the leaves of Microtrichia perotitii*

The approximate weights, nature and melting points of A (C₁),B (C₂) and C (C₃) is summarised in Table 4.18.

Table 4.18: weights and melting point range of A,B and C

Constituent	Weight(mg)	Nature	Melting point range(^o C)	R _f
A(C ₁)	13.0	milky white powder	224-226	0.31
B(C ₂)	9.0	milky(powder)	172-174	0.73
C(C ₃)	5.0	chocolate brown(gummy)	90-92	0.70

4.11.3 Spectroscopic analysis of compounds A(C₁), B(C₂) and C(C₃) soluble fraction C(BuOH) of methanol crude extract (MPME) of the powdered leaves of *Microtrichia perotitii*.

The spectroscopic data of compound A(C₁) are as follows:

4.11.3.1 FTIR spectroscopy of compound A(C₁) isolated from Microtrichia perotitii

In the Infrared spectrum, prominent peaks appeared at 1726.35 (C=O stretch), 1654.98 (C=C stretch) and 1374.33 cm⁻¹ (O=C-O) for acetate moiety. These observations are shown in Figure 4.10 and tabulated in Table 4.20.

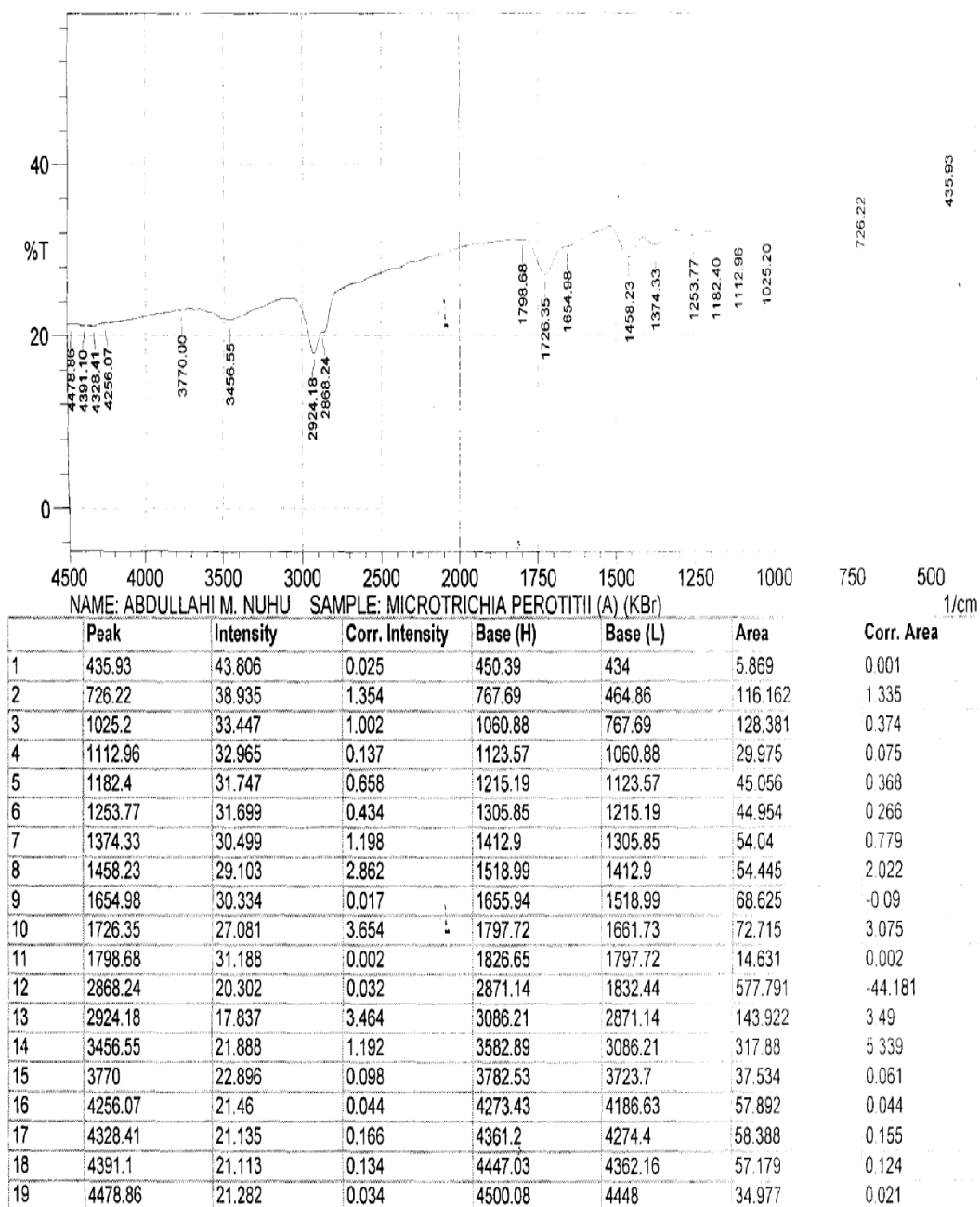


Figure 4.10: FTIR Spectrum of compound A(C₁) isolated from *Microtrichia perotitii*

4.11.3.2 ¹H-NMR for compound A(C₁) isolated from *Microtrichia perotitii*

The ¹H-NMR spectrum of compound A(C₁) showed signals for eight singlet methyl groups centered at δ 0.83 (3H,s,H-28), 0.90, 0.84, 0.86, 0.87 (12H,s, H-23 H-24, H-29, H-30), 0.98 (3H,s, H-26), 0.91(3H,s, H-25) and 1.04(3H,s,H-27), one acetoxy group at δ 1.57(s), a signal at δ 4.47 (1H,dd,H-3α) for a methine proton attached to an acetoxy group and the signals at δ 4.83(1H,H-12) for an olefinic proton. These observations are shown in Figure 4.10i and ii and Tabulated in Table 4.11.

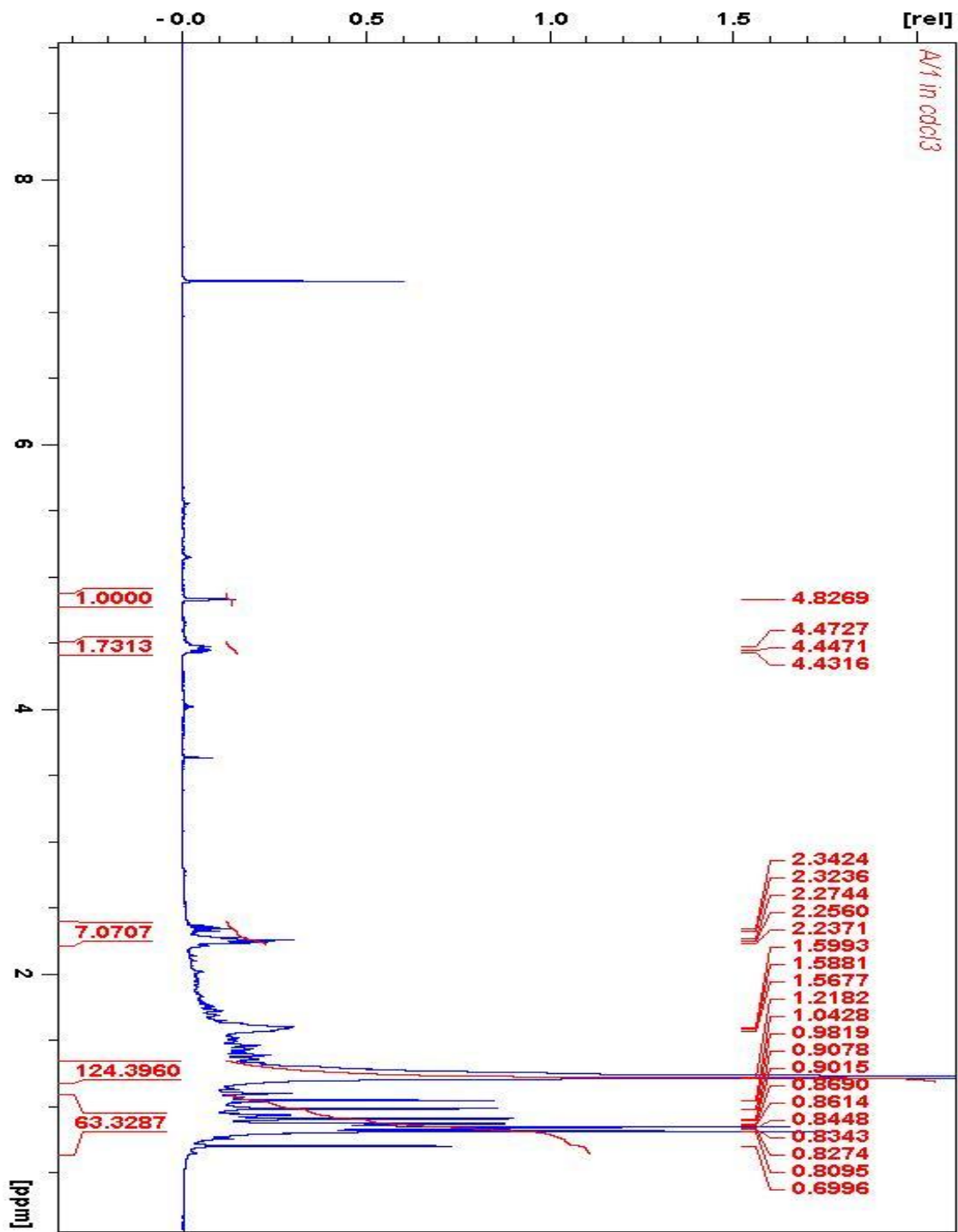


Figure 4.11i: $^1\text{H-NMR}$ Spectrum of compound A(C₁) isolated from *Microtrichia perotii*

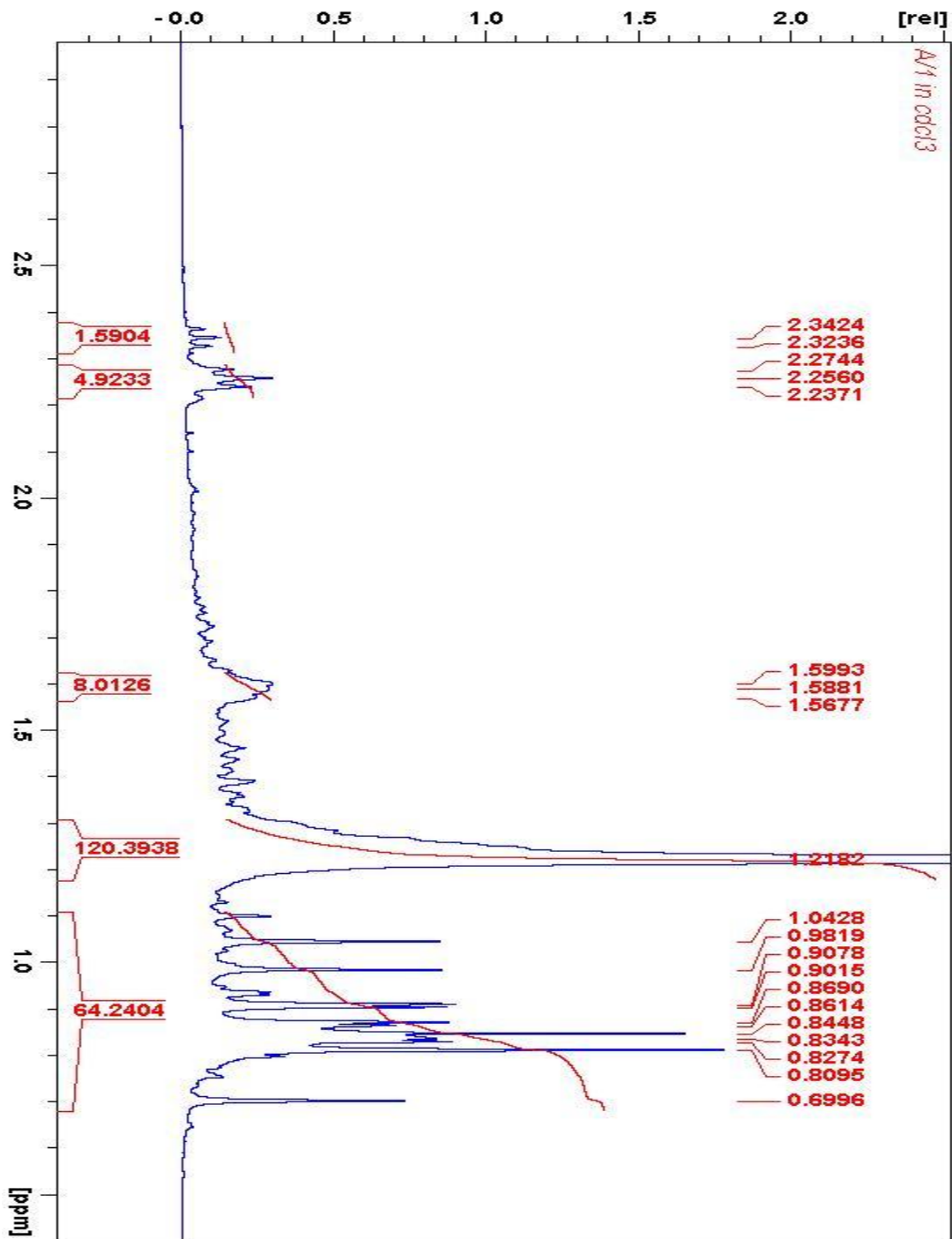


Figure 4.11ii: ¹H-NMR Spectrum of compound A(C₁) isolated from *Microtrichia perotitii* (expanded)

4.11.3.3. ^{13}C -NMR for compound A(C_1) isolated from *Microtrichia perotitii*

In ^{13}C -NMR spectrum the absorption at δC 173.7 (C=O) and δC 21.2 (COCH₃). The absorption at δC 80.6 also indicated that acetyl group is attached to the C-3. The protons present at C-3 appeared as triplet at δH 4.47. Absorption at δH 4.83 has been assigned to one olefinic proton at C-12. It was also supported by the absorption at δC 129.8 and δC 142.7 in ^{13}C -NMR spectrum, assigned to C-12 and C-13 carbon atoms respectively. These observations are shown in Figure 4.12i,ii and iii and tabulated in Table 4.21.

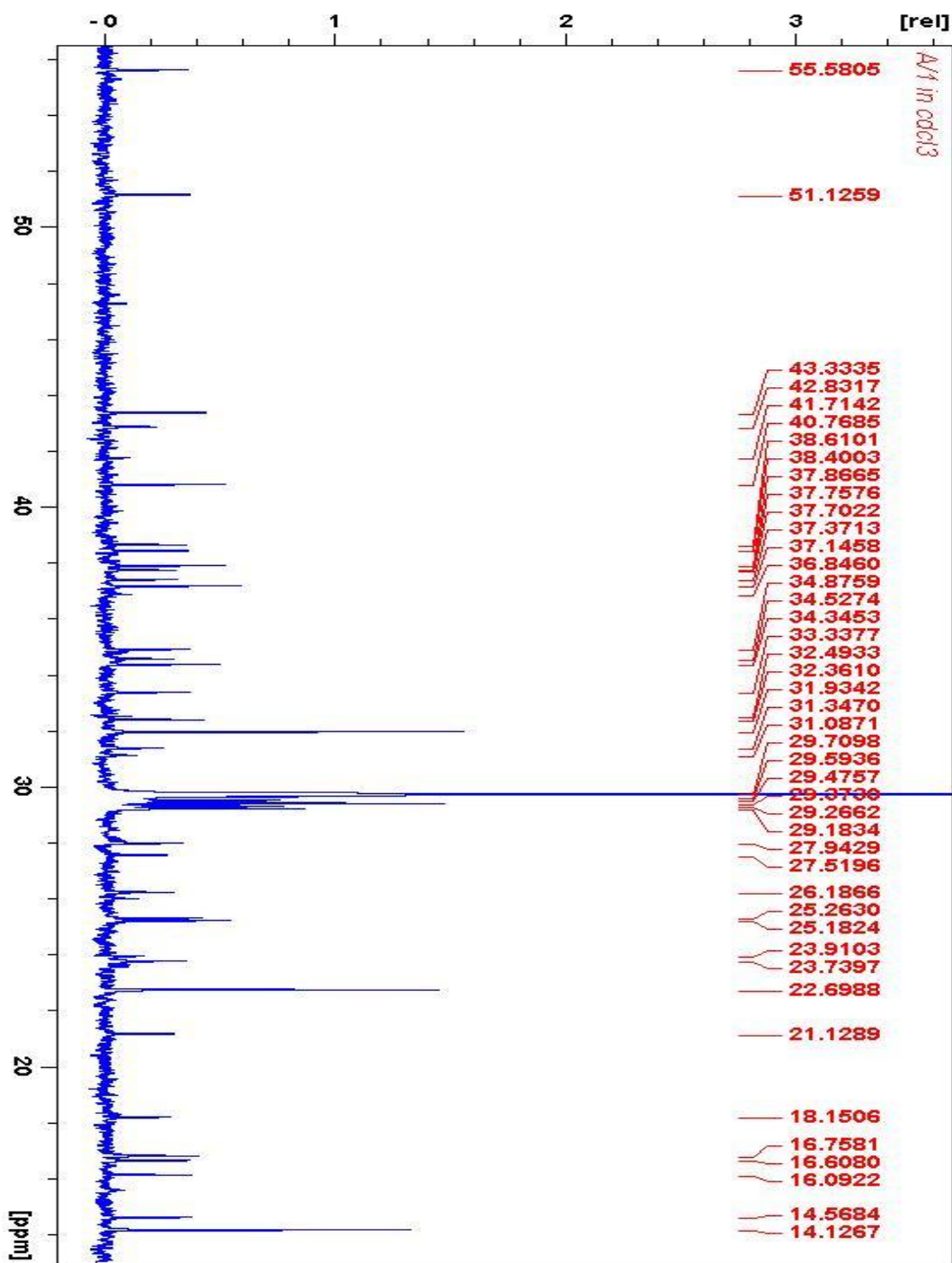


Figure 4.12i: ^{13}C -NMR Spectrum of compound A(C₁) isolated from *Microtrichia perotitii*

