EXTRACTION, ISOLATION AND CHARACTERIZATION OF COMPOUNDS FROM *Euphorbia prostrata* AND THEIR ANALGESIC PROPERTIES

BY

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DECLARATION

Declaration by Student

This thesis is my original work and has not been presented for a degree in any other University or elsewhere. No part of this thesis may be produced without the prior written permission of the author and/or Moi University.

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Declaration by Supervisors

This thesis has been submitted for examination with our approval as University supervisors.

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DEDICATION

I dedicate this work to my loving parents, Rose Chemutai Chebochok and the late Antony Kibiwott Chebochok.

ABSTRACT

Pain is a symptom that results from particular physiological processes as injurious stimuli characteristic of cell injury or disease. Several herbs including *Phyllanthus lawii*, *Diallium guineense*, *Jathropha gossypiofolia linn*, *Pangamia pinnata linn* and *Papaver somniferrum* have been reported for use to relieve various aches, such as headaches, toothache, lower back pain and neuralgia. The objectives of this study were to extract, isolate, characterize and determine the analgesic efficacy of compounds from *Euphorbia prostrata*. The plant was collected from its natural habitat in Uasin-Gishu County and samples processed as whole plant by air drying before grinding. Serial extraction was carried out by soaking ground powder in three organic solvents (hexane, ethyl acetate and acetone) of increasing polarity each for 48 hours before filtering and rotary evaporation to dryness. Testing for analgesic activity was then carried out by Tail Immersion Model using albino rats. The active extracts were packed on silica gel column for chromatographic separation. Thin Layer Chromatography (TLC) was used to monitor the fractions with the help of visualization reagents. Further bioassay was carried out for the isolated fractions before purification by crystallization. Pure isolated fraction with the highest analgesic activity was then characterized by UV-Vis, Fourier Transform-Infra Red spectroscopy (FT-IR) and Gas Chromatography-Mass Spectroscopy (GC-MS). Results showed that ethyl acetate crude extracts afforded best yield of 5.80%. The highest analgesic activity was from ethyl acetate crude extract which significantly ($p > 0.05$) increased the pain reaction time (PRT) to10.40 seconds compared to positive control (Declofenac) that was 8.04seconds. Isolation by column chromatography afforded four fractions: H_1, H_2, H_3 and H_4 from hexane crude extract and five fractions: E1, E_2, E_3, E_4 and E_5 from ethyl acetate crude extracts. Further bioassay and purification of the fractions was done to yield fraction A which had optimum PRT of 6.21 seconds and UV λ_{max} of 259 nm. By using spectroscopic methods, fraction A showed steroidal characteristics. This was confirmed from FT-IR strong peaks of the fraction A at 3480 cm^{-1} (OH group, alcohols), 3150-3400 cm^{-1} (C-H stretching, aliphatic), 1650 cm^{-1} (C=C, alkene), 1400 cm^{-1} (CH_2), 1050 cm^{-1} (cycloalkane) and 870 cm^{-1} (C-H vibration for unsaturated part) as characteristic functional groups for steroids. GC-MS results showed pronounced molecular peaks at m/z 412, 414 and 426 for retention time of 12.26, 13.04 and 13.21 minutes respectively. With the help of data obtained, fraction A is most probably having a mixture of stigmasterol, β-sitosterol and cholest-5-en-3-ol, 24 propylidene. Analgesic activity of *Euphorbia prostrata* is attributed to the presence of steroids. *E. prostrata* is worth consideration for exploration in the quest for lead compounds in drug discovery particularly analgesics.

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LIST OF ABBREVIATIONS

AE	-	Acetone crude extract
ANOVA	-	Analysis of variance
DCM	-	Dichloromethane
E. prostrata	-	*Euphorbia prostrata*
EE	-	Ethyl acetate crude extract
EIMS	-	Electron impact mass spectroscopy
EtOAc	-	Ethyl acetate
EtOH	-	Ethanol
FT-IR	-	Fourier Transform-Infra Red
GC	-	Gas chromatography
HE	-	Hexane crude extract
Kg	-	Kilograms
MeOH	-	Methanol
mL	-	Milliliter
Mp	-	Melting point
NSAID	-	Non steroidal Anti-inflammatory Drug
Sec	-	Seconds
Spp	-	Species
TLC	-	Thin Layer Chromatography
UV-Vis	-	Ultra violet and visible Spectroscopy
WHO	-	World Health Organization
R_f	-	Retention factor

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CHAPTER ONE

INTRODUCTION

1.1 Background of the Study

1.1.1 Medicinal plants

The use of medicinal plants involves the use of plant extracts as medicines. Plants have been used therapeutically since time immemorial (Chelaiah & Muniappan, 2006). The oldest written verification of medicinal plants' usage for preparation of drugs was found on a Sumerian clay slab from Nagpur, approximately 5000 years old (Sumboonnanonda & Lertsithichai, 2004). This Sumerian clay slab showed 12 recipes for drug preparation referring to over 250 various plants, some of the plants include poppy, henbane, and mandrake. Many of these recipes were also found in the ancient Chinese book on roots and grasses "Pen T'Sao," written by Emperor Shen Nung circa 2500 BC. This book details 365 drugs from dried parts of medicinal plants, many of which are used even currently (Tesfahun *et al.*, 2014).

