

STUDY OF ANTIMICROBIAL, ANALGESIC AND TOXIC PROPERTIES OF *VERNONIA*
HYMENOLEPIS (A. Rich).

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I dedicate this research work to my beloved Parents Mr. Lawrence Okindo and Mrs. Aschah Okindo and my grandparents who continually supported me both financially and morally throughout my education and during my research.

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LIST OF ABBREVIATIONS

W H O	World Health Organization
AU	African Union
LD ₅₀	Median and Lethal dose
Fig	Figure
BC	Before Christ
AD	Anno domini
USD	United States Dollar
USA	United State of America
ATCC	American Type Culture Collection
DCM	Dichloromethane
ATC	Acute Oral Toxic Class
OECD	Organization for Economic Co-operation and Development
GHS	Global Harmonized Classification System
ED	Effective dose
DMSO	Dimethyl sulfoxide
CLSI	Clinical Laboratory Standards Institute

ANOVA	One-way analysis of variance
MIC	Minimum inhibitory concentration
MBC	Minimum bactericidal concentration
W.H.O	World Health Organization
I.A.S.P	International association for the study of pain
M.H.R.A	Medicine and Healthcare products Regulatory Agency
S.E.M	Standard error of mean
I.P	Intraperitoneal
P.O	Oraly

ABSTRACT

Traditional medicines have been used since ancient time for treatment of various ailments including dental diseases. Inadequacy of dental facilities and high cost of modern treatment has resulted to increased use of alternative forms of treatment. *Vernonia hymenolepis* is a species in the genus *Vernonia* that has widely been used traditionally by herbalist and communities in Trans Nzoia County for treatment of various infections including toothache. However the plants pharmacological and toxicology efficacy has not been adequately established. The aim of the study is to evaluate the antimicrobial, analgesic properties and toxicity of *Vernonia hymenolepis* leaves in order to elucidate its use for the treatment of toothache. Leaves of *Vernonia hymenolepis* were collected from Trans Nzoia County, Kenya and identified at the University of Nairobi Herbarium in the Department of Botany School of Biological Sciences and voucher specimens deposited (RO2011/001). It was shade dried, ground and organic and water extraction carried out. Antimicrobial test was done to determine minimum inhibitory concentration against standard bacteria culture *Staphylococcus aureus* (ATCC 25923), *Pseudomonas aeruginosa* (ATCC 27853), *Escherichia coli* (ATCC 25922), *Bacillus cereus* (ATCC 11778) and *Candida albicans*, using standard microbial techniques. Analgesic property at a dose of 100 mg/kg was determined using formalin test. Cytotoxicity of the plant extracts were determined by use of Brine Shrimp lethality test with the concentration of 10-1000 ppm. The lethal concentration (LC₅₀) was determined using Finney computer program. The Acute Oral Toxicity Testing was performed as described by the Organization for Economic Co-operation and Development (OECD) guideline. Aqueous extract and dichloromethane /methanol extract yielded 32% in powder form and 21.5% in paste form respectively. Aqueous

extract was inhibitory to only *Staphylococcus aureus* at dose of 400 mg/ml of plant extract. While DCM/M extract had inhibitory effect against *Staphylococcus aureus* at a dose of 100 mg/ml, *Pseudomonas aeruginosa* and *Escherichia coli* both at a dose of 400 mg/ml and *Bacillus cereus* at a dose of 200 mg/ml.

The plant extracts showed antinociceptive activities for early phase of aqueous $p < 0.0005$ and organic extract $p < 0.05$, while the late phase of both extracts had $p < 0.01$. It significantly reduced the time spent in pain behavior in both the early phase ($56.92 \pm 7.013s$ verses $143.75 \pm 11.9s$ control) and late phase ($32.33 \pm 1.97s$ verses $84 \pm 3.19s$ control) for aqueous extract compared to early phase (74.33 ± 19.4 verses 111.17 ± 11.4) and in late phase (9.907 ± 1.59 verses $62 \pm 17.91s$ control) for dichloromethane/methanol extract of formalin test. Both the organic and aqueous extracts were in category 5 of Global Harmonization System GHS ($>2000-5000$ mg/kg b.w.t) of acute oral toxic class method with LD_{50} of 2500 mg/ml and thus not toxic at the dose of 2500 mg/ml. Dichloromethane: methanol extract (1:1) had (LC_{50}) of 482 $\mu\text{g/ml}$ and water extract (LC_{50}) of 492 $\mu\text{g/ml}$ which indicate moderate cytotoxicity.

In conclusion *Vernonia hymenolepis* has antimicrobial and antinociceptive activities. However, the plant leaves exhibited more analgesic potency compared to antimicrobial activity. Both water and Dichloromethane/Methanol extracts have moderate cytotoxicity. The plant leaves is also not toxic at the dose of 2500 mg/ml. The result validates the use of these plants for toothache by herbalists in Trans Nzoia County, Kenya. However, further studies may provide information about phytochemistry, mechanism of action of the plant and shelf life.

CHAPTER ONE: INTRODUCTION

1.1 Background information

Ethnomedicine is a field of study that involves healthcare systems that includes beliefs and practices that are related to various diseases and health. It uses the indigenous and cultural development and not modern medicine. The World Health Organization does recognize the use of traditional medicine as a primary health care service that involves the people either individually or collectively in the planning and implementation of their healthcare needs (WHO, 2009). It therefore promotes and advocates its usage alongside modern health care systems around the world. It defines traditional medicine as the sum total of knowledge, skills and practices based on the theories, beliefs and experiences that are used to maintain health, (WHO, 2008). Alternative medicine or Complementary medicines are the terms used to describe the users of traditional medicine which includes herbal materials, herbal preparations and finished herbal products that possess novel active ingredients (WHO, 2008).

The plant kingdom contains much of untapped reservoir of new and exciting chemical compounds that contain lead compounds for drug development (Deraniyagala *et al.*, 2003). Plants are medicinal agents even as crude unprocessed materials and are considered to be having vital healing energy that exceeds the measurable effects of their physical properties (William *et al.*, 2005).

There are approximately 60,000 plant species in Africa, which represents roughly a quarter of the world plant population. Despite the vast wealth and endemicity of the Africa plant biodiversity and associated cultures, Africa has only contributed 83 of the world's 1100 leading commercial medicinal plants. In part of African countries, up to 90% of the population still relies mostly on plants as a source of medicines (Hostettmann *et al.*, 2000, Miaron, 2004). According

to World Health Organization, out of a population of 700 million of sub Saharan Africa only less than 30% have access to modern health care and pharmaceuticals. The remaining 70% still depend entirely on traditional herbal remedies (dharani *et al.*, 2013). In Kenya is reported by the WHO that at least 80% of the Kenyan population has used herbal medicine before (Business Daily Newspaper, 2010).

Plants provide an option of alternative drugs against resistant pathogens since the plant kingdom provides a useful source of principal compounds of novel structure (Hostettmann *et al.*, 1994). Herbal medicine has been used together with conventional drugs and other products like tooth-paste so as to counteract the antimicrobial resistance of conventional medicine and products in oral healthcare systems. Oral health forms an integral part of general health and oral conditions affect all age groups and are universal in prevalence (WHO, 2009).

Vernonia is the largest genus of the tribe *Vernonieae* which has close to 1000 species and it occurs mainly in Africa. More than 300 species have been described from Africa (Isawumi, 1993). A total of 12 *Vernonia* species were identified to be used in ethnoveterinary medicine while 2 species are used in self medication practices by chimpanzees and gorillas (Ngeh *et al.*, 2013).

Vernonia hymenolepis has been widely used in herbal medicine in Kenya and more especially in communities in Trans Nzoia and Marakwet for treatment of malaria, wound healing, stomach conditions like constipation and for the treatment of oral diseases (Kokwaro, 1993). The oral cavity contains a wide variety of oral bacteria, but just a few specific bacteria are believed to cause oral infections and this includes; *Streptococcus mutans*, *Lactobacillus acidophilus*, *Actinomyces viscosus*, *Nocardia* species and *Streptococcus mutans* (Bartelstone *et al.*, 1947). Based on herbalist information on the use of *Vernonia hymenolepis*, the plant have been used as traditional herb by herbalists in communities of Trans Nzoia County in treatment of various

infections including toothache. However, its efficacy and toxicity have not been established. The purpose of this study was to evaluate the anti-microbial, analgesic activity and toxicity and of the plant.

1.2 Research problem and justification

Oral health problems affect people of all age groups especially in rural communities because of lack of availability of good oral health services. This is due to high cost of tooth paste and resistance of conventional antimicrobials that are available for treatment of oral conditions.

The widespread poor oral health and poor oral hygiene practices implies that there is a high demand of oral health care. These have resulted to many people in rural community seeking for alternative treatment which is herbal medicine. Traditional healers do not understand the scientific rationale behind their medicines, but they know from personal experience that these plants can be highly effective if used at therapeutic doses. Scientists have a better understanding of cell biology and hence have a better position to explain the healing powers of plants.

Many people in various communities have used plants and other natural products for treatment of various ailments including oral diseases. In this study the anti-microbial, analgesic and toxicity effects of *Vernonia hymenolepis* one of the medicinal plant used in rural communities in Trans Nzoia County in Kenya for treatment of toothache will be determined.

1.3 Objectives:

1.3.1 General objective

To determine the antimicrobial, analgesic and safety of *Vernonia hymenolepis* obtained from Trans Nzoia County.

1.3.2 Specific objective

1. To determine antimicrobial activity of aqueous and DCM/M extracts of *Vernonia hymenolepis* against selected bacterial isolates.
2. To determine the analgesic properties of *Vernonia hymenolepis* leaves for aqueous and dichloromethane/Methanol extract.
3. To screen *Vernonia hymenolepis* leaves extracts for cytotoxicity using brine shrimps lethality test for and to determine acute oral toxicity.

CHAPTER TWO: LITERATURE REVIEW

2.1 Background on the usage of medicinal plants

Since ancient time nature has bestowed its benefits on human being by providing food, shelter, medicine and other resources (Cragg and Newman 2005). Traditional medicine includes skills, knowledge and practices that are based on the theories from various cultures that are used in diagnosis, treatment and prevention of various diseases (WHO, 2009). Disruption of biodiversity, loss of forest and plant resources has resulted in less resilience in our ecosystem causing it to become more vulnerable to shocks and disturbances. Human health extremely depends on the quality of the environment in which we live in. For people to be healthy, good environments and a proper medical care system are required to provide eco-friendly, bio-friendly, cost effective and relatively safe treatments. Increase in pollution, poor lifestyles, stress, loss of traditional medicine practices and loss of plant biodiversity has increased and has resulted in alarming disturbance in the structure and function of nature (Alves and Rosa, 2007 and Dubey *et al.*, 2004).