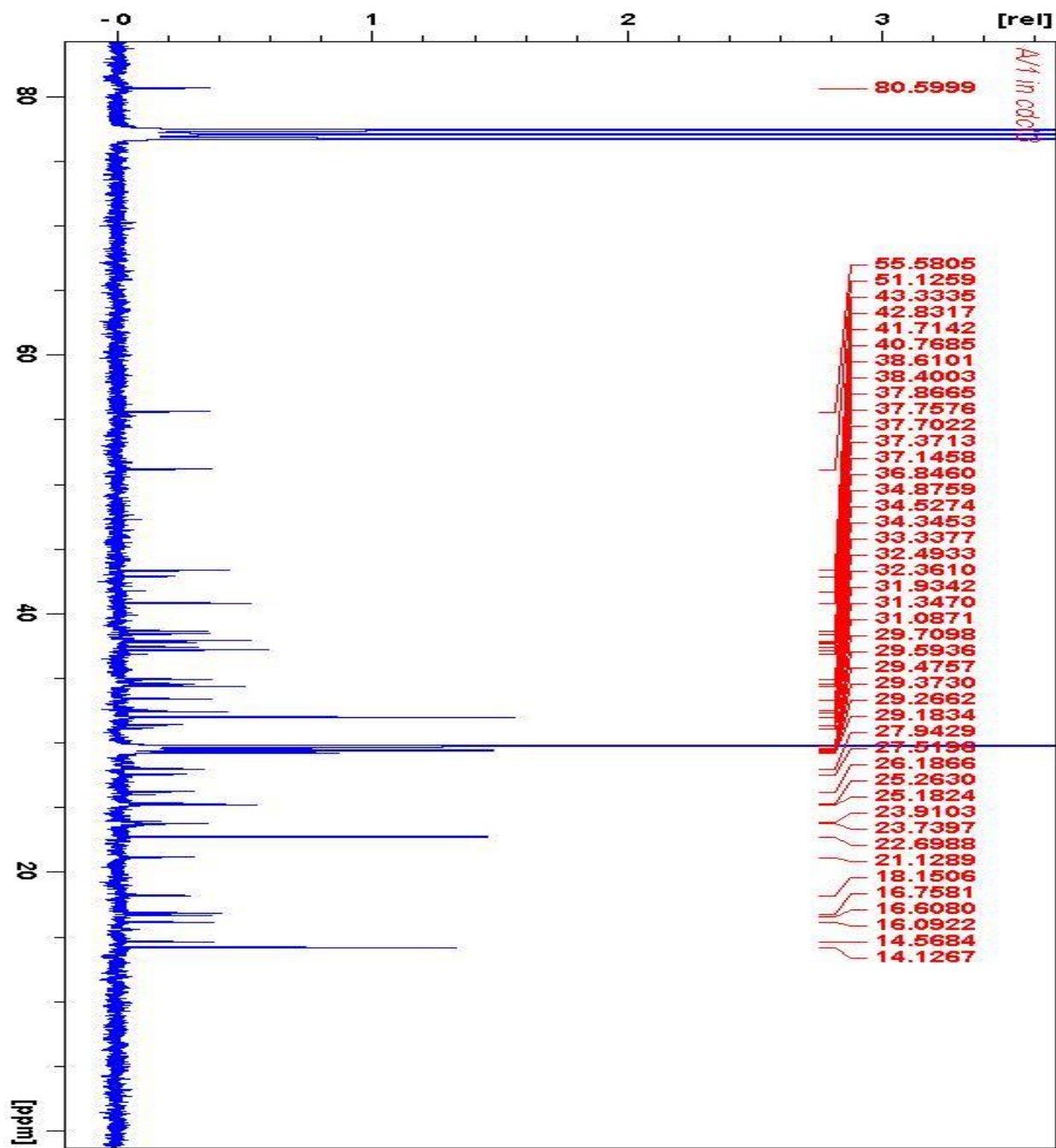


Figure 4.12ii: ^{13}C -NMR spectrum of compound A(C₁) isolated from *Microtrichia perotitii*

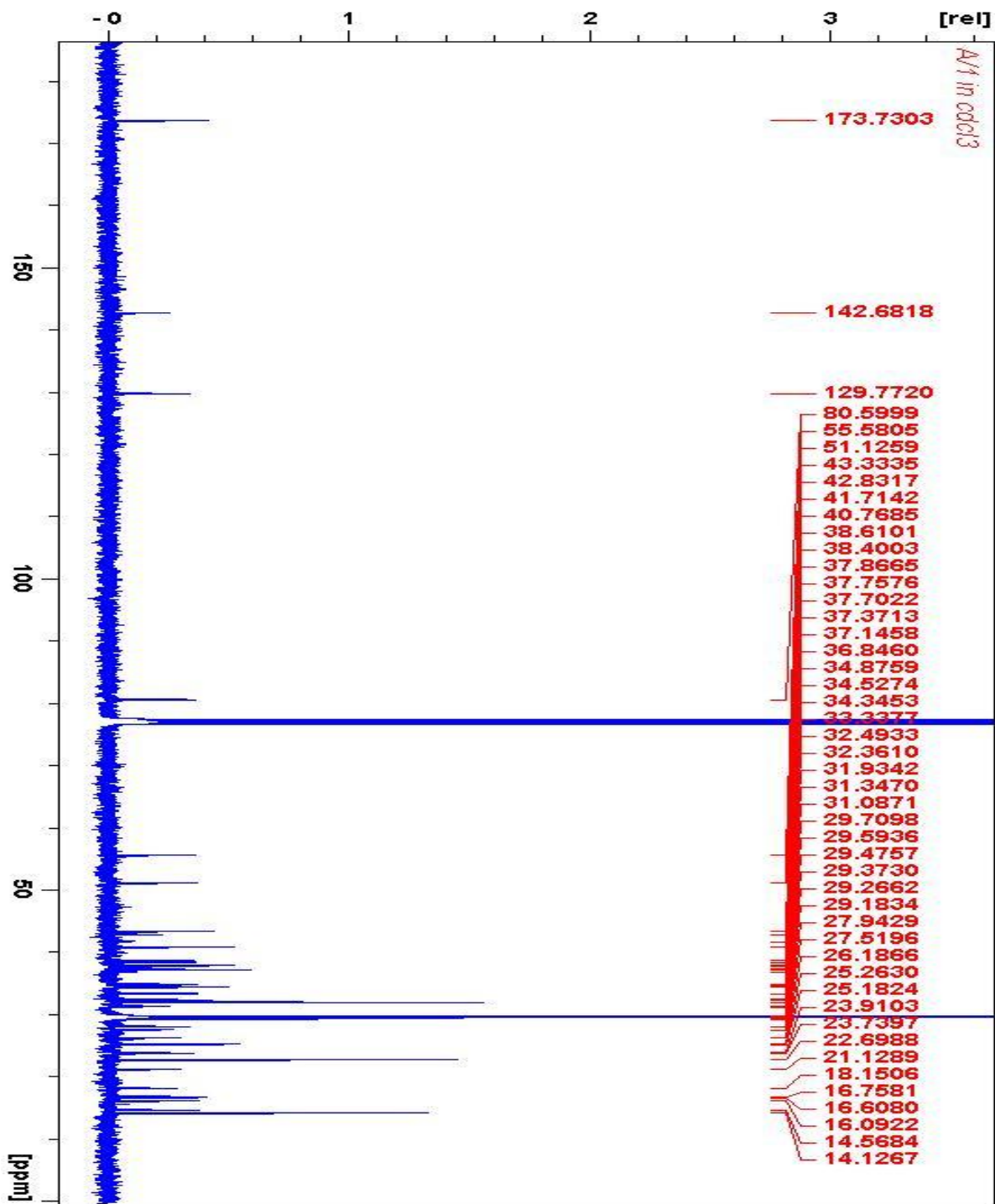


Figure 4.12iii: ^{13}C -NMR Spectrum of compound A(C₁) isolated from *Microtrichia perotitii* (expanded)

4.11.3.4: DEPT Spectra of compound A(C₁) isolated from Microtrichia perotitii

In the DEPT spectra of compound A(C₁) the following type of carbons were observed that is methyls, methylenes and methines especially at the DEPT-135. Details are as presented in Figure 4.13 i-iii.

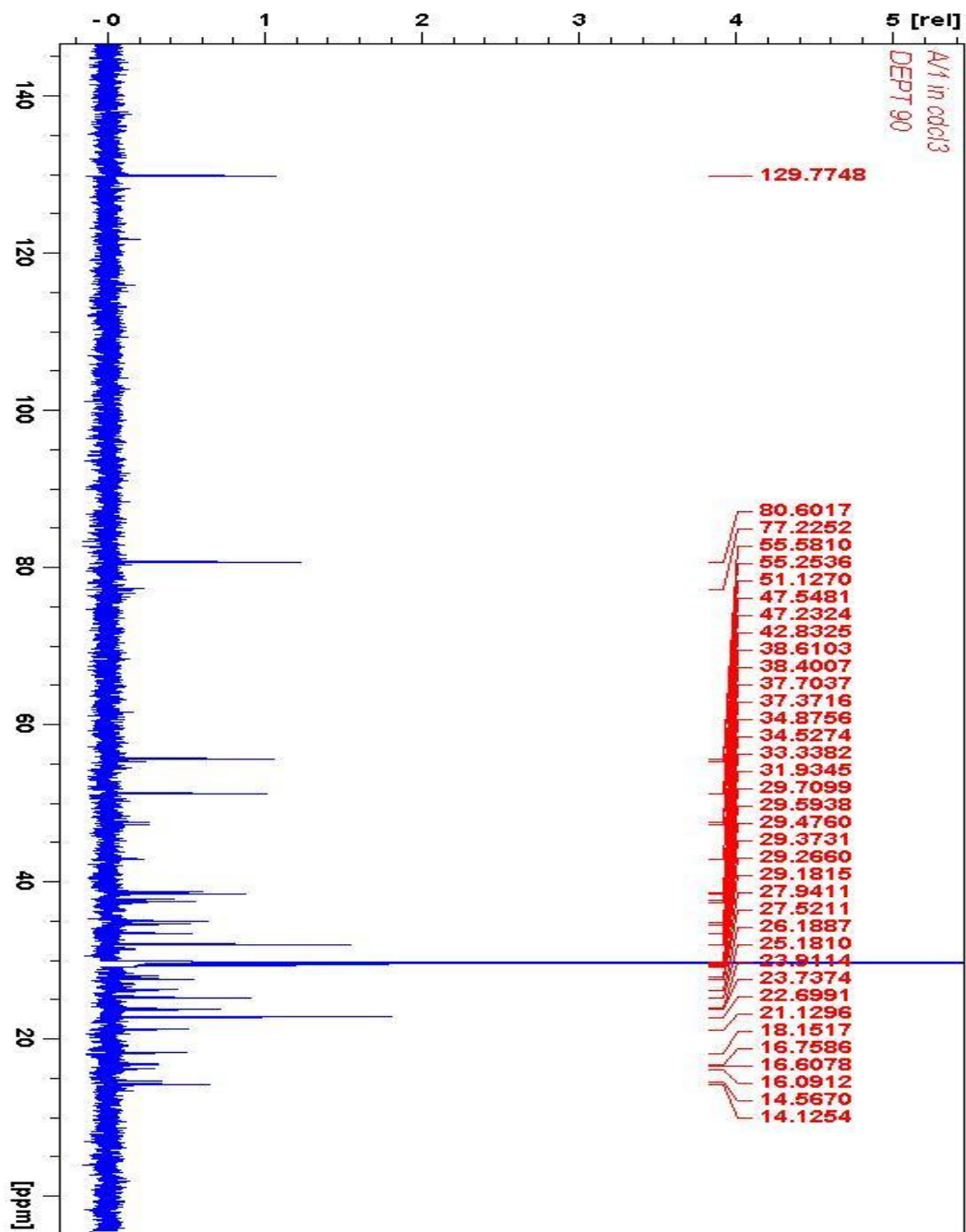


Figure 4.13i: DEPT Spectrum of compound A(C₁) isolated from *Microtrichia perotitii*

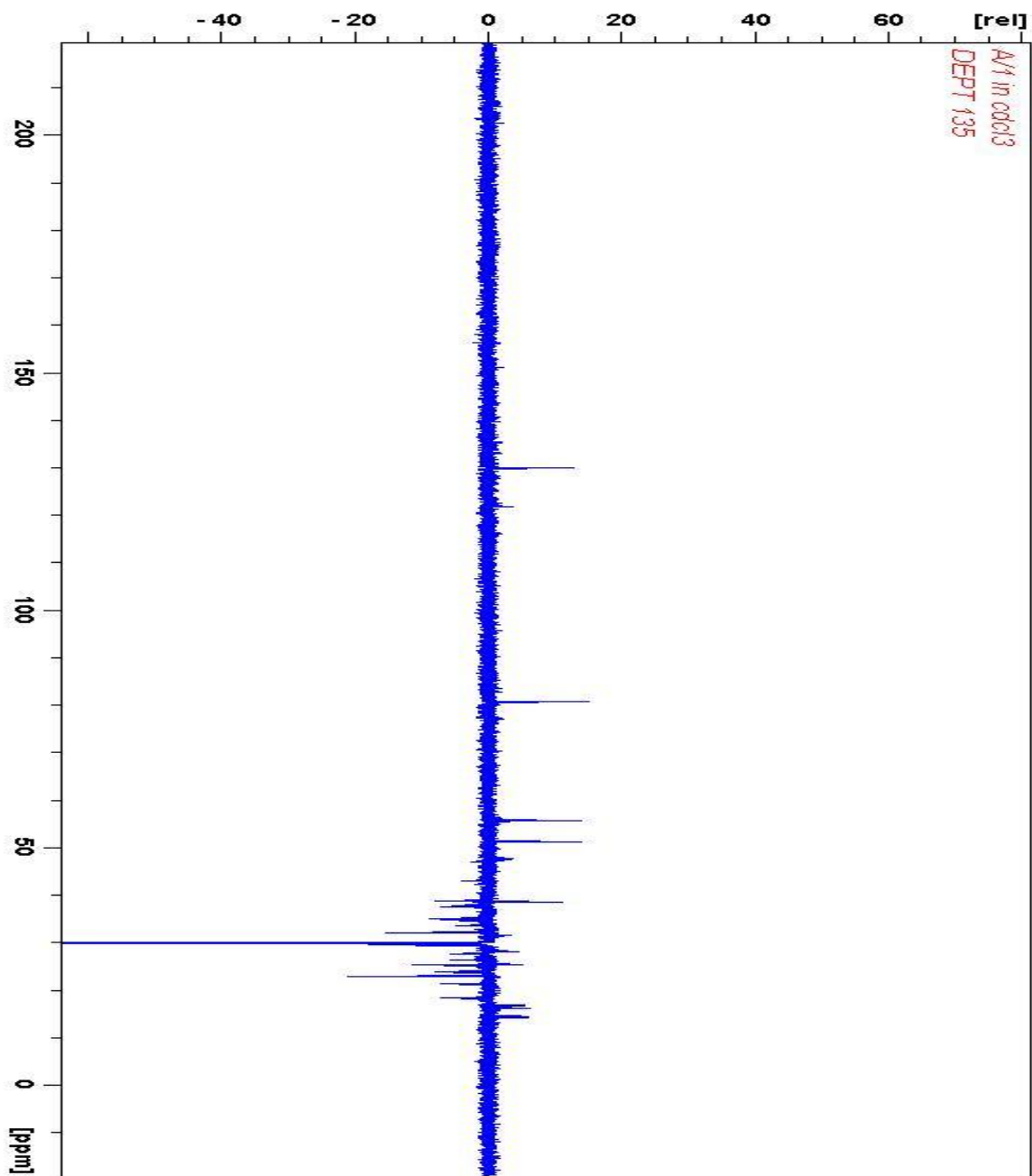


Figure 4.13ii: DEPT Spectrum of compound A(C₁) isolated from *Microtrichia perotitii*