While the people in the past used medicinal plants primarily as simple pharmaceutical forms (infusions, decoctions and macerations), the demand for compound drugs was constantly increasing with time. Plants have metabolites which have been utilized by humans for making medicines (Daniel *et al.*, 2012). The interest in medicinal value of trees has sharply risen over the past few years mainly due to the rise in demand for chemical diversity in screening programs that seek therapeutic drugs from natural products (Sasidharan *et al.*, 2011). According to World Health Organization (WHO), 80% of people in the world currently rely on herbs for their primary healthcare, and to

generate income and for livelihood improvement (Ozbilgin & Saltan, 2012). This is because herbs are considered to be accessible, safer and affordable compared to the synthesized products which are regarded to have adverse side effects to humans and the environment. Currently, biosynthetic approaches where plant extracts are used are preferable because they are easily available, safe to handle and possess a broad viability of metabolites (Gunjan *et al.,* 2013). In addition, most of the secondary metabolites from plants provide protection against infections and are source of plant survival (Lauren & Peter, 2011).

1.1.2 Use of medicinal plants as analgesics

The use of plants as medicine for relieving pain has been reported in many studies (Fabricant & Farnsworth, 2001; Kennedy, 2004; Scheid, 2007; Khare, 2007). Researchers have identified a number of plants whose extracts have shown analgesic activity (Ezeja *et al.,* 2011; Panda *et al.,* 2009; Kondangala *et al.,* 2011; Rahul *et al.,* 2011) and this has especially been prompted by the fact that many synthesized analgesic drugs that relieve pain such as morphine are known to be associated with adverse effects like ulceration, gastrointestinal bleeding, addictive potential, respiratory distress, drowsiness and nausea (Modi *et al.,* 2012). Many phytochemicals from herbal plants have been found to provide analgesic solutions which are safe and broadly effective with fewer side effects (Sasidharan *et al.,* 2011; Ali *et al.,* 2014). For instance, phytochemical from *Pimenta officinali* (Priya *et al.,* 2012), *Andrographis paniculata, Argemone mexicana, Pimenta racemosa, Monarda didyma, Citrus bergamia, Boswellia thurifera* have been used as herbal solutions to relieve pain (Dana, 2010; Remedy, 2015).

1.2 Problem statement

Synthetic drugs have been used to treat diseases but they are expensive, inaccessible and some are known to have adverse side effects. For example, tramadol, a painkiller, has been found to cause slow breathing system, nausea and diarrhea (National Prescribing Service Limited, 2008). Medications with opioids results in numerous physical and psychological effects. Sometimes, providers of medications do not consider fully the harmful side effects in their prescriptions (Huntsman Cancer Institute, 2013). There is an increase in prescription of opioid drugs because medical professionals believe that opioid drugs are normally much safer. Among the side effects of opioids are; cognitive impairment, respiratory depression, gastrointestinal e.g. constipation, nausea & vomiting and gastrointestinal bleeding. Rosiglitazone which is used to treat diabetes is known to increase the risk of heart failure (Martinez, 2006).

Pain is an accompaniment of many medical conditions and pain control is among important therapeutic priorities (Ezeja *et al.*, 2011). It is mostly a warning signal that causes discomfort which leads to many adverse effects (Raquibul, 2010). Since most of the analgesic drugs available in the market have serious side effects, it is necessary to heighten the search for new analgesic drugs with minimal side effects. This study thus aimed to extract and isolate active components of *E. prostrata* plant and determine their analgesic properties.

1.3 Justification

About 80% of the world's population depends on herbal medicines for therapeutic purposes and useful information about medicinal plants is with the local people. The total population of plant species in the world is estimated at between 250,000 and 500, 000 and a large proportion has not been explored for medicinal purposes (Mehesh & Satish, 2008) and (Shinwari *et al.,* 2013). About 35,000-75,000 medicinal plants could make a substantial contribution in health gap (Khalil *et al.,* 2013). Due to the changing dynamics of human life style, indigenous knowledge which is relevant to using plants has decreased. The main hindrance for herbal therapies is the blending of native knowledge in the modern medical practices because of little or no scientific data available regarding the safety and efficacy of the herbal drugs (Ali *et al.,* 2014). Among the strategies employed by WHO, is to promote the safety, efficacy and quality of traditional medicine practices (WHO, 2013).

Up-coming health threats', ranging from drug resistance to strains of AIDS necessitates the pharmaceutical industry to continue the push for discovery and development of new drugs (Rachel *et al.,* 2010). This calls for documentation and authentication of the available indigenous knowledge.

Non-steroidal analgesic drugs reduce pain by suppressing the formation of prostaglandin, by inhibiting the activity of the enzyme cyclooxygenase. However, their use is limited due to their significant side effects that affect stomach, kidney and other body tissues (Debasis, 2011).

