PHARMACOGNOSTIC AND ANTI-INFLAMMATORY STUDIES ON THE LEAF OF *LUDWIGIA ABYSSINICA* RICH. (ONAGRACEAE)

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APRIL, 2017

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(ONAGRACEAE)

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P13PHPD8007

A DISSERTATION SUBMITTED TO THE SCHOOL OF POST-GRADUATE STUDIES, AHMADU BELLO UNIVERSITY, ZARIA IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE AWARD OF A

MASTER'S DEGREE IN PHARMACOGNOSY

DEPARTMENT OF PHARMACOGNOSY AND DRUG DEVELOPMENT

FACULTY OF PHARMACEUTICAL SCIENCES

AHMADU BELLO UNIVERSITY, ZARIA

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APRIL, 2017

DECLARATION

I declare that the work in this dissertation entitled Pharmacognostic and Anti-inflammatory Studies on Leaf of *Ludwigia abyssinica* Rich (Onagraceae) has been carried out by me in the Department of Pharmacognosy and Drug Development, Faculty of Pharmaceutical Sciences, Ahmadu Bello University Zaria, under the supervision of Dr. G. Ibrahim, and Dr. U.H. Danmalam. The information derived from literature has been duly acknowledged in the text and a list of references provided. No part of this dissertation has been previously presented for another higher degree or diploma at this or any other institution.

Aisha Barau, Ibrahim _____ _____

Signature **Date**

CERTIFICATION

This dissertation entitled "PHARMACOGNOSTIC AND ANTI-INFLAMMATORY STUDIES ON THE LEAVES OF *LUDWIGIA ABYSSINICA* RICH (ONAGRACEAE)" by Aisha Barau IBRAHIM, meets the regulations governing the award of the degree of Master of Science in Pharmacognosy of the Ahmadu Bello University, Zaria, and is approved for its contribution to knowledge and literary presentation.

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DEDICATION

This work is dedicated to my lovely parents, may Allah (SWT) reward them and continue to bless them, ameen.

ACKNOWLEDGEMENT

All praise is due to Allah (SWT) the Lord of the Universe for granting me the opportunity to carry out this research to completion. My sincere and profound gratitude goes to my supervisors Dr. G. Ibrahim and Dr. U. H. Danmalam for their efficient supervision and sound academic contributions to the success of this research work despite their busy and numerous commitments.

My sincere appreciation and profound gratitude to my parents Alhaji Ibrahim Barau and Hajiya Safiya Sani for their love, prayers, care, encouragement and financial support. I wish to acknowledge the support of all members of my family especially my dearest sister Hannatu Ibrahim for their support in one way or the other during the course of my studies.

My sincere gratitude also goes to all the members of staff (academic and non-academic) of the Department of Pharmacognosy and Drug Development who have assisted in teaching and guiding me with the laboratory work and those contributed one way or the other during the course of my programme.

I would like to recognize the assistance of Mrs. Aisha Shehu, Mal. Abubakar and Mal. Aliyu all in Pharmacology and Therapeutics Department Ahmadu Bello University Zaria, in carrying out the Anti-inflammatory studies during the course of my work.

My special thanks go to my lovely family especially my son (Nasar) for their patience, support and encouragement during the course of this work.

My special appreciation to all my friends and course mates for their prayers, love, advices and encouragement, Nuhu Aliyu, Zakir Abdulhamid, Nafisa Salihu, Pharm Sani, Khadijah Imam, Pharm. Tabitha, Pharm. Nafisa Y., Mal. Bashir, Zainab M.Hassan, and tothose that are not mentioned here due to limited space but, their efforts are duly acknowledged. I say thank you all.

ABSTRACT

Ludwigia abyssinica Rich (Onagraceae) is a plant native to South America but now widely distributed in Africa. It has been exploited for its medicinal and economic importance. It is traditionally used for the treatment of a number of ailments such as cough, skin diseases, sores, diarrhoea, rheumatism, constipation, liver diseases and intestinal worm infestations. Aqueous decoction of the leaves is taken orally for its analgesic effect in the treatment of generalized pain in some parts of Africa, and economically, the cooked leaves provide a black liquid that is used for dyeing straw and fibres. *L. abyssinica* is considered s an ornamental plant because of its showy flowers which are yellow in colour. Evaluation of the fresh, anatomical sections, powdered and extract of the leaves of *L. abyssinica* were carried out to determine its micromorphological, chemomicroscopic, some physicochemical parameters. The extracts were obtained successively from hexane, ethylacetate and methanol using Soxhlet apparatus, to determine the phytochemical profiles, acute toxicity, and anti-inflammatory properties. Microscopical evaluation revealed the presence of anomocytic type of stomata in both adaxial (upper) and abaxial (lower) epidermis with subsidiary cells. The physical constants values were: moisture content (7.00%), total ash value (7.80%), water soluble ash (4.67%), acid insoluble ash (2.33%), ethanol extractive value (17.00%) and water extractive value (19.7%). Phytochemical analysis of the leaf extract (hexane, ethylacetate, and methanol) revealed the presence of alkaloids, tannins, flavonoids, cardiac glycosides, saponins (steroids / triterpenes) and carbohydrate. Thin layer chromatographic analysis visualized with specific reagents confirmed the presence of steroids/triterpenes, flavonoids and other phenolic compounds. The median lethal dose (LD_{50}) via oral route of the extracts was found to be greater than 5000 mg/kg when administered orally in rats. The extracts of *L. abyssinica* leaf demonstrated significant decrease ($p<0.05$) against carrageenan

induced paw oedema in rats, reaching its peak at the 3rd hour and the highest inhibitory value of (28.7, 29.1, 26.7) was shown at the 5th hour for hexane, ethylacetate and methanol extracts at a dose of 1500mg/kg which was higher than the standard, piroxicam (10 mg/kg) having (25.9%). Hexane extract (500-1500 mg/kg) was observed to have the highest activity compared to the other extracts. The results provided basic pharmacognostic standards for the plant and scientific basis for traditional uses of leaves in the treatment of inflammation.

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ACRONYMS AND ABBREVIATIONS

B.H. P: British Herbal Pharmacopoeia

Fig: Figure

FAA: Formalin Acetic acid Alcohol

g: Gram

b.wBody weight

G.A.A: Glacial Acetic Acid

HCl: Hydrochloric acid

H_2SO_4: Sulphuric acid

LD_{50}: Median Lethal Dose

kg: Kilogram

ml: Milliliter

mm: Millimeter

mg: Milligram

TLC: Thin Layer Chromatography

UV: Ultraviolet Light

Vol.: Volume

w/w: Weight per Weight

W.H.O: World Health Organization

COX: Cyclo-oxygenase

%: Percentage

CHAPTER ONE

1.0 INTRODUCTION

Traditional medicine includes diverse health practices, approaches, knowledge and beliefs incorporating plant, animal and mineral based medicines, spiritual therapies, manual techniques and exercises applied singularly or in combination to maintain well-being, as well as to treat, diagnose or prevent illness (WHO, 2002). It is the sum total of all knowledge and practices, whether explicable or not, used in diagnosis, prevention and elimination of physical, mental or social imbalance and relying exclusively on practical experience and observation handed down from generation to generation whether verbally or in writing (WHO, 2002).

Plant materials are of wide use in traditional systems of medicine in several communities of the developing world, and may be the only resources available for the treatment of different infections. In some Asian and African countries, 80% of the population depends on traditional medicine for primary healthcare and more than 100 countries have regulations for herbal medicines (Tagboto and Toronson, 2001). Medicinal plants are believed to be an important source of new chemical substances with potential therapeutic effects. Plant based traditional remedies are generally used all over the world for the treatment of diseases. In developing countries, plants are used for the treatment of all forms of diseases including both major and minor implication. They have been used by indigenous population all over the world for various therapeutic purposes (Philipson, 1998).

Traditional and folk remedies have provided humanity with important drugs in the treatment of diseases and are being increasingly subjected to scientific studies. The family of anti-

inflammatory drugs is no exception. Inflammatory diseases such as rheumatoid arthritis, inflammatory bowel disease, multiple sclerosis and other connective tissue diseases are a major cause of morbidity (Gross, 2011).

The research into plants with alleged folkloric use as anti-inflammatory agents should therefore be viewed as a fruitful and logical research strategy in the search for new anti-inflammatory drugs. These anti-inflammatory agents contain various classes of phytoconstituents including flavonoids, alkaloids, glycosides, terpenoids, steroids and polyphenolic compounds which may be acting singly or collectively in relieving inflammation (Gupta *et al.*,2006; Muhammad *et al.*, 2011).

Anti-inflammatory agents have been traditionally evaluated by studying their effect on inflammation produced in animals by injecting foreign or noxious agents (Ghosh*et al*., 2011). Though there are standard drugs like aspirin, indomethacin, phenylbutazone, etc., these drugs are not entirely free from side effects (Borris, 1996). These synthetic drugs reported to be used for the treatments of inflammatory disorders are of least interest due to their potential side effects and serious adverse effects in humans and animals (Green *et al.*, 2004; Rocca *et al.*, 2005; Abatan *et al.*, 2006; Freidman and Kaiser, 2007). In the last few decades, alternative anti-inflammatory and analgesic agents have regained their popularity in the treatment against several human ailments such as inflammation (Bawa and Khanum, 2009; Tripathy *et al.*, 2010).

1.1 Pharmacognostic Evaluation of Medicinal Plant

Pharmacognosy is the study of medicines derived from natural sources, mainly from plants. It basically deals with standardization, authentication and study of natural drugs. Most of the research in pharmacognosy has been done in identifying controversial species of plants,

authentication of commonly used traditional medicinal plants through morphological, phytochemical and physicochemical analysis. The importance of pharmacognosy has been widely felt in recent times. Unlike taxonomic identification, pharmacognostic study includes parameters which help in identifying adulteration in dry powder form also. This is again necessary because once the plant is dried and made into powder form, it loses its morphological identity and easily prone to adulteration. Pharmacognostic studies ensure plant identity, lays down standardization parameters which will help in the detection and prevention of adulterations. Such studies will help in authentication of the plants and ensures reproducible quality of herbal products leading to guarantee in the safety and efficacy of natural products (Sumitra, 2014).

Pharmacognostic evaluation includes the macroscopic, microscopic, physico-chemical, fluorescence and phytochemical studies of whole plant parts or powdered drug. Herbal raw material shows a number of problems when quality and authentication aspects are considered.This is because of the nature of herbal parts, ingredients and different phytochemicals present in crude drugs (WHO, 2011). To ensure quality of herbal medicines, proper control of starting raw material is very important. The physico-chemical evaluation includes qualitative and quantitative tests, assays and instrumentation analysis. Qualitative and quantitative chemical tests include the presence or absence, quantity, number, values and identification of various phtyochemicals like flavonoids, glycosides, saponins, alkaloids etc (Brain and Turner, 1975; Harborne, 1992; Evans, 2002).

Standardization of crude drug is a system that ensures a predefined amount of quantity, quality, and therapeutic effect of ingredients in each drug (Zafar *et al.,* 2005). Standardization of herbal formulations is essential in order to assess the quality of drugs, based on the concentration of their active principles, and *in-vitro*, *in-vivo* parameters. The

quality assessment of herbal formulations is of paramount importance in order to justify their use acceptability in modern system of medicine (Satheesh *et al.,* 2011).

1.2 Plants as Sources of Anti-inflammatory Agents

Owing to the numerous adverse effects associated with the use of synthetic drugs, attention is being drawn on exploring anti-inflammatory agents of plant origin which are considered to be equally effective with minimal or no side effects. Plants have contributed to the development of some anti-inflammatory drugs such as *Salix alba*(white willow tree) from which salicin was obtained and it is believed to be less toxic than aspirin (Shehu *et al*., 2016) Plant secondary metabolites have provided an important source of drugs since time inmemorial and now part of the practical drugs used are obtained from natural sources and many of these herbal constituents are being prescribed widely for the treatment of inflammatory condition (Su, 2011).

Pharmacological value of phenolic compounds have been reported, with reports of some having anti-inflammatory properties. Different types of phenolic compounds such as flavonoids, condensed tannins, have been reported to inhibit some molecular targets ofproinflammatory mediators in inflammatory responses and inhibit particular enzymes such as cyclooxygenase -2- (COX-2) enzymes (Mona *et al.*, 2014).

Some flavonoids, such as quercetin have been reported to have the ability to block both the cyclooxygenase and lipooxygenase pathways at relatively high concentrations. Other metabolites from plants reported to show potential anti-inflammatory activities are alkaloids, saponins, sterols, terpenoids, coumarins and essential oils and these may provide important sources of anti-inflammatory agents which may have the capacity to modulate the expression

of pro-inflammatory signals thereby assessing their capacity as anti-inflammatory agents (Mona *et al.,* 2014).

1.3 STATEMENT OF RESEARCH PROBLEMS

Pain is the main reason of visiting the emergency department in more than 50% of cases and is present in 30% of family practice visits all over the world (Hasselstorm *et al.,* 2002). Several epidemiological studies from different parts of the world have reported prevalence rates for chronic pain, ranging from 12 - 80% of population. It becomes more common as people approach death (Perquin *et al.,* 2002). Chronic pain is a general complaint in the world and more common in industrialized countries constituting major public health and socio-economic problem (Bishaw, 2007). Data from U.S.A. suggests that chronic pain is responsible for more than 150 billion dollars spent on health care and disability related costs. In Nigeria, for individual experiencing pain, the human cost is incalculable, but can only be evidenced in decreased quality of life, activity limitation, reduced functional capacity and increase financial burden arising from increased use of health services and medication (Igumbar *et al.,* 2011).

In spite of the progress made in medicinal research during the past decades, the treatment of many serious diseases is still problematic. Chronic inflammatory diseases remain one of the world's major health problems (Shehu *et al*., 2016). Many anti-inflammatory drugs have been developed for the treatment of inflammatory diseases. However, most of such drugs are associated with many side effects and heavy cost (Shah *et al.*, 2011). As a result, there is a great interest in the search for alternative, plant-based medicines with anti-inflammatory activity which are safer and accessible. Therefore, the drugs as alternative substitutes are required to address these problems.

1.4 JUSTIFICATION

Medicinal plants have continued to play dominant role since time immemorial for the protection, prevention and treatment of diseases. Traditional herbal therapy has the potential of contributing to a better health care system (WHO, 1998).

In various countries across tropical Africa, *L. abyssinica* has been studied without proper standardization, despite its use by rural populace as an herbal remedy for various diseased conditions (Oyedeji *et al.*, 2010).

Extracts from *L. abyssinica* plant have been used among traditional healers for the treatment of inflammatory diseases. It has been consumed traditionally as anti-rheumatic agent and many other ailments in Northern part of Nigeria (Oyedeji, 2010). It becomes extremely important to make an effort towards standardization of the plant as crude drug and also to establish scientific evidence for its traditional use as anti-inflammatory agent.

1.5 GENERAL AIM

The overall aim of the research is to set some pharmacognostic standards and provide scientific evidence for the use of *Ludwigia abyssinica* in ethnomedicine as anti-inflammatory agent.

1.5.1 Specific objectives

(i) To evaluate some pharmacognostic features of *L. abyssinica* leaves with a view to providing parameters for its proper identification and prevention of adulteration;

(ii) To develop thin layer chromatographic profile of the leaf extracts of the plant; and

(iii) To investigate the anti-inflammatory activity of the leaf extracts of the plant in animal

experimental model.