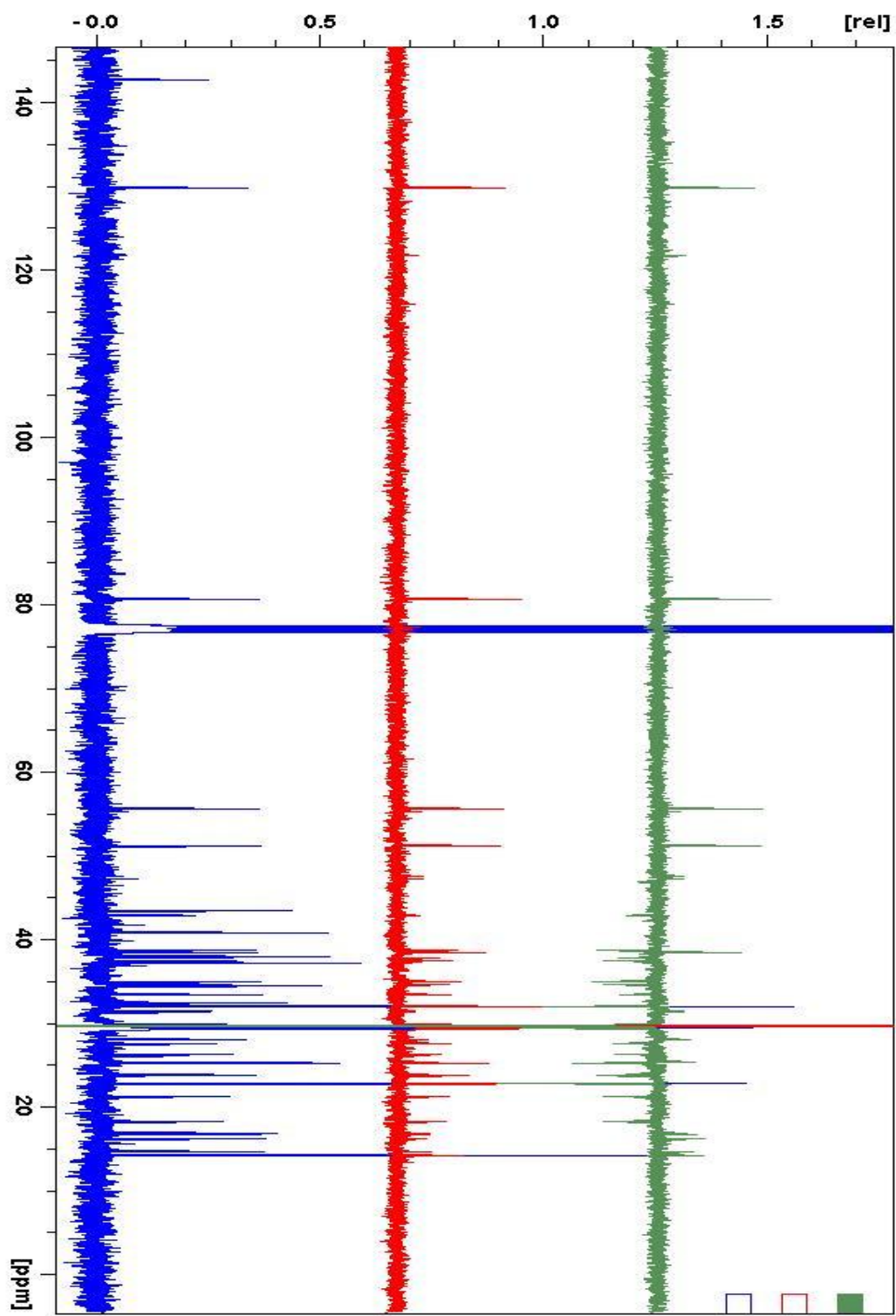


Figure 4.13iii: DEPT Spectrum of component A(C₁) isolated from *Microtrichia perotitii*

*4.11.3.5: ^1H - ^1H COSY spectrum of compound A(C₁) isolated from *Microtrichia perotitii**

The 2D COSY spectrum shows off-diagonal peaks which identify protons coupled to other protons often via three bonds. In the COSY spectrum of A(C₁) cross links were observed between protons such as between δH 4.8 and δH 2.2, δH 4.0 and δH 2.0.

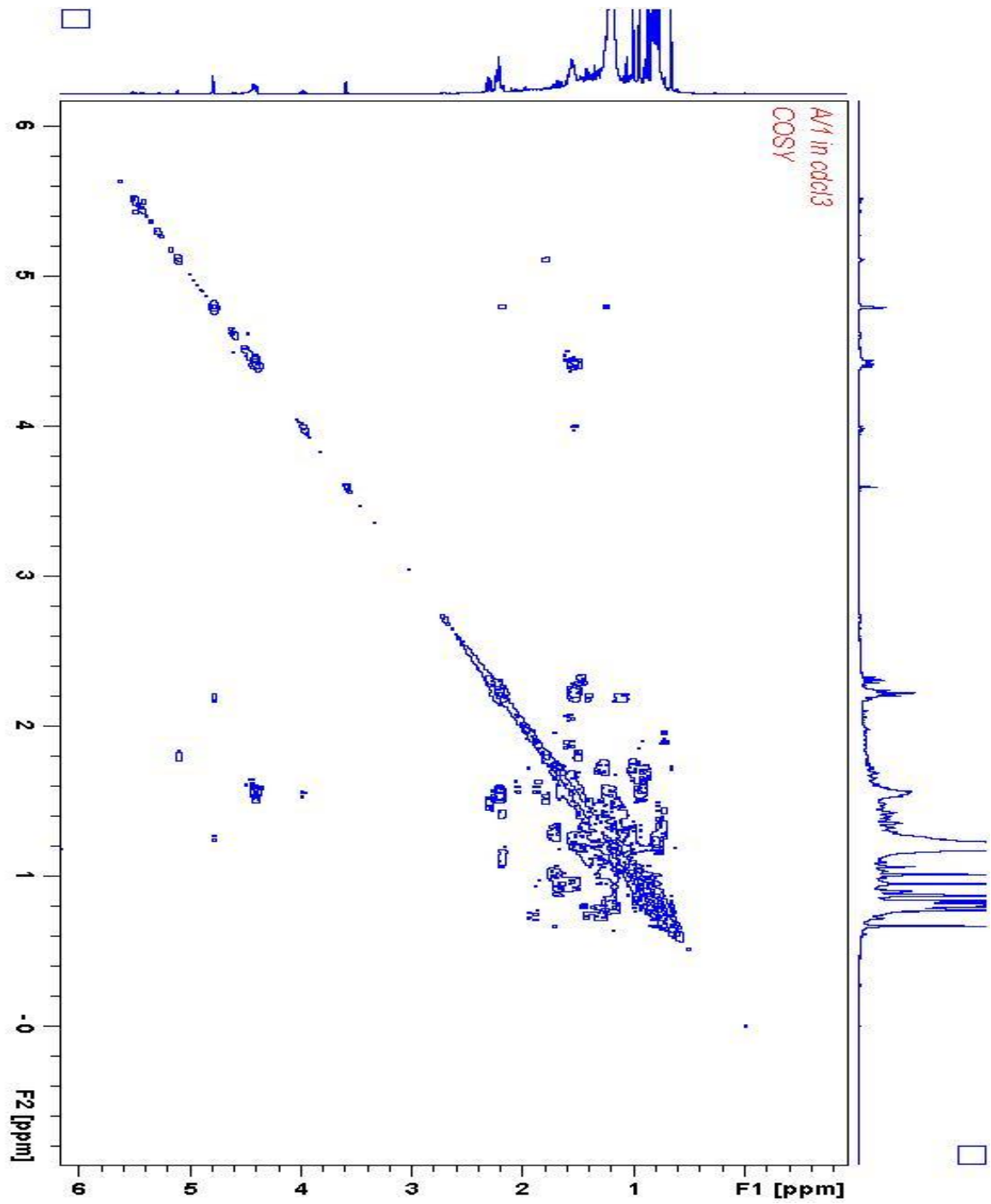


Figure 4.14: COSY Spectrum of compound A(C₁) isolated from *Microtrichia perotitii*

4.11.3.6: NOESY spectrum of component A isolated from Microtrichia perotitii

The NOESY spectrum indicates which ^1H 's are close to which other ^1H 's in space. For instance such interaction exists between δ H 4.47 (C-3) and δ H 0.09(C-23) in the spectrum of compound A(C₁).

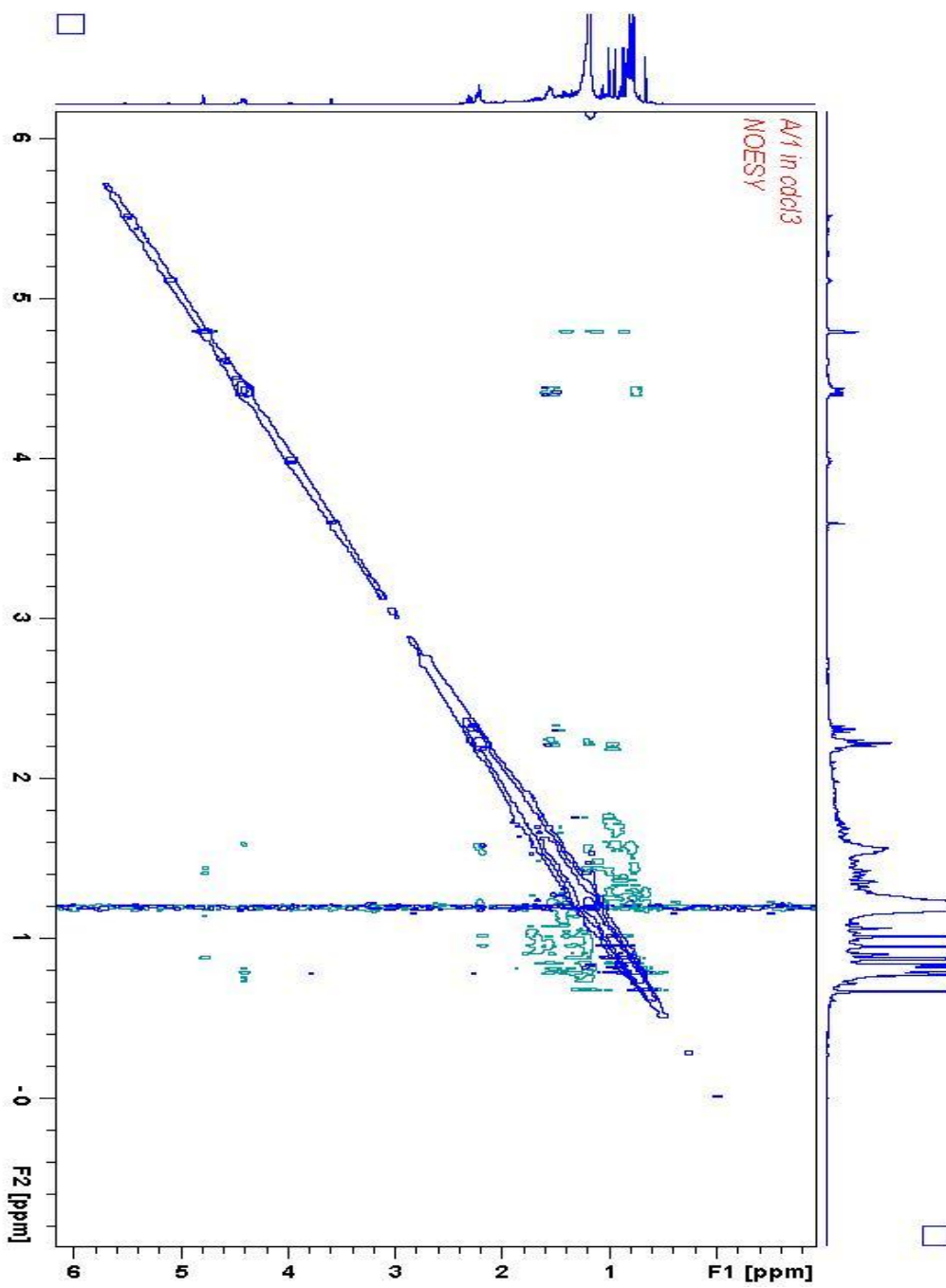


Figure 4.15: NOESY Spectrum of compound A(C₁) isolated from *Microtrichia perotitii*

4.11.3.7: HMBC Spectrum of compound A(C₁) isolated from Microtrichia perotitii

The HMBC spectrum indicates long range connectivity correlations between carbons and protons that are separated by two or three bonds. Such observation existed between signals at δ H 2.2 and δ C 173.7, δ H 4.4 and δ C 16.1, δ H and δ C 25.3 in the HMBC spectrum of compound A(C₁).

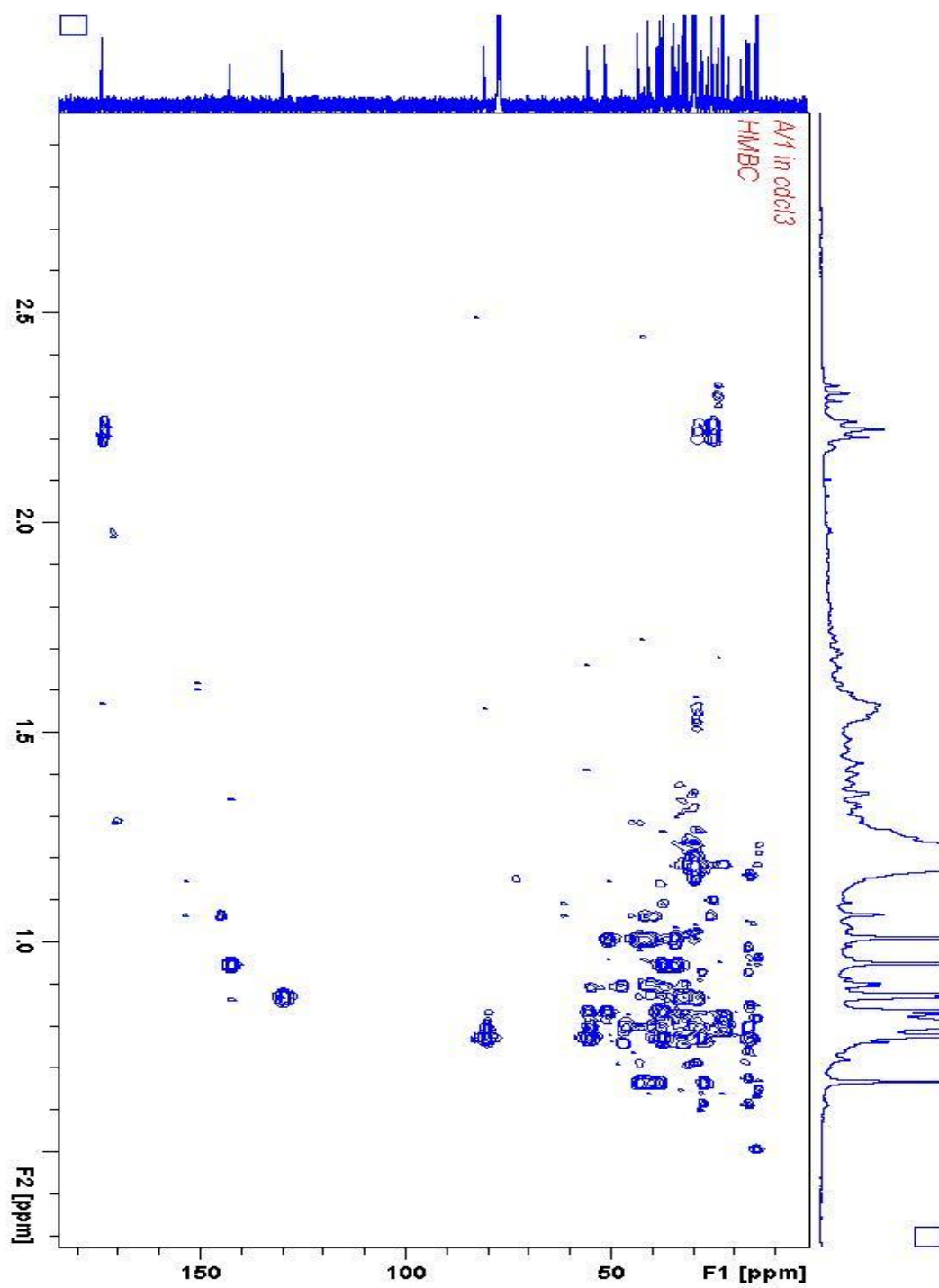


Figure 4.16i: HMBC Spectrum of compound A(C₁) isolated from *Microtrichiaperotitii*