In the 21st century traditional medicine is gaining importance in mainstream healthcare since most people seek relatively safe remedies and approaches to healthcare. The need for herbal medicines, herbal health products, herbal pharmaceuticals, nutraceuticals, food supplements and herbal cosmetics is increasing globally due to the growing recognition of these products as they are thought to be non-toxic, have less side effects, good compatibility with physiological flora, and are easily available at affordable prices (Dubey *et al.*, 2004 and Sharma and Sharma 2010). Medicinal plants form an important component of traditional medicinal systems and this has been documented in the Indian, Chinese, Egyptian, Greek, and Roman medicinal systems for about 5000 years. Traditional herbal medicine has also been practiced from ancient times in

American and Arabian countries, as well as Japan. Medicinal plants provide healthcare system that imparts a significant role in economic development.

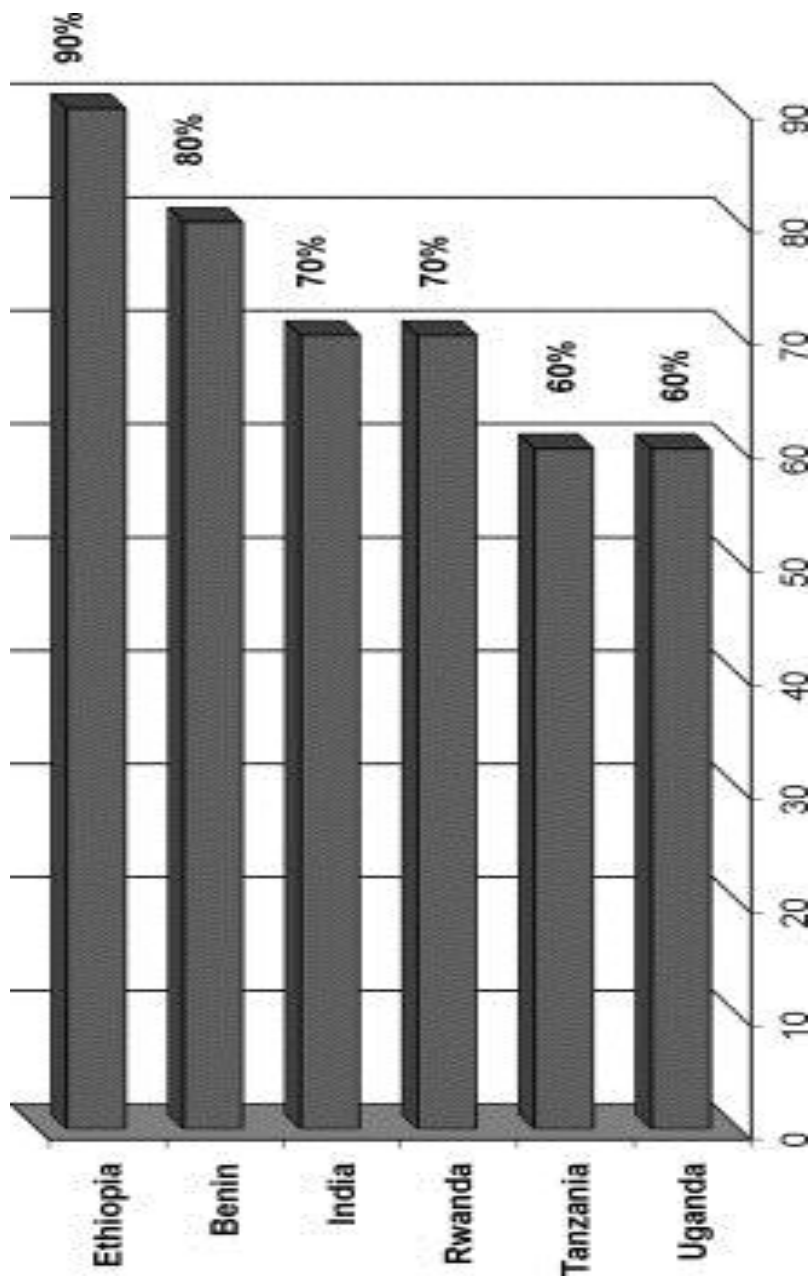
Many valuable medicinal plants are on the verge of extinction due to degradation of biodiversity (Alves and Rosa, 2007). Maintaining plant biodiversity and proper sustainable use will build a healthy and economic society. It was estimated that approximately 5–15% of the total 250,000 species of higher plants that have been scientifically investigated to have novel bioactive ingredients that are yet to be tapped (Cragg and Newman, 2005).

Plant products and herbal medicine play a vital role in the healthcare system, mainly in rural areas of developing countries. Medicinal plants have a great history as the source noble bioactivity, for example reserpine, deserpidine, rescinnamine, vinblastine, vincristine, codeine, morphine, etoposide, guggulsterone, teniposide, nabilone, plaunotol, z-guggulsterone, lectinan, artemisinin and ginkgolides, which have been incorporated into modern medicine (Kamboj, 2000; Mukherjee *et al.*, 2010 ; Verma and Singh 2008). Plant-derived products have become a primary choice for biological and pharmacological research and have served as leads for the development of synthetic drugs. It is estimated that nearly 75% of the herbal drugs used worldwide were incorporated from indigenous medicine (Verma and Singh, 2008).

Investigation and modification of herbal drugs takes comparatively much less time and expenses compared to synthetic drugs (Wakdikar, 2004). The use of synthetic drugs could sometimes be unwanted and can lead to unpredictable side effects which can be more dangerous than the diseases they claim to treat. Generally natural drugs are less toxic and produce fewer side effects than synthetic drugs. In contrast with synthetic medicine, herbal medicines contain natural substances that support health and alleviate illness. It's believed that herbal products are

relatively safer, possess better patient tolerance, relatively less expensive and globally competitive.

The use of plants as food or herbal products in the diet as supplements has also proved effective in keeping the body healthy and free from disease (Sen *et al.*, 2010). Dietary supplements from plant sources are now gaining importance. The percentage uses of traditional medicine for primary healthcare in some developing countries are given in Fig 2. 1 (W HO, 2002).



African countries

Figure 2.1: Percent use of herbal medicine for primary health care in some developing countries.

Adapted from WHO 13/July/2013

2.2 Types of traditional medicine

Modern commercialized medicine originates from ancient medicine and it is likely that many important new remedies will be discovered and commercialized in the future by following the leads provided by indigenous knowledge and experiences. Traditional medicine in some countries is often associated with witchcraft and superstition since people did not have the scientific basis to validate the curative action of plants (WHO, 2002).

The concept of the Doctrine of Signatures on healing cultures is based on the appearance of plants which gives clues to their medicinal properties. For example, red juice and sap is associated with blood and menstrual ailments, yellow flowers with bile and jaundice, the human shape of certain roots with the female form of fertility etc. This concept showed significant relationships for example, *Chelidonium majus* which contains yellow flowers and its alkaloid latex which is yellow in color has been used successfully to treat jaundice (Gurib-Fakim, 2006)

2.2.1 African traditional medicine

African traditional medicine is the oldest and with the most biodiversity in all systems of medicine in both biological and cultural diversity in healing practices. However, the systems of medicines are poorly recorded even up-to date. There is need of documentation of medicinal uses of African plants though there is a rapid loss of the natural habitats due to anthropogenic activities since African continent is reported to have one of the highest rates of deforestation in the world. Despite the depletion Africa is a continent with a high rate of endemism with the Republic of Madagascar topping the list at 82% (WHO, 2010).

African traditional medicines are holistic in nature and involves both the body and the mind. It involves diagnosing and treating the psychological illness before prescribing medicines to treat the symptoms. Famous African medicinal plants include *Acacia senegal*, *Agathosma betulina*,

Aloe ferox, Aloe vera, Artemisia afra, Aspalanthus linearis, Boswellia sacra, Catha edulis, Commiphora myrrha, Harpagophytum procumbens, Hibiscus sabdariffa, Hypoxis hemerocallidea, Prunus africana.

2.2.2 Indian traditional medicine

Ayurveda, Naturopathy, Unani, Siddha and folk medicine are the most known healthcare systems in Indian society which uses natural resources. India has rich repository of medicinal plants from ancient civilizations. Ayurveda is the most ancient of all medicinal traditions since ancient Hindu writings on medicine contain no references to any other medicines whereas Greek and Middle Eastern texts do refer to ideas and drugs of Indian origin. Dioscorides (who influenced Hippocrates) is thought to have taken many of his ideas from India (Gurib-Fakin 2006).

Ayurveda is derived from the Indian words ‘*Ayar*’ (Life) and ‘*veda*’ (Knowledge or Science) and hence means the Science of Life. This knowledge and wisdom have been passed on from one generation to the next through songs and poems while scholars and physicians had to learn by heart and recite. The *Veda* has four parts (Rig Veda, Sama Veda, Yajur Veda and Atharva Veda. Famous Ayurvedic medicinal plants include *Azadirachta indica, Centella asiatica, Cinnamomum camphora, Elettaria cardamomum, Rauwolfia serpentina, Santalum album, Terminalia* species and *Withania somnifera* (Dev, 2006). Unani medicine depends on the diagnosis of disease based on clinical features like signs, symptoms, laboratory features and temperament. After diagnosing, the disease is managed on the basis of elimination of the cause, normalization of humors and normalization of tissues or organs. Naturopathy medicine belief in a special "vital energy" which is based on metabolism, reproduction, growth, and adaptation. It involves the use of non-invasive treatment and avoids the use of surgery and drugs. Siddha refers to a being who has achieved a

high degree of physical and spiritual perfection and it had a special power to fly, Siddha medicine is the oldest medical systems known to mankind (Atwood, 2003; Immermann and marion, 2003).

2.2.3 Chinese traditional medicine

The civilizations of China were flourishing when only modestly sophisticated cultures were developing in Europe. Expectedly writings on medicinal plants and the aesthetics of vegetation were numerous. This ancient system of medicine, believed to be more than 5000 years old, is based on two separate theories about the natural laws that govern good health and longevity, namely *yin* and *yang*, and the five elements (*wu xing*). The most complete reference to Chinese herbal prescription is the Modern Day Encyclopedia of Chinese *materia medica* published in 1977. It lists nearly 6000 drugs out of which 4800 are of plant origin (Ramawat, 2009).

Yin and Yang denotes opposites that complement each other. The five-element theory is similar to the four humours and elements of the Greeks or the three humours of Ayurveda. The five elements are earth, metal, water, wood and fire each of which is linked to the main organ systems of the body (respectively the spleen, lungs, kidney, liver and heart), the emotions (reflection, grief, fear, anger, joy), the climates (damp, dry, cold, windy, cold), the seasons (late summer, autumn, spring, summer) and tastes (sweet, pungent, salty, sour, bitter) and so on (Tang *et al.*, 2008). Medicine is used to restore or maintain balance between these elements and to grant vital energy (*qi*) which has both yin and yang aspects. Treatment is therefore based not only on symptoms but also on pattern of imbalances, often detected by taking the pulse or observing the patient's tongue. Warming or hot herbs such as ginger, and cinnamon, are used to treat ailments associated with cold symptoms such as cold hands, abdominal pains and indigestion. In common with Western and African traditional medicines, Chinese herbs are usually given in fixed

mixtures or formulas of up to 20 herbs, carefully prepared according to traditional recipes contained in ancient compendia. There are hundreds such recipes being used alongside with Western Medicines. Traditional recipes are used mainly against chronic illnesses while acute or serious illnesses are cured by Western Medicines (Hongyi *et al.*, 2003).