1.6 RESEARCH HYPOTHESIS

Ludwigia abyssinica has diagnostic characters that are unique and can be used in its identification and standardization. It also has anti-inflammatory activity.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 The Family: Onagraceae

Onagraceae family, also known as the willow herb family or evening primrose family of floweringplantswas named after the genus Onagra (now known as Oenothera) in 1836 by John Lindley in his second edition of 'A Natural System of Botany'. They include about 650 species of herbs, shrubs, and trees in 17 genera (Ford and Gottlieb, 2007). The family is widespread, occurring on every continent from boreal to tropical regions and includes a number of popular garden plants, such as evening primrose (*Oenothera*) and fuchsias (*Fuchsia*). Some, particularly the willow herbs *(Epilobium)* are common weeds in gardens and in the wild (Chen *et al.,* 1992; Fadoup *et al.,* 2014).

The family is characterized by flowers with usually four sepals and petals. In some genera, such as *Fuchsia*, the sepals are as brightly coloured as the petals (Ruaux, 2009).

The seeds are generally very small. In some genera, such as *Epilobium*, they have tufts of hairs and are dispersed by wind. In others, such as *Fuchsia*, the seeds develop in juicy berries dispersed by animal. The leaves are commonly opposite, but are spirally arranged in some species; in most, they are simple and lanceolate in shape. The pollen grains in many genera are loosely held together by viscin threads. Most bees cannot collect it, and only bees with specialized morphologies can effectively pollinate the flowers; nearly all bee taxa that visit the flowers are oligoleges specialized on the family Onagraceae (Ford and Gottlieb, 2007).

2.2 The Genus: *Ludwigia*

The genus *Ludwigia* comprises of about 75 species with 21 genera that are submerged or growing in ditches, shallow marshy areas, river banks and slow moving streams as annual, biennial or perennial herbs or shrubs which are cosmopolitan and pantropical in distribution (Raven 1963; Mabberley 1987). It is a native to South America but is now well distributed in Africa (Van der Burg, 2004).

The plant is widespread in Africa occurring from Guinea, Ethiopia, and Nambia, Botswana, South Africa, (Natal), also in Madagascar. The plant when grown is found abundant in Benin, Cote d'ivoire and Ghana. It's also frequently grown in Burkina faso, Chad, Mali, Nigeria and Tanzania (Akobundu and Agyakwa, 1998).

2.3 Description of*Ludwigia abyssinica*

2.3.1 Taxonomical classification

Kingdom: Plantae

Order: Myrtales

Family: Onagraceae

Genus: *Ludwigia*

Species: *L. abyssinica* (A. Rich).

*L. abyssinica*Richis a flowering plant locally called '*Allayyahun Fadama*' in Hausa, 'Ako ewuro oda' in Yoruba, and commonly known as water primrose. It is an erect annual or perennial herb (Plate I) about 1-2 m high that reproduces from seed. The stem is more or less

angled, many branched,smooth. The leaves are alternate in arrangement and lonceolate in shape, about 10-12 cm long and up to 4 cm wide. They have short triangular stipules in the axils. The leaf margins are smooth on both surfaces and have many laterally ascending nerves on the lower surface. It is made up of solitary yellow flowers that have 4-5 persistent sepals and corresponding 4-5 yellow petals. The fruit is an elongated capsule 1-2 cm long, about 1-2 mm wide, crowned by the persistent sepals. The seeds are ellipsoid, 0.5-1 mm long, and brownish in colour. Itis a common weed of lowland and the plant varies in colour from green to red (Grubben, 2004).



Plate I: ***Ludwigia abyssinica*** **in its Natural Habitat (around Yankarfe, Sabon Gari Local Government, Kaduna State)**

2.3.2 Ethno-medicinal and Economic Importance of *Ludwigia abyssinica*

It is one of the important plants with presence of phytochemical compounds used for medicinal and other purposes. Extracts of roots and leaves are used in treating rheumatism (Baerts and Lehmann, 2012). In Sudan and Congo the plant is used in traditional medicine to treat ailments such as cough, skin diseases, dysentery, flatulence, and constipation (Van der Burg, 2004). Aqueous decoction of the leaves is taken orally for its analgesic effect in the treatment of generalized pain in some parts of Africa (Oyedeji *et al.,* 2014). Leaf decoction of the plant is also used in the treatment of fungal infections by the Haya people of North-Western Tanzania (Mainen *et al.,* 2009).

The leaf sap is taken orally to prevent abortion (Van der Burg, 2004). Its roots decoction is used against diarrhoea, dysentery, flatulence and leucorrhoea (Das *et al.,* 2007). In East Africa, a root decoction of the plant is used to treat liver diseases and intestinal worm infestations in children while the leaves are eaten as cooked vegetables for nutritional values in Congo, Malawi, parts of Nigeria and Tanzania (Igoli *et al.,* 2005).

The cooked leaves and stem provide a black liquid that is used for dyeing straw and fibres. It is useful as an ornamental plant because of its showy flowers which are yellow in colour, and sold in many part of Europe (Ruaux, 2009). The seeds are rich in oil (Burkill, 1997).

In Nigeria *L. abyssinica* is used traditionally for the treatment of various skin infections, gastrointestinal, wound and bone joint disorders (Grubben and Denton, 2004). Stem and root extracts of the plant are taken orally for the treatment of snake bites/poisoning by ethnic groups from Southern Ethiopia (Baerts and Lehmann, 2012). Leaf infusion of the plant is used in treatment of inflammatory disorders (Oyedeji, 2010). The whole or parts of the plant

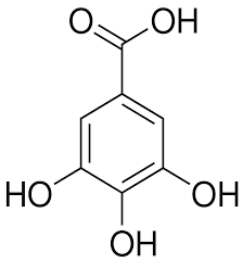
are also consumed for nutritional purposes (Van der Burg , 2012; Igoli *et al.,* 2005; Abulude, 2005).

2.3.3 Chemical Constituents Reported from *Ludwigia abyssinica*

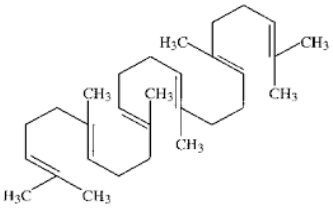
Literatures reports on the isolation of some compounds from the plant, include phenolic compounds such as gallic acid **(1)** which was isolated from the n-butanol fraction from the leaves extract. (Oyedeji *et al.,*2010).Fadoup *et al.,* 2014 reported that GC-MS analyses of the oil fraction from the crude leaf extract of *L. abyssinica* revealed the presence of some compounds including: squalene **(2)** (Fig 2.1).

2.4 Reported Chemical Constituents from other Speciesof *Ludwigia*

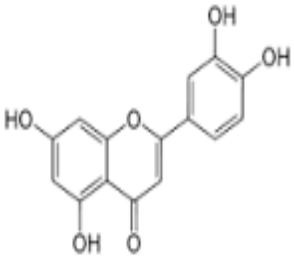
Phytochemical investigation of methanol extract of*L. octovalvis*has reportedly led to the isolation of some compounds from extract such asquercetin **(3)**, and apigenin **(4)** which are known to have antibacterial and anti-oxidant activity (Yan and Yang 2005; Chang and Kuo, 2007; Wu *et al.*, 2010).Ayinampudi and Ramchander, (2012) reported that some compounds were isolated from stem of *L. alternifolia* which include vitexin **(5)** isovitexin **(6)**, orientin **(7)**, iso-orientin **(8)** and ellagitannin **(9).**Previous phytochemical investigation of *L. hyssopifolia* alsoyielded piperine, vitexin, isovitexin, orientin and iso-orientin **(**Mohammad *et al.,* 2003). Report on chemical studies of the ethanol extract of *Ludwigia hyssopifolia*whole plant led to isolation of some pentacyclic triterpenoids compounds. The compounds were elucidated as 6*β*, 24 hydroxy tormentic acid by spectroscopic data. The known compounds were identified as xanthyletin (**10**), sitosterol (**11**), hydroxytormentic acid (**12**) by using comparison with available data and spectroscopic studies (Ayinampudi *et al.,* 2013).


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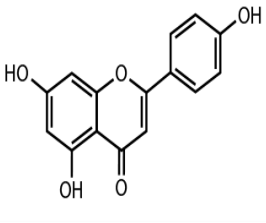
(1)


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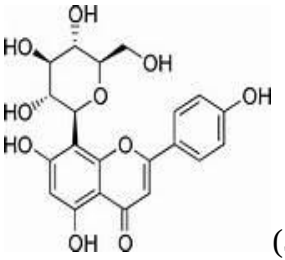
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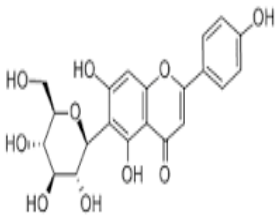
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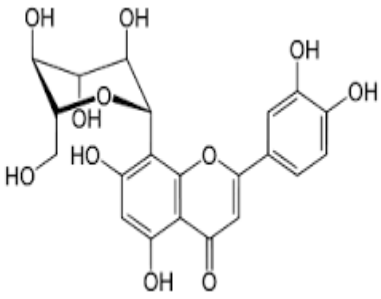
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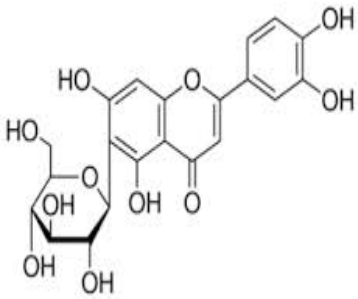
(5)


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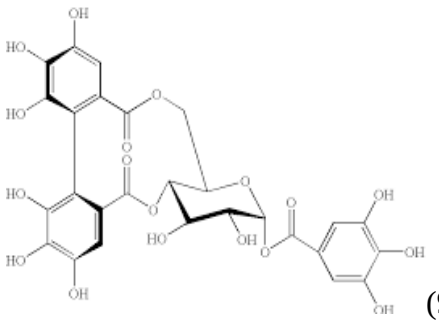
(6)


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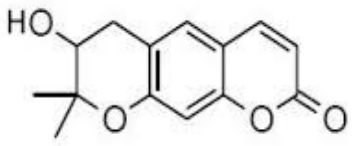
(7)


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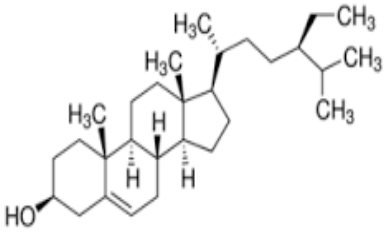
(8)



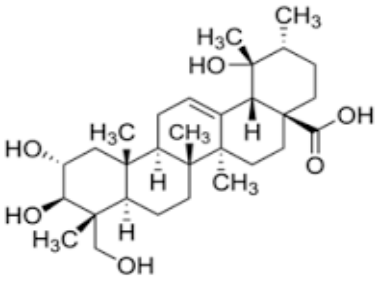
(9)



(10)



(11)



(12)

Fig. 2.1 Chemical Structures of Some Compounds Isolated From *Ludwigia* Species Sources: (Oyedeji *et al.,* 2010, Fadoup *et al*., 2014; Ayinampudi and Ramchander 2012; Yan and Yang 2005.,Chang and Kuo, 2007, Wu *et al.,* 2010).

2.5 Reported Biological Activities of *Ludwigia* species

Different biological activities have been reported for various *Ludwigia* species ranging from antioxidant, antifungal, anti-bacterial and anti-inflammatory properties.Scientific investigations involving a number of various *Ludwigia* species including *L. abyssinica* proved numerous activities such as antimicrobial and antioxidant.The gallic acid isolated from n-butanol fraction of *L. abyssinica* leaf was found to exhibit considerable antimicrobial effects when compared with standard antibiotic and antifungal agents. This polyphenolic compound exhibited broad spectrum antimicrobial activities against all test bacterial and fungal species (Oyedeji *et al*., 2014). The compound also exhibited strong antioxidant activity based on DPPH radical scavenging capacity. The plant could therefore be a potential source of natural antimicrobial and antioxidant agents for the treatment of microbial infections and prevention of various oxidative stress-associated diseases, such as cancer and other degenerative human diseases (Oyedeji *et al.,* 2014).n-Hexane, ethylacetate and methanol extracts of *Ludwigia hyssopifolia* were reported to show analgesic and anti-inflammatory activity by inhibiting inflammation, pain and induce diuresis in animal models (Das *et al.*, 2014). Antidiarrheal activity of the methanol extract of *Ludwigia hypossifolia* Linn was also reported (Mohammad *et al.*, 2003).

Also, *L. octovalvis* methanolic extract was reported to be active against *Escherichia coli* and other pathogenic bacteria (Haidar *et al.*, 2012). Crude extract of *L. octovalvis* was observed to show potent anti-oxidant effectbased on DPPH radical scavenging capacity as reported by Lie-Fen *et al.*, (2005).Ahmad *et al.*, (2005) reported that methanol extract of *L. adscendens* showed a broad spectrum of anti-bacterial activity against some bacteria's.

Extracts of *L. abyssinica* and *L. decurrens* leaves were active against broad spectrum of bacterial and fungal species (Oyedeji *et al.*, 2010).

2.6 Inflammation and Anti-inflammatory Compounds in Plants

Inflammation is a dynamic process that is elicited in response to mechanical injuries, burns, microbial infections, and other noxious stimuli that may threaten the well-being of host (Gautam and Jachak, 2009). The classical key features of inflammation are redness, swelling and pain. This process involves change in blood flow, increased vascular permeability, destruction of tissues via the activation and migration of leucocytes with synthesis of reactive oxygen derivatives and other local inflammatory mediators such as prostaglandins (PGs), leukotrienes, platelet-activating factors induced by phospholipase, cycloxygenases (COXs), and lipoxygenases (Wiart, 2006). Inflammation can be classified as either acute or chronic.

2.6.1 Acute inflammation

Acute inflammation is the initial response of the body to harmful stimuli and is achieved by the increased movement of plasma and leukocytes from the blood into the injured tissues (Danesh *et al.*, 2004). A cascade of biochemical events propagates and matures the inflammatory response, involving the local vascular system, the immune system and various cells within the injured tissue (Abbas and Litchman, 2009).

2.6.2 Chronic inflammation

Chronic inflammation is a prolonged inflammation that occurs when the inflammatory response is out of proportion resulting in damage to the body. The examples of disorders related with inflammation include: rheumatoid arthritis, asthma, pelvic inflammatory

disease, acne vulgaris, autoinflammatory diseases, inflammatory bowel diseases (Shailasree *et al.,* 2012).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Materials, Chemicals, Solvents, Reagents/Solutions and Equipments

3.1.1 List of Chemicals and Reagents

N-Hexane,Ethylacetate, and Methanol (JHD, Sci-Tech Co., Ltd China),Ferric chloride, Sulphuric acid (Sigma-Aldrich, St. Lous, MO, USA),Anisaldehyde (Sigma-Aldrich, St. Lous, MO, USA), Aluminium chloride, Sodium hypochlorite, Chloralhydrate, Phloroglucinol, Glycerol, Carageenan (Sigma-Chemical, St. Lous, MO, USA), Piroxicam (Pfizer, Pakistan), Normal saline, Hydrochloric acid, Sudan red, Zinc chloride, TLC silica gel 60 F_{254} pre-coated plates (Merk-Germany)

3.1.2 List of Equipment

Melting point Apparatus (Gallencamp, USA), Soxhlet apparatus, Compound microscope (Fisher Scientific, UK), Camera Lucida, Stage Micrometer and Ocular Lens (Graticules Ltd, Ton ridge, Kent. England), Glass Slides and Cover slips, Water bath (HHS, Mc Donald Scientific International, England), Mechanical shaker (Stuart Scientific Flask Shaker, Great Britain), Dessicator, Laboratory glass wares (Funnel, Conical flask, Beakers, Measuring cylinder), Metallic cages and feeding bottles for rats, TLC tanks (Uni kit® TLC Chromatank® , Shandon Germany), Disposable syringes (1ml, 5ml, and 10ml), Slide dryer (Hospital and Lab. Supply Ltd, London, UK), Microtome (C 740527, Cambridge Instrument Company Ltd, London and Cambridge, England), Digital Varnier caliper.