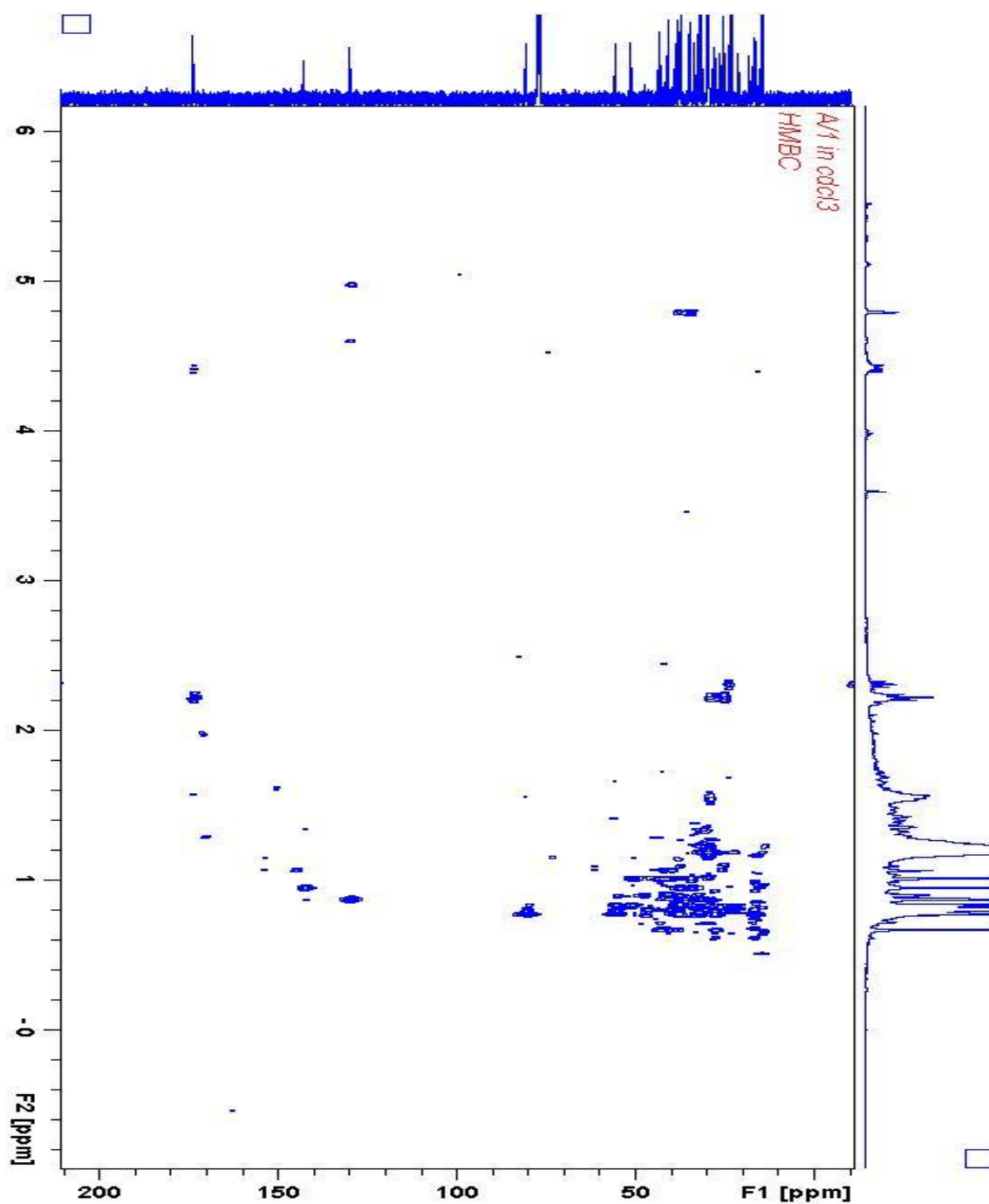


Figure 4.16ii: HMBC Spectrum of compound A(C₁) isolated from *Microtrichia perotitii*

4.11.3.8: HSQC Spectrum of compound A(C₁) isolated from Microtrichia perotitii

The HSQC correlates the chemical shift of proton with the chemical shift of the directly bonded carbon (H-C). Such correlations existed between δ H 4.83 and δ C 129.8, δ H 4.47 and δ C 80.6, δ H 1.60 and δ C 23.9. Details of the HSQC spectra are as presented in Figures 4.17i and 4.174ii.

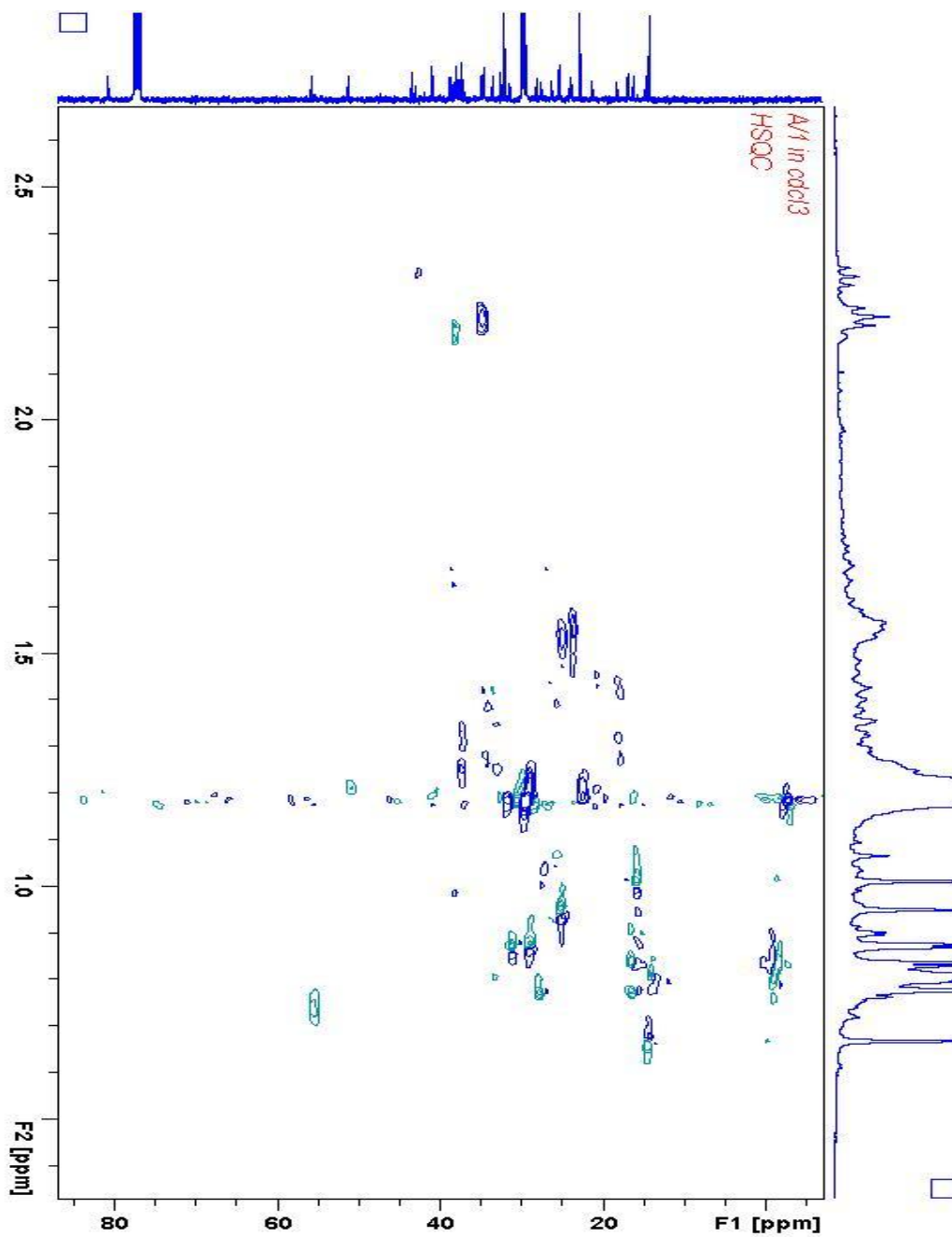


Figure 4.17i: HSQC Spectrum of compound A (C_1) isolated from *Microtrichia perotitii*

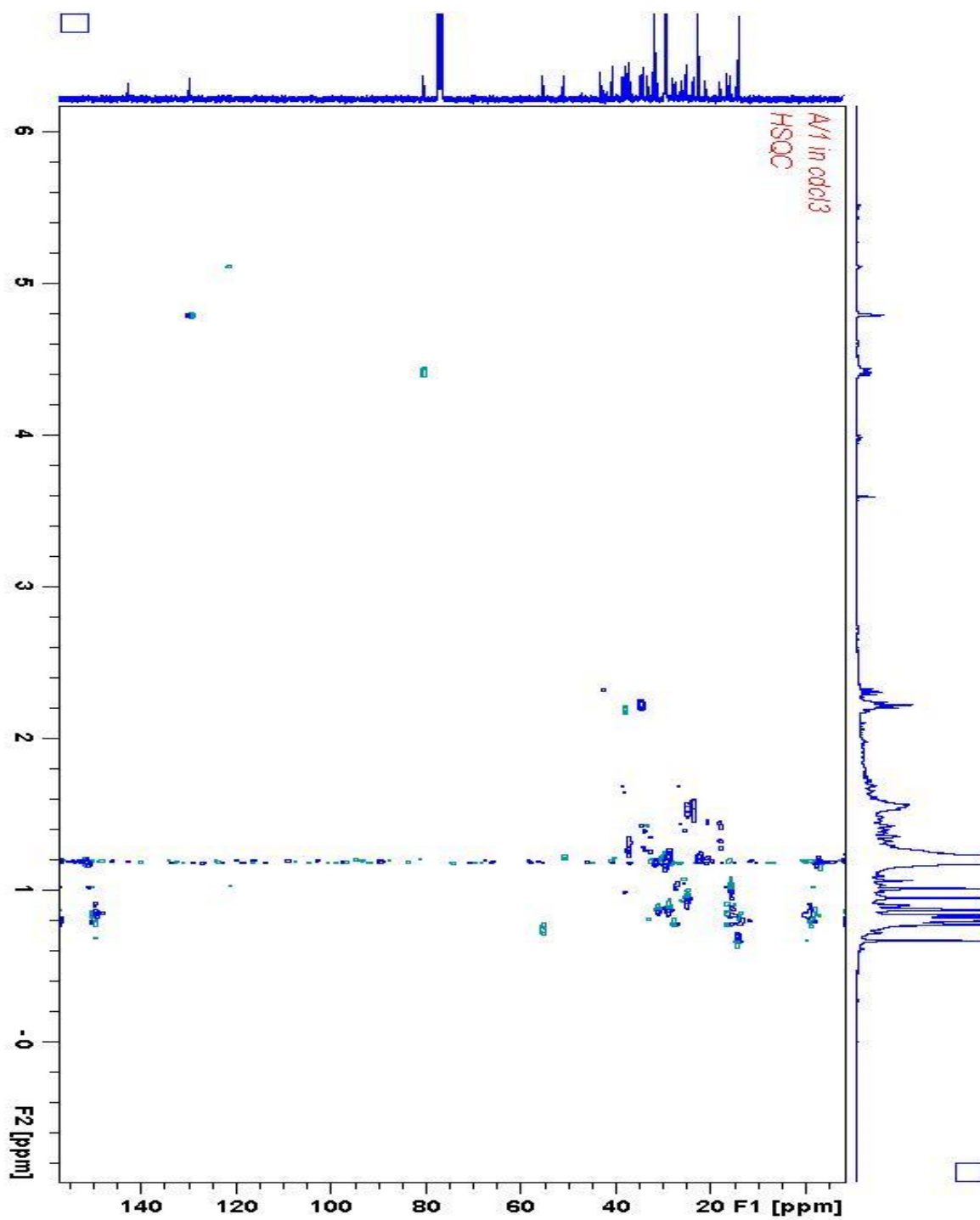


Figure 4.17ii: HSQC Spectrum of compound A(C₁) isolated from *Microtrichia perotitii*

The FTIR spectrum for compound A(C₁) showed prominent absorption signals for CH (asymmetric) for aromatic system (1458,23 cm⁻¹),CH(symmetrical) for aliphatic systems (2868.24 cm⁻¹) and for C=O (stretch) functionality (1726.36 cm⁻¹ and 1654.98cm⁻¹).

Table 4.19: FTIR spectrum of compound A(C₁) of soluble fraction C of crude methanol crude extract of the powdered leaves of *Microtrichia perotitii* (MPME)

Vmax (cm⁻¹)	Functional group
2924.18	CH
2868.24	CH
1726.36	C=O
1654.98	C=O
1458.23	C=C
1374.33	CH ₃
1253.77	CH ₃ -/CH ₃ CO
1182.40	-CH ₂ -
1112.96	CH
1025.20	C-O
726.22	CH

Table: 4.20: NMR spectroscopic data of compound A(C₁) of solvent fraction C of crude methanol extract of the leaves of *Microtrichia perotitii* (MPME)(chemical shifts and multiplicity)

Carbon No	δ H(ppm) (Exptal)	δ H(ppm) (literatue) ^a	δ C(ppm) (Exptal)	δ C(ppm) (literature) ^b	Multiplicity (DEPT)	HMBC (H→C)
1	-	-	38.6	38.5	CH ₂	-
2	1.60	1.66	23.9	23.4	CH ₂	2,3
3	4.47	4.48	80.6	80.9	CH	1,2,4,23,24,1'
4	-	-	38.6	37.7	C	-
5	0.81	0.81	51.5	55.3	CH	4,6,10,23,24
6	1.57	1.51,1.34	18.2	18.3	CH ₂	-
7	-	-	33.3	32.9	CH ₂	-
8	-	-	37.8	39.7	C	-
9	1.58	1.54	43.3	47.7	CH	-
10	-	-	36.8	36.8	C	-
11	2.25	1.89	22.7	22.8	CH ₂	-
12	4.83	5.10	129.8	124.2	CH	9,11,14,18
13	-	-	142.7	139.5	C	-
14	-	-	42.8	42.1	C	-
15	-	-	27.9	28.2	CH ₂	-
16	-	-	26.2	26.7	CH ₂	-
17	-	-	32.5	33.8	C	-
18	2.23	1.29	55.6	59.0	CH	-
19	2.24	1.38	40.8	39.5	CH	18,20
20	2.27	1.98	41.7	38.9	CH	-
21	-	-	31.3	33.6	CH ₂	-
22	-	-	42.8	41.6	CH ₂	-
23	0.90s	0.85	29.7	28.1	CH ₃	3,4,5,24

24	0.84s	0.84	16.7	15.8	CH ₃	3,4,5,23
25	0.91s	0.96	16.6	14.2	CH ₃	1,5,9,10
26	0.98s	0.98	16.1	16.8	CH ₃	7,8,9,14
27	1.04s	1.04	18.1	17.6	CH ₃	8,13,14,15
28	0.83s	0.78	27.9	28.8	CH ₃	16,17,18,22
29	0.86s	0.77	27.5	23.3	CH ₃	18,19,20
30	0.87s	0.83	25.3	24.4	CH ₃	19,20,21
-COCH ₃	1.57bs	2.02	21.2	21.5	CH ₃	1'
-C=O	-	-	173.7	170.8	C	-

a = Ali 2003; b = Vincent *et al*, 2015

4.11.4. Proposed structure of an isolated compound from the leaves of *Microtrichia perotitii*

Analysis of the spectral data of the leaves and comparison with an authentic sample (literature values) has led to the proposal of an α -amyrin acetate as the active compound in the leaves of *Microtrichiaperotitii*.

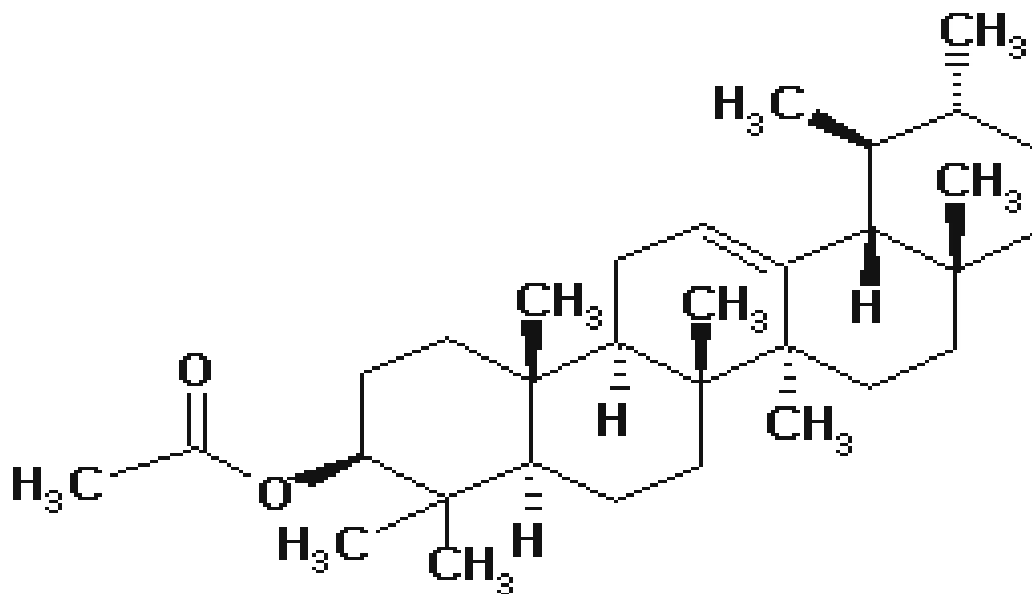


Figure 4.18: Proposed structure of α -amyrin acetate isolated from the leaves of *Microtrichia perotitii*

CHAPTER FIVE

5.0

DISCUSSION

The herb *Microtrichia perotitii* has been used in folk medicine to treat diseases associated with pains and inflammation which included toothaches, rashes in children and rheumatism in elderly persons. It is therefore important to establish the exact identity, purity, safety and adulteration of the herb before its inclusion in pharmacopeia. Therefore, the present evaluation was to set certain standards for its botanical diagnosis in order to differentiate it from other species (Alice, *etal.*, 2003).