Chinese medicine has contributed to herbal medicines throughout the world. Examples Chinese medicinal herbs are *Angelica polymorpha* var. *sinensis* (dang gui), *Artemisia annua* (qing hao), *Ephedra sinica*, *Paeonia lactiflora*, *Panax ginseng* and *Rheum palmatum* (Zhang and Zia, 2007).

2.2.4 American traditional medicine

Native American traditional medicine approaches illnesses by addressing both the physical and spiritual dimension of diseases. They involve Shamanistic ceremony that includes chanting, dancing and other rituals to expel evil forces from the patients (Mehl-madrona, 1999).

The native American herbal remedies were adopted and eventually formed the basis of the Pharmacopeia of the United States. The famous medicinal plants of the United States are the Echinacea (*Echinacea purpurea*), Goldenseal (*Hydrastis canadensis*), *Cinchona pubescens*, *Erythroxylum coca*, *Ilex paraguariensis*, *Myroxylon balsamum*, *Paullinia cupana*, *Peumus boldus*, *Psidium guajava*, *Spilanthes acmella*, *Tabebuia impetiginosa* and *Uncarina tomentosa* (Rotblatt and Ziment, 2002).

2.2.5 Australian and Southeast Asian medicine

The Aborigines of this region had a complex healing system although much of the traditional knowledge was lost and could not be well recorded. The medicinal plants used in this region are *Croton tiglium*, *Duboisia hopwoodii*, *Eucalyptus globulus*, *Melaleuca alternifolia*, *Myristica*

fragens, *Piper methysticum*, *Strychnos nux-vomica*, *Styrax benzoin* and *Syzygium aromaticum* (Gurib-Fakin, 2006).

2.2.6 European medicine

The European traditional healing systems were originated by Hippocrates (460–377 BC) and Aristotle (384–322 BC) of whom most of their ideas were rooted in ancient beliefs from India and Egypt. Dioscorides who is a Greek physician (100 AD) recorded the collection, storage and the use of medicinal herbs. Greek and Roman medicine just like the Chinese traditional medicine was based on the belief that the world is composed of four elements; earth, wind, fire and water of which Each of these has its corresponding humours that is linked to the four vital fluids in the body. The four humours are; blood, phlegm, black bile and yellow bile which influence both health and temperament (Lutz and Peter, 2002).

European tradition had many regional influences on local folk practices and traditions e.g. the influences of the famous book *De Materia Medica* which was written by the Greek physician Dioscorides in the first century AD. It became the standard reference in Europe for many years giving the base for most of the later herbals (Ramawat, 2009).

2.2.7 Classical Arabic and North African traditional medicine

The most ancient written information in the Arabic traditions originated from the Sumerians and Akkadians of Mesopotamia hence originating from the same part as the archeological records of Shanidar IV (Heinrich *et al.*, 2004). The common plants that were presumably used as medicines are: *Centaurea solstitialis* (Asteraceae), *Ephedra altissima* (Ephedraceae), *Althea* sp. (Malvaceae) amongst others (Gurib-Fakin, 2006).

2.3 Economic importance of herbal plants

Traditional and alternative healthcare systems are growing hence the global market for herbal drugs is becoming very lucrative and the world herbal trade is expected to reach USD 7 trillion by 2050. According to available data, the value of the herbal medicine market in European countries in 1991 was about USD 6 billion which became USD 10 billion in 1996, and was expected to exceed USD 20 billion by 2000. The contribution to the world herbal medicine market from the USA in 1996 was about USD 4 million and in other countries was USD 5 billion. Herbal sales increased in the USA by 101% in mainstream markets between May 1996 and May 1998 (Kamboj, 2000).

According to (WHO) on Traditional medicine, Japan spent over USD 1 billion in 2006 while Australia spent a total of USD 1.86 billion between 2004-2005 on herbal medicine. Traditional medicine in China constitute between 30% and 50% of the total consumption of medicine which is equivalent to a sale value of USD 14 billion in 2005. Kenya exports 1923 tons of *Prunus africana* per year while Uganda and Madagascar exports 193 and 800 tons respectively; the bark of this plant is used for treatment of prostate cancer and hypertrophy (Evans, 2005; Hostetmann *et al.*, 2000). Globally the market for traditional medicine was USD 83 billion in 2008, which is increasing exponentially while the product sales amounts to between 5% and 18% per annum (Robinson and Zhang, 2011).

2.4 Challenges to the advancement of herbal medicine use

Forest degradation is a major threat to medicinal plants. Rapid increase in population, pollution, modern civilization, industrialization and unsustainable resource use are the major reason for degradation of plant biodiversity. This has resulted to extinction of numerous medicinal plant species around the world (De *et al.*, 2010). Proper regulation worldwide is still a big challenge. Regulation and legislation of herbal remedies has been enacted in very few countries especially

in Africa where most countries do not have any proper regulation of botanicals thus, the quality of herbal products sold is generally not guaranteed (W H O, 2002 ; Warude and Patwardhan, 2005).

For future advancement in traditional medicine usage, scientific validation, risk associated and technological standardization of herbal medicine should be addressed. Research into herbal products is also inadequate and very few trials produce satisfactory results that are included in the healthcare system. Policies regarding various issues like legislation, regulation for herbal products, practice of therapies, training and licensing of providers, research development and allocation of financial and other resources needs to be addressed (Kamboj, 2000 and W H O, 2002).

Biopiracy of indigenous traditional knowledge and natural resources is also challenge hence intellectual property right should be protected (W H O, 2002). Unscientific collection results in the destruction of medicinal plants causing threat to genetic stocks and medicinal plant diversity, therefore sustainable use of natural products is necessary (Sharma, 2010). Standardization of crude drugs and herbal medicines are another challenge for the growth of herbal medicine. Lack of quality controls for herbal material and formulations which is vital for acceptability just like in modern medicine, are the limitations for standardization of herbal medicine (Verma and Singh, 2008).

Conservation of medicinal plants and their sustainable use are necessary for the growth of herbal medicine. Cultivation of wild medicinal plants is an important approach to safeguard the herbal industry. However, environmental factors like temperature, rainfall day length and soil characteristics are some of the factors that affect the effectiveness of some cultivated medicinal plants. Biotechnological techniques like plant cell or tissue culture, biochemical conversions and

clonal propagation of indigenous medicinal plants is another potential strategy in improving herbal medicine (Dubey *et al.*, 2004).

2.5 Plants used for treatment of toothache

There is still growing literature on traditional use of medicinal plants in the treatment of oral diseases (Hadissa and Jean-pierre, 2006). Toothache is mainly due to bacterial infections. There is high reliance on the use of traditional plant products for the management of oral health related conditions not only in African countries such as Equitorial Guinea (Akandengue,1992), Madagascar (Novy, 1997), and South Africa (Lin *et al.*, 1999) but also other parts of the world like Palestine (Ali-Shtayeh *et al.*, 2000) and Nepal (Manandhar, 1998). Table 2.1 below shows some of medicinal plant species, parts used and there usage in treatment of tootache.

The table 2.1: Plants species, parts used and there usage in the treatment of toothache

Plant	Part used	Usage
<i>Mangifera indica</i>	Bark	Powdered inner bark is kept in mouth for about half an hour to an hour thrice a day to get relief from toothache
<i>Acrus calamus</i>	Rhizome	Rhizome is made into paste and applied to painful teeth and gums
<i>Calatropic gigantean</i>	Latex	Cotton is wetted with the latex of this plant and placed on the painful tooth area
<i>Pergularia daemia</i>	Latex	Latex of this climber is placed on affected area for relief of toothache
<i>Lannea acidic</i>	Leaves and Bark	Bark decoction is used as regular mouthwash until recovery
<i>Lannea microcarpa</i>	Bark	Bark is boiled with indigenous salt and used as mouth wash in toothache

Sources: Hadissa and Jean-pierre 2006; Hedge *et al.*, 2004

2.6 Oral health care

Medicinal plants are important part of Primary healthcare systems especially where access to clinics that dispense modern medicine is limited and costly, hence traditional herbal remedies are still the primary means of meeting the health and medicinal needs of most rural communities. Prevention of oral diseases is easier than a cure. Proper oral care is necessary to prevent oral diseases. Knowledge about oral diseases and their causes will help to maintain proper oral health.

Oral conditions are usually associated with the teeth, tongue or gums. Dental plaque and caries, pyorrhea, toothache and naphtha are common oral ailments. Dental plaque is a colorless sticky mixture of bacterial products while dental caries is the decalcification process carried out by micro-organisms which grows on remnants of food materials between the teeth. *Streptococcus mutans* and *Streptococcus sobrinus* are the primary etiologic agents of dental caries. These bacteria metabolize carbohydrates like sucrose, glucose, and fructose and produce lactic acid as by-products in oral cavity (Takahashi and Nyvad, 2008).

When carbohydrates are present in excess, cariogenic bacteria can synthesize intra-cellular polysaccharides leading to dental caries. Periodontal diseases include gingivitis and periodontitis which are multifactorial chronic infections. Gingivitis is an inflammation of the unattached gingiva, while periodontitis is a progressive destruction of all supporting tissues of the teeth including the alveolar bone (O'Brien-Simpson *et al.*, 2004).

The treatment for periodontitis is done by removing dental plaque and calculus as well as oral hygiene to prevent dental biofilm. This can be done by mechanical, chemical, and surgical methods. Non-surgical methods together with oral hygiene can help reduce tissue inflammation and improve clinical periodontal attachment (Cobb, 2002; Suvan, 2005).

Pyorrhoea is a disease of the tooth socket manifested by bleeding of the gums, which is due to bacterial activities that produce toxins that often affect the gums (Farooqi *et al.*, 1998). Oral candidiasis is an opportunistic infection of the oral cavity caused by *Candida albicans*. This yeast is a normal flora in the mouth and generally causes no infections in healthy people except when one is immunocompromised (McCullough and Savage, 2005; Samaranayake *et al.*, 2009).

The use of local and systemic antibiotics, anti-inflammatory drugs, or sub-anti-microbial low-dose doxycycline has been reported to provide additional treatments of dental conditions. (Haffajee *et al.*, 2003; Preshaw *et al.*, 2004).

Ethnomedical treatments especially of oral diseases and conditions have no well documented catalog that describes the ethnography, ethnomedicine, ethnopharmacology, and evidence-based clinical applications of traditional medicines used for dentistry. Most publications describe anecdotal dental therapeutic applications of the current medicinal uses of plants (Ocasio *et al.*, 1999). Epidemiological analysis of the ethnopharmacological oral medicine efficacy and safety is an emerging science worldwide. Only a few publications document use of traditional use of plants in dentistry, oral medicine, in the treatment and cure of oral and maxillofacial conditions and oral pain (Ogura *et al.*, 1982).