3.2 Collection and Identification and Preparation of Plant Material

Whole plant sample of *L. abyssinica* were collected from Yankarfe Village, Sabon Gari Local Government Area, Kaduna State, in May, 2015 and were taken to the Herbarium unit, Department of Biological Sciences, Ahmadu Bello University, Zaria for identification by the taxonomists. The plant sample was taxonomically identified and authenticated by Namadi Sanusi with voucher specimen number 801. Sufficient quantity of the leaves was then obtained dusted, cleaned and all foreign matter removed.They were air dried to constant weight, and pulverized into powder and stored in air-tight container for subsequent use.

3.3 Pharmacognostic Studies of *Ludwigia abyssinica* Leaves

3.3.1 Experimental Design

Detailed pharmacognostic studies of leaves of *L. abyssinica* were carried out by examining the microscopical and chemomicroscopical characteristics of the leaves in order to establish some Pharmacopoeial standards of the plant.

3.3.2 Microscopical examination on the Leaves of *Ludwigia abyssinica*

3.3.2.1 Surface preparation and anatomicalsection

Anatomical section of the leaf sample was examined under the microscope and features were described by using the terms according to Dutta (2003) and Evans, (2002).

The transverse section across the midrib of the fresh leaf of *L. abyssinica* was prepared. Leaf epidermises (upper and lower) were peeled.

The prepared sections were cleared using 70% chloral hydrate solution. The cleared sample was mounted on a microscope slide by using dilute glycerol. This was then observed under the microscope and the diagnostic features were noted and documented (WHO, 2011).

3.3.2.2 Micrometric evaluation

Measurements of dimensions (length and width) of the various diagnostic microscopic characters of the leaf; namely stomata, epidermal cells were carried out by using a binocular microscope with the aid of calibrated eyepiece graticles (Kokate, 2003).

3.3.2.3 Quantitative Leaf Microscopy

Evaluation of some physical constants of the leaves of *L. abyssinica*namely stomatal number and index, palisade ratio, veinislet and veinlet termination number were carried out.

(i) Stomata Number

Sections from the upper and lower epidermis of the plant were cleared with boiling in 70% choral hydrate solution and mounted on a clean microscope slide with dilute glycerol. A camera Lucida was set up and with the aid of a stage micrometer a paper was divided into squares of $1mm^2$ by using x10 objective. The stomata were traced and counted in the fields on a single section of the leaf of the plant and the average number stomata of five counts per mm^2 of epidermis was recorded (Evans, 2009)

(ii) Stomatal Index

Sections of the epidermal portion of the leaves were mounted and examined as in stomata number determination, except that here both stomata and epidermal cells were counted. The stomatal index was calculated in accordance with Evans (2009) as:

$$S\,I = \frac{S}{E + S}\ X\ 100$$

Where S I = Stomatal Index,

S= Number of stomata per unit area and

E= Number of epidermal cells per unit area

(iii) Vein-islet number

It was determined by boiling pieces of leaf of the plant in a test-tube containing 70% chloral hydrate solution. This was followed by treatment with 10% hydrochloric acid to remove calcium oxalate crystals to enhance visibility. A camera Lucida was set up and by means of a stage micrometer the paper was divided into squares of 1 mm^2 by using x10 objective. The stage micrometer was then replaced by the cleared preparation of the leaf and the veins traced in four contiguous squares that is a rectangle 1 x 4^2. Each vein weretraced and areas which were completely enclosed by veins were counted and those that were not completely enclosed were excluded (Evans, 2009).

(iv) Vein-let Termination Number

It was determined for *L. abyssinica* leaves using a camera Lucida set up as in vein-islet number but here the terminal ends of the veins not completely enclosed were counted in each square and noted (Evans, 2009).

(v) Palisade Ratio

Section from the upper epidermis of the plant was cleared by boiling in 70% choral hydrate solution and mounted on a clean microscope slide with dilute glycerol and examined with x40 objective. A camera Lucida was set up and the palisade ratio determined in groups of four and the average taken (Evans, 2009).

3.3.3 Chemo-microscopic Studies on the Leaves of *Ludwigia abyssinica*

Small amount of the finely ground powdered leaves was cleared in a test-tube containing 70% chloral hydrate solution. It was boiled on a water-bath for thirty minutes to remove obscuring materials. The cleared sample was mounted on a microscope slide by using dilute glycerol. Using various detecting reagents, the presence of some cell inclusions and cell wall materials were detected in accordance with Brain and Turner (1975); Evans (2002).

3.3.3.1 Cell wall materials

i) Test for Cellulose: Two drops of iodinated zinc chloride were added to the cleared sample on a slide, and this was allowed to stand for 2 minutes. One drop of sulphuric acid was added, cover- slipped and observed under the microscope for blue colour which indicate the presence of cellulose in the cell walls.

ii) Test for Lignin: Two drops of phloroglucinol were added to the cleared sample and allowed to stand until almost dry. A drop of hydrochloric acid was added and cover slip was applied. This was observed under the microscope.

iii) Test for Cutin: Two drops of Sudan red were added to the cleared powdered sample on a slide, cover slip was applied and this was gently heated over hot water bath for 2 minutes.

The slide was then observed under the microscope for orange red coloration which indicates the presence of suberin in the cell wall.

iv) Test for Gum and Mucilage: A drop of ruthenium red was added to the cleared sample on a slide, cover slipped and observed under the microscope. The appearance of pink colouration is an indication for the presence of gums and mucilage**.**

3.3.3.2 Cell Inclusions/ Cell Contents

i) Test for Starch: Two drops of N/50 iodine solution were added to the cleared sample and cover slip was applied. This was observed under the microscope for the appearance of blue-black or reddish-blue colouration on some grains to indicate the presence of starch.

ii) Aleurone grains: Few drops of iodine solution were added to the powdered leaf sample cover slipped and observed under the microscope. Appearance of yellowish brown to brown colour indicates the presence of aleurone grains.

iii) Test for Calcium oxalates: To the cleared sample, cover slip was applied and this was observed under the microscope. Two drops of hydrochloric acid were then added, and observed. Dissolution of shining crystals in powdered samplethe of the leaves indicates the presence of calcium oxalates.

iv) Test for Calcium carbonates: The appearance of effervescences on addition of concentrated hydrochloric acid to the prepared slide of the powdered plant on the slide showed the presence of calcium carbonate.

iv) Inulin: Two drops of 1-naphthol and that of sulphuric acid were added to the powdered leaf of the plant and cover slip was applied and observed under the microscope. Spherical

aggregations of crystalsof inulin turns to brownish red and dissolve which indicates the presence of inulin.

v) Test for Tannins: A single drop of ferric chloride was added to the cleared plant sample and cover slip was applied and this was observed under the microscope. The appearance of greenish black coloration on some cells of the anatomical sections of the leaves indicates the presence of tannins.

3.4 Determination of Physicochemical Constants of the powdered Leaves of *Ludwigiaabyssinica*

The physicochemical examinations for the powdered leaf of the plant were determined according to the method outlined by WHO (2011).

3.4.1 Determination of Moisture Content

The moisture content was determined by "Loss on drying" method (gravimetric determination). Air-dried leaf (3 g) in a dried and weighed crucible was weighed using KERN EW Electronic Balanced. The crucible was transferred into a hot air sterilizing oven, which was set at 105^0C. After one hour, the crucible containing the powdered plant was removed, placed in a desiccator over phosphorous pentoxide under atmospheric pressure at room temperature. After 30 minutes in the desiccator, the weight of the powder and crucible were quickly determined and the crucible returned to oven. The heating and weighing were repeated until a constant weight was obtained and noted. Three determinations were conducted and the average of these was taken as the moisture content of the plant material. The moisture content (loss of weight) was calculated using the formula:

Moisture content (%) = $\frac{InitialWeig\ htofPowder\ -FinalWeig\ htofPowder}{InitialWeig\ htofPowder} X\ 100$

3.4.2 Determination of total ash value

A platinum crucible was heated red hot, cooled in a desiccator and quickly weighed. Exactly (2 g) of the air-dried leaf powder was weighed into the crucible and heated in a furnace at 450^0, until it became white, indicating absence of carbon and was then cooled in a dessicator and weighed. The procedure was repeated three times to obtain average value. The total ash content of the air-dried powder was calculated in percentage using the formula:

Ash Value (%) =$\frac{Weig\ htofResidualAs\ \ h}{OriginalWe\ \ ightofPowder} X\ 100$

3.4.3 Determination of Acid-insoluble Ash

To the crucible containing the total ash, 25 ml of dilute hydrochloric acid was added and covered with a watch glass and boiled gently for 5 minutes. Hot water (5 ml) was used to rinse the cover glass into the crucible. The insoluble matter was collected on an ashless filter paper and washed with hot water until the filtrate was neutral. This was then transferred back to the crucible and dried on a hot plate and ignited to a constant weight. The residue was allowed to cool in a dessicator for 30 minutes and quickly weighed. The acid insoluble ash was calculated as follows:

Acid insoluble Ash (%) =$\frac{Weig\ htofResidualAs\ \ h}{OriginalWeig\ \ htofPowder} X\ 100$

3.4.4 Determination Water Soluble Ash

To the crucible containing the total ash, 25 ml of water was added and boiled for 5 minutes. The insoluble matter was collected in a sintered glass crucible. It was then washed with hot

water and ignited to a constant weight. The weight of the residue was subtracted from the weight of the total ash. The water soluble ash of air dried powder was calculated using the formula:

$$\text{Water Soluble Ash (\%)} = \frac{Weig\,htofTotalAs\;\;h - Weig\,htofResidualAs\;\;h}{Orig\,inalWeig\;\;htofPowder} X\ 100$$

3.5 Extractive Values

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

3.5.1 Water extractives value

Exactly 4 g of air-dried leaves powder was weighed into a 250 ml glass stoppered conical flask and chloroform- water (1: 400),100 ml was used to macerate the powder for 6 hours with frequent shaking by using mechanical shaker and was allowed to stand for 18 hours. It was then filtered rapidly and 25 ml of filtrate was transferred into a previously dried and weighed evaporating dish and evaporated to dryness on a hot water bath. This was further dried in the oven at 105^0C for 6 hours, cooled in a desiccator for 30 minutes and then weighed without delay. The percentage water extractive value was calculated using the formula:

$$\text{Water Extractive Value (\%)} = \frac{Weig\,htofresiduein\;\;\;25mlExtract}{OriginalWeig\;\;htofPowder} X\ 100$$

3.5.2 Ethanol extractive value

The procedure above (subsection 3.5.1) was repeated with ethanol in place of water and the percentage ethanol extractive value was calculated using the following formula:

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

3.6 Extraction of the Leaves of *Ludwigiaabyssinica*

Extraction of the plant material was done using the method described by (Kokate, *et al.*, 2003) with slight modification. The pulverized plant material (1 kg) was extracted with n-hexane, ethyl acetate and methanol successively in a Soxhlet apparatus (Fig. 3.1). The extract obtained was concentrated via rotary evaporator to recover some solvent and final evaporation to dryness of the extracts were done via the water bath after which each extract was stored in desiccator for subsequent use. The percentage yield was calculated using the formula:

$$\text{Yield of extracts (\%)} = \frac{\text{Weight of total extract}}{\text{Weight of powdered material}} \text{ X } 100$$

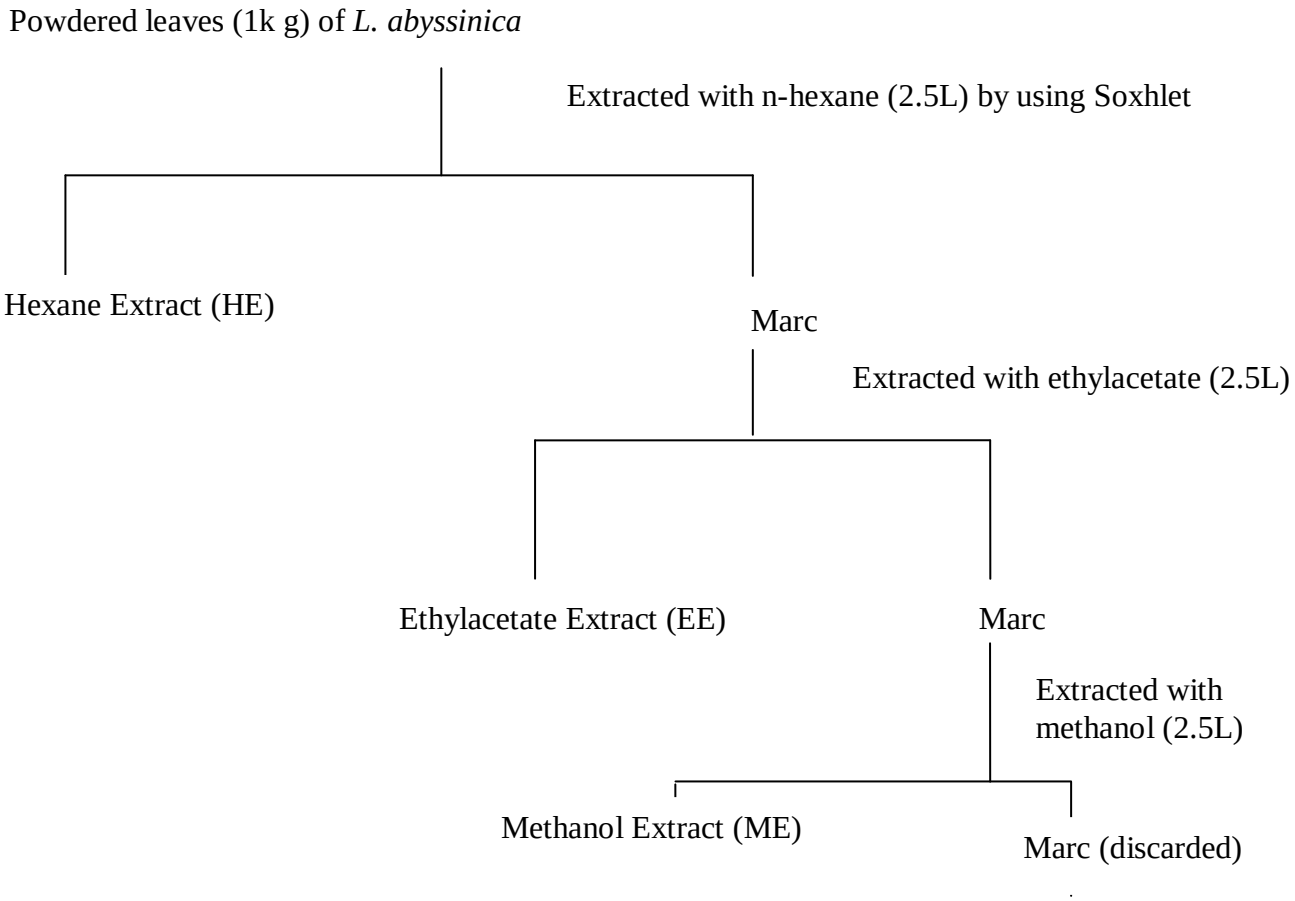


Fig. 3.1: Flowchart of Extraction Profile of *Ludwigia abyssinica* Leaves

3.7 Phytochemical Screening of the Leaf Extracts of *Ludwigia abyssinica*

The leaf extracts (HE, EE and ME) were subjected to phytochemical screening in order to identify the phytochemical constituents present in each of them (Evans, 2002; Musa, 2005; Sofowora 2008).

3.7.1 Test for Saponins

3.8.1.1 Frothing test: Five hundred milligrams of the hexane extract was dissolved in 10 ml of water and shaken vigorously for 30 seconds and allowed to stand for one hour, the occurrence of frothing column or honey comb-like of at least 1 cm in height and persisting for at least 30 minutes indicates the presence of saponins. The procedure above was repeated for ethyl acetate and methanol extracts (Sofowora, 2008).