The leaves of *Microtrichia perotitii* were observed to be green in colour with slightly bitter taste and an unpleasant odour (Table 4.1, Plates 4.1 – 4.3 and Figure 4.1) (Cronquist, 1981^a, 1988^b; Suseela and Prema, 2007). Some of these characteristics were reported for some members of the family and these included *Vernonia amygdalina* Del (Chavan *et al.*, 2011; Priyanka and Nayak, 2011), *Lonchocarpus cyanescens* (Schum & Thonn) Benth (Dinesh *et al.*, 2012; Trivedi *et al.*, 2012), *Tridax procumbens* Linn (Sharma and Pracheta, 2013), *Leptoderris micrantha* Dunn (Mubo *et al.*, 2014; Avinash *et al.*, 2015) and *lagascea mollis* Cav (Gopi, 2009; Mahundra, 2009; Shaikat *et al.*, 2010). (Plates 4.1-4.3 and Figure 4.1).

The microscopic evaluation of leaves of *Microtrichia perotitii* showed the typical structure of upper and lower epidermis with palisade and spongy parenchyma while the powder revealed the presence of ladder like xylem vessels (spiral), anomocytic stomata, covering trichomes that appeared on both upper and lower epidermis and starch granules. The anomocytic stomata occur more frequently on the lower epidermis of the leaves of

Microtrichia perotitii. The anomocytic and anisocytic stomata types occur more frequently in the Asteraceae family and they contribute greatly in the taxonomic, pharmacognostic and medicinal values of the family (Metcalf and Chalk, 1950; Popescu *et al.*, 2010; Shaukat *et al.*, 2010). Multicellular type trichomes were observed at both adaxial and abaxial surfaces of the leaves of *Microtrichiaperotitii*. Presence and types of trichomes are useful diagnostic features and apart from being of taxonomic significance, leaf trichomes contribute to plant resistance against herbivory and reduction of the rate of transpiration in the plants (Ahlam and Bouran, 2011; Priyanka *et al.*, 2011)^{a,b}.

From the transverse section of the leaf calcium oxalate crystals were observed and they may be involved in the dispersing of light to the chloroplasts in the photosynthetic parenchyma cells of the leaves of *Microtrichiaperotitii*. The crystals also were thought to have a physiological role sequestering excess calcium within plant cells. Thus, the constancy of crystals, type and distribution may be considered a taxonomic character for classification of species in the Asteraceae family (Bhattacharjee and De, 2003; Ciler and Feruzan, 2004; Ciler, 2008; Popescu *et al.*, 2010) (Figure 4.5, 4.6, 4.7, 4.8, 4.9, 4.10, 4.11 and 4.12).

Quantitative microscopic examination of the leaves of *Microtrichiaperotitii* provided values for palisade ratio, stomatal number, epidermal layer, stomatal indices, vein islet and veinlet as shown in Table 4.2. The differences in the number of stomata at both the upper and lower epidermis may be of diagnostic importance. The average number of

stomata is about 300 per square mm of any leaf surface (Veeranjaneyulu and Rama, 1984). These informations set genuity and standard for the herb as well as distinguishing it from co-generic species that may be closely related that cannot easily be characterized by general microscopy.

The chemo-microscopic examinations of the leaves of *Microtrichia perotitii* revealed the presence of starch grains, calcium oxalate crystals, tannins, cellulose, lignin and oils & fats which may confirm the presence of alkaloids, flavonoids and glycosides in the family as well as justifying the use of the herb in ethnomedicine (Prabhu *et al.*, 2009). Some of these observations were reported for *Tridax procumbens* L., *Bidens pilosa* L. *Tithonia diversifolia* Hemsl (A. Gray) in the Asteraceae family (Marbry *et al.*, 1968; Mahundra *et al.*, 2009; Prabhu *et al.*, 2009; Gunaselvi *et al.*, 2010; Jain *et al.*, 2010; Salahuddin *et al.*, 2010; Chavan *et al.*, 2011; Ashok *et al.*, 2014).

The study of the physicochemical constants of the leaves of *Microtrichia perotitii* showed total ash 20.67 %, acid-insoluble ash 2.73 % and water-soluble ash 8.18 % thus indicating total ash to be the highest. The total ash value for *Microtrichia perotitii* lies within fair limits and thus signified its quality and purity and gives idea about the total inorganic content in it (Ugur and Selima, 2011; Woratouch *et al.*, 2011). The acid-insoluble ash value of 2.73 ± 0.13 % when compared with the total ash value of 20.33 ± 0.02 % for the herb showed a differentiation between contaminating materials and variations of the natural ash of the sample. The acid-insoluble ash particularly indicates contamination with silicious materials (earth and sand) and provides information about

non-physiological ash produced due do adherence of inorganic dirt and dust to the crude drug. The water- soluble ash was 8.18 ± 0.30 % and this parameter is used to detect the presence of materials exhausted by water.(Schoffstall,2000; Singh and Sharma, 2010; Adedapo *et al.*, 2011; Pratima and Pratima, 2011; Ahamad *et al.*, 2012; Kamalakannan *et al.*, 2012 ; Kunle *et al.*, 2012; Veena and Pracheta, 2013; Sangram *et al.*, 2015).

The water and alcohol soluble extractive values for the leaves of *Microtrichia perotitii* were found to be 40.90 ± 1.73 % and 25.45 ± 1.24 % respectively. The leaves therefore, exhibited more amount of water soluble components compared to alcohol extract.The extractive values obtained would be useful in evaluating the chemical constituents present in the leaves (crude drug) and may provide estimation of specific constituents soluble in a particular solvent (Periyanayagam *et al.*, 2013;Vipin *et al.*, 2015).Similar observations were reported for *Elephantopus scaber* L. and some species of Wedelia genus in the Asteraceae family (Meena *et al.*, 2010; Mohan *et al.*, 2010; Trivedi *et al.*, 2012).

The moisture content for *Microtrichia perotitii* was calculated by loss as 12.67%. This value was less than 14 % standard requirement for crude drugs. It will also provide referential information for the correct identification of the herb and since it is within the range.It therefore means that the powder of this herb can be stored for a longer period of time without spoilage(Ahmad *et al.*, 2012; Kadam *et al.*, 2012;Mubo *et al.*, 2014). The values reported for some members of the Asteraceae family were: 18.79 % for *Calendula*

offininaliss L., and 15.7 % for *Aspilia Africana*. Pers (Mahan *et al.*, 2010;Sneha *et al.*,2011;Tairoet *al.*, 2011;Saha and Rahaman, 2013).

The results of the acute toxicity tests demonstrate that LD₅₀ values of compounds vary depending on the species and ways of administration and it is carried out in order to provide safety margin for the use of the plant. The aqueous extract of the leaves of *Microtrichia perotiti* using Lorke's method produced 2 deaths at a dose of 1000mg/kg *b.w* after 24 hrs of the first phase of intraperitoneally administration while in the second phase 1 death was recorded at a dose of 1600mg/kg *b.w* as shown in table 4.12. The LD₅₀ was calculated as 1131.37 mg/kg *i.p.* and according to the toxicity classification, compounds with an LD₅₀ value in the range of 1000 -5000 mg/ kg are considered slightly toxic and those with an LD₅₀ value of more than 5000 mg/kg may be considered practically nontoxic (Kenned *et al.*, 1986). The *i.p.* LD₅₀ value of aqueous extract of the leaves was in the range of 1000-5000 mg/kg; thus, *Microtrichia perotiti* should therefore be considered slightly toxic in acute intraperitoneal route (Lorke, 1983;WHO, 1992;Monteiro *et al.*, 2001; Akhila, *et al.*, 2007).An LD₅₀ of 11,748 and 11,885 (non-toxic) were reported for both ethanolic and aqueous extracts of the leaf of *Tithonia diversifolia* Hemsl (Asteraceae) respectively.

The results from this work revealed that both aqueous(MPAE) and methanol (MPME) crude extracts of the leaves exhibited dose-dependent significant inhibitory activity against acetic acid induced writhings in mice. Acetic acid induced abdominal constriction is widely used model for the evaluation of peripheral analgesic activity (Gene *et al.*,

1998). This test is very sensitive and capable of detecting analgesic effects of compounds at doses that may appear inactive in other screening techniques like the tail flick test (Collier *et al.*,1968). Acetic acid is believed to stimulate the release of cyclooxygenase enzymes (COX-1 and COX-2) that are normally found in damaged inflamed tissues and it is the therapeutic target for the NSAIDs in inflammatory pains, both peripherally and centrally (Abbasi *et al.*, 1997^a, 2001^b). The effectiveness of NSAIDs in inflammatory pains further supports the involvement of prostaglandins (PGs) biosynthesis in nociceptive mechanism of acetic acid (Franzotti *et al.*, 2000; Abdullah *et al.*, 2011). The observed effect on both aqueous (MPAE) and methanol (MPME) crude leaf extracts may therefore be due to inhibition of the synthesis of prostaglandins (PGs) (Table 4.7, Figure 4.5, Appendix A)(Guadalupe *et al.*, 2011). Similar results were reported for some members of the Asteraceae such as *Matricaria aurea* L, *Wedelia chinensis* (Osbeck) Merr and *Sphearanthus indicus* L (Shanmugam *et al.*, 2006; Lingaraju *et al.*, 2011; Varsha and Bharatkumar,2011 Masoume *et al.*, 2014). Similarly, soluble fractions A,B ,C and D of the methanol crude extract (MPME) of the powdered leaves of *Microtrichia perotitii* exhibited dose-depended significant inhibition against acetic acid induced writhings in mice (Table 4.8; Figure 4.6, Appendix A). The observed effects of the soluble fractions may also be due to inhibition of the synthesis of prostaglandins (PGs) (Ronaldo, *et al.*, 2002; Voilley, 2004; Thankarajan and Thangaraj, 2014). Significant inhibition activities of soluble fractions were reported for some members of the Asteraceae such as *Blumea membranacea* DC (Jimuty *et al.*, 2014;Muhammad, *et al.*, 2014).

The hotplate test is thought to involve the spinal reflex and is regarded as one of the suitable models for determining the involvement of central antinociceptive mechanism and also considered to examine compounds acting through the opioid receptor. The exposure of animal to thermal stimuli in this method will lead to the development of a non-inflammatory, acute nociceptive response and the ability of the extract to inhibit the thermal induced nociceptive indirectly indicates its ability to inhibit non-inflammatory pain (Table 4.9 ; Table 4.10 and Figure 4.7; Figure 4.8, Appendix A). The mediators prostaglandins and bradykinin were suggested to play important role in the analgesia (Hosseinzadeh and Younesi, 2002; Shanmugam *et al.*,2006; Sabina *et al.*, 2009;Zimudzi *et al.*, 2013). It has been reported that extracts containing phenolics, flavonoids or alkaloids can enhance the centrally mediated responses (Thankarajan and Thangaraj, 2014). The analgesic activities of the extracts of *Microtrichia perotitii* could largely be due to the presence of polar compounds in the plant. These include tannins, flavonoids or saponins which were detected in the plant (Parke and Sapota, 1996; Ahmadiani *et al.*, 1998). Based on these results it can be concluded that the aqueous (MPAE) , methanol (MPME) crude extracts of *Microtrichia perotitias* well as the soluble fractions of MPME whose possible mechanism of action might be similar to that of standard drugs used (Shinde, 2009; Biplab *et al.*,2014; Ruchiet *al.*, 2015).

The formalin–induced inflammatory response is usually biphasic. The initial phase of oedema is attributed to the release of histamine, kinins and serotonin while the second phase of oedema is due to the release of prostaglandins, protease and lysosomal enzymes(Wheeler–Aceto and Cowan, 1991; Wibool *et al.*, 2008; Yi *et al.*,

2008;Thankararaja and Thangara, 2014). Anti-inflammatory activity was exhibited by both crude aqueous (MPME) and methanol (MPME) extracts (Figure 4.9) as well as the solvent fractions A, B, C and D of the crude methanol extract of *Microtrichia perotitii* (Table 4.11; Table 4.12). This may be due to their ability to inhibit the release of the mediators of inflammation since the herb contains flavonoids, saponins and tannins. Therefore these compounds might have acted via blocked of prostaglandins. The analgesic and anti-inflammatory activities exhibited by the crude extracts and soluble fractions of the leaves of *Microtrichia perotitii* in this study may be due to the individual or synergistic actions of various phytochemical compounds present in the herb (Tables 4.8; 4.9 (Ahmadiani, *et al.*,1998;Ahmadiani *et al.*, 2000 ; Meena *et al.*,2011; Thankarajan and Thangaraj, 2014).

The solubility percentage of the dried leaves of *Microtrichia perotitii* in aqueous extraction was higher (6.67 %) when compared to methanol extraction(6.00 %). The difference arose due to the fact that water is more polar than methanol and is an indication that the herb contained more polar constituents (Ankit *et al.*, 2012; Ashok *et al.*,2012 ; Amita and Shalini, 2013; Binu and Jagrati, 2013).These values could be a veritable tool in estimating the chemical constituents, idea about their nature and guide towards evaluating a definite constituent(s) that could be soluble in a given solvent (Larson *et al.*, 2001;Hsu *et al.*, 2006;Sukhdev *et al.*, 2008; Bushra, *et al.*, 2009).

Preliminary phytochemical screening of both aqueous and methanol crude extracts of the leaves of *Microtrichia perotitii* revealed the presence of tannins, flavonoids, alkaloids,

carbohydrates, cardiac glycosides and saponins while anthraquinones were absent. Tannins and flavonoids have been reported to possess anti-inflammatory property. In particular the flavonoids are widespread and have low toxicity compared to other active plant compounds. The bitter taste could be due to the presence of tannins and alkaloids in the herb (Motar *et al.*, 1985; Li and Wang, 2003; Bushra, *et al.*, 2009). Alkaloids have been reported to possess analgesic properties in addition to anti-inflammatory properties which were reported for most alkaloids derived from medicinal plants (Abdul *et al.*, 2013; Erum *et al.*, 2015; Misonge *et al.*, 2015). The carbohydrates though the main components of cell wall and protoplasm have not been indicated in major pharmacological activities but in recent years, they have been widely recognized to play important roles in diverse biological processes, including viral and bacterial infections, cell growth and proliferation, cell-cell communication, as well as immune-response (Chen and Fakudu, 2006; Murrey and Hsieh-W, 2008; Walker *et al.*, 2008; Majaw and Moirangthem, 2009). Saponins have been reported to possess analgesic and anti-inflammatory activities and they protect plants against attack by potential pathogens (Just *et al.*, 1998; Ramesh *et al.*, 1998; Rajnarayana *et al.*, 2001; Yadav and Munin, 2011; Jakir *et al.*, 2013). The cardiac glycosides have direct action on heart thereby helping to support its strength and rate of contraction when it is failing (Yadav and Munin, 2011). Flavonoids and tannins compounds in general have been known to possess antioxidants, astringency, bitterness and colour properties (Jin and Russell, 2010; Mamta *et al.*, 2013). Triterpenes were detected from solvent fractions C and D of the crude methanol extract of the leaves. Triterpenes in general have been reported to possess analgesic, anti-inflammatory, anti-cancer, anti-malaria, anti-viral, anti-bacterial activities and also they inhibit cholesterol

syntheses (Inamake *et al.*, 2010; Amin *et al.*, 2013; Ramazan *et al.*, 2013; Abdulet *et al.*, 2013; Abdelmohsen *et al.*,2014). These compounds therefore could be responsible for the analgesic and anti-inflammatory activities of the leaves of *Microtrichia perotitii*DC as studied. Some of these compounds were reported for some members of the Asteraceae family: *Achillea tenuifolia* LAM , *Elephantopus seaber* L,*Sphaeranthus indicus* L,*Sonchus cornatus*Hochst,*Sonchus ocleraceus* L and the aerial parts of *Ambrosia maritime*L (Geuns, 2003; Hatil, 2009 ; Venkata *et al.*,2010; Raymond *et al.*, 2011; Adinortey *et al.*, 2012; Azadeh *et al.*, 2012). Thus the results of phytochemical screening obtained will always be helpful in prediction of nature of herb(drug) and would also be helpful in discovering the actual value of folkloric remedies(Farnsworth, 1966; Robert, 1976; Mojab *et al.*, 2002; Sayeed, 2007; Sukhdev *et al.*, 2008; Shweta, *et al.*, 2011).