Many phytochemicals from plants have been found to provide medical solutions which are safe and broadly effective with fewer side effects (Ali *et al.,* 2014; Sasidharan *et al.,*

2011). Since many diseases are accompanied by pain, and because a good number of people currently seek for herbal therapy (Maliheh *et al.*, 2013), there is need for the search of bioactive compounds from natural products especially from medicinal plants for use as alternative analgesics with little or no side effects.

Euphorbiceae is one of the families that are used widely as medicinal plants to treat various aches. This family has therapeutic characteristics that arise due to secondary metabolites present in the plant (Serkan & Giilcin, 2012). This is caused by worldwide distribution of the plant species needing adaptations to all kinds of habitats. As a result of this adaptation, there is stimulation of many chemicals to help them survive the conditions such as different salinity and temperatures (Lai *et al.*, 2004). This leads to many secondary metabolites which may be reasons behind *Euphorbiaceae* family's medicinal use (Julius & Patrick, 2011). Species from this family are being utilized by herbalists and locals to cure ailments e.g skin disease, parasites, migraine and wounds (Ramezani *et al.*, 2008). Compounds in *Euphorbia* genera have various biological activities like; anticancer, anti-microbial, cytotoxic, antioxidants and anti-proliferative (Nazemiyeh *et al.*, 2010). In contrast, some of the species in this family are poisonous to human, domestic animals and fishes (Julius & Patrick, 2011).

Euphorbia prostrata belongs to *Euphorbiceae* family and it is used globally because of its medicinal properties. In India, it is used to treat first and second grade internal hemorrhoids (Serkan & Giilcin, 2012). In Africa, it is used to treat pregnancy problems, wasp & scorpion stings. It is also used to treat female sterility and painful menstruations (Gupta, 2011), amoebic dysentery, inflammations and as poultice for broken arms (Masango, 2008).

Traditionally, communities living in the Rift Valley region of Kenya have used *Euphorbia prostrata* to treat toothaches. In this study, *Euphorbia prostrata* was subjected to phytochemical and biological screening for its analgesic properties.

1.4 Objectives

1.4.1 General Objective

To study properties of *E. prostrata* compounds for analgesic activity.

1.4.2 Specific Objectives

The specific objectives of the study are to:

i. Extract and quantify crude extracts of *E. prostrata* from different solvents

ii. Perform analgesic bioassay of the crude extracts from *E. prostrata*

iii. Isolate the active compounds from the crude extracts of *E. prostrata*

iv. Perform analgesic bioassay of the isolated compounds from *E. prostrata*

v. Characterize the isolated active compounds from *E. prostrata*

1.5 Hypothesis

i. *Euphorbia prostrata* plant contains compounds that have analgesic activity.

ii. Bioactive compounds are stable and can be isolated and their structures elucidated.

CHAPTER TWO

LITERATURE REVIEW

2.1 Medicinal Plants

Medicinal plants are considered to be important part of current pharmaceutical drugs because they are a rich source of ingredients which can be used to develop and synthesize drugs. These plants produce a range of substances that play important roles in the body (Ramezani *et al.,* 2008) and are characterized depending on their therapies. For example, synergic medicine is when all the plant ingredients interact simultaneously and their uses harmonize or damage or deactivate the possible negative effects (Che *et al.,* 2013). With the support of conventional medicine in the treatment of complex cases such as in cancer diseases, plant ingredients have been proven to be very effective (Madana *et al.*, 2014), while preventive medicine prevents the appearance of some diseases leading to reduction of the side effects of synthetic treatment (Carlos, 2007).

2.2 Pharmacology of medicinal plants

2.2.1 Antioxidants

Free radicals of oxygen induce damage to tissues hence causing diseases (Abheri *et al.*, 2010). Antioxidants deactivate free radicals thus help in preventing diseases (Funda & Hakan, 2008). Synthetic anti-oxidants such as butylated hydroxyl anisole (BHA) have been reported to be dangerous to human health (Lobo *et al.,* 2010). Most medicinal plants offer a good source of antioxidant phytochemicals and thus have received a growing attention especially as chemo preventive agents (Ramhat *et al.*, 2012). Epidemiological reports show that antioxidants have protective role in most health

diseases and they have been shown to be vital to human health and possess less or no toxicity (Pollyana *et al.,* 2014). Replacing synthesized antioxidants with plants possessing anti-oxidant activity will be of great benefit to health (Andres *et al.,* 2001). *Curcuma domestica* (Turmeric) is known to have compounds with anti-oxidant activity due to presence of curcumin (**1**) and β- sitosterol (**2**) (Ramya, 2013). *Daucas carota* (carrot) is known to have terpineol (**3**) (Figure 2.1) which act as anti-oxidant too (Kamlesh *et al.,* 2012).

Figure 2.1. Some of the anti-oxidative compounds from plants.

2.2.2 Anti-microbial

Resistance of important antibiotic by pathogens has increased (WHO, 2013