3.7.1.2 Haemolysis test: Two ml of sodium chloride (1.8% solution in distilled water) was added to two test tubes A and B. two ml of distilled water were added to test tube A, 2 ml of the hexane extract was added to test tube B. five drops of blood were added to each tube and the tubes were inverted gently to mix the contents. Haemolysis in tube B containing the hexane extract but not in tube A (i.e. control), indicated the presence of saponins in the extract. The procedure above was repeated for ethyl acetate and methanol extracts (Brain and Turner, 1975).

3.7.2 Test for steroids/terpenes

3.7.2.1 Lieberman-Burchard test:One ml of acetic anhydride was added to 0.5 g of the hexane extract dissolved in one ml of chloroform. Concentrated sulphuric acid was then added gently by the side of the test tube to form lower layer and at the junction of the two liquids.

Formation of reddish brown or violet brown ring, the upper layer bluish green or violet indicates the presence of sterols and or triterpenes. The procedure above was repeated for ethyl acetate and methanol extracts (Evans, 2002).

3.7.2.2 Salkowski test:2 ml of chloroform was added to 0.5 g of the hexane extract and one ml of concentrated sulphuric acid was carefully added to the side of the test tube to form a lower layer. A reddish brown coloration at the interface indicated the presence of steroidal nucleus. The procedure above was repeated for ethyl acetate and methanol extracts (Sofowora, 2008).

3.7.3 Test for flavonoids

3.7.3.1 Shinoda test:0.5 g of the hexane extract was dissolved in the extracting solvent, 2 ml of 50% methanol. Pieces of magnesium fillings and 3 drops of hydrochloric acid were added and a pink, rose or red colouration indicated the presence of flavonoids. The procedure above was repeated for ethyl acetate and methanol extracts (Evans, 2002).

3.7.3.2 Sodium hydroxide test:0.5 g of the hexane extract was dissolved in water and filtered. 2 ml of 10% aqueous sodium hydroxide solution was then added. The solution was observed for the presence of yellow colour. A change in colour from yellow to colourless on addition of dilute hydrochloric acid was used as an indication for the presence of flavonoids. The procedure above was repeated for ethyl acetate and methanol extracts (Evans, 2002).

3.7.4 Test for Tannins

3.7.4.1 Ferric chloride test: 0.5 g of the extracts (hexane, ethyl acetate and methanol) were dissolved in 5 ml of water each and filtered. Two drops of ferric chloride solution were

added to the filtrate. Appearance of blue-black or green or blue-green (condensed/cathehic tannins) precipitate indicates the presence of tannins (Musa, 2005).

3.7.4.2 Lead sub-acetate test:To 0.5 g of the hexane extract, 2 ml of ethanol was added followed by two drops of lead sub-acetate solution; appearance of whitish-yellow precipitate indicates the presence of tannins. The procedure above was repeated for ethylacetate and methanol extracts (Musa, 2005).

3.7.5 Test for Alkaloids

Hexane extract (1.0 g) of the extracts was stirred with 20 ml of 1% aqueous hydrochloric acid on water bath and filtered. The filtrate was basified with concentrated NH_4OH and extracted with chloroform. The chloroform layer was then extracted with 2 ml of 1% HCl. The aqueous layer was divided into four portions for the following tests: To the first portion, 1 ml of freshly prepared Dragendorff's reagent was added drop-wise and observed. To the second portion 1 ml of Mayer's reagent was added drop-wise and observed. To the third, 1 ml of Wagner's reagent was also added. The fourth portion was used as control. Appearance of rose red to brownish, white to yellowish or cream color and brown or reddish–brown precipitates respectively indicates the presence of alkaloids. Ethylacetate and methanol extracts were treated similarly (Evans, 2002).

3.7.6 Test for anthraquinones

3.7.6.1 Bontrager test:To 0.5 g of the hexane extract, 10 ml of chloroform was added and shaken. This was then filtered and 5 ml of 10% ammonia solution was added to the filtrate. The presence of pink or cherish red colour in the lower layer indicates the presence of

anthracenes. The procedure above was repeated for ethyl acetate and methanol extracts (Evans, 2002).

3.7.6.2 Modified Borntrager's test: 0.5 g of the hexane extract was boiled with 10 ml of aqueous sulphuric acid and filtered hot. The filtrate after cooling to room temperature was shaken with 5 ml chloroform, the chloroform layer was separated and half of its volume, 10% ammonium hydroxide was added. A pink, red or violet colouration in the ammonia phase (lower phase) is an indication for the presence of combined anthracene or anthraquinone derivatives. The procedure above was repeated for ethyl acetate and methanol extracts (Evans, 2002).

3.7.7 Test for cardiac glycosides

***3.7.7.1 Keller-Killiani test*:** 0.5 g of the hexane extract was dissolved in glacial acetic acid containing ferric chloride and one drop of sulphuric acid was added to the solution. The appearance of reddish-brown colouration at the interphase indicates the presence of deoxysugar. The procedure above was repeated for ethyl acetate and methanol extracts (Sofowora, 2008).

***3.7.7.2 Kedde's test*:** 0.5 g of the hexane extract was treated with 1ml of 2% solution of 3, 5-dinitrobenzoic acid in 95% alcohol. The solution was made alkaline by the addition of 5% NaOH. The presence of purple- blue colour indicates the presence of cardenolides. The procedure above was repeated for ethyl acetate and methanol extracts (Evans, 2009).

3.7.8. Test for carbohydrates:

3.7.8.1 Molisch's Test:0.5 g of the extracts (hexane, ethyl acetate and methanol) were separately dissolved in 5 ml of distilled water each and filtered. The filtrates were treated

with 2 drops of alcoholic α-naphthol solution in a test tube, followed by 1 mL of concentrated H_2SO_4 down the side of the test tube. Formation of the violet ring at the junction indicates the presence of carbohydrates (Evans, 2002).

3.7.8.2 Fehling's Test:0.5 g of the extracts (hexane, ethyl acetate and methanol) were separately dissolved in 5 ml of distilled water each and filtered. The filtrates were hydrolysed with dilute HCl, neutralized with alkali and heated with mixture of equal volume of Fehling's A & B solutions. Formation of brick red precipitate indicates the presence of reducing sugars (Evans, 2002).

3.8 Thin Layer Chromatographic Profile of the of *Ludwigia abyssinica* Leaf Extracts

TLC plates of 20 × 20 cm coated with silica gel 60 F_{254} were used and one way ascending technique was employed for the analysis. The extracts (HE, EE and ME) were dissolved in the initial extraction solvent. The plates were cut into size of 5×10 cm and spots were applied manually on the plates using capillary tube after which plates were dried and developed using Hexane - Ethyl acetate (9:1, 7:3), Chloroform - Methanol (9:1), Ethyl acetate - Methanol -Water (10:1:0.5) and n-Butanol - Acetic acid - Water (10:1:1) as the case maybe in chromatographic tank. Developed plates were sprayed using general detecting reagent (*p*-Anisaldehyde/H_2SO_4) and specific detecting reagents (Ferric chloride, Lieberman-Buchard and Aluminium chloride) and heated at 110ºC where applicable. Number of spots, colours and retardation factors (R_f values) for each of the spots were determined and recorded (Gennaro, 2000; Stahl, 2005).

3.9 Anti-inflammatory Study of the *Ludwigia abyssinica* Leaf Extracts

3.9.1 Source and maintenance of experimental animals

Wistar albino rats of both sexes weighing 150 – 200g were obtained from the Animal House of the Department of Pharmacology and Therapeutics, Ahmadu Bello University, Zaria. The animals were maintained under standard conditions at room temperature. The rats were fed with standard (grower) mash (Vital feed, Jos, Nigeria) and water was given *ad-libitum* (Nwafor, 2000).

3.9.2 Acute toxicity study of *Ludwigia abyssinica* leaf extracts

The acute toxicity study was carried out using rats of both sexes according to Lorke (1983)

Method:

In the first phase, nine rats were divided into three groups of three rats each. The groups received 10, 100 and 1000 mg/kg of the hexane extract respectively. The administration of the extracts was carried out orally and observed for signs and symptoms of toxicity including death for 24 hours. In the second phase, four groups of one rat each were treated, the groups received 1200, 1600, 2900 and 5000mg/kg doses of hexane extract via oral route respectively. Signs and symptoms of toxicity such as rolling, stress, diarrhea and death were observed for 24 hours. The Acute toxicity was calculated as the geometric mean of the lowest dose that caused death and the highest dose that did not cause death. The procedure above was repeated for ethylacetate and methanol extracts.

3.9.3 Anti-inflammatory Activity of extracts of *Ludwigia abyssinica* leaf extracts using Carrageenan Induced Paw Oedema Model

The anti-inflammatory activities of hexane, ethylacetate and methanol extracts were evaluated using the carrageenan induced paw oedema in rats (Winter *et al*., 1962, Ahmadu *et al.*, 2015). The extracts of n-Hexane and ethylacetate were suspended in Acacia gum suspension while the methanol extract was dissolved in distilled water before administration.

Fifty-five (55) albino rats of either sex weighing between 150-180g were used for this experiment and the rats were divided into 11 groups of five animals each. Group I received 10 ml/kg normal saline as (negative control), group II received 10 mg/kg piroxicam as (positive control), groups III, IV and V were treated with 500, 1000 and 1500 mg/kg n-hexane extract respectively, groups VI, VII, and VIII received ethyl acetate extract at doses of 500, 1000, and 1500mg/kg respectively and group IX, X and XI were also treated with methanol extract at same doses of 500, 1000 and 1500mg/kg of the extract respectively. All treatments were done orally. After 1 hour, acute inflammation was induced by sub-planter injection of 0.1ml of sterile saline solution of 1% freshly prepared suspension of carrageenan into the sub planter right hind paw of all animals. Measurements of left hind paw was taken using varnier caliper to determine the diameter of oedema immediately after the injection (0h) and followed by every hour for 5 hours (i.e. 1, 2, 3, 4 and 5 hr) after the carageenan administration to each group and the increase in the paw diameter was recorded (Ahmadu *et al.*, 2015). The differences between the readings at time 0 hour and different time intervals were taken as the thickness of oedema.

The results were expressed in terms of mean increase in paw diameter at 0- 5hr and anti-inflammatory activity was expressed in terms of percentage inhibition of paw at 5hr. The inhibitory activity was calculated according to the formula below (Owoyele *et al.*, 2005).

$$\% \text{ inhibition} = \frac{T_{o\,(control)} - T_{t(test)}}{T_{o\ control}} X\ 100$$

Where $T_o$is paw size for negative control and T_t is paw size for test groups at various time.

3.10 Statistical Analysis

The results were expressed as mean ± standard errors of the mean (SEM) for all values. The data was statistically analyzed using repeated measures ANOVA followed by Bonferroni *post hoc* test. Results were considered to be significant when P values were less than 0.05 (P<0.05) (Okasha *et al*., 2008).

CHAPTER FOUR

4.0 RESULTS

4.1 Pharmacognostic Evaluation of *Ludwigia abyssinica* Leaves

4.1.1 Microscopic Studies on the Leaf of *Ludwigia abyssinica*

Microscopical examination of the leaf of *L. abyssinica* revealed the presence of important diagnostic characters on both upper and lower epidermal layers whichinclude; Anomocytic type of stomata in both adaxial and abaxial surfaces with irregular cell and deeply wavy anticlinal walls on the upper epidermis and sinous-walled anticlinal walls and polygonal shaped epidermal cells, on the lower epidermis. The transverse section of the leaf through the midrib tissue was examined and revealed different anatomical features namely upper and lower epidermis, mesophyll cells, vascular bundle composed of phloem and xylem tissues as presented in Plate II and III, Table 4.1.

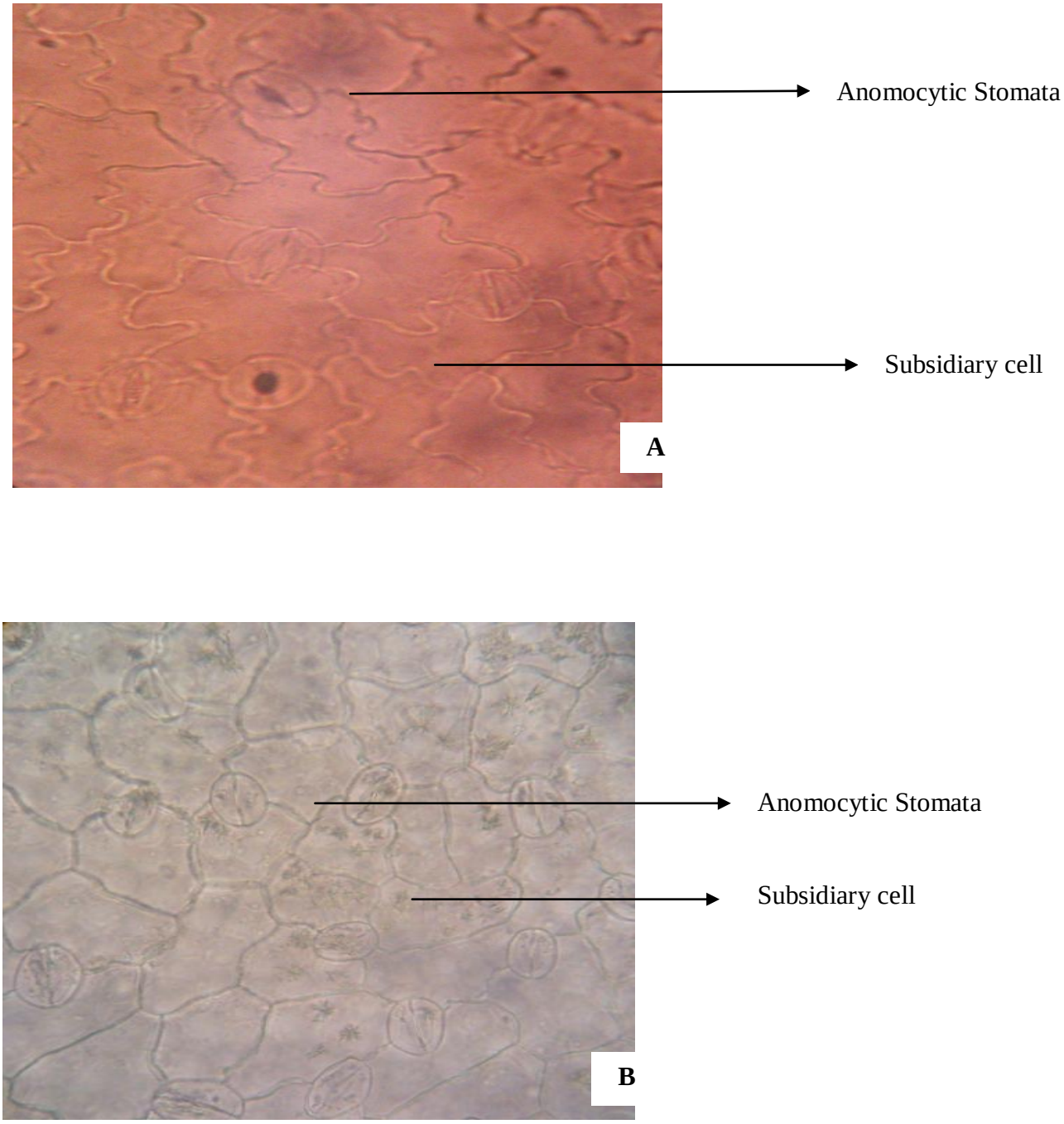


Plate II: Photomicrograph of the Upper (A) and Lower (B) epidermal Layer of *Ludwigia abyssinica* Leafshowing Anomocytic stomata with Subsidiary cells (Mag. ×100)