The n-butanol fraction C of the methanol crude extract of the leaves (MPME) showed more activity in both analgesic and anti-inflammatory studies conducted. Its TLC in different solvent systems of Butanol: Acetic acid: Water and Hexane : Ethylacetate produced profiles of components whose R_fvalues ranged from 0.25 – 0.89 (Plate 4.9 & 4.10). The R_fvalues compared well with those observed for alkaloids, flavonoids, terpenes, triterpenoids and steroids (Tables 4.11.1 and 4.11.2) (Kalita and Saikia, 2004; Das *et al.*, 2010 ; Dutta, 2013 ;Neha *et al.*, 2013; Pratibha *et al.*, 2013; Rajendra and Estari, 2013;Sanjay and Bhagyashri,2013;Singh *etal.*, 2014). Three compounds were isolated, A(C₁), B(C₂) and C(C₃) with different melting points (Plate 4.11; Table 4.12). However, compound A(C₁) has a melting point close to those of amyryns (Atta-ur, 1998; Kyun *et al.*, 2008; Gururaj *et al.*,2011; Sharma and Sharin, 2012; Neelima *et al.*, 2013).

The IR spectroscopic data of component A(C₁) showed observed absorptions signal at 2924.18 cm⁻¹ for CH asymmetric for aromatic system and 2868.24 cm⁻¹ for C-H stretching in CH₃ and CH₂, 1726.35 cm⁻¹ and 1654.98 cm⁻¹ (C=O stretching), 1458.23 cm⁻¹ (C-H in CH₃) and 1374.33 cm⁻¹ (CH₃-/CH₃CO, 1112.96 cm⁻¹ C-H scissor for CH₂, 1025.20 cm⁻¹ C-H rocking for CH₂ and 726.22 cm⁻¹ wagging for CH₂ as shown in table 4.10 and Figure 4.5 (Sultana and Saleem, 2010; Krishnaveni and Thakur, 2012; Niaz, 2013; Arun *et al.*, 2014; Punnam *et al.*, 2014; Gopukumar *et al.*, 2016).

The ¹H-NMR of component A (C₁) showed signals for methyls, methylenes and methine protons. In the up-field region of the spectrum singlets each of three protons integration resonating at different wave numbers were assigned to eight methyl singlets thus; δ 0.83s, 3H-28; 0.84,s,3H-24; 0.86,s,3H-29; 0.87,s,3H-30; 0.90,s,3H-23; 0.98s, 3H-26, & δ 0.91,s, 3H-25; 1.04,s,3H-27 while at the downfield region of the spectrum, a triplet proton signal at δ 4.83 ppm was for the olefinic proton H-12, a multiplet at δ 4.47 ppm was observed for oxymethine proton (H-3) which suggested the attachment of an acetate moiety at position 3. One of the methyl singlets at δ 1.57,s, was assigned to methyl of the acetate group attached at C-3 (Table 4.11 and Figures 4.10i and 4.10ii (Segovlet *et al.*, 2011; Chander *et al.*, 2014; Goyal and Sharama, 2014).

The ¹³C-NMR spectrum of component A showed thirty two prominent carbons resonance of 32 carbon atoms in the compound. The up-field resonance at δ 29.7, 16.7, 16.6, 16.1, 18.1, 27.9, 27.5, and δ 25.3 ppm were due to the tertiary methyls while the signal at δ 21.2 ppm was for the carbon of methyl acetate group. In the downfield region, the peak at δ C

81.1 was assigned to the acetate-containing carbon at position 3 with the carbonyl of the acetate resonating at δC 173.7 and its terminal methyl at δC 21.2. The presence of the acetate at C-3 was observed by the chemical shift of C-2 that resonated at δC 23.9. Two single protonated carbons had distinct signals at δC 51.5 and δC 55.6 for the carbons C-5 and C-18, respectively. The signal at δC 129.8 was ascribed to C-11 and that at δC 142.7 was attributed to C-12. The chemical shifts of C-12 and C-13 gives evidence to the position and orientation of the methyl C-29 and C-30. This is provided by the resonance of the 19 *p*-methyl at δC 40.8 in the equatorial position, which is in close proximity to the double bond (Table 4.13 and Figures 4.1i-4.7iii). The DEPT showed signals for the nine methyls, nine methylenes, seven methines and seven quaternary carbons (Figure 4.13i-iii).

A confirmation of the structure of component A was accomplished through 2D NMR experiments thus, 1H - 1H COSY (Figure 4.14), NOESY (Figure 4.15), HMBC (Figure 4.16i; Figure 4.16ii), HSQC (Figure 4.17i; Figure 4.17ii) spectra (Niaz, 2013; Lea, *et al.*, 2014; Ragasa, *et al.*, 2014). On the basis of above observations along with some reported literature data compound A was identified as α -amyrin acetate (Figure 4.18) (Feleke and Brehani, 2005; Razbor *et al.*, 2007; Catharina *et al.*, 2013; Lea *et al.*, 2014). The positive test of the solvent fraction C to Libermann-Burchard test was an indication of the presence of a triterpenoid in the plant. If this proposed compound is confirmed as an α -amyrin acetate, it will be the first time it is isolated from the leaves of *Microtrichia perotitii*.

Although no biological activity tests were conducted on the isolated compound, literature search revealed that it has diverse bioactivities such as anti-inflammatory, anti-oxidant,

sedative, anti-pyretic, anticonvulsant and anti-bacterial properties (Aragao *et al.*, 2009; Manguro *et al.*, 2009; Sob *et al.*, 2010; Dias *et al.*, 2011; Joyce *et al.*, 2011; Ali, 2013; Catharina, *et al.*, 2013; Lea, *et al.*, 2014; Singh *et al.*, 2014; Virgilio *et al.*, 2015). α -amyrin acetate was isolated from the aerial roots of *Ficus benghalensis* L (Asteraceae) for its antidiabetic activity, *Hoya multiflora* from its sedative, anxiolytic and anticonvulsant properties as well as significant anti-inflammatory activity in egg albumen-induced paw edema (Aragão *et al.*, 2009; Jahan, *et al.*, 2014; Ebajo, *et al.*, 2015).

CHAPTER SIX

SUMMARY, CONCLUSION AND RECOMMENDATION

6.1

SUMMARY

Pharmacognostic studies revealed that the leaves of *Microtrichia perotiti* contains anomocytic stomata (27 x 16.2; 27 x 13.5 μm) and uniseriated multicellular trichomes (135 x 21.6; 132 x 21.6 μm) both located at upper and lower layers of the leaves respectively while prism and cylindrical calcium oxalate crystals were also found in the leaves. The physical constant revealed moisture content of 12.67 ± 0.62 , water-soluble extractives ($40.9 \pm 1.73\%$), alcohol-soluble extractives ($25.45 \pm 1.24\%$), ash content of $20.33 \pm 0.20\%$, acid-insoluble ash ($2.73 \pm 0.13\%$) and water-soluble ash ($8.18 \pm 0.30\%$). The leaves contain starch, cellulose, calcium oxalate, tannins, oils & fats and tannins. These values are very useful for the preparation of the monograph of the herb as well as inclusion in pharmacopeia.

The aqueous, methanol and its solvent fractions exhibited analgesic and anti-inflammatory activities in laboratory animals that were either dose-dependent or independent at varying concentrations.

The aqueous, methanol and its solvent fractions revealed the presence of tannins, flavonoids, alkaloids, cardiac glycosides, saponins, carbohydrates and terpenoids which are of medicinal values.

Spectroscopic characterization of the solvent fraction of the methanol crude extract of leaves lead to the isolation of an α -myrin acetate from the leaves.

6.2

CONCLUSION

The pharmacognostic standards for the leaves of *Microtrichia perotitii* has been established from the study carried out and could be used for the preparation of its monograph. The leaves contain vital compounds which are responsible for its analgesic and anti-inflammatory activities. A triterpenoid which has a lot of biological activities was isolated from the leaves.

6.3

RECOMMENDATION

There is virtually no reported work on this herb and therefore it is recommended that more research should be conducted in order to;

- a. isolate more active phytoconstituents.
- b. identify more biological activities.
- c. package it as a form herbal drug for easy dispensing

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APPENDICES

Appendix A

Effects of crude leaves extract of *Microtrichia perotitii* and solvent fractions against acetic acid induced writhings in mice and formalin induced paw Oedema in rats.

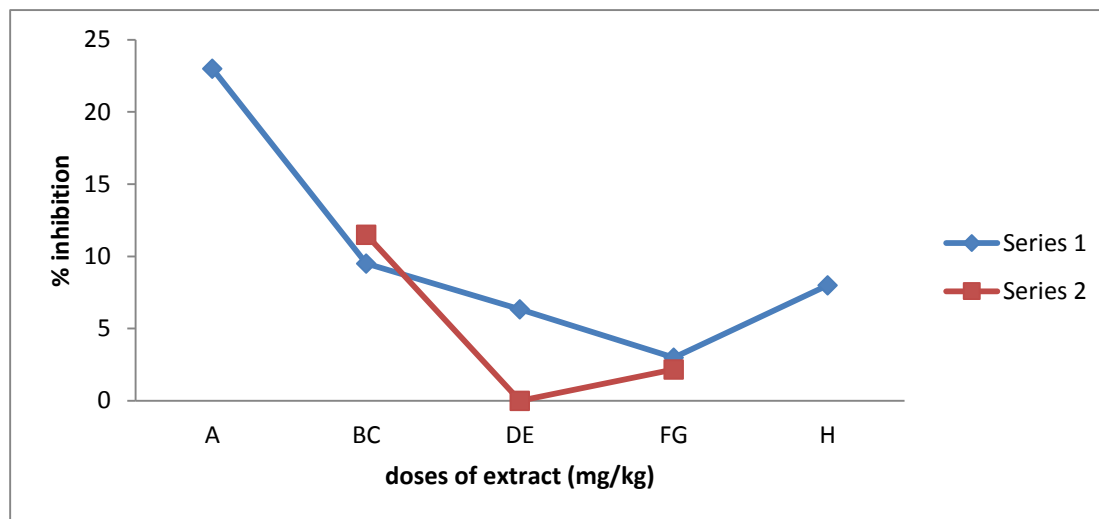


Figure 4.5: Effects of extracts Aqueous and methanol leaves extracts of *Microtrichia perotitii* against acetic-acid induced writhings in mice

- Key:
- A - Normal Saline
 - B - M.P.A.E at 25mg/kg
 - C - M.P.M.E at 25mg/kg
 - D - M.P.A.E at 50mg/kg
 - E - M.P.M.E at 50mg/kg
 - F - M.P.A.E at 100mg/kg
 - G - M.P.M.E at 100mg/kg
 - H - Proxicam at 10mg/kg

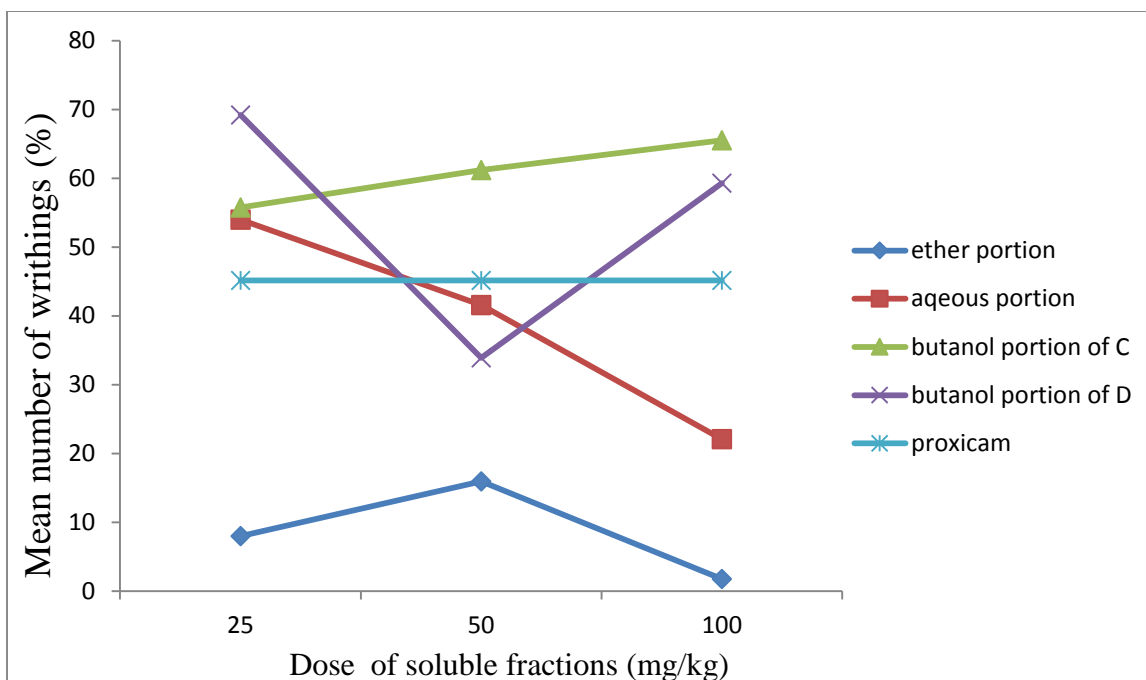


Figure 4.6: Effects of solvent fractions (A, B, C, and D) of methanol crude extract of the *Microtrichia perotitii* (MPME) against Acetic acid. Data was presented as mean \pm SEM and were analysed by ANOVA followed by the student's *t*-test.

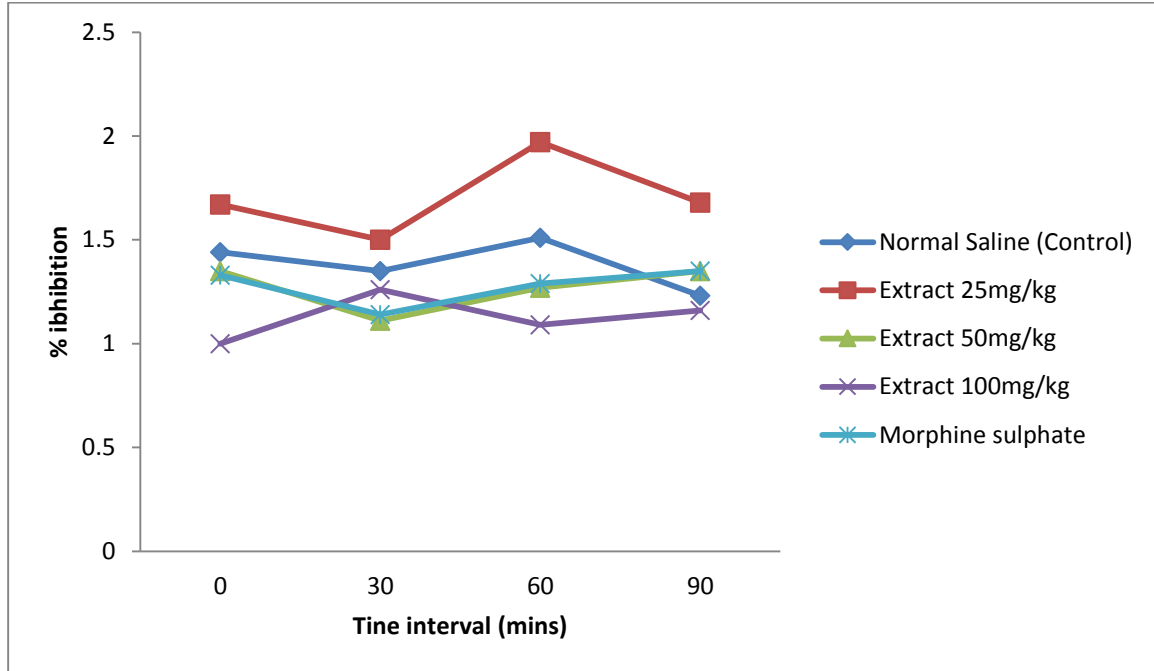


Figure 4.7: Effect of aqueous crude extract(MPAE) of the powdered leaves of *Microtrichia perotitii* in mice when exposed to a Hot-plate. Data was presented as mean \pm SEM and were analysed by ANOVA followed by the student's *t*-test.