Clinical trials, which can verify indications, safety, and efficacy of herbal products and compounds, must increase in order to justify their role in contemporary oral medicine. Most of the oral diseases are due to bacterial infections and medicinal plants provide considerable antibacterial activity against various micro-organisms. Effective antimicrobial agents against the oral pathogens could play an important role in the treatment and prevention of oral infections. However, antibiotics such as penicillin and erythromycin have been found to be effective in both animal and humans but are not used clinically for oral conditions since they cause many adverse

effects such as hypersensitivity reactions, supra-infection and teeth staining. Furthermore bacteria such as *viridians* groups, *streptococci* such as *Streptococcus mitis*, *Streptococcus sanguis* and *Streptococcus mutans* are resistant to conventional antibiotics (Yanagida *et al.*, 2000).

This drawback gives the gap for further research and development of natural anti-bacterial that are safe for the host and specific to the oral pathogens. The natural phytochemical could offer an effective alternative treatment and prevention than conventional antibiotics for oral infection.

2.7 Description and distribution of *Vernonia hymenolepis*

Vernonia is a genus in the family *Asteraceae* and has about 1000 species. Some species are known as ironweed. This genus is named after William Vernon, an English botanist who collected and identified this genus in Maryland in the late 1600s before his death in 1711 (Quattrocchi, 1999). Some species are edible and of economic value. It has numerous distinct subgenera and subsections (Beentje, 2000). *Vernonia hymenolepis* has serrated green leaves with pink and mauve white flowers as shown in figure 2.1, it is an ever green shrub that grows up to 5 meters high. The stem has spines and its green in color, the leaves are leathery pubescent and tomentose beneath.

Vernonia hymenolepis is normally found in habitat that is disturbed by human activities and it thrives well at temperatures of less than 30°C, altitudes of between 600–3000 m and a minimum rainfall of 840 mm per year. It flourishes well in loose and moist soil rich in humus. In Kenya it is widely distributed in highlands.

Vernonia species has a wide habitats of broad ecological diversity and climatic conditions that ranges from tropical forest, marshes and wet areas, dry plains, tropical savannahs, desert sites to

even frosty regions of eastern North America (Gleason, 1923; Keeley and Jones, 1979).

Vernonia is annuals, herbaceous perennials, shrubs and also trees.

Vernonia hymenolepis occurs wild in mountainous and high plateau regions of west, central, East and South Africa. It is a leafy indigenous vegetable that is commonly cultivated by farmers in Nigeria and Cameroon (Afui *et al.*, 2008). *Vernonia hymenolepis* also occurs along rivers, roadsides, in forest margins, old cultivation areas, in bushed grassland and also in mountain forest.



Figure 2.2: Aerial view of *Vernonia hymenolepis* showing serrated green leaves with pink and mauve white flowers. Adapted from <http://www.google.com> on 15/June/2013

2.8 Uses of *Vernonia hymenolepis*

The genus of *Vernonia* has widely been used as food and medicine but no comprehensive review has been carried out to document and analyze its contribution to the nutrition and health of humans and animals (Johri *et al.*, 1997). Only six species have been identified and reviewed on their use while about twenty other species were indicated to be featured in various traditional *materia medica* with insignificant details. In Cameroon *Vernonia hymenolepis* is known as *Baying* which means bitter leaf because of its taste. Its leaves are consumed fresh and in dry form as garnish, potherb or salad. It is used to cure pneumonia, hypertension and also to heal and stop bleeding wounds. Juice from its crashed leaves is used to treat diarrhea in babies and jaundice (Kupchan *et al.*, 1968).

Furthermore, the juice of the leaf has been used traditionally by herbalist to treat oral infections including dental caries and for pain relief in toothache conditions. The use of leaf in treatment of malaria, typhoid, Amoebiasis and abdominal conditions like constipation in communities in Trans Nzoia and Uasin Ngishu Counties in Kenya has been documented (Kokwaro, 1993). Table 2.2 shows some of the species of *Vernonia* that has been documented to treat various conditions including toothache.

Table 2.2: Conditions treated by of some species of *Vernonia*

Types	Conditions treated	References
<i>Vernonia serratuludes</i>	Toothache, rheumatic pains, heart problems	Heinrich,1996
<i>Vernonia thomsoniana</i>	Antimicrobials	Mungarulire 1990
<i>Vernonia venosa</i>	Antinociceptive,anti-inflammatory,anti-microbial	Al malgboul <i>et al.</i> , 1988
<i>Vernonia aemulans</i>	Anti-bacterial,Viral	Vermani and Garg 2002
<i>Vernonia auriculifeta</i>	Toothache	Giday <i>et al.</i> , 2009
<i>Vernonia conferta</i>	Pain	Mengome <i>et al.</i> ,2010
<i>Vernonia guineensis</i>	Toothache,pain,sores,jaundice,purgatives	Burkill,1985
<i>Vernonia kotschyana</i>	Gingivitis,gonorrhoea,arthritis	Ibrahim <i>et al.</i> ,2009
<i>Vernonia patula</i>	Oral infection and lesions, respiratory tract infections	Mollik <i>et al.</i> ,2010
<i>Vernonia leiocapa</i>	Antibacterial	Meckes <i>et al.</i> ,1995
<i>Vernonia lasiopus</i>	Bacteria ,Viral	Vlietinck <i>et al.</i> ,1995

2.9 Bioactivity and the active principles of *Vernonia*

Various compounds have been isolated from genus of *Vernonia* with the flavonoids being among the major classes of compounds (Carvalho *et al.*, 1999). They occur as yellow or white pigments in plants and are responsible for yellow, orange and red in flowers (Pengelly, 2004). Flavonoid strengthens the capillaries through membrane stabilization hence used in respiratory system.

Studies have been shown that they possess anti-inflammatory, hepatoprotective, antiallergic and antihypertensive effects. Rutin, Quercetin, Kaempferol and Nomilin are examples of flavonoids with medicinal value (Akaneme, 2008; Middleton and Kandaswami, 1993; Pengelly, 2004). The flavones and phenolic compounds have been isolated from *Vernonia amygdalina* and *Vernonia cinerascens* and have potent antioxidant (Igile *et al.*, 1994).

Alkaloids have been identified in very few species of *Vernonia*. They have been reported in *Vernonia ambigua* (Aliyu *et al.*, 2011), *Vernonia amygdalina* (Nwanjo, 2005; Ayoola *et al.*, 2008; Sharma and Sharma, 2010), *Vernonia cinera* and *Vernonia colorata* (Neuwinger, 1996). Alkaloids are generally white or colorless crystalline solids and exist as salts of organic acids. Studies have shown that they possess antispasmodic, antibacterial, decongestant, stimulant, antihypertensive and analgesic activities. Examples of alkaloids are Quinine, Morphine, Nicotine, Cocaine, Sparteine and Reserpine (Goodwin, 2003; Kisangau, 1999; Kokwaro, 1993; Pengelly, 2004).

Various other compounds have been isolated from *Vernonia hymenolepis* which includes; sesquiterpene dilactone, vernolepin and Glaucolides which have been reported to have properties such as smooth muscle relaxants and phyto-growth inhibitors (Campos *et al.*, 2003).

The Vernolides is a class of sesquiterpene lactone which is the most studied compounds from the genus *Vernonia* and has bioactivity in antiplasmodial, antileishmanial, antischistosomal, cytotoxicity, antimicrobial and anti-inflammatory assays. (1)-Vernolepin is a sesquiterpene dilactone which was isolated from the Ethiopian plant *Vernonia hymenolepis* with spasmolytic, anti-aggregating, de-aggregating activities and antitumor activity *in-vitro* and *in-vivo* (Kupchan *et al.*, 1968). The unique structure and biological activity of vernolepin is of great interest to synthetic organic chemists.

Terpenes have functional diversity of compounds in plants. There more than 30,000 terpenoids identified (Buckingham, 1998). They are identified by isoprene rule which stipulates that all terpenes have fundamental repeating 5-carbon isoprene units (Croteau 1998) and are the largest class of secondary metabolites (Connolly *et al.*, 1991). They help plant to protect itself from environmental factors. They have been shown to possess antibiotic, cytotoxic, antimalarial, antifungal, insecticides and herbicidal properties (Zhang *et al.*, 2005; Robert, 2007; Gershenzon and Dudareva, 2007; Kaur *et al.*, 2009). Terpenes are vital targets for finding novel leads for medicines. Terpenoids are subdivided into monoterpenes, sesquiterpenes, diterpenes, triterpenes and carotenes. Monoterpenes and sesquiterpenes are major essential oils found in *Vernonia* genus (Ogumbinu *et al.*, 2001).

Sesquiterpenes is a C₁₅ compounds that have 3-isoprene units and it exist in aliphatic, bicyclic and tricyclic frameworks. Sesquiterpene lactone isolated from *Artemisia annua* is currently used as an antimalarial drug. The genus of *Vernonia* is good sources of sesquiterpene lactones (SLs). Sesquiterpene lactones are in the family of highly oxygenated group such as glaucolides and hirsutinolides (Costa *et al.*, 2005). Hirsutinolides have been reported as having cytotoxicity

(Chen *et al.*, 2005), antibacterial and anti-inflammatory properties (Kos *et al.*, 2006), as well as antiplasmodial activities (Pillay *et al.*, 2007). Glaucolides have smooth muscle relaxants, phyto-growth inhibitors (Campos *et al.*, 2003), as well as weak cytotoxic (Williams *et al.*, 2005) and molluscicidal effects (Alarcon *et al.*, 1990).

2.9.1 Antinociceptive assays

Antinociceptive activity testing can be performed by any of the following methods:

2.9.1.1 Writhing test induced by acetic acid

This method evaluates pain response through nociception induction by chemical stimulation of 0.1 ml/10 g of 0.6% acetic acid through intraperitoneal (i.p.) pathway. Food is withheld but not water two hours prior to the experiment. Oral treatments (p.o.) with the test material (drug) is given one hour prior to acetic acid injection (n = 6 per group), then each animal is isolated and placed in a box (30 x 30 x 30 cm) for observation. The numbers of writhes is counted over a period of thirty minutes recorded and expressed as the percentage (Garcia *et al.*, 2004).

2.9.1.2 Formalin test

The formalin test is used to determine the potential analgesic effects of compounds for states of persistent pain in which tissue damage occurs. The nociceptive response produced by formalin is biphasic, the early and late phases. It produces a dual response of pain in rodents and also helps in the elucidation of the action mechanism of the drugs. According to Bentley *et al.*, (1983), the advantage of this protocol is that even compounds with weaker antinociceptive activity can be detected. The initial response (phase 1) is caused by a burst of activity from pain fibers (particularly C fibers). Phase 2 is mediated by peripheral inflammation and central sensitization. Formalin test was carried out as described by Abbot *et al.*, (1999) and Bannon and Malmberg,

(2001).The advantage of the formalin model of nociception is that it could discriminate pain in its central and/or peripheral components.