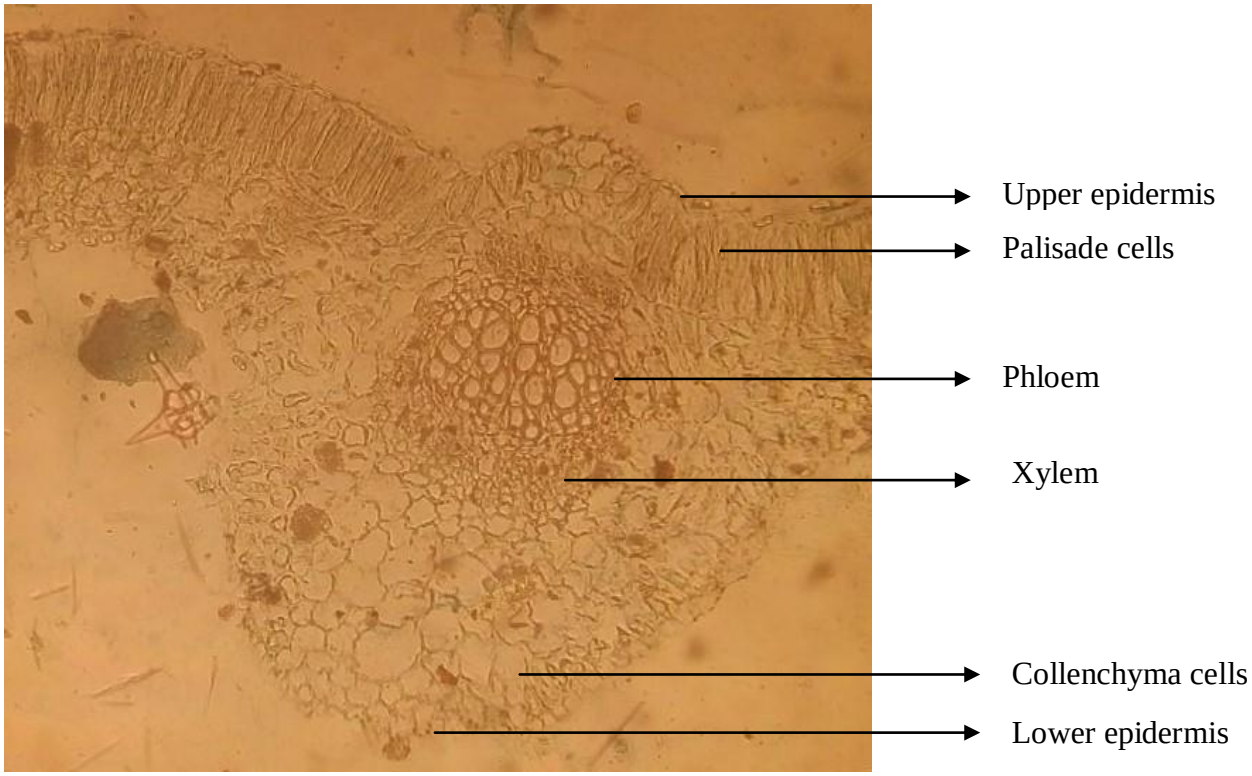


Plate III: Photomicrograph of the Transverse Section of *Ludwigia abyssinica* Leaf showing some cells (Mag. ×100)

Table 4.1: Microscopic Features of the Upper and Lower Epidermis of *L. abyssinica* Leaf

CHARACTERS	RESULTS	OBSERVATIONS
Upper epidermal cells	Shape	Irregular in shape
Anticlinal walls	Type	Deeply wavy
Stomata	Type	Anomocytic
	Position	Adaxial (upper) epidermis
	Frequency	Few
	Size	(18.35 ×13.60µm^2)
Lower epidermal cells	Shape	Polygonal in shape
Anticlinal walls	Type	Sinuous
Stomata	Type	Anomocytic
	Position	Abaxial (lower) epidermis
	Frequency	Numerous
	Size	(14.13 ×09.74µm^2)

4.1.2 Quantitative Leaf Microscopy of *Ludwigia abyssinica*

On the average, the leaf was observed to have stomatal number (8.00) and (20.0) upper and lower surfaces respectively. The stomatal indices were discovered to be (12.12) and(16.8) for each of the epidermis. The palisade ratio was (13.0), whereas the vein islet and veinlet termination number were (12) and (11) respectively (Table 4.2).

Table 4.2: Quantitative Microscopical Values for the Upper and Lower epidermis of *Ludwigia abyssinica*Leaf

Evaluative Parameter	Mean± SEM	Lower Range	Upper Range
Upper epidermis			
Stomatal number	8.00 ± 0.57	6.80	9.20
Stomatal index	12 .12 0.01	10.30	13.94
Lower epidermis			
Stomatal number	20.00 ± 0.67	17.00	23.00
Stomatal index	16.80 ± 0.39	14.28	19.32
Palisade ratio	13.00 ± 0.76	11.05	14.95
Vein islet number	12.00 ± 0.33	10.20	13.80
Veinlet termination no.	11.00 ± 0.58	09.35	12.60

Average Values of five counts

4.1.3 Chemomicroscopical studies of the powdered leaves of *Ludwigia abyssinica*

Chemomicroscopical examination of the powdered leaves of *L. abyssinica* revealed the presence of several types of cell wall material and cell wall inclusions as:

Table 4.3: Chemomicroscopical Features of *Ludwigia abyssinica* Powdered Leaf

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

4.1.4 Determination of physical constants of powdered Leaves of *Ludwigia. abyssinica*

The result of average moisture contents using loss on drying method was calculated to be 7.0% and the percentage yield of total ash, acid insoluble and water soluble matter were recorded in percentage values as 7.8, 2.33 and 4.67% respectively. The solvent extractive values obtained were 17.0 and 19.7 % for alcohol and water respectively (Table 4.4).

Table 4.4: Physicochemical Constants of *Ludwigia abyssinica* Leaf Powder

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Parameters	Values (%w/w) ± SEM*
Moisture content	7.00 ± 0.43
Total ash value	7.80 ± 0.31
Acid Insoluble ash	2.33 ± 0.17
Water Soluble ash	4.67 ± 0.00
Ethanol Extractives	17.00 ± 0.33
Water Extractives	19.70± 0.33

*****Average values of three determinations.

4.2 Percentage Yield from Extraction of the Leaf of *Ludwigia abyssinica*

One kilogram (1 kg) of the powdered Leaves of *L. abyssinica* extracted in order of increasing polarity with n-hexane, ethyl acetate and methanol using Soxhlet apparatus yielded 21.09, 32.00 and 152.53 g of the extracts respectively. These implied 2.11, 3.2, and 15.25 % of the plant material (Table 4.5).

Table 4.5: Mass and percentage yield for the extracts of *Ludwigia abyssinica*

S/No.	Extracts	Mass (g)	Percentage Yield (%w/w)
1.	Hexane	21.09 g	2.11
2.	Ethylacetate	32.00 g	3.2
3.	Methanol	152.53g	15.25

4.3 Preliminary Phytochemical Constituents of *Ludwigia abyssinica*

Preliminary Phytochemical screening on the n-Hexane (HE), Ethyl acetate (EE) and Methanol extracts (ME) of the *L. abyssinica* leaves using standard methods gave the following results; Saponin, steroids/triterpenes, flavonoids, tannins, alkaloids and cardiac glycoside as shown in the table 4.6.

Table 4.6: Preliminary Phytochemical Screenings of *Ludwigia abyssinica* Leaf Extracts

Tests	Hexane Extract	Ethylacetate Extract	Methanol Extract
Saponin			
Frothing	Absent	Absent	Present
Haemolysis	Absent	Absent	Present
Steroids/Triterpenes			
Salkowski	Present	Present	Present
Lieberman-Burchard	Present	Present	Present
Flavonoids			
Shinoda	Absent	Absent	Present
Sodium Hydroxide	Absent	Absent	Present
Tannins			
Ferric chloride	Absent	Present	Present
Lead sub acetate	Absent	Present	Present
Alkaloids			
Dragendorff	Absent	Present	Present
Mayer	Absent	Present	Present
Wagner	Absent	Present	Present
Cardiac glycosides			
Keller- kiliani	Absent	Present	Present
Kedde's	Absent	Absent	Absent
Anthraquinones			
Borntrager	Absent	Absent	Absent
Modified Borntrager	Absent	Absent	Absent

4.4 Thin Layer Chromatographic Profiles of *Ludwigia abyssinica* Leaf Extracts

4.4.1 TLC profile of hexane extract (HE)

HE was noted to have eleven (11) spots (PlateIV) in hexane - ethyl acetate (9:1) sprayed with *p*-Anisaldehyde and heated at 110ºC for 2 minutes. TLC of HE developed in Hexane - Ethylacetate (9:1) sprayed with Borntragers, Dragendorff, Lieberman-Buchard, Ferric chloride and Aluminium chloride reagents showed various spots with colours (Plate V-VI). The R_f values of this spots is given in (Table 4.5 and 4.6)

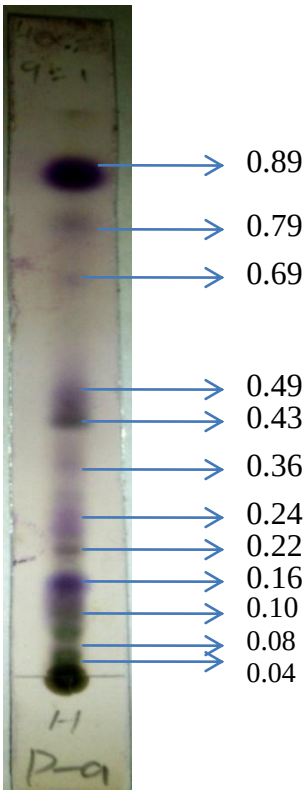


Plate IV

Plate IV: Chromatograms of n-Hexane extract on precoated silica gel plate developed in Hexane - Ethylacetate (9:1) sprayed with *p*-Anisaldehyde/H_2SO_4.Showing 12 distinct spots.

A **B**

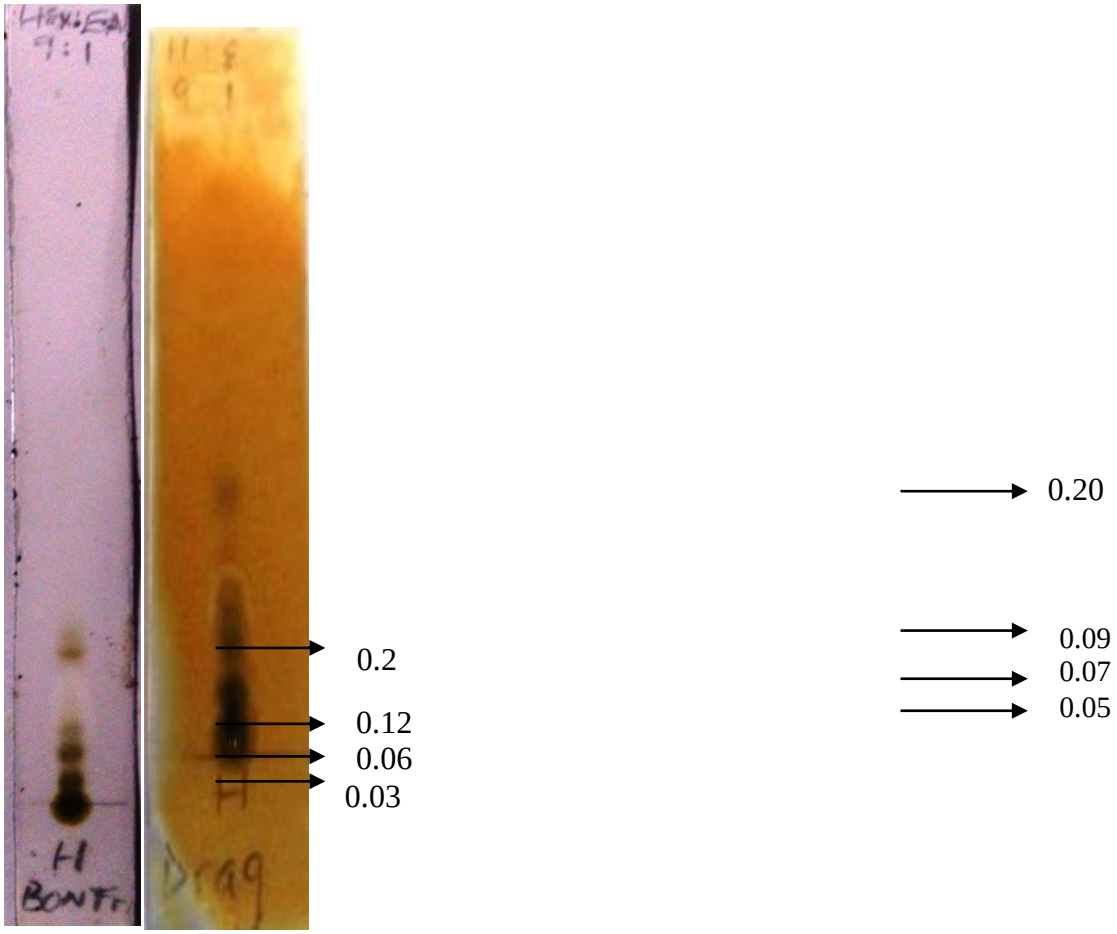


Plate V: Chromatograms of LAHE on precoated silica gel plate developed in Hexane - Ethylacetate (9:1) sprayed with Bontragers spray (A) and Dragendorff reagent (B) Showing 4 coloured spots withR_f values respectively.

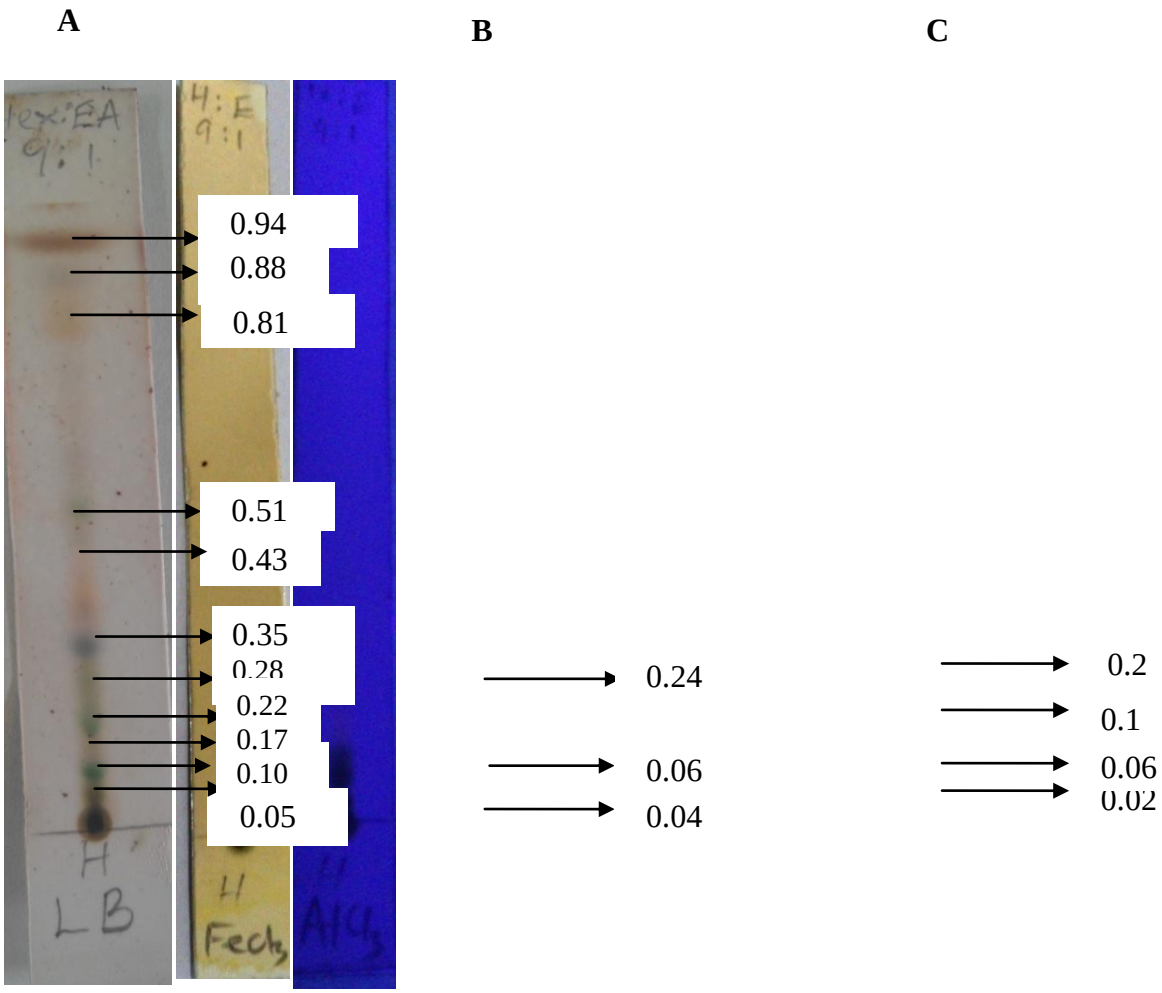


Plate VI: Chromatograms of LAHE on precoated silica gel plate developed in Hexane - Ethylacetate (9:1) sprayed with Lieberman-Buchard (A) heated at 110ºC for 2 minutes, showing 11 coloured spots, Ferric- Chloride reagent (B) showing 3 coloured spot and Aluminium chloride reagent (C) observed under UV light at 245nm, showing 4 spots with their R_f values respectively.