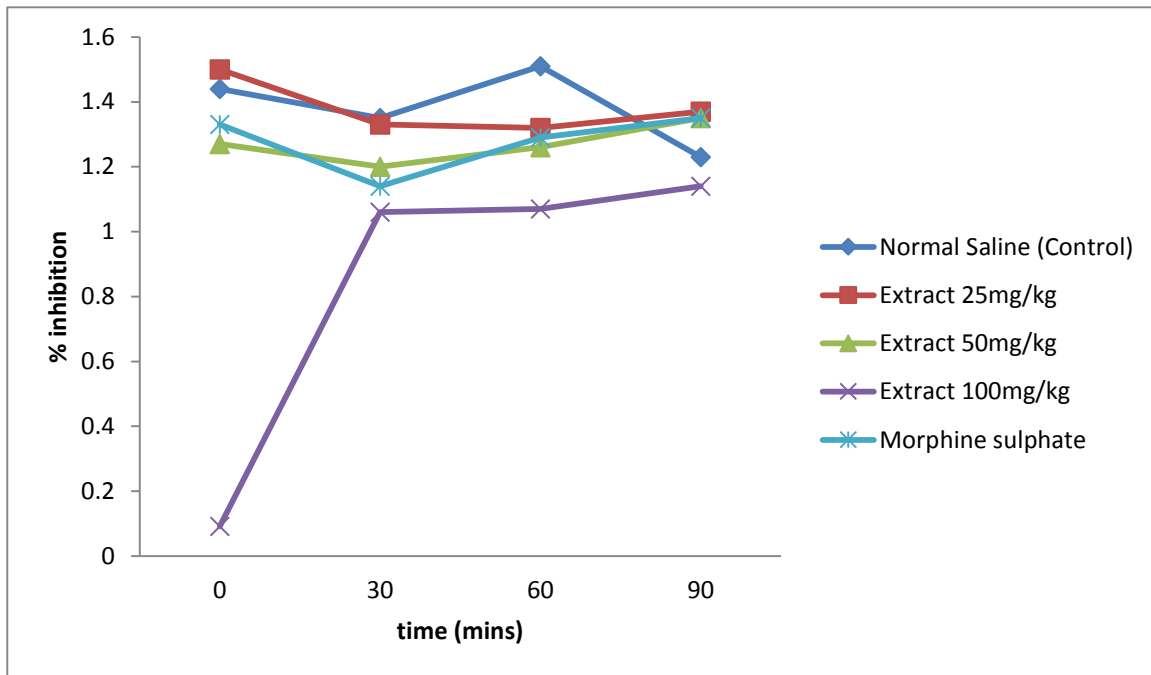


Figure 4.8: Effect of Methanol crude extract (MPME) of the powdered leaves of *Microtrichia perotitii* in mice when exposed to hotplate thermal stimuli. Data was presented as mean \pm SEM and were analysed by ANOVA followed by the student's *t*-test.

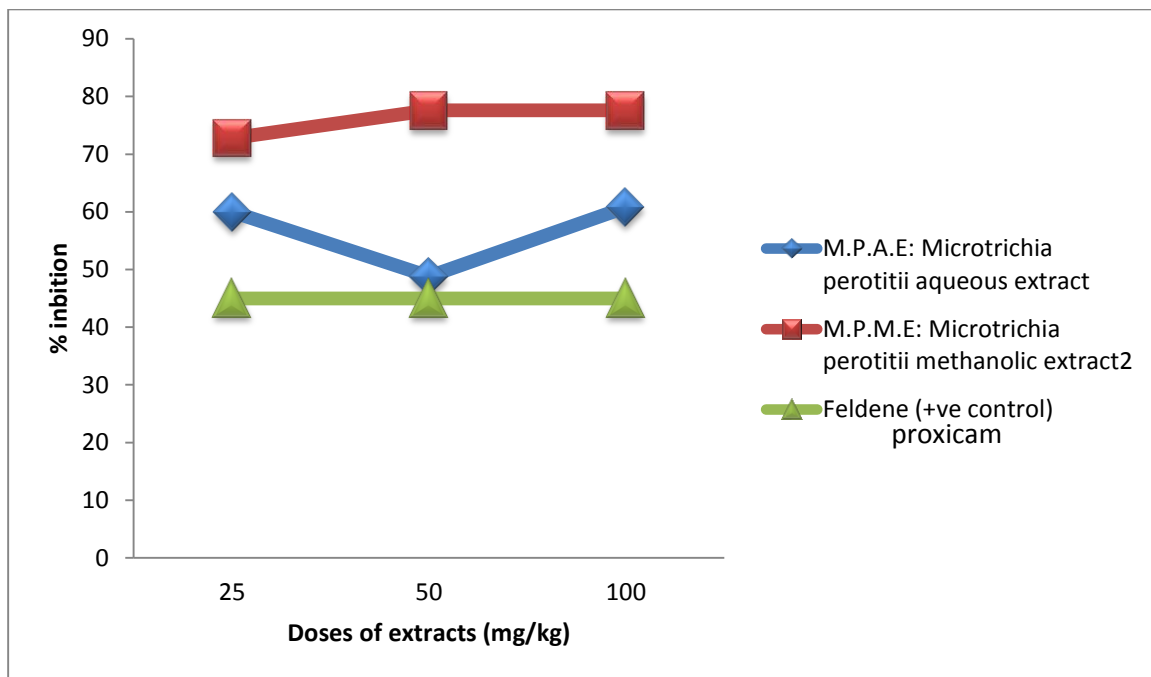


Figure 4.9: Effects of crude aqueous (MPAE) and methanol (MPME) extracts of the powdered leaves of *Microtrichia perotitii* against formalin induced paw Oedema in rats.

* significant at $p < 0.05$. Data was presented as mean \pm SEM and were analysed by ANOVA followed by the student's *t*-test.

Appendix B

Microscopical characters of *Microtrichia perotitii*

1. Stomatal number

Stomata number = $\frac{\text{average number of stomata}}{\text{Square micrometer of epidermis}}$

a: $\frac{4+5+6+3}{4} = 4.5$

b: $\frac{3+5+2+1}{4} = 2.8$

c: $\frac{5+5+6+4}{4} = 5.0$

d: $\frac{4+4+4+5}{4} = 4.3$

Sample calculation:

Stomata number = 4.5

2. Stomatal index

%Stomatal index = $\frac{\text{Total number of stomata in a given area of leaf}}{\text{Total number of epidermal cells in the same area of leaf}} \times 100$

a. $\frac{4.5}{6 + 4.5} \times 100$

%SI = 42.88 %

b. $\frac{2.8}{3 + 2.8} \times 100$

%SI = 48.28 %

c. $\frac{5.0}{7 + 5} \times 100$

%SI = 41.67 %

d. $\frac{4.3}{6 + 4.3} \times 100$

$\%SI = 41.75 \%$

3. Vein-islet and Veinlet

a. Vein-islet = $6 + 7.8 + 7 + 8 : 6 - \underline{7.4} - 8$

b. Veinlet = $8 + 9.4 + 10.4 + 11 : 8 - \underline{9.9} - 11$

Appendix C

a. Determination of total ash value of *Microtrichia perotiti*

Description	1	2	3
Weight of crucible(g)	56.13	54.23	57.20
Weight of plant material used(g)	3.00	3.00	3.00
Weight of crucible + powder(g)	59.13	57.23	60.20
Weight of crucible + ash(g)	56.74	57.82	60.82
Weight of ash(g)	0.61	0.60	0.62
Ash value (%)	20.33	20.00	20.67
Average mean total ash value (%)	20.33 %		
Standard error of mean	20.33 ± 0.02		

Sample calculation:

$$\begin{aligned}
 \% \text{ total ash value} &= \frac{\text{final weight of crucible + ash}}{\text{Weight of ash}} \times 100 \\
 &= \frac{0.61}{3.00} \times 100 \\
 &= \underline{\underline{20.33 \% \text{ w/w}}}
 \end{aligned}$$

b. Determination of Acid insoluble ash of *Microtrichia perotitii*

Description	1	2	3
Weight of crucible(g)	52.21	49.57	51.40
Weight of plant material used(g)	3.00	3.00	3.00
Weight of crucible and acid-insoluble ash(g)	52.29	49.65	51.48
Weight of acid-insoluble ash(g)	0.08	0.08	0.08
Acid insoluble ash(%)	2.73	2.73	2.73
<hr/>			
Average mean ash value (%)	2.73 %		
Standard error of mean	2.73 ± 0.13		
<hr/>			

Sample calculation:

$$\begin{aligned}
 \% \text{ Acid insoluble} &= \frac{\text{weight of acid insoluble ash}}{\text{Initial weight of drug}} \times 100 \\
 &= \frac{0.08}{3.00} \times 100 \\
 &= \underline{\underline{2.73 \% \text{ w/w}}}
 \end{aligned}$$

c. Determination of water soluble ash of *Microtrichia perotitii*

Description	1	2	3
Weight of crucible(g)	52.210	49.570	51.400
Weight of plant material used(g)	3.000	3.000	3.000
Weight of crucible and ash(g)	52.290	49.650	51.480
Weight of ash (g)	0.610	0.610	0.610
Weight of water insoluble ash(g)	0.365	0.366	0.366
Weight of water soluble ash (g)	0.245	0.244	0.244
Water soluble ash(%)	8.17	8.15	8.19
Average mean water soluble ash (%)	8.18 %		
Standard error of mean	8.18 ±0.30		

Sample calculation:

$$\begin{aligned}
 \% \text{ water soluble ash} &= \frac{\text{weight of total ash} - \text{weight of insoluble ash}}{\text{Initial weight of drug}} \times 100 \\
 &= \frac{0.610 - 0.365}{3.00} \times 100 \\
 &= \underline{\underline{8.17 \% \text{ w/w}}}
 \end{aligned}$$

d. Determination of water soluble extractive values for *Microtrichia perotitii*

Description	1	2	3
Weight of dish(g)	56.13	50.43	57.20
Weight of plant material used(g)	3.00	3.00	3.00
Weight of crucible and content after heating(g)	57.36	51.65	58.43
Weight of extractible content(g)	1.23	1.22	1.23
Water extractive value (%)	41.00	40.66	41.00
Average mean of extractive value (%)	40.90 %		
Standard error of mean	40.90 ± 1.73		

Sample calculation:

$$\begin{aligned}
 \% \text{ total ash value} &= \frac{\text{Wt of dish \& content after heating} - \text{constant wt of dish}}{\text{Initial wt of drug}} \times 100 \\
 &= \frac{(57.36 - 56.14)}{3.00} \times 100 \\
 &= \frac{1.23}{3} \times 100 \\
 &= \underline{\underline{41.00 \% w/w}}
 \end{aligned}$$

e.Determination of Alcohol soluble extractive values for *Microtrichia perotitii*

Description	1	2	3
Weight of dish(g)	50.45	52.33	57.20
Weight of plant material used(g)	3.00	3.00	3.00
Weight of crucible and content after heating(g)	51.22	53.08	60.20
Weight of extractible content(g)	0.77	0.75	0.77
Water extractive value (%)	25.67	25.00	25.67
Average mean of extractive value (%)	25.45 %		
Standard deviation (n=3)	25.45 ± 1.24		

Sample calculation:

$$\begin{aligned}
 \% \text{ total ash value} &= \frac{\text{Wt of dish\& content after heating} - \text{constant Wt of dish}}{\text{Initial wt of drug}} \times 100 \\
 &= \frac{0.77}{3.00} \times 100 \\
 &= \underline{\underline{25.67 \% \text{ w/w}}}
 \end{aligned}$$

f.Determination of moisture content of the leaves of *Microtrichia perotitii*

Description	1	2	3
Initial weight of crucible(g)	56.13	54.33	56.43
Weight of plant material used (g)	3.00	3.00	3.00
Initial wt of crucible + powder(g) (before heating)	59.13	57.33	59.43
Weight of crucible + powder after heating(g) 1 st 30mins, 2 nd 30 mins and 3 rd 30 mins respectively)	58.75	57.71	59.81
Loss in weight(g)	0.38	0.38	0.38
Loss in weight(%)	12.66	12.66	12.66
Average moisture content(%)	12.67		
Standard error of mean	12.67 ± 0.62		

Sample calculation:

$$\% \text{ loss on drying} = \frac{\text{wt of crucible + powder (before heating)} - \text{wt of crucible + powder (after heating)}}{3} \times 100$$

$$\begin{aligned} \% \text{ loss on drying} &= [59.13 - 58.75/3] \times 100 \\ &= 12.67 \% \text{ w/w} \end{aligned}$$

Appendix D

Percentage yields of extracted materials from *Microtrichia perotitii*.

	Aqueous extraction (MPAE)	Methanol extraction (MPME)
Weight of container (g) W_1	65	71
Weight of extract and container (W_2)	85	89
Weight of dried sample used (g) W_0	300	300
Weight of extract (g)	20	18
Yield (%)	6.67	6.00

Sample calculation:

$$\begin{aligned}
 \text{Yield (\%)} &= \frac{W_2 - W_1}{W_0} \times 100 \\
 &= \frac{85 - 65}{300} \times 100 \\
 &= \frac{20}{300} \times 100 \\
 &= \mathbf{6.67}
 \end{aligned}$$

ii. soluble fractions

a. aqueous extract

fraction	ether	aqueous	n-butanol (C)	n-butanol (D)	Hcl
W ₁ (g)	20	20	32	23	21
W ₂ (g)	22.11	23.01	33.10	24.03	21.07
W ₀ (g)	10	10	10	10	10
Wt of extract (g)	2.11	3.01	1.10	1.03	0.07
Yield (%)	21.1	30.1	11.0	10.3	0.7

Key: W₁ = Weight of container

W₂ = Weight of extract and container

W₀ = Weight of dried sample used

Sample calculation:

$$\text{Yield (\%)} = \frac{W_2 - W_1}{W_0} \times 100$$

$$= \frac{22.11 - 20}{300} \times 100$$

$$= \frac{2.11}{10} \times 100$$

$$= 21.1 \%$$

a. methanol extract

fraction	ether	aqueous	n-butanol (C)	n-butanol (D)	Hcl
W ₁ (g)	23	21	34	25	21
W ₂ (g)	25.08	25.73	35.05	25.09	21.05
W ₀ (g)	10	10	10	10	10
Wt of extract (g)	2.08	2.72	1.05	0.09	0.05
Yield (%)	20.8	27.2	10.5	0.9	0.5

Key: W₁ = Weight of container; W₂ = Weight of extract and container
W₀ = Weight of dried sample used

Sample calculation:

$$\begin{aligned}\text{Yield (\%)} &= \frac{W_2 - W_1}{W_0} \times 100 \\ &= \frac{25.08 - 23}{10} \times 100 \\ &= \frac{2.08}{10} \times 100 \\ &= 20.8 \%\end{aligned}$$

Appendix E

Median lethal dose (LD₅₀) OF *Microtrichia perotitii* leaves extract

1 st Phase			
s/n	Groups (wt)	Dose(mg kg ⁻¹)	No of deaths/ no of survivals
	Wt of rats	1000 mgkg ⁻¹	
1.	19	"	2/3
2.	20	"	
3.	20	"	
4.	21	100 mgkg ⁻¹	
5.	20	"	0/3
6.	18	"	
7.	16	10 mg/kg ⁻¹	
8.	22	"	0/3
9.	18	"	
2 nd Phase			
1.	18	1600 mg/kg ⁻¹	1/1
2.	18	800 mgkg ⁻¹	0/1
3.	18	400 mgkg ⁻¹	0/1
4.	16	200 mgkg ⁻¹	0/1

$$LD_{50} = \sqrt{(D_0 - D_{100})}$$

$$\begin{aligned} LD_{50} &= \sqrt{(\text{highest dose that gave no mortality} \times \text{lowest dose that produced mortality})} \\ &= \sqrt{(1600 \times 800)} \\ &= \sqrt{(1280,000)} \\ &= 1131.37 \text{ mgkg}^{-1} \end{aligned}$$

Appendix F

Analgesic activities of *Microtrichia perotitii*

i. Crude aqueous extract (MPAE)

Treatment mgkg ⁻¹	Number of writhings	Mean ± SEM
Normal saline	14, 15, 23, 28, 30, 28	22.2 ± 2.7
100	4, 3, 2, 2, 3, 4	3.00 ± 0.4
50	5, 5, 6, 9, 5, 8	6.33 ± 0.72
25	10, 12, 9, 4, 15, 7	9.50 ± 1.56
Proxicam (10)	9, 8, 7, 6, 12, 6	8.40 ± 1.0
Number of animals = 6		

ii. Crude methanol extract (MPME)