Twenty micro liters (20 μ l) of 5% formalin is injected intradermally on the plantar surface of the hind paw of each mouse one hour after administration of the test samples/drug. The duration of paw licking as an index of painful response is determined at 0 – 10 min in early phase and 15 – 60 min in late phase after formalin injection. Then latency of licking the injected paw is measured and recorded. (Bannon and Malmberg, 2001).

2.9.1.3 Hot plate test

In this test mice is placed in a hot plate at a temperature of 50 ± 1 °C and the first nociceptive responses like licking of paw, jumping or shaking is recorded as the reaction time. Mice are divided into groups, negative control to receive saline plus Tween 80 (1%), positive control to receive standard analgesic drug subcutaneously and another group to receive the test drug. The administrations are made 1 hour prior to the nociceptive test. A cut-off time of 20 seconds is chosen to indicate complete analgesia and to avoid tissue injury. The latencies for paw licking or jumping were recorded for each animal in seconds (Yamamoto *et al.*, 2002).

2.9.1.4 Tail flick test

In this test the lower two-thirds of the tail of the mice is immersed in a beaker containing water kept at 50 ± 0.5 °C). The time in seconds until the tail is withdrawn from the hot water is defined as the reaction time or tail flick latency. The reaction time is then measured at 0, 30, 60, and 120 min after the oral administration of the drug to be tested. The mice are exposed to hot water for no longer than 20 seconds to avoid tissue injury. Tail flick response is calculated and compared with the control groups (Wang *et al.*, 2000b).

2.9.2 Antimicrobial susceptibility assay

2.9.2.1 Broth dilution method

Serial two fold dilution of the test sample are prepared and placed in sterile tubes with Muller Hinton broth to allow for growth of microorganisms. After 24 hours of incubation, the test strains are suspended in sterile physiological saline to give a final density of 10^6 . The last tubes containing Muller Hinton broth without extract and 0.1 ml inocula is used as a negative control. Standard antibiotic is also used as a positive control. The minimum inhibitory concentration is the lowest concentration that inhibited any visible bacterial growth on the culture plates (Shahindi, 2004).

2.9.2.2 Well diffusion assay

Wells of 0.5 cm diameter is made in the inoculated media and placed at room temperature for an hour to allow diffusion of extract into the agar and then incubated for 24 hours at 37°C. The antimicrobial activity is determined by measuring the zone of inhibition around each well at the end of 24–48 h (Hettiarachchi, 2006; Kisangau *et al.*, 2007; Mbaria *et al.*, 2006).

2.9.2.3 Disc diffusion method

The disc diffusion assay uses cellulose discs. These discs are impregnated with test drug and then placed onto the inoculated plates. These are then incubated for 24 hour and antimicrobial activity is determined by measuring the diameters of zones of inhibition (Hettiarachchi, 2006; Kisangau *et al.*, 2007; Moshi *et al.*, 2009; Mothana *et al.*, 2008; Parekh and Chanda, 2006).

2.9.3 The oral acute toxicity testing

Acute oral toxicity testing was done according to standard methods Acute Oral Toxic Class Method (ATC method) OECD guidelines 423. This method was developed as an alternative to replace LD₅₀ test, this method reduces the number of animals compared to LD₅₀ test. It is a sequential testing procedure that uses three animals of one sex, usually female per step.

According to ATC protocol the starting doses are 5, 50,300 or 2000 mg/kg b.w.t based on the class limits of the Global Harmonized Classification System (Commission, 2004; OECD, 2001d). The outcome of the test such as the number dead or moribund mice will determine if dosing of three additional mice with the same dose is to be done or dosing of three additional mice at the next higher or the next lower dose, or if test is terminated at that dose (Bliss,1934). Figure 2.3 shows Sequential stepwise dosing of mice according to acute Oral Toxic Class Method.

ANNEX 2c: TEST PROCEDURE WITH A STARTING DOSE OF 300 MG/KG BODY WEIGHT

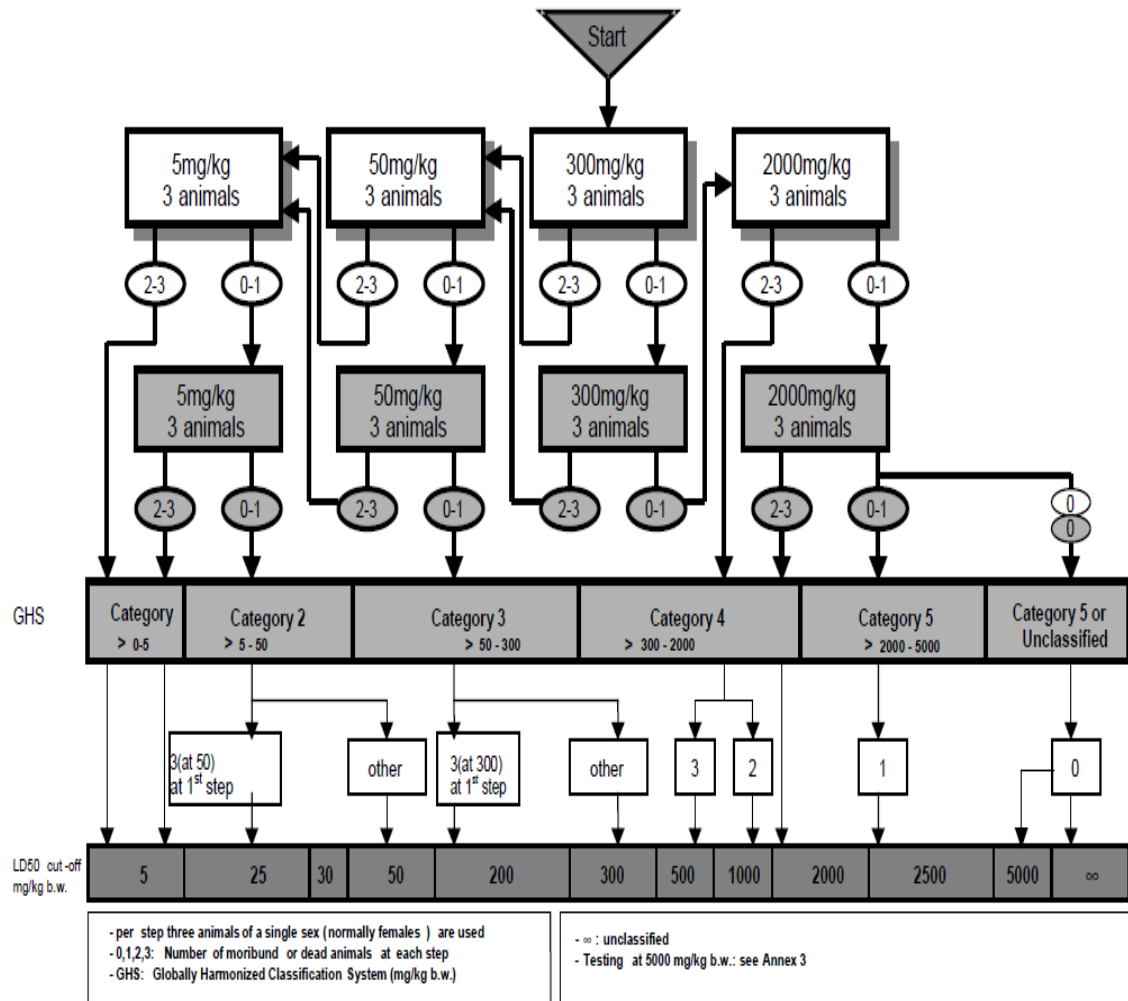


Figure 2.3: Sequential stepwise dosing of mice in ATC method. Adapted from OECD guidelines 423

CHAPTER THREE: MATERIALS AND METHODS

3.1 Study area

The plant samples were obtained from Trans Nzoia County, Kenya. The County is in the former Rift Valley Province and it's located between the Nzoia River and Mount Elgon. It has three constituencies Cherangani, Kwanza and Saboti Constituency. Trans-Nzoia County covers a total area of 2,469.9 Km² with a total estimated population of 818,757 people as per 2009 census. The temperature ranges between 10°C and 37°C while rainfall averages 11,200mm per annum. Figure 3.1 shows the location of Trans- Nzoia County in Kenya. The county is an agricultural epitome of the country and is considered as one of the most food secure regions in Kenya. The laboratory assays were conducted at the University of Nairobi, Department of Public Health Pharmacology and Toxicology University of Nairobi.

3.2 Plant material

3.2.1 Collection and identification of plant material

Leaves of *Vernonia hymenolepis* were collected from Trans Nzoia County, Kenya and identified at the University of Nairobi Herbarium in the Department of Botany School of Biological Sciences and voucher specimens deposited (RO2011/001).

3.2.2 Preparation of plant extracts

The plant sample (Leaves) was shade dried at the Department of Public Health, Pharmacology and Toxicology, University of Nairobi, and milled into powder after removal of any foreign matter. The powder was packed in clean air tight polythene papers.

3.2.3 Dichloromethane/Methanol extract

For the preparation of Methanol: Dichloromethane (1:1) extraction, 100 grams of the plant material were dissolved in DCM:M 1:1 i.e. 500 mls of dichloromethane and 500 mls of methanol then placed in a soxhlet evaporator and extracted at 60°C for 8 hours. The resulting extract was then evaporated to dryness in a rotary evaporator (Ugo Basile, Italy) at 40°C and a pressure of 376 Pascals, the extract was weighed and percentage yield was 21.5%.

3.2.4 Water extracts

One hundred grams of ground leaves was soaked in one liter of distilled water for 72 hours in a conical flask. Filtration was done using Whatman No.1 filter paper and the filtrate collected in sterilized beaker then covered by aluminum foil. The filtrate was then freeze dried at International Livestock Research Institute (ILRI) and 32% (w/w) was yielded. The extracts were placed in airtight amber colored sample bottles. The extracts were stored in a refrigerator at 4°C for further tests.

3.3 Experimental animals

Thirty Male albino mice (Wister) aged 6-8 weeks and weighing 25-30 grams were used for formalin test while twelve female mice were used for acute oral toxicity test. Food and water were given *ad libitum*. Animals were allowed 7 days for acclimatization. They were kept at temperature of 22° C to 25° C and relative humidity of 50%. Diurnal rhythms were regulated with a twelve hour light: twelve hour dark cycle. Each mice was used only once in the experiment.

3.4 Determination of antimicrobial susceptibility

3.4.1 Test organisms

Strains of bacteria and a fungus were obtained from stock cultures at the Department of Public Health, Pharmacology and Toxicology, University of Nairobi for antimicrobial assays. The gram positive bacterial strains used were *Staphylococcus aureus* (ATCC 25923) and *Bacillus cereus* (ATCC 11778) while gram negative bacteria strains used were *Escherichia coli* (ATCC 25922) and *Pseudomonas aeruginosa* (ATCC 27853). *Candida albicans* was also used. The positive control was amoxicillin.