Table 4.7: TLC Profile of Hexane Extract (HE) of *Ludwigia abyssinica* leaf sprayed with *p*-Anisaldehyde/H_2SO_4

Extract	Solvent System	No. of Spots	Colour of Spots	R_f values
Hexane	H:E (9:1)	12	Green	0.04
			Green	0.08
			Purple	0.10
			Purple	0.16
			Purple	0.22
			Green	0.24
			Purple	0.36
			Green	0.43
			Green	0.49
			Grey	0.69
			Grey	0.79
			Purple	0.89

Key: H-E = Hexane - Ethylacetate

Table 4.8: TLC Profile of Hexane (HE) of *L. abyssinica* leaf Sprayed with Specific Detecting Reagents

Detecting Reagents	Solvent System	No. of Spots	Colour of Spots	R_f Values
Bontragers	H:E (9:1)	4	Blue-black, Brown,Grey, Brown	0.03, 0,06, 0.12, 0.20
Dragendorff	H:E (9:1)	4	Green, Green Orange, Brown	0.05, 0.07, 0.09, 0.20
Libermann-buchard	H:E (9:1)	10	Brown, Green Green, Brown Purple, Brown Brown, Green Brown, Brown	0.05, 0.10 0.17, 0.22 0.28, 0.30 0.33, 0.51 0.81, 0.94
Ferric chloride	H:E (9:1)	3	Blue-black, Blue-black, Blue-black	0.04, 0.06, 0.24
Alluminium chloride	H:E (9:1)	4	Blue-black, Blue-black, Yellow-flourescence, Brown	0.02, 0.06 0.10, 0.20

Key: H-E = Hexane - Ethylacetate

4.4.2 TLC profile of ethylacetate extract (EE) of *L. abyssinica* leaf

EE was noted to have ten (10) spots (Plate VII) in hexane: ethyl acetate (7:3) visualized with *p*-Anisaldehyde H_2SO_4 and heated at 110ºC for 2 minutes. TLC of EE developed in hexane - ethylacetate (7:3) sprayed with Bontragers, Dragendoff, Lieberman-Buchard, Ferric chloride

and Aluminium chloride reagents showed various spots with colours (Plate VIII– IX). The $R_f$values of the spots is given in (Table 4.7 and 4.8).

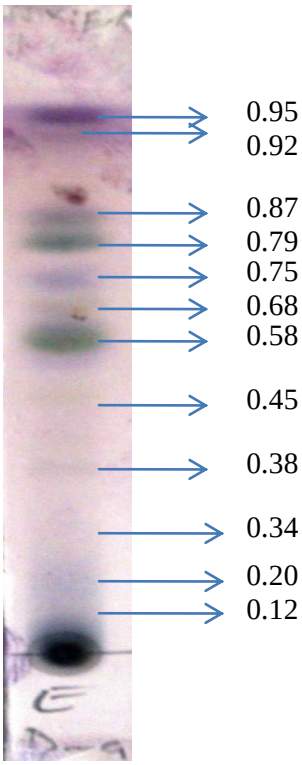


Plate VII

Plate VII: Chromatograms of Ethylacetate extract on precoated silica gel plate developed in Hexane - Ethylacetate (7:3) sprayed with *p*-Anisaldehyde/H_2SO_4. Showing 12 spots with R_f values.

A **B** **C**

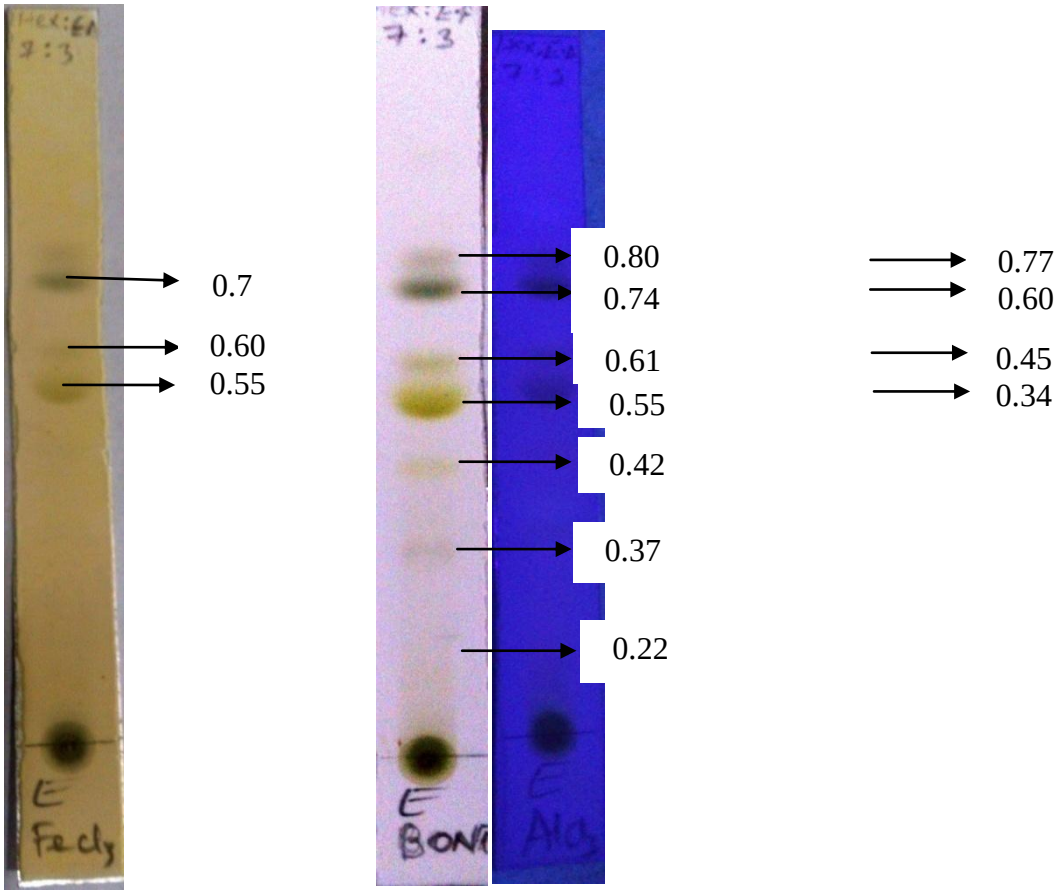
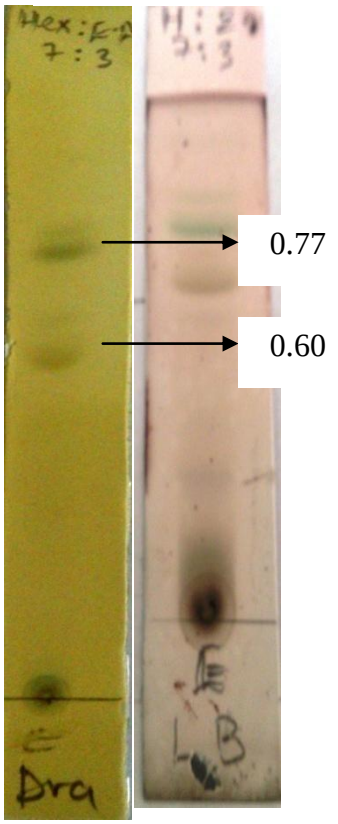


Plate VIII: Chromatograms of LAEE on precoated silica gel plate developed in Hexane - Ethylacetate (7:3) sprayed with Ferric-chloride (A) and heated at 110ºC for 2 minutes, showing 3 coloured spots,Bontragers reagent (B), Showing 7 coloured spots, andAluminium chloride reagent (C) observed under UV light at 254nm showing 4 spots with their R_f values respectively.

A **B**



0.77
0.68
0.58

0.22
0.15

Plate IX: Chromatograms of LAEE on precoated silica gel plate developed in Hexane - Ethylacetate (7:3) sprayed with Dragendorff reagent (A), Showing 2 coloured spots, Libermann-Buchard reagent (B) and heated at 110ºC for 2 minutes, Showing 5 coloured spots with their R_f values.

Table 4.9: TLC Profile of Ethylacetate Extract (EE) of *L. abyssinica* leaf sprayed with *p*-Anisaldehyde/H_2SO_4

Extract	Solvent System	No. of Spots	Colour of Spots	R_f values
Ethyl acetate	H:E (7:3)	12	Brown	0.12
			Purple	0.20
			Purple	0.34
			Grey	0.38
			Grey	0.45
			Green	0.58
			Purple	0.68
			Green	0.75
			Purple	0.79
			Purple	0.87
			Pink	0.92
			Pink	0.95

Key: H-E= Hexane - Ethylacetate

Table 4.10: TLC Profile of Ethylacetate Extract (EE) of *L. abyssinica* leaf Sprayed with Specific Detecting Reagents

Detecting Reagents	Solvent System	No. of Spots	Colour of Spots	R_f Values
Bontragers	H:E (7:3)	7	Brown, Brown, Brown, Yellow, Yellow, Yellow Blue-black	0.22, 0.37, 0.42, 0.55, 0.61, 0.74, 0.80
Dragendorff	H:E (7:3)	2	Blue-black, Blue-black	0.60, 0.77
Libermann-buchard	H:E (7:3)	5	Green, Green, Green, Black-black, Green	0.15, 0.22 0.58, 0.68 0.77
Ferric chloride	H:E (7:3)	3	Green, Blue-black, Blue-black	0.55, 0.60, 0.70
Alluminium chloride	H:E (7:3)	4	Brown, Brown, Blue-black, Blue-black	0.34, 0.45, 0.60, 0.77

Key: H-E= Hexane - Ethylacetate

4.4.3 TLC Profile of Methanol Extract (ME) of *L. abyssinica* Leaf

ME was noted to have ten (10) spots (Plate X) in chloroform: methanol (9:1) visualized with *p*-Anisaldehyde and heated at 110ºC for 2 minutes. TLC of ME developed in chloroform:

methanol (9:1) sprayed with Lieberman-Buchard, Ferric chloride and Aluminium chloride reagents showed various spots of brown, pink, blue and yellow fluorescence for Lieberman-Buchard, Ferric chloride, and Aluminium chloride spray reagents (Plate XI - XII). The $R_f$values of the spots is given in (Table 4.9 and 4.10).

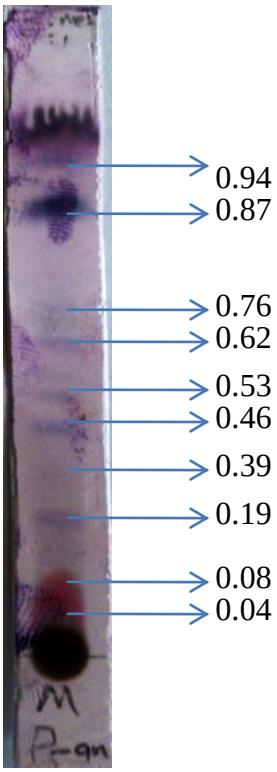


Plate X

Plate XVII: Chromatograms of Methanol extract on precoated silica gel plate developed in Chloroform-Methanol (9:1) sprayed with *p*-Anisaldehyde/H_2SO_4, Showing 10 spots with R_f values.

A

B

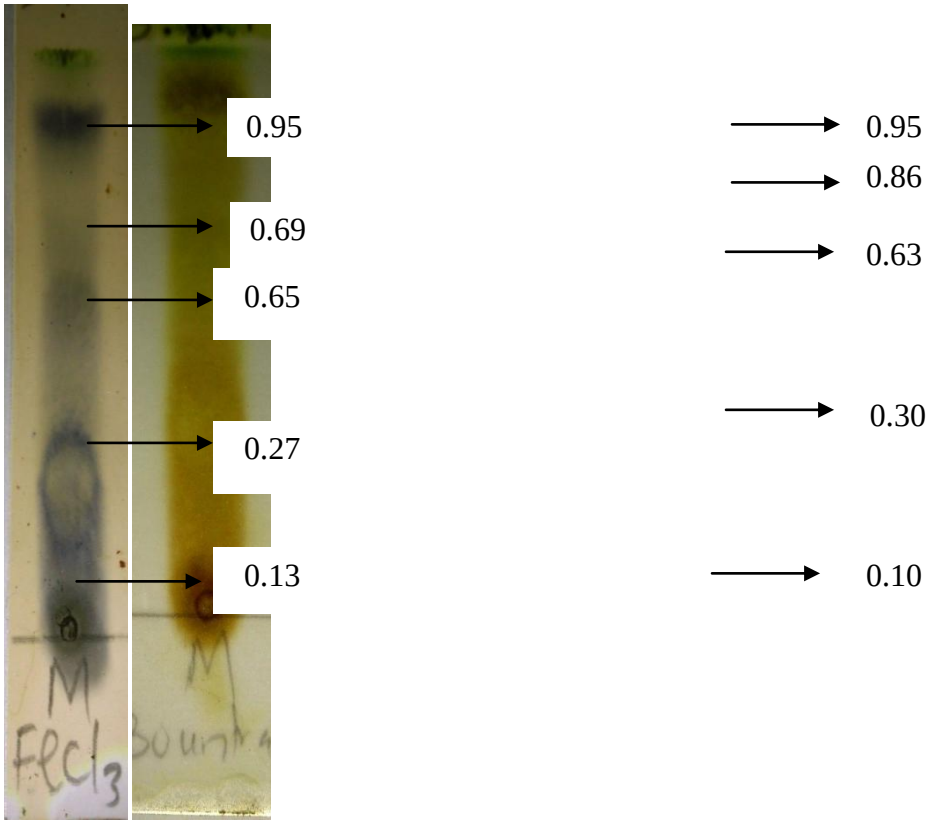
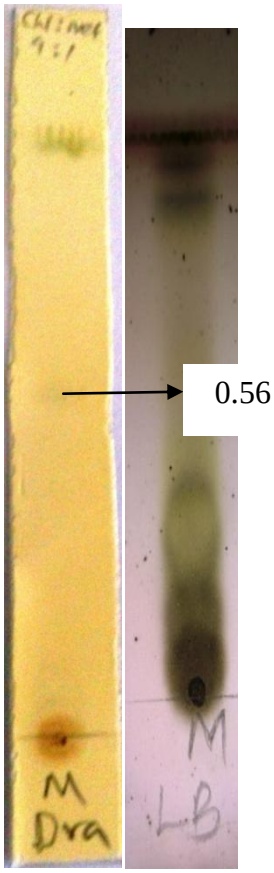


Plate XI: Chromatograms of LAME on precoated silica gel plate developed in Chloroform: Methanol (9:1) sprayed with Ferric-chloride reagent (A) and heated at 110ºC for 2 minutes, Showing 5 coloured spots, Bontragers reagent (B), Showing 5 coloured spots with their R_f values respectively.