Treatment mgkg ⁻¹	Number of writhings	Mean ± SEM
Normal saline	14, 15, 23, 28, 30, 28	23.00 ± 2.85
100	13, 0, 0, 0, 0, 0	2.17 ± 2.20
50	0, 0, 0, 0, 0, 0	0.00 ± 0.00
25	9, 7, 19, 8, 9, 17	11.50 ± 2.09
Proxicam	9, 8, 7, 6, 12, 6	8.40 ± 1.0
Number of animals = 6		

iii. Soluble fractions

Portion	Treatment (mgk ⁻¹)	Number of writhings	Mean ± SEM
A(ether)	100	19, 20, 25, 11, 10, 26	18.5 ± 2.77
	50	25, 15, 17, 9, 11, 18	15.8 ± 2.07
	25	29, 11, 21, 12, 19, 30	20.3 ± 3.30
B(aqueous)	100	7,26, 20, 7, 13,1 5	14.7 ± 3.04
	50	16, 6, 12, 6, 10, 16	11.0 ± 1.84
	25	4, 12,5, 12, 7,12	8.7 ± 1.54
C(BuOH)	100	11, 5, 8, 5, 9, 6	6.50 ± 0.91
	50	4, 7, 13, 5, 8, 7	7.33 ± 1.28
	25	9, 5, 6,7,12,11	8.33 ± 1.15
D(BuOH)	100	7, 5, 18, 7, 4, 5	7.67 ± 2,12
	50	17, 12,7, 11, 7, 21	12.50 ± 2.28
	25	12, 7, 7, 3, 4, 2	5.83 ± 1.49
Normal saline (-ve control)		19, 11, 18, 27, 19, 19	18.83 ± 2.07
Proxicam (+ve control) 10		8, 13, 8, 21, 8, 4	10.33 ± 2.43
Number of animals = 6			

Sample calculation:

$$\begin{aligned}
 \% \text{ inhibition} &= \frac{\text{mean no of writhings (control,N/S)} - \text{mean no of writhings treated}}{\text{mean number of writhings (control)}} \times 100 \\
 &= \frac{23.0 - 3.0}{22.2} \times 100 \\
 &= \mathbf{86.96 \%}
 \end{aligned}$$

iv. Hotplate Analgesia

a. crude aqueous crude extract (MPAE)

Treatment (mgkg ⁻¹)	Time (minutes)			
	0	30	60	90
100	1.00	0.62	0.50	1.58
	1.00	0.20	0.50	1.85
	1.00	1.00	1.05	1.50
	1.00	1.65	1.65	0.95
	1.00	1.05	1.08	0.79
	1.00	3.05	1.09	1.04
Mean + SEM	1.50	1.00	1.09	1.16
	± 0.16	±0.23	±0.15	±0.17
50	1.00	0.65	1.21	1.54
	0.99	0.90	0.96	1.26
	1.00	1.95	0.73	0.91
	1.09	0.65	1.50	1.37
	2.00	1.37	1.22	1.27
	2.00	2.12	1.99	1.72
Mean ± SEM	1.35	1.11	1.27	1.35
	±0.21	±0.23	±0.18	±0.11
25	2.00	1.00	2.45	2.03
	1.00	2.00	2.67	2.21
	2.00	2.00	1.96	1.12

	2.00	1.00	2.02	1.13
	2.00	2.00	1.05	1.67
	1.00	1.00	1.69	1.94
Mean ± SEM	1.67	1.50	1.97	1.68
	±0.21	±0.22	±0.23	±0.19
Normal saline	2.05	2.20	1.84	1.24
(-ve control)	1.65	1.03	0.92	1.10
(10mgkg ⁻¹)	1.45	1.30	2.01	1.32
	1.00	0.91	1.25	1.43
	1.45	1.68	1.90	1.13
	1.05	0.99	1.14	1.14
Mean±SEM	1.44	1.35	1.51	1.23
	±0.16	±0.21	±0.19	±0.52
Morphine	2.00	1.40	1.65	1.89
Sulphate	1.20	1.20	1.20	1.42
(4 mgkg ⁻¹)	1.00	1.02	1.34	1.34
(+ve control)	1.40	1.00	1.40	1.05
	1.05	1.14	1.06	1.20
	1.35	1.06	1.06	1.20
Mean ±SEM	1.33	1.44	1.29	1.35
	±0.15	±0.06	±0.09	±0.12

b. Crude methanol extract (MPME)

Treatment (mgkg ⁻¹)	Time(minutes)			
	0	30	60	90
100	1.00	0.62	0.84	1.10
	0.90	0.95	1.10	1.25
	1.00	1.05	1.20	1.20
	1.05	1.02	1.25	1.00
	0.85	0.88	1.00	1.02
	0.75	1.10	1.02	1.25
	Mean + SEM	1.33	1.00	1.07
	± 0.15	±0.03	±0.06	±0.06
50	1.30	1.05	1.35	1.26
	1.20	1.20	1.20	1.20
	1.45	1.30	1.38	1.60
	1.10	1.35	1.30	1.10
	1.40	1.20	1.10	1.35
	1.20	1.10	1.20	1.60
	Mean ± SEM	1.27	1.20	1.26
	±0.54	±0.05	±0.43	±0.09
25	2.00	1.45	1.89	2.00
	1.50	2.00	1.35	1.20
	1.50	1.35	1.05	1.06
	1.89	1.00	1.08	1.32
	1.08	1.10	1.35	1.35
	1.08	1.10	1.20	1.30
	Mean ± SEM	1.50	1.33	1.32
	±0.16	±0.15	±0.13	±0.13
Normal saline	2.05	2.20	1.84	1.24
(-ve control)	1.65	1.03	0.92	1.10
(10mgkg ⁻¹)	1.45	1.30	2.01	1.32

	1.00	0.91	1.25	1.43
	1.45	1.68	1.90	1.13
	1.05	0.99	1.14	1.14
Mean±SEM	1.44	1.35	1.51	1.23
	±0.16	±0.21	±0.19	±0.52
Morphine	2.00	1.40	1.65	1.89
Sulphate	1.20	1.20	1.20	1.42
(4 mgkg ⁻¹)	1.00	1.02	1.34	1.34
(+ve control)	1.40	1.00	1.40	1.05
	1.05	1.14	1.06	1.20
	1.35	1.06	1.06	1.20
Mean ±SEM	1.33	1.44	1.29	1.35
	±0.15	±0.06	±0.09	±0.12

Sample calculation:

$$\begin{aligned}
 \% \text{ Inhibition} &= \frac{\text{post-treatment latency} - \text{pre-treatment latency}}{\text{cut-off time} - \text{pre-treatment latency}} \times 100 \\
 &= \frac{1.56 - 1.44}{15 - 1.44} \times 100 \\
 &= \frac{0.06}{13.56} \times 100 \\
 &= \mathbf{0.44 \%}
 \end{aligned}$$

Appendix G

Anti-inflammatory activities of *Microtrichia perotitii* in formalin induced Oedema in Rats

i. Crude aqueous extract (MPAE)

Treatment(mg ^{kg⁻¹})	non-inflamed limb	inflamed limb	wt of Oedema	mean±SEM
25	1.34	0.84	0.50	0.50±0.03
	1.38	0.81	0.57	
	1.42	0.95	0.47	
	1.60	0.95	0.65	
	1.30	0.82	0.48	
	1.18	0.83	0.35	
50	1.46	1.01	0.45	0.09±0.02
	1.71	1.25	0.46	
	1.68	1.13	0.56	
	1.81	0.92	0.89	
	1.66	1.00	0.66	
	1.85	1.04	0.81	
100	1.72	1.35	0.30	0.49±0.03
	1.42	0.82	0.50	
	1.56	1.14	0.42	
	1.76	1.16	0.60	
	1.76	1.23	0.53	
	1.56	1.06	0.50	
Normal saline(+ve control)	1.90	0.80	1.10	1.25±0.04
	2.20	1.03	1.17	
	2.43	1.13	1.30	
	2.41	1.02	1.39	
	2.20	0.94	1.26	
	2.12	0.86	1.26	
Feldene(-ve control)	1.66	1.00	0.66	0.66±0.07
	1.90	1.13	0.77	
	1.92	1.12	0.80	
	1.42	1.03	0.39	
	1.76	1.23	0.53	
	1.89	0.98	0.81	

ii. crude methanol extract(MPME)

Treatment(mg ^{kg⁻¹})	non-inflamed limb	inflamed limb	wt of Oedema	mean±SEM
25	1.20	0.90	0.30	0.33±0.01
	1.25	0.92	0.33	
	1.25	0.92	0.33	
	1.18	0.88	0.30	
	1.17	0.80	0.37	
	1.18	0.79	0.39	
50	1.07	1.19	0.04	0.28±0.05
	0.86	0.26	0.07	
	1.13	0.33	0.11	
	1.04	0.22	0.05	
	1.31	0.27	0.07	
	1.20	0.45	0.20	
100	1.30	0.90	0.40	1.25±0.04
	1.03	0.90	0.13	
	1.27	0.87	0.40	
	1.11	0.92	0.19	
	1.06	0.87	0.19	
	1.27	0.92	0.35	
Normal saline(-ve control)	1.90	0.80	1.10	0.66±0.07
	2.20	1.03	1.17	
	2.43	1.13	1.30	
	2.41	1.02	1.39	
	2.20	0.94	1.26	
	2.12	0.86	1.26	
Feldene(+ve control)	1.66	1.00	0.66	0.66±0.07
	1.90	1.13	0.77	
	1.92	1.12	0.80	
	1.42	1.03	0.39	
	1.76	1.23	0.53	
	1.89	0.98	0.81	

Sample calculation:

$$\begin{aligned}
 \% \text{ Inhibition} &= \frac{\text{mean paw diameter}(-\text{ve control}) - \text{mean paw diameter}(\text{treated})}{\text{Mean paw diameter}(-\text{ve control})} \times 100 \\
 &= \frac{(1.25 - 0.49)}{1.25} \times 100 \\
 &= \frac{0.76}{1.25} \times 100 = \mathbf{60.80 \%}
 \end{aligned}$$

iii. anti-inflammatory activities of soluble fractions of crude *Microtrichia perotitii*

treatment(cm) in hours (mgkg ⁻¹)	paw diameter					
	0	1	2	3	4	5
Normal saline	0.21	0.17	0.17	0.16	0.15	0.16
(-ve control)	0.22	0.18	0.15	0.18	0.15	0.12
	0.22	0.22	0.23	0.20	0.18	0.1
	0.14	0.14	0.15	0.16	0.10	0.12
	0.23	0.22	0.16	0.16	0.11	0.07
Mean ± SEM	0.20	0.18	0.14	0.17	0.14	0.15
	±0.02	±0.13	±0.01	±0.11	±0.02	±0.02
A(ether) 25	0.16	0.14	0.14	0.15	0.16	0.16
	0.14	0.14	0.17	0.15	0.16	0.15
	0.15	0.16	0.17	0.14	0.15	0.16
	0.14	0.13	0.14	0.15	0.16	0.13
	0.15	0.15	0.16	0.16	0.15	0.15
Mean ± SEM	0.16	0.16	0.16	0.14	0.14	0.15
	±0.01	±0.01	±0.02	±0.02	±0.01	±0.02
50	0.19	0.18	0.20	0.19	0.19	0.19
	0.18	0.18	0.19	0.18	0.19	0.20
	0.17	0.17	0.18	0.17	0.16	0.14
	0.18	0.18	0.19	0.15	0.16	0.17
	0.19	0.18	0.16	0.16	0.15	0.15
Mean ± SEM	0.19	0.18	0.18	0.15	0.15	0.16
	±0.02	±0.02	±0.01	±0.01	±0.02	±0.01
100	0.20	0.20	0.18	0.15	0.16	0.17
	0.19	0.19	0.18	0.20	0.19	0.17
	0.19	0.20	0.19	0.19	0.18	0.20
	0.19	0.19	0.20	0.19	0.19	0.19
	0.19	0.20	0.19	0.19	0.17	0.17
Mean ±SEM	0.23	0.20	0.18	0.16	0.16	0.16

		±0.02	±0.02	±0.03	±0.02	±0.01	±0.02
B(aqueous) 25		0.20	0.20	0.19	0.19	0.20	0.19
		0.20	0.20	0.19	0.17	0.18	0.19
		0.23	0.23	0.19	0.20	0.18	0.18
		0.19	0.19	0.23	0.22	0.22	0.21
		0.22	0.22	0.21	0.21	0.21	0.22
Mean±SEM		0.19	0.17	0.15	0.19	0.19	0.21
		±0.01	±0.02	±0.01	±0.01	±0.01	±0.02
	50	0.22	0.23	0.21	0.19	0.18	0.21
		0.19	0.19	0.18	0.23	0.23	0.21
		0.19	0.18	0.18	0.20	0.20	0.19
		0.20	0.21	0.19	0.19	0.21	0.19
		0.23	0.23	0.22	0.21	0.21	0.18
Mean±SEM		0.20	0.17	0.20	0.20	0.19	0.18
		±0.01	±0.01	±0.01	±0.01	±0.01	±0.01
	100	0.22	0.22	0.21	0.22	0.19	0.19
		0.19	0.17	0.18	0.19	0.18	0.18
		0.17	0.18	0.19	0.19	0.20	0.18
		0.22	0.22	0.17	0.16	0.19	0.19
		0.18	0.18	0.19	0.16	0.19	0.20
Mean±SEM		0.17	0.17	0.16	0.16	0.17	0.21
		±0.01	±0.02	±0.01	±0.01	±0.01	±0.01
C(BuOH) 25		0.17	0.12	0.13	0.12	0.12	0.13
		0.18	0.14	0.13	0.13	0.11	0.09
		0.22	0.20	0.16	0.17	0.16	0.13
		0.15	0.16	0.14	0.13	0.11	0.09
		0.14	0.13	0.12	0.13	0.10	0.09
Mean±SEM		0.17	0.17	0.14	0.13	0.12	0.11
		±0.01	±0.01	±0.01	±0.01	±0.01	±0.01
	50	0.17	0.15	0.16	0.10	0.09	0.10
		0.11	0.10	0.12	0.11	0.10	0.08

		0.10	0.15	0.15	0.12	0.12	0.10
		0.11	0.10	0.09	0.07	0.05	0.04
		0.17	0.16	0.12	0.11	0.10	0.10
	Mean±SEM	0.15	0.15	0.14	0.13	0.11	0.09
		±0.02	±0.03	±0.02	±0.03	±0.02	±0.03
	100	0.12	0.14	0.16	0.14	0.18	0.17
		0.16	0.16	0.13	0.13	0.11	0.10
		0.13	0.17	0.16	0.17	0.17	0.16
		0.16	0.11	0.11	0.10	0.12	0.12
		0.08	0.08	0.07	0.06	0.05	0.05
	Mean±SEM	0.13	0.13	0.12	0.12	0.11	0.10
		±0.01	±0.02	± 0.03	±0.02	±0.02	±0.02
	D(BuOH) 25	0.16	0.15	0.15	0.14	0.11	0.10
		0.15	0.17	0.17	0.11	0.12	0.09
		0.15	0.15	0.16	0.12	0.10	0.08
		0.14	0.15	0.14	0.13	0.09	0.08
		0.13	0.11	0.15	0.15	0.13	0.11
	Mean±SEM	0.15	0.15	0.14	0.11	0.10	0.13
		±0.01	±0.03	±0.01	±0.01	±0.01	±0.01
	50	0.12	0.12	0.13	0.13	0.11	0.10
		0.16	0.16	0.15	0.17	0.16	0.14
		0.17	0.18	0.16	0.16	0.14	0.12
		0.18	0.19	0.14	0.12	0.11	0.12
		0.17	0.15	0.14	0.14	0.13	0.11
	Mean±SEM	0.19	0.18	0.19	0.12	0.13	0.14
		±0.02	±0.02	±0.01	±0.01	±0.01	±0.01
	100	0.16	0.14	0.14	0.13	0.12	0.11
		0.17	0.16	0.15	0.16	0.14	0.13
		0.17	0.15	0.14	0.15	0.13	0.14
		0.15	0.15	0.14	0.13	0.12	0.16
		0.16	0.16	0.15	0.14	0.13	0.11

Mean±SEM	0.23	0.18	0.18	0.14	0.14	0.15
	±0.03	±0.02	±0.02	±0.01	±0.02	±0.02
Ketoprofene	0.13	0.12	0.12	0.11	0.09	0.07
	0.12	0.13	0.14	0.10	0.09	0.05
	0.14	0.13	0.12	0.09	0.11	0.06
	0.14	0.11	0.10	0.12	0.09	0.10
	0.12	0.11	0.12	0.10	0.11	0.09
	0.16	0.16	0.17	0.12	0.16	0.14
	±0.02	±0.02	±0.01	±0.01	±0.01	±0.01

Sample calculation:

$$\begin{aligned}
 \% \text{ Inhibition} &= \frac{\text{mean paw diameter}(-\text{ve control}) - \text{mean paw diameter}(\text{treated})}{\text{Mean paw diameter}(-\text{ve control})} \times 100 \\
 &= \frac{(0.16 - 0.13)}{0.16} \times 100 \\
 &= \frac{0.03}{0.16} \times 100 \\
 &= \mathbf{18.75 \%}
 \end{aligned}$$

Appendix H

Calibration of Eyepiece Micrometer

Under x 40 objective, the 40th small eyepiece micrometer division coincided with the 10th small stage micrometer division. The calibration factor was calculated thus:

30 small eyepiece micrometer divisions = 10 small stage micrometer division

Since, 1 small stage micrometer division = 10 μ m

\therefore 30 small eyepiece micrometer divisions = 100 μ m (i.e 10 x 10)

\therefore 1 small eyepiece micrometer division = $\frac{100\mu\text{m}}{30}$

\therefore Calibration factor = 2.7