3.4.2 Broth dilution method

Five species of Standard microbials namely; *Pseudomonas aeruginosa*, *Escherichia coli*, *Bacillus cereus*, *Staphylococcus aureus* and *Candida albicans* strains were cultured overnight at 37C° in blood agar for 18 hours. The test organisms were suspended in physiological saline to equal that of 0.5 McFarland standards. Then serial two-fold dilution of plant extract was done to test the activity against micro-organisms by broth dilution method.

The lowest concentration that inhibited any visible bacterial growth on the culture tubes is known as MIC (Shahindi, 2004). The MIC test was conducted as per the Clinical Laboratory

Standards institute (CLSI, 2010). One thousand six hundred milligrams of plant leaves extracts were weighed and added into 4 ml of sterile Mueller Hinton broth to make a master dilution of 400 mg/ml to be used for both gram positive and gram negative. Eight culture tubes containing 2ml Mueller Hinton broth were arranged in duplicate, and then two fold serial dilutions were made from the master dilution.

The concentrations made were 400mg/ml, 200mg/ml, 100mg/ml, 50 mg/ml, 25mg/ml, 12.5mg/ml, 6.25mg/ml, and 3.125mg/ml. For organic extract 2% DMSO was added to make 4ml Mueller Hinton broth for the master dilution. The plant extract was inoculated with 0.1 ml of individual microorganism into the tubes. All inoculated dilutions were incubated at 37C° for 24 hours. 2% DMSO and Mueller Hinton broth was used as negative control to study the impact of the solvent itself (without plant components) on growth of the micro-organisms (Bauer *et al.*, 1966). The Positive control was Amoxicillin (Botelho *et al.*, 2007). For determination of MBC, 100 µl of broth from all tubes that showed no turbidity and two tubes with turbidity was removed aseptically and then placed on TSA plates using pour plate method. The plates were incubated at 37C° for 24 hours. After incubation the lowest concentration of the plant extracts showing no bacteria growth was recorded as MBC.

Minimum bactericidal concentration is the lowest concentration at which 99.9% or more of the initial bacteria inoculums were killed. All the experiment was performed in duplicate (Akinyemi *et al.*, 2005; Pavithra *et al.*, 2010).

3.5 Determination of analgesic activity

3.5.1 The formalin test

Six mice per group (5 groups) were randomly assigned to receive intraperitoneally 0.5ml of 100 mg/kg of aqueous extract of *Vernonia hymenolepis* and Dichloromethane: Methanol (ratio 1:1).

Physiological saline, acetylsalicylic acid and 0.5 % dimethylsulfoxide (DMSO) was used as controls. Twenty micro liters (20 μ l) of 1% formalin was injected intradermally on the plantar surface of the hind paw of each mouse one hour after administration of the test samples/extracts and control. The time in seconds spent in paw licking as an index of painful response was determined at 0 – 10 min (Early phase) and 15– 60 min (late phase) after formalin injection. The results were observed and recorded. The mean pain response was calculated by comparing with control. Pain experiments were done according to the guidelines issued by the International Association for the Study of Pain for animal pain experimentation (Zimmermann, 1983).

3.5.2 Sensorimotor activity testing

The pull-up test (Deacon and Gardner, 1984) was performed in order to verify the presence of any antinociceptive activity in the extracts were independent muscle relaxant and sensorimotor retardation effects. The mice were fully extended in an inverted position one hour after administration of extract/control. The end point of the experiment was set when the mouse was attempting to gain an upright position by touching the hand or fingers of the experimenter with both forepaws simultaneously (Githinji *et al.*, 2012). The end point was recorded using a stop watch, while the cut-off point of experiment was set at fifteen seconds.

3.6 Toxicity testing

3.6.1 Cytotoxicity study

In-vitro lethality assay of brine shrimp was used to detect cell toxicity of the plant extracts (Meyer *et al.*, 1982; McLaughlin *et al.*, 1991).

3.6.1.1 Preparation of the marine salt solution

Thirty three grams of marine salt was weighed and dissolved in one liter of distilled water (3.3%) in a conical flask (Gakuya, 2001; Wagate, 2008).

3.6.1.2 Hatching the brine shrimp

A shallow brine shrimp hatching tank with two chambers and a divider with several holes were filled with 3.3% marine salt. Sterile spatula was used to scoop one gram of brine shrimp eggs and applied on a large compartment of the improvised tank. Five milligrams of yeast was also added to the chamber to feed the hatched nauplii, and then the large compartment was covered with aluminum foil. The smaller compartment was illuminated by a 40 watt electric bulb for 48 hours for incubation. The hatched nauplii were collected by use of sterile pasteur pipette to be used in brine shrimp lethality assay (Pisutthanan *et al.*, 2004).

3.6.1.3 Preparation of stock solution

One hundred milligrams (100 mg) of both water and dichloromethane/methane extracts were used. Dichloromethane/methane extract was dissolved in 0.5% DMSO then topped up to ten milliliters of marine salt in universal bottle then mixed with electric mixture to dissolve the extract completely. Water extract was dissolved in 0.5% distilled water in marine salt. This gave a final stock solution of 10,000 µg/ml that was used for serial dilution (Gakuya, 2001; Wagate, 2008).

3.6.1.4 Cytotoxicity test

Ten nauplii were placed in vials containing 5ml of seawater and increasing concentrations of *Vernonia hymenolepis* leaves extract (10-1000 ppm). Control was done using the same volume

of 0.5% DMSO. Live nauplii were counted and recorded after 24 h and the percentage mortality calculated for the plant extracts and controls. The lethal Concentration (LC_{50}) was calculated using the probit analysis at 95% confidence interval (Finney, 1971).

3.6.2 The acute oral toxicity testing

Twelve female albino mice were fasted prior to dosing for 3-4 hours but water was given *ad libitum*. Three concentrations of the extracts (50 mg/kg, 300 mg/kg, and 2000 mg/kg) were prepared. The test substance was administered in a single dose by gavage using intubation canula. The animals were weighed and the plant extracts were administered at a starting dose of 300mg/kg body weight according to animal welfare recommendation. This dose was repeated with three mice.

The same number of mice were dosed at the next higher dose level of 2000mg/kg for both the extracts and dose repeated.

3.7 Statistical analysis

The data obtained in this study was evaluated using the one-way analysis of Variance (ANOVA) test between two mean groups: control and test groups, followed by turkey test for analgesic test. Differences were considered statistically significant at $p < 0.05$.

CHAPTER FOUR: RESULTS

4.1 Antimicrobial susceptibility (MIC and MBC)

The MBC and MIC value of aqueous extract was similar on *Staphylococcus aureus* at a dose of 400 mg/ml while for DCM/M extract was similar on *Pseudomonas aeruginosa* and *Escherichia coli* at a dose of 400 mg/ml. The result shows that the aqueous extract had an inhibitory activity against *Staphylococcus aureus* and had no significant effect on *Pseudomonas aeruginosa*, *Escherichia coli*, *Bacillus cereus* and *Candida albicans* at concentration of 400 mg/ml of plant extract. The DCM/M extract had inhibitory effect against *Staphylococcus aureus* at a dose of 100mg/ml, *Pseudomonas aeruginosa* and *Escherichia coli* both at a dose of 400 mg/ ml, *Bacillus cereus* at a dose of 200 mg/ml.

Bactericidal activity was exhibited by aqueous extract at a dose of 400 mg/ml on *Staphylococcus aureus* while DCM/M extract exhibited at a dose of 400 mg/ml on *Pseudomonas aeruginosa*, *Escherichia coli*, *Bacillus cereus* and a dose of 200 mg/ml on *Staphylococcus aureus*.

Table 4.1 and 4.2 shows the antibacterial activity of aqueous extract and DCM/M extracts against the bacterial species.

Table 4.3 shows minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the plant extracts at various concentrations.

Table 4.1: Antimicrobial activity of aqueous extract of *Vernonia hymenolepis*

Bacteria Species	Growth of test organisms at different concentration of extracts							
	400 mg/ ml	200 mg/ ml	100 mg/ ml	50 mg/ ml	25 mg/ ml	12.5 mg/ ml	6.25 mg/ ml	3.125 mg/m l
<i>Escherichia coli</i>	+	+	+	+	+	+	+	+
<i>Pseudomonas aeruginosa</i>	+	+	+	+	+	+	+	+
<i>Bacillus cereus</i>	+	+	+	+	+	+	+	+
<i>Staphylococcus aureus</i>	-	+	+	+	+	+	+	+
<i>Candida albicans</i>	+	+	+	+	+	+	+	+

Key: + Growth; -No growth (inhibited)

Table 4.2: Antimicrobial activity of dichloromethane/Methanol extract

Bacteria Species	Growth of test organisms at different concentration of extracts							
	400 mg/ ml	200 mg/ ml	100 mg/ ml	50 mg/ ml	25 mg/ ml	12.5 mg/ ml	6.25 mg/ ml	3.125 mg/ ml
<i>Escherichia coli</i>	-	+	+	+	+	+	+	+
<i>Pseudomonas aeruginosa</i>	-	+	+	+	+	+	+	+
<i>Bacillus cereus</i>	-	-	+	+	+	+	+	+
<i>Staphylococcus aureus</i>	-	-	-	+	+	+	+	+
<i>Candida albicans</i>	+	+	+	+	+	+	+	+

Key: + Growth; - No Growth

Table 4.3: Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the plant extracts and the standard antibiotic

Plant extract	Bacteria Species	MIC(mg/ml)	MBC(mg/ml)
Aqueous extracts	<i>Staphylococcus aureus</i>	400	400
Dichloromethane/methanol	<i>Pseudomonas aeruginosa</i>	400	400
	<i>Bacillus cereus</i>	200	400
	<i>Staphylococcus aureus</i>	100	200
	<i>Escherichia coli</i>	400	400
Amoxicillin	<i>Escherichia coli</i>	3.125	6.25

4.2 Analgesic effect

The plant leaves extracts showed analgesic activities in both early phase of aqueous and organic extract $p < 0.0005$ and $p < 0.05$ respectively, while the late phase of both extracts was significant at $p < 0.01$. The late phase of water extract and organic extract showed better analgesic effects compared to the Standard drug (Acetylsalicylic acid). There is a significant difference between the negative controls and the extracts.

Table 4.4: Shows the summary of means, standard error of mean and significant levels of aqueous extract, Dichloromethane: Methanol ratio 1:1, Physiological saline and DMSO of the time spent in seconds in behavior of formalin test

Test sample	Mean and Standard error of mean (Early phase)	Mean and Standard error of mean (Late phase)
Acetylsalicylic acid	31.67±9.67 *	44.85±6.99*
Aqueous extract	56.92±7.013***	32.33±1.97**
Physiological saline	143.75±11.9	84±3.19
DCM/M extract	74.33±19.4*	9.907±1.59**
DMSO	111.17 ±11.4*	62±17.91**

*p< 0.05; **p< 0.01; *** = p< 0.0005 Compared to control; n=6

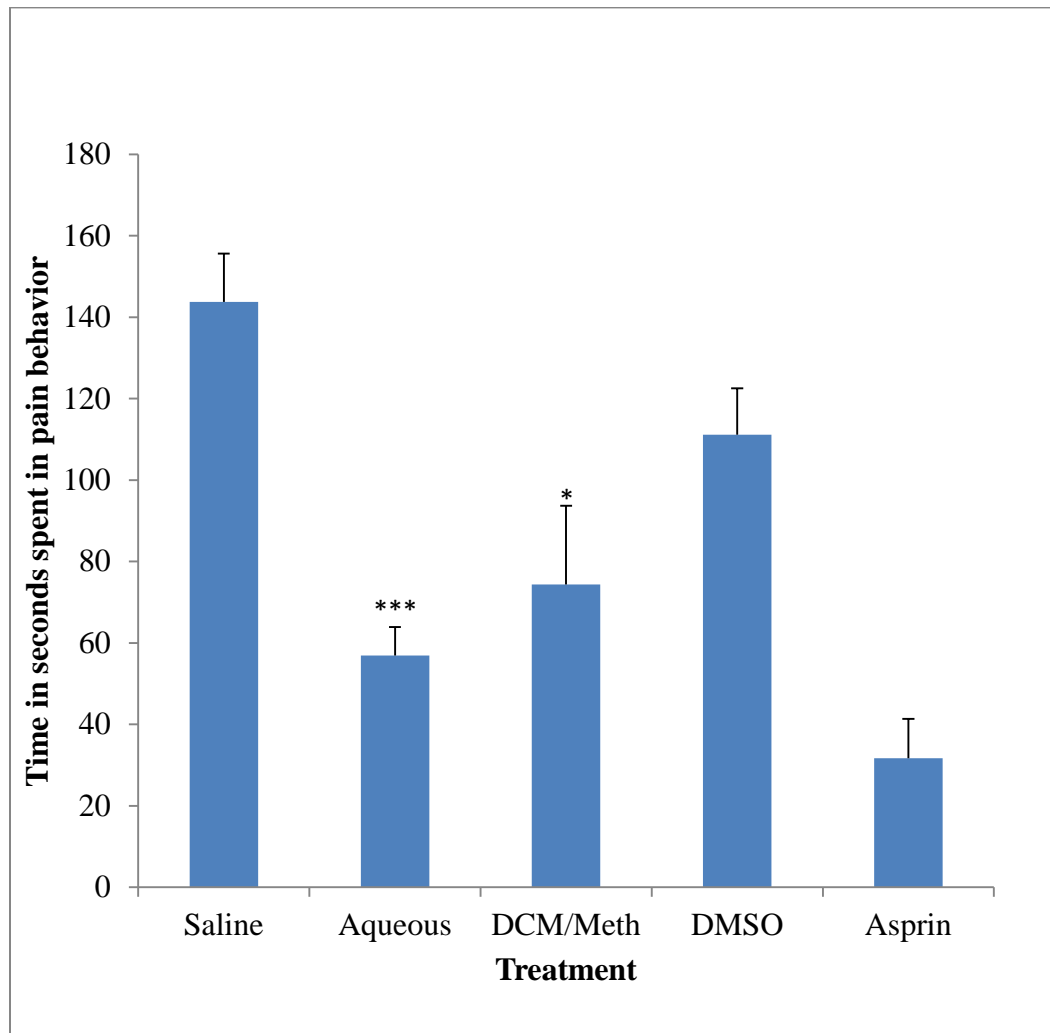


Figure 4.1: Effect of the intraperitoneal administration of 100 mg/kg dichloromethane/methanol and aqueous leaf extracts of *Vernonia hymenolepis* in the formalin test in early phase. Key: *** = $p < 0.0005$; * = $p < 0.05$; a = (physiological saline); b =Aqueous extract; c = Acetylsalicylic acid; d= DCM/M 1: 1; e = DMSO

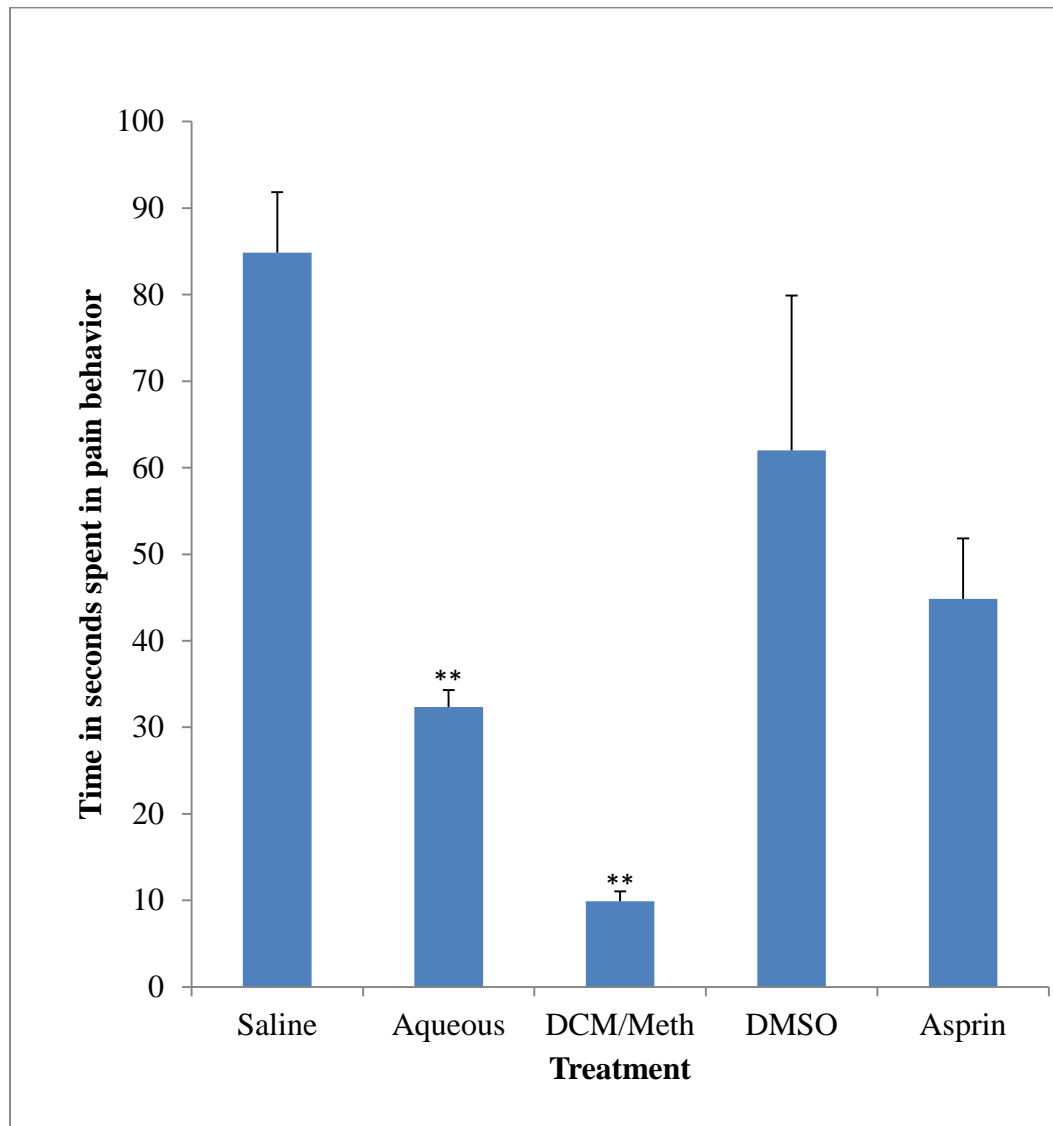


Figure 4.2: Effect of the intraperitoneal administration of 100 mg/kg dichloromethane/methanol and aqueous leaf extracts of *Vernonia hymenolepis* in the formalin test in late phase. Key: ** = $p < 0.01$; a=Control (physiological saline); b=Aqueous extract; c=Acetylsalicylic acid; d = DCM/M 1: 1; e = DMSO.

4.3 Toxic effects of plant extracts

4.3.1 Brine Shrimp Lethality Test

The LC₅₀ (µg /ml) of plant extracts for various concentrations (10-1000µg /ml) were 491.8 (µg /ml) and 481.7 (µg /ml) for water extract and Dichloromethane/Methanol extract respectively.

There was no mortality to brine shrimp in both marine salt and DMSO at the tested concentration.

Table 4.5: Percentage mortality of brine shrimp in serial dilution of dichloromethane/methanol and water extract

Plant extracts	Part used	Concentration of DCM/M and Water extract ($\mu\text{g} / \text{ml}$)		
		10	100	1000
Water extract of <i>Vernonia hymenolepis</i>	Leaves	2	12	70
DCM/Methanol extract of <i>Vernonia hymenolepis</i>	Leaves	0	4	76

Table 4.6: Lethal concentration LC_{50} of *Vernonia hymenolepis* extracts

PLANT EXTRACT	LC_{50} ($\mu\text{g} / \text{ml}$)	95% CI
Water extract	491.8	186.3-1501.9
Dichloromethane/Methanol 1:1	481.72	165.44-3183.4

4.3.2 Acute Oral Toxicity

There was no mortality in the mice at 300 mg/kg (b.t.w) even on repeating the same dose as per OECD guidelines. At a dose of 2000 mg/kg (b.t.w) none of the three mice died at the first set of dosing in both the aqueous and Dichloromethane: Methanol 1:1 plant extract but on repeating the dose with the three set of mice one died in DCM: M extract. Hence both the aqueous and organic extract are in category 5 of Global Harmonization System (>2000-5000 mg/kg b.w.t) with LD₅₀ of 2500.

CHAPTER FIVE: DISCUSSION, CONCLUSION AND RECOMMENDATIONS

5.1 Discussion

5.1.1 Anti-microbial effects of *Vernonia hymenolepis*

The aqueous extract of the plant showed significant inhibitory effect on *Staphylococcus aureus* while organic extract showed inhibitory effects on all the tested micro-organisms *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Bacillus cereus* and *Candida albicans*. This suggested that organic extract (dichloromethane/methane 1:1) was a good solvent to extract active ingredients from the plant leaves.

Other studies have shown that some species of *Vernonia* such as *Vernonia cruda* (Van puyvelde *et al.*, 1983), *Vernonia colorata* (Kelmanson *et al.*, 2000), *Vernonia amygdalina* (Ogundare *et al.*, 2006), *Vernonia cinerea* (Yoga Latha *et al.*, 2009), *Vernonia scorpioides* (Bardon *et al.*, 2007) and *Vernonia leopoldii* (Mothana *et al.*, 2009) have been shown to have antimicrobial activity.

Even though the aqueous extract preparation aimed to mimic the traditional use, very little noteworthy antimicrobial activity was observed in this experiment. These findings correlate with that found previously where aqueous extracts showed low or no antimicrobial activity (Bamuamba *et al.*, 2008, and Kariuki and Njoroge, 2011) which suggested that water is not the most suitable solvent for extracting the active compounds from plants.

5.1.2 Analgesic properties of *Vernonia hymenolepis* in formalin test

In this study, Dichloromethane/methanol and aqueous extracts of *Vernonia hymenolepis* leaves significant analgesic activity in both phases of formalin test. The plant caused reduction in the duration of time spent in pain behavior for aqueous extract in both the early phase ($56.92 \pm 7.013s$ versus. $143.75 \pm 11.9s$ control) and late phases ($32.33 \pm 1.97s$ versus. $84 \pm 3.19s$ control) and for

Dichloromethane/Methanol extract in early phase (74.33 ± 19.36 s versus 111.17 ± 11.4 s control) and (9.91 ± 1.59 versus $62. \pm 17.9$ s control) in late phase. The result implies that the use of plant leaf by herbalist for treatment of toothache is due to analgesic bioactive ingredients that are in the leaf.

Other studies have shown that various species of *Vernonia* such as *Vernonia lasiopus* (Johri *et al.*, 1995), *Vernonia tenoreana*, *Vernonia cinerea* (Iwalewa *et al.*, 2003) and *Vernonia galamensis* (Johri *et al.*, 1995) have analgesic properties. *Vernonia aurilulifera* leaves are used in Cameroon to treat cataract and in Ethiopia to treat toothache (Focho *et al.*, 2009b; Giday *et al.*, 2009).

Formalin test is a widely used tonic pain model that is often used in the assay of antinociceptive activity (Coderre and Melzack, 1992). It is generally accepted that centrally acting analgesics have effects on both phases whereas peripherally acting analgesics affects only the first phase (Shibata *et al.*, 1989; Tjølsen *et al.*, 1992). This is because the injection of formalin resulted in the release of various neurotransmitters including glutamate and aspartate in the dorsal horn (Skilling, 1990).

This study has shown that *Vernonia hymenolepis* extracts has activities that inhibit pain in both phases just like acetylsalicylic acid hence; the extract could be acting as a central analgesic like acetylsalicylic acid. Acetylsalicylic acid is an analgesic that relieves aches and it is metabolized to integral part of human and animal metabolism (John *et al.*, 2008). It inhibits cyclooxygenase enzyme irreversibly unlike other NSAIDs since they affects more of COX-1 variant than the COX-2 variant of the enzyme (Garrat *et al.*, 2006). They also have ability to suppress the production of prostaglandins and thromboxanes due to its irreversible inactivation of the cyclooxygenase enzyme required for prostaglandin and thromboxane synthesis.

The early phase of the formalin test represents the transmission of nociceptive impulses while the second phase of the formalin test represents the events of central sensitization and wind-up phenomena (Coderre and Melzack, 1992; Vaccarino *et al.*, 1993).

Central analgesic drugs like narcotics inhibits both phases, while peripherally acting drugs like steroids (hydrocortisone and dexamethasone) and NSAIDs (indomethacine) acts mainly in the late phase (Trongsakul *et al.*, 2003).

5.1.3 Toxicity level

Studies have shown that brine shrimp lethality assay is an excellent cytotoxicity test. It has been used to detect fungal toxins, pesticide and cytotoxicity of dental materials (Alluri *et al.*, 2005). The interpretation of cytotoxicity was done (Meyer *et al.*, 1982; Santos *et al.*, 2003) such that LC₅₀ of between 500-1000 µg /ml is weak cytotoxicity, 100-500µg /ml is moderate cytotoxicity, 0-100 µg /ml is strong cytotoxicity while >1000 µg /ml non toxic. In the present study the plant extracts having LC₅₀ 491.8 (µg /ml) and LC₅₀ 481.7 (µg /ml) for water extract and Dichloromethane/Methanol extract respectively have moderate cytotoxicity.

Acute oral toxicity ATC method showed that the plant extracts in both extracts i.e. aqueous and Dichloromethane: Methanol 1:1 were not toxic at a high dose of 2000 mg/kg hence in category 5 of Global Harmonization System (>2000-5000 mg/kg b.w.t) with LD₅₀ of 2500. LD₅₀ of 2500 validates that the plant is not toxic even at high dose of 2000 mg/kg b.w.t (Eva *et al.*, 2005). Other studies have shown that acute oral toxicity ATC method is an alternative to the oral LD₅₀ test (Schlede *et al.*, 1995).

5.2 Conclusion

The use of *Vernonia hymenolepis* leaves should be encouraged by herbalist in various communities as herbal medicine to relieve pain in toothache since both extracts exhibited significant analgesic activity. The antimicrobial activity shown by the study suggests that the plant extracts can be incorporated into modern oral health care products as antimicrobial. This study has also shown that the plant extracts has a moderate cytotoxicity. Developing countries like, Kenya should include courses on alternative medicine in their institutions of higher learning to build capacity in natural products and to utilize the broad biodiversity that the country is endowed with. This will reduce leaving traditional medicine practice in the hands of less knowledgeable practitioners who may pose risks of deaths especially due to toxicity as it is reported in many parts of our country.

There is an urgent need of integrating herbal medicine into conventional health care system and also collaboration between the herbal practitioners and conventional practitioners to improve health care.

The antinociceptive and antimicrobial activities exhibited by this plant in the current sthought it was toxic.

5.3 Recommendations

1. Future studies should be done to involve the possible isolation and identification of the active Compounds responsible for the antinociceptive and antimicrobial activities in both the aqueous and organic extracts of *Vernonia hymenolepis*.
2. Herbalist should be encouraged to cultivate the plant to conserve it for sustainability.
3. The plant has shown not to be toxic hence herbalist should not have a course of alarm when patient swallow some juice.

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APPENDICES

Appendix 1: Mean, standard error of mean and range of the time spend in behavior of aqueous extract in formalin test

TIME	Mean and std	Range
0-5	97.17±48.75	(60-194)
5-10	45.33±25.41	(22-92)
10-15	37.833±15.70	(19-55)
15-20	44.67±18.39	(20-63)
20-25	38.67±16.633	(22-64)
25-30	40.33±14.28	(27-60)
30-35	34.83±18.52	(7-58)
35-40	28.83±14.36	(13-52)
40-45	22.33±11.18	(12-42)
45-50	22.33±11.91	(6-42)
50-55	27±10.62	(14-45)
55-60	19.5±8.69	(4-29)

Appendix 2: Mean, Standard error of mean and range of the time spend in

behavior of Acetylsalicylate in formalin test

Time	Mean and std	Range
0-5	62.17±15.77	(46-92)
5-10	1.17±1.17	(0-3)
10-15	15.17±22.94	(1-60)
15-20	21.67±24.91	(1-55)
20-25	36.83±35.58	(2-91)
25-30	32.17±39.83	(1-92)
30-35	58.5±39.83	(6-172)
35-40	35.33±41.13	(0-105)
40-45	86.17±71.12	(0-144)
45-50	22.33±11.91	(0-216)
50-55	70.67±82.32	(3-73)
55-60	22.33±27.61	(1-76)

Appendix 3: Mean, standard error of mean and range of the time spend in behavior of physiological saline in formalin test

Time	Mean and std DEV	Range
0-5	161.17±30.93	(120-200)
5-10	143.67±61.99	(55-220)
10-15	114.67±39.49	(83-170)
15-20	66.5±51.88	(8-140)
20-25	80.33±47.84	(13-146)
25-30	81.67±37.64	(16-120)
30-35	81.33±30.41	(53-120)
35-40	90.17±35.68	(57-156)
40-45	93.83±42.03	(51-173)
45-50	86.83±42.03	(45-134)
50-55	88.17±18.24	(72-120)
55-60	69.33±16.05	(38-82)

Appendix 4: Mean, standard error of mean and range of the time spend in

behavior of DMSO in formalin test

TIME	Mean and std DEV	Range
0-5	106.83±38.09	(43-150)
5-10	116±39.09	(85-170)
10-15	4.5±2.66	(3-7)
15-20	32.33±46.90	(3-122)
20-25	50.83±44.90	(8-64)
25-30	73.56±3.87	(15-81)
30-35	32±39.12	(3-109)
35-40	50.83±44.63	(3-118)
40-45	51.67±30.08	(13-80)
45-50	67.87±23.87	(25-75)
50-55	40.17±11.91	(26-58)
55-60	84.67±41.33	(3-106)

Appendix 5: Mean, standard error of mean and range of the time spend in behavior

DCM/M in formalin test

TIME	Mean and std DEV	Range
0-5	134.83±63.76	(36-227)
5-10	51.67±66.20	(0-164)
10-15	8.67.67±9.24	(2-26)
15-20	5.17±10.26	(0-26)
20-25	16.67±34.51	(0-87)
25-30	18.83±38.90	(0-98)
30-35	10.5±18.02	(0-45)
35-40	22.83±16.93	(2-42)
40-45	42.5±56.32	(4-147)
45-50	18.83±22.26	(1-56)
50-55	16.33±15.63	(0-31)
55-60	20±22.28	(2-63)

Appendix 6: Number of brine shrimp before and after exposure to test sample/control

	Tube 1		Tube 2		Tube 3		Tube 4		Tube 5	
	No. Shrimps		No. Shrimps		No. Shrimps		No. Shrimps		No. Shrimps	
	Dichloromethane/ Methanol extract									
Conc.	Before	After	Before	After	Before	After	Before	After	Before	After
1000	10	3	10	2	10	3	10	2	10	5
100	10	8	10	9	10	9	10	8	10	10
10	10	10	10	9	10	10	10	10	10	10
	Aqueous extract									
	Before	After	Before	After	Before	After	Before	After	Before	After
1000	10	1	10	3	10	2	10	4	10	2
100	10	10	10	9	10	9	10	10	10	10
10	10	10	10	10	10	10	10	10	10	10
	Marine salt and 0.5% DMSO									
1000	10	10	10	10	10	10	10	10	10	10
100	10	10	10	10	10	10	10	10	10	10
10	10	10	10	10	10	10	10	10	10	10

Appendix 7: Preparation of blood agar medium

Suspend 40g of blood agar in one liter of distilled water and boil to dissolve. This is autoclaved to sterilize at a temperature of 121 ° c for 15 minutes. It's then cooled to 45-55 ° c either 7% of sterile blood is warmed to 37 ° c then added to the media. This is placed into sterile dishes.

Appendix 8: Preparation of Muller Hinton broth medium

Typical formula (g/l)

Beef dehydrated infusion	300
Starch	1.5
Casein hydrosylate	17.5

Dissolve 21 grams in one liter of distilled water. This was boiled to dissolve. This is sterilized by autoclaving at 121 ° c for 15 minutes.