A



B

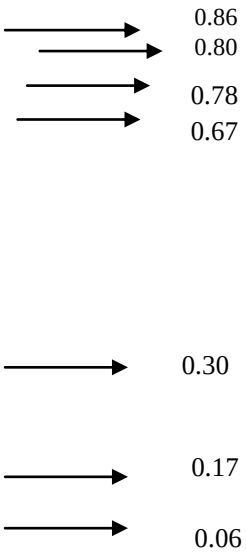


Plate XII: Chromatograms of LAME on precoated silica gel plate developed in Chloroform: Methanol (9:1) sprayed with Dragendorff reagent (A), Showing coloured spot, Libermann-Buchard reagent and heated at 110ºC for 2 minutes, Showing 7 coloured spots with their R_f values respectively.

Table 4.11: TLC Profile of Methanol Extract of *L. abyssinica* Leaf sprayed with *p*-Anisaldehyde/H_2SO_4

Extract	Solvent System	No. of Spots	Colour of Spots	R_f values
Methanol	C: M (9:1)	10	Brown	0.04
			Pink	0.08
			Grey	0.19
			Grey	0.39
			Purple	0.46
			Brown	0.53
			Brown	0.62
			Purple	0.76
			Pink	0.87
			Purple	0.94

Key: C-M= Chloroform-Methanol

Table 4.12: TLC Profile of Methanol Extract (ME) of *L. abyysinica* Leaf Sprayed with Specific Detecting Reagents

Detecting Reagents	Solvent System	No. of Spots	Colour of Spots	R_f Values
Bontragers	C:M (9:1)	5	Brown, Yellow Yellow, Yellow Blue-black	0.10, 0.30, 0.63, 0.86, 0.95
Dragendoff	C:M (9:1)	1	Green	0.56
Libermann-buchard	C:M (9:1)	7	Green, Green, Purple, Purple, Blue-black, Blue-black Blue-black	0.06, 0.17 0.30, 0.67, 0.78, 0.80, 0.86
Ferric-chloride	C:M (9:1)	5	Blue-black Blue-black, Blue-black Blue-black Green	0.13, 0.27, 0.65 0.68, 0.95

Key: C-M= Chloroform - Methanol

4.5 Results of Acute Toxicity test of *Ludwigia abyssinica* Leaf Extracts

The acute toxicity (LD_{50}) of the extracts (HE, EE and ME) of *L. abyssinica* showed that the LD_{50} were greater than 5000 mg/kg for each of the extracts. There were no death or any sign of toxicity recorded.

4.6 Results of Anti-inflammatory Activities of (Hexane, Ethylacetate and Methanolic) Extracts of *Ludwigia abyssinica* Leaves

In the distilled water treated rats, sub-planter injection of 1% carrageenan suspension produced a local edema reaching its maximum at 3hour. Meanwhile for animals treated with piroxicam 10 mg/kg, HE EE and ME the peak of edema was reached at the 2nd hour. All the extracts showed significant dose dependent anti-inflammatory activity when compared to the negative control group.The percentage inhibition in all groups is quite remarkable and increased with increase in dose (Tables 4.13, 4.14 and 4.15). At 3rd hour post-carrageenan injection, inhibition in the treated groups and that of reference group was beyond 50%. The effect of HE,EE, and ME on carrageenan induced paw oedema in rats revealed a dose dependent inhibition of the paw size when compared to the reference drug. The concentrations of the hexane extracts 500, 1000, 1500 mg/kg showed 23.6%, 26.8%, and 28.7% inhibition at the 3rd hour and 16.7%, 19.8%, and 21.3% at the 4th hour while 25.9%, 27.3%, 28.7% at 5th hourrespectively which were statistically significant $p<0.05$. This indicates dose dependent anti-inflammatory activity of the extract.

EE administered with 500, 1000 and 1500 mg/kg also showed a dose dependent percentage inhibition of 23.6%, 24.3%, 25.2% at the 3rd hour, 19.1%, 18.4%, 18.7%, at 4th hour and 14.3%, 28.8%, 29.1% at the 5th hour. While ME atsame doses were recorded to be 23.0%, 23.6%, 23.9%, at 3rd hour, at 4th hour it was recorded to be 16.5%, 24.6%, 25.8%, and 24.5%,

25.9%, 26.7% at 5th hour respectively when compared to piroxicam (26.8%), while the least percentage inhibition of (13.3%, 12.6% and 26.8%) for HE, EE, and ME was at 500 mg/kgwhen compared to the standard piroxicam (16.4%) at 2nd h.All results were in dose dependent manner.

Table 4.13: Effect of n-Hexane Extract and Percentage Inhibition of the Leaves of *L. abyssinica*on Carrageenan Induced Paw Oedema in Rats

Treatment / Dose (mg/kg)	**Mean paw Edema Diameter ± SEM (mm) and (% Inhibition of Paw Edema)**					
	0 h	**1 h**	**2 h**	**3 h**	**4 h**	**5 h**
D/W 10 ml	2.15±0.08	2.83±0.18	2.93±0.14	3.17±0.21	2.72±0.09	2.85±0.09
LAHE 500	2.05±0.05	2.36±0.09* (16.6%)	2.54±0.05 (13.3%)	2.42±0.05* (23.6%)	2.23±0.05* (16.7%)	2.09±0.04* (25.9%)
LAHE 1000	2.07±0.05	2.33±0.03* (16.6%)	2.53±0.06 (13.6%)	2.32±0.04* (26.8%)	2.18±0.04* (19.8%)	2.07±0.02* (27.3%)
LAHE 1500	2.09±0.02	2.26±0.04* (20.2%)	2.45±0.09* (16.4%)	2.26±0.04* (28.7%)	2.14±0.04* (21.3%)	2.09±0.02* (28.7%)
Piroxicam 10	2.00±0.05	2.31±0.09* (18.4%)	2.45±0.06* (16.4%)	2.32±0.07* (26.8%)	2.12±0.05* (21.0%)	2.11±0.05* (25.9%)

Data were analyzed using repeated measures ANOVA followed by Bonferroni post hoc test,* = $p<0.05$, Significant statistical decrease in mean paw edema size as compared to negative control and time zero, Values are presented as Mean±SEM (n=5), D/W = Distilled water, LAHE= *L. abyssinica* Leaves Hexane extract.

Table 4.14: Effect of Ethylacetate Extract and Percentage Inhibition of *L. abyssinica* Leaves on Carrageenan Induced Paw Oedema in Rats

Treatment/ Dose(mg/kg)	Mean paw Oedema Diameter ± SEM (mm) and % Inhibition in Parenthesis					
	0 h	1 h	2 h	3 h	4 h	5 h
D/W 10 ml	2.15±0.08	2.83±0.18	2.93±0.14	3.17±0.21	2.72±0.09	2.85±0.09
LAEE 500	1.93±0.18	2.40±0.10 (15.2%)	2.56±0.11 (12.6%)	2.42±0.08* (23.6%)	2.20±0.03* (19.1%)	2.12±0.03* (14.3%)
LAEE 1000	2.03±0.05	2.38±0.04* (16.6%)	2.51±0.19 (14.3%)	2.40±0.04* (24.3%)	2.22±0.04* (18.4%)	2.03±0.01* (28.8%)
LAEE 1500	2.02±0.06	2.39±0.06* (16.8%)	2.48±0.05* (15.9%)	2.37±0.04* (25.2%)	2.21±0.03* (18.7%)	2.02±0.06* (29.1%)
Piroxicam 10	2.00±0.05	2.31±0.09* (18.4%)	2.45±0.06* (16.4%)	2.32±0.07* (26.8%)	2.12±0.05* (21.0%)	2.11±0.05* (25.9%)

Data were analyzed using repeated measures ANOVA followed by Bonferroni post hoc test, * = $p<0.05$, Significant statistical decrease in mean paw edema size as compared to negative control and time zero, Values are presented as Mean±SEM (n=5), D/W = Distilled water, LAEE= *L. abyssinica* Leaves Ethylacetate extract.

Table 4.15: Effect of Methanol Extractand and Percentage Inhibitionof*Ludwigia abyssinica* Leaves on Carrageenan Induced Paw Oedema in Rats

Treatment / Dose (mg/kg)	**Mean paw Oedema Diameter ± SEM (mm) and Percentage Inhibition in Parenthesis**					
	0 h	**1 h**	**2 h**	**3 h**	**4 h**	**5 h**
N/S 10 ml	2.15±0.08	2.83±0.18	2.93±0.14	3.17±0.21	2.72±0.09	2.85±0.09
LAME 500	2.01±0.10	2.47±0.08 (13.4%)	2.54±0.08 (13.3%)	2.44±0.06* (23.0%)	2.27±0.05* (16.5%)	2.15±0.02* (24.5%)
LAME 1000	2.10±0.02	2.48±0.09 (12.4%)	2.46±0.07* (16.0%)	2.42±0.05* (23.6%)	2.25±0.05* (24.6%)	2.11±0.02* (25.9%)
LAME 1500	2.09±0.04	2.45±0.11 (13.4%)	2.42±0.06* (17.4%)	2.41±0.03* (23.9%)	2.18±0.02* (25.8%)	2.09±0.02* (26.7%)
Piroxicam 10	2.00±0.05	2.31±0.09* (18.4%)	2.45 ±0.06* (16.4%)	2.32±0.07* (26.8%)	2.12±0.05* (21.0%)	2.11±0.05* (25.9%)

Data were analyzed using repeated measures ANOVA followed by Bonferroni post hoc test, * = $p<0.05$, Significant statistical decrease in mean paw edema size as compared to negative control and time zero, Values are presented as Mean±SEM (n=5), D/W = Distilled water, LAME= *L. abyssinica* Leaves Methanol extract.

CHAPTER FIVE

5.0 DISCUSSION

Microscopically, the presence of anomocytic stomata on both adaxial and abaxial surfaces is commonwith most vegetable drugs. The epidermal cells were observed to be irregular in adaxial surface and polygonal in shape in the abaxial surface with anticlinal walls. The occurrence of the above mentioned characteristic features was observed among members of the Onagraceae family as reported by (Kadiri and Olowokudejo, 2010). He also stated that the geometry in all species of *Ludwigia* varies from irregular to polygonal in shape, reporting that the epidermal cells in *L. Africana* and *L. erecta* were irregular and that of *L. brenanii* was found to be polygonal in shape while the anticlinal walls pattern varies from sinuous-undulate to straight and curve in the genus.

Transverse section of the leaf of *L .abyssinica* across the midrib portion has shown that the leaves are dorsiventral, showing an upper and lower epidermis consisting of cells that were unequal in size. The vascular bundle tissues (xylem and phloem tissues) are of the conjoint type (Plate IV). This is a good distinguishing and diagnostic anatomical feature for the leaf because most dicotyledonous leaves are known to be dorsiventral (Dutta, 2003). These characteristics are observed among members of the Onagraceae (Kadiri and Olowokudejo, 2010). Anatomical features of the internal structures of plant drugs provides an important diagnostic features for the identification of both entire and powdered crude drugs and detection of adulterants in plant materials (Ghani, 1990). Macro and microscopical evaluation of crude drugs are aimed at identification of right variety and search for adulterant in plant materials (WHO, 2000).

Quantitative microscopy was used to study microscopic characters not easily characterized by general microscopy. The average for stomatal number, stomatal index, palisade ratio, vein islets and veinlet termination numbers have all been investigated and reported for the first time from the leaves of this plant. Stomatal number on the upper surface was (6.80 – 8.00 – 9.20) and lower surface was found to be (17.00 –20.00– 23.00). Unfortunately, the limits of the numbers are wide and have been shown to vary quite widely according to the environmental conditions in which the plant was grown (Sunita *et al.*, 2010). Evans stated that early investigation by Timmerman indicated the stomatal numbers are useless in distinguishing between closely allied species (Evans, 2009). The stomatal index of upper surface was found to be (10.30 – 12 .12– 13.94) and that of the lower surface was found to be (14.28 – 16.80– 19.32). This result is in line with the statement of Kadiri and Olowokudejo (2010), who stated that the stomatal index has been useful in diagnosis of some of the species in this genus; generally it ranges from 8.7% to 36.7%. The stomatal index is a more useful value and supportive evidence which when taken together with other factors, can make a positive identification possible and it is less subjected to variations with external conditions (Brain and Turner, 1975).

The vein islets number (10.20 –12.00 – 13.80) and veinlet termination number (9.35 – 11.00 - 12.65) of the plant were of diagnostic importance. The vein islet and termination number may appear to vary according to the preliminary treatment the leaf has received (Evans, 2009).

The palisade ratio (11.05– 13.00 - 14.95) observed is important. Palisade ratio can be determined even on quite fine powders unlike the veinlet termination and vein islet numbers which require fresh and large portions of leaves and are preferably determined on a

particular part of a leaf. For these reasons, it is an important parameter that is used primarily for evaluating intact leaves rather than powder (WHO, 2011).

Chemomicroscopical studies of the powdered leaves of *L. abyssinica* were found to have cellulose cell, lignin, calcium oxalate, tannins, cutin, starch and mucilage. The structures, chemical reactions and colour changes are most valuable in the identification of powdered drug as their identification is largely based on the form, the presence or absence of certain cell wall types and cell inclusions (Eggeling *et al.,* 2000). Most of these cell wall materials such as cellulose, ligning and cutin perform the functions of protection, insulation, strengthening and reinforcing vascular plants without which they will topple over (Graca *et al.,*2015). Cell inclusions such as starch, lipids, and proteins are food storage in the plant.

The Physicochemical constants are useful criteria to judge the identity and purity of crude drug (WH0, 2011). It also indicates presence of various impurities like carbonate, oxalate and silicate in plant materials (Kaneria and Chanda, 2011). Quantitative evaluation is an important parameter in setting standard of crude drugs and the physical constant parameters could be useful in detecting any adulterant in the drug (Musa, 2005). Moisture content (7.00%) is not high which indicated less chances of microbial degradation of the drug during storage. The general requirement of moisture content in crude drug is that, it should not be more than 14% (B. H. P, 1990) and the value obtained in this research work was within the accepted range. Moisture is considered an adulterant because of its added weight as well as the fact that excess moisture is conducive to the promotion of mold and bacterial growth, up to 5% is usually not considered excessive and low moisture content in a crude drugs suggest better stability against degradation of product (WHO, 2011).

Total ash value (7.80%) represents both the physiological and non-physiological ash from the plant. The non-physiological ash is an indication of inorganic residues after the plant drug is incinerated. The acid insoluble ash values (2.33%) obtained in this study indicated that the plant was in good physiological condition and it contained little extraneous matter such as sand, silica and soil. The total ash value is used as criteria to judge the identity and purity of drugs (WHO, 1996; Prasad *et al*., 2012).

Estimation of extractive values determines the amount of the active constituents in a given amount of plant material when extracted with a particular solvent. The extractions of any crude drug with a particular solvent yield a solution containing different phytoconstituents. The compositions of these phytoconstituents depend upon the nature of the drug and the solvent used. It also gives an indication whether the crude drug is exhausted or not (Tatiya *et al.*, 2012).

This study indicated that the water had high extractive value of (19.7% w/w) compared to ethanol which had extractive value of (17.0% w/w). The resultimplies that extraction of *L. abyssinica*leaf with water have the ability to extract more constituents than ethanol. This is in agreement with the work of Ajazuddin and Shailendra, (2010) who stated that havinghigher water extractive value implies that: is a better solvent of extraction than ethanol.

Despite that water is a universal solvent and its use primarily as solvent by traditional healers, alcohol is still given preference in terms of choice of solvent when it comes to medicinal plant researches. The choice of solvent in a research involving plants depend on so many factors among which include the diversity of different phytochemicals to be extracted and also what is intended with the extract. Methanol was chosen as part of the solvent of

extraction in this study. This is due to higher activities of methanol extracts (Tiwari and Mishra, 2011).

Powdered *L. abyssinica* leaf was extracted with n-hexane, ethylacetate and methanol solvents in order of increasing polarity in a Soxhlet apparatus with the aim of separating its components on the basis of polarity, produced the following yield in % w/w, n-hexane; 2.11, ethylacetate; 3.2 and methanol extract was 15.25. The result of this study revealed that methanol had the highest yieldwhich was followed by ethylacetate and then n-hexane (Table 4.5). These could be attributed to the ability of highly polar solvent to attract more of the phytochemical constituents present in the leaf; it is also an indication that most of the constituents present in the leaf are more soluble in methanol than the other solvents.

Preliminary phytochemical screening gives a brief idea about the qualitative nature of active phytochemical constituents present in plant extracts, which will help the future investigators regarding the selection of the particular extract for further investigation or isolating the active principle (Mishra *et al.*, 2010).

The findings of phytochemical analysis of the leaf extracts had revealed the presence of saponins (steroids/triterpenes), alkaloids, tannins, flavonoids, cardiac glycosides,and carbohydrate in the methanol extract, this is in line with the work of Firoj *et al.,* (2005) and Minal *et al.,* (2012) who reported that methanolic extract of *Ludwigia adscendens* leaf and stems contain flavonoids, terpenes, phenols, tannins, triterpenoids, carbohydrate and alkaloids.However, ethylacetate extract revealed the presence of phenolic compound, steroids and triterpenes, and cardiac glycosides while hexane extract showed the presence of steroids and triterpenes only.

The information on the presence or absence and the type of phytochemical constituents especially the secondary metabolites are useful taxonomic keys in identifying a particular species and distinguishing it from a related species, thus helping in the delimitation of taxa (Jonathan and Tom, 2008).

Each plant family, genus, and species produces a characteristic mix of these chemicals, and they can sometimes be used as taxonomic characters in classifying plants (Biology Encyclopaedia, 2015)**.** In addition, certain terpenoids, flavonoids, phenolics, cyanogenic glycosides and non–protein amino acids are illustrated to be of systematic use in particular cases (Jonathan and Tom, 2008)**.** This system of plant classification is called chemotaxonomy.

Thin layer chromatographic profiling of n-hexane, ethylacetate and methanol from *L. abyssinica* leaf in different solvent systems at different ratios gave various degree of separation. Thechromatogram of hexane extract was developed in hexane: ethylacetate (9:1), this revealed 12 spots and colour observed were green and purple (Table 4.5). ethylacetate extract developed in hexane: ethylacetate (7:3), 12 spots were seen. The colous observed were purple, brown and pink (Table 4.7) while the chromatogram of methanol extract was developed in chloroform: methanol (9:1), revealed a clear separation with 10 spots which are brown and pink colours (Table 4.9). All these chromatograms were sprayed with p-anisaldehydebefore visualization.The successful separation of phytochemicals by chromatographic technique depends upon suitable solvent system which is ideal for each target compounds (Ito, 2005). More chromatograms of all the extracts were developed for specific spraying, all the extracts tested positive to Libermann-Buchard reagent which revealed the presence of steroids and triterpenoids, however, ethylacetate and methanolic extract tested positive to the following reagents; ferric chloride for phenolic compounds,

bontragers for anthraquinone, and alluminium chloride for flavonoids. Thin layer chromatographic analysis is a simple and cheap method of detection of active constituents in plants due to its good selectivity, providing convincing results (Patra *et al.,* 2012), this is considered a reliable technique for qualitative phytochemical screening of plants active constituents. Using the TLC technique, organic compounds can be separated based on their molecular weight and polarity. Haugland and Johnson, (1999) described the technique as an ideal method for the separation of natural substances and have huge applications in analyzing biological and chemical samples for identification and determination of their composition. Phenolic compounds such as tannins and flavonoids possess diverse pharmacological properties such as anti-inflammatory, anti-oxidant and antibacterial activities (Sen *et al.,* 2009). In this study, phenolic compounds, unsaturated steroids, triterpenes and flavonoids were observed to be the most bioactive constituents in *L. abyssinica* leaf. However, these constituents are commonly found in other species in the genus *Ludwigia* as reported by Oyedeji *et al.,* (2014).

In order to determine the safety margin of drugs and plant products for human use, toxicological evaluation is usually carried out in experimental animals using Lorke's method to predict toxicity and to provide guidelines for selecting a "safe'' dose in animals and also used to estimate the therapeutic index (LD_{50}/ED_{50}) of drugs (Olson *et al.,* 2000; Rang *et al.,* 2003; Maikai *et al.,* 2008). In this study, median lethal dose (LD_{50}) of the extracts (hexane, ethyl acetate and methanol) of the leaf of *L. abyssinica* was carried out orally in rats. The LD_{50} was found to be greater than 5000 mg/kg when administered orally in rats. These studies showed the extracts of *L. abyssinica* leaf are practically non-toxic when administered using the oral route. This is based on the toxicity classification which states that substances

with LD_{50} values of 5000 to 15,000 mg/kg body weight are practically non-toxic (Loomis and Hayes, 1996).

Carrageenan-induced paw oedema is a suitable model for evaluating anti-inflammatory activities of natural products and is a significant predictive test for anti-inflammatory agents acting by mediators of acute inflammation (Panthong *et al.,* 2003; Sawadogo *et al*., 2006; Ndebia *et al.,* 2007).

The result of anti-inflammatory study showed that hexane and ethylacetate extract at doses 500, 1000 and 1500 mg/kg body weight were significant ($p<0.05$) and produce anti-inflammatory effect (at the 1^{st} to 5^{th} hour), and reached the peak of inflammation at the 2^{nd} hr while methanol extract showed its effect at (2^{nd} to 5^{th} hour) in comparison with the distilled water (negative control) group.

Also all the treatment groups (HE, EE, and ME) showed that the activity increases with increase in dosage at the $3^{rd}$to 5^{th} hour. Thus the effect was dose dependent. Therefore the results of this study are indication that all the extracts can be effective in acute inflammatory disorders. It also showed that the edema paw size reduction of all the extracts at all doses increases with increase in time.

The probable mechanism of action of carrageenan-induced inflammation is biphasic. The first phase is attributed to the release of histamine, serotonin and kinins in the 1^{st} h, while the second phase is attributed to the release of prostaglandins, bradykinins and lysosomal enzymes in 2-3 h (Gupta *et al*., 2006; Nwaehujor *et al.,* 2014). The extracts significantly inhibited the carrageenan-induced inflammation in the 1^{st} - 5^{th} h. Therefore mechanism of action may be due to inhibition of histamine or prostaglandin synthesis. So, it is possible that

The constituents found present in the extracts such as steroids and triterpenes, saponins and flavanoids might be influencing both stages of carrageenan. Usually most anti-inflammatory drugs produced antipyretic action through the inhibition of prostaglandin (Hayare *et al.*, 2000).

HE at all doses showed significant ($p<0.05$) decrease in mean paw oedema size at the 5^{th} hour, whereas EE at 500 and 1500 mg/kg showed significant reduction at the 5^{th} hour and ME at dose 1500 mg/kg also showed a significant ($p<0.05$) reduction in paw size when compared to the distilled water group (negative control)and standard drug piroxicamat 5^{th} hour (Table 4.11, 4.12, and 4.13). This implied that HE was found to have highest activity than EE and ME. This result therefore, may be due to the presence of steroid and triterpenes present in the hexane extract as discussed in the phytochemical screening result. Anti-inflammatory activity of two triterpene saponins from *Quercus imbircaria* have been reported (Mona *et al.*, 2014).Plants have different kinds of secondary metabolites such as flavonoids, tannins, saponins and these have been found to have anti-inflammatory activities both *in vivo* and *in vitro* (Mona *et al.*, 2014).

Thus the above inhibitory effect of *L. abyssinica* on inflammation in this study could be due to the presence of phytoconstituents such as flavonoids, other phenolic compounds and saponins in the extracts and this is in line with statement of Kiss *et al.*, (2011); Parades *et al.*, (2013) who stated that extracts from the different members of the Onagraceae family were reported by researchers to have diverse biological effects and these properties are ascribed to the high content of polyphenols, such as tannins. Also analgesic and anti-inflammatory effects have been observed and reported in flavonoids as well as tannins (Ahmadiani *etal.*, 2000). Flavonoids have also been proven to have anti-inflammatory effects.

Though over 4000 different flavonoids have been documented, these polyphenolic compounds were said to have a variety of biological effects in numerous mammalian cell systems both *in-vitro* and *in-vivo* (Guardia *et al.,* 2001; Owoyele, 2015).

Different types of saponins isolated from plants like *Phytolocca americana, Madhucalongifolia* and *Carissa edulis* have reportedly exhibited significant anti-inflammatory activity (Hassan *et al.,* 2010; Shehu *et al.,* 2016). Steroids have also been reported to inhibit inflammation against carrageenan (Ravichandran and Panneerselvam, 2004).

CHAPTER SIX

6.0 SUMMARY, CONCLUSION AND RECOMMENDATION

6.1 Summary

Some pharmacognostic standards of the leaves of *Ludwigia abyssinica* were established for the first time in this study to the best of our knowledge and these data could be used as diagnostic tool for the standardization and proper identification of this medicinal plant.

The research began with the microscopic examination of the transverse section, upper and lower epidermis of *L. abyssinica* leaves which revealed some prominent features like the anomocytic stomata on both upper (18.35 µm x13.60µm) and lower (14.13 µm x 9.74 µm) epidermis. Chemomicroscopical features of powdered leave of *L. abyssinica* revealed the presence of cellulose cell wall, lignified cell wall, mucilage, tannins, cutin and calcium oxalate crystals.

The physical parameters (%w/w) of the powdered leaves of *L. abyssinica*was found include moisture content (7.0 %), total ash value (7.8 %), acid insoluble ash (2.33 %), water soluble ash (4.67 %), ethanol extractive value (17.00 %) and water extractive value (19.70 %).

Phytochemical analysis of the leaf extracts revealed the presence of some secondary metabolites namely alkaloids, tannins, flavonoids, carbohydrate, cardiac glycosides, saponins, and steroids/triterpenes.

Thin layer chromatographic analysis visualized with specific reagents confirmed the presence of steroids/triterpenes, flavonoids and other phenolic compounds in the extracts.

The median lethal dose (LD_{50}) of the extracts was found to be greater than 5000 mg/kg when administered orally in rats and considered practically non-toxic.

The leaf extracts (Hexane, Ethylacetate and Methanol) of *L. abyssinica* at doses 500, 1000 and 1500 mg/kg produced significant anti-inflammatory effect starting from the 1^{st}- 5^{th} hour in HE and EE while in ME the effect started from 2^{nd} – 5^{th} h with the effect being dose dependent at the 3^{rd} - 5^{th} hour. Hexane extract was observed to have best activity compared to ethylacetate and methanol extract.

6.2 Conclusion

The present study hasestablished:

i) Microscopic, chemomicroscopic, physicochemical constants and phytochemical analysis of*L. abyssinica*leaf that provided information which are useful in the preparation of the monograph of the plant.

ii) The thin layer chromatographic profiles of the leaf extracts of the plant have shown that it contains chiefly flavonoids, carbohydrates, steroids/triterpenes and other phenolic compounds.

iii) Also the extracts of *L. abyssinica*were observed to significantly showed anti-inflammatory activity with the highest percentage inhibition at dose 1500mg/kg for all extracts, which support the basis of its use in traditional medicine in the management of inflammation and related inflammatory disorders.

The results can be usefulfor the proper identification, authentication and compilation of the monograph of *L. abyssinica*.

6.3 Recommendations

- It is recommended that further study needs to be done on *L. abyssinica* so as to isolate and identify the active compounds responsible for anti-inflammatory activity in the plant and formulate a standardized herbal preparation
- Sub-acute and chronic toxicity test should be carried out in order to determine the long-term effects of the extract

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APPENDIX A

a). Determination of moisture content of powdered leaves of *L. abyssinica*

3g of the powdered plant material was used

Description	1	2	3
Constant weight of crucible (g)	44.58	46.72	44.32
Initial weight of powder (g)	47.58	49.72	47.32
Final weight of powder	47.37	49.51	47.11
Loss in weight (g)	0.21	0.21	0.21
Moisture content (%)	7.00	7.00	7.00
Average mean (%)		7.00	

Sample calculation

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

$$\%\ Moisture\ content = \frac{(47.58 - 47.37)}{3}\ X\ 100$$

$$= 7.0\ \%w/w$$

APPENDIX B

b). Determination of Ash Value of powdered leaves of *L. abyssinica*

Description	1	2	3
Constant weight of crucible (g)	39.17	50.72	39.17
Weight of crucible and content (g)	41.17	52.72	41.17
Weight of crucible and Ash (g)	39.33	50.72	39.34
Weight of Ash (g)	0.16	0.14	0.17
Ash Value (%)	8.0	7.00	8.50
Average mean (%)		7.80	

Sample calculation

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

Weight of Residue = 39.33 – 30.17

= 0.16

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

$$\text{Ash Value} = \frac{0.16}{2} \; X \; 100$$

= 8.0 % w/w

APPENDIX C

c) Determination of Acid insoluble Ash of powdered Leaves of *L. abyssinica*

Description	1	2	3
Constant weight of crucible (g)	39.16	50.72	39.16
Weight of crucible and Acid insoluble ash (g)	39.21	50.76	39.21
Weight of Acid insoluble ash (g)	0.05	0.04	0.05
Acid Insoluble Ash Value (%)	2.50	2.00	2.50
Average mean (%)		2.33	

Sample calculation

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

$$\text{Acid Insoluble Ash value} = \frac{0.05}{2} X\ 100$$

$$= 2.5\ \%w/w$$

APPENDIX D

d). Determination of water–soluble extractive value of Leaves of *L. abyssinica*

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

Description	1	2	3
Constant weight of dish (g)	144.75	64.76	84.24
Weight of crucible and content after heating (g)	144.95	64.96	84.43
Water extractive content (g)	0.20	0.20	0.19
Water extractive Value (%)	20.00	20.00	19.00
Average mean (%)		19.70	

Sample calculation

$$\text{Water extractive value} = \frac{\text{Wt of dish \& content after heat (g)} - \text{Constant wt.of dish (g) X 4}}{\text{Initial weight of drug}} X\ 100$$

$$\text{Water extractive value} = \frac{(144.95 - 144.75)\ \text{X}\ 4}{4} X\ 100$$

$$= 20.0\ \%\text{w/w}$$

APPENDIX E

e). Determination of alcohol – solubleextractive value of Leaves of *L. abyssinica*

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

Description	1	2	3
Constant weight of dish (g)	84.24	64.76	123.08
Weight of dish and content after heating (g)	84.42	64.92	123.24
Alcohol extractive content (g)	0.18	0.17	0.16
Alcohol extractive Value (%)	18.00	17.00	16.00
Average mean (%)		17.00	

Sample calculation

$$\text{Alcohol extractive value} = \frac{\text{Wt of dish \& content after heat (g)} - \text{Constant wt.of dish (g) X 4}}{\text{Initial weight of drug}} X\ 100$$

$$\text{Alcohol extractive value} = \frac{(84.42 - 84.24)\ \text{X}\ 4}{4} X\ 100$$

$$= 18.00\ \%\text{w/w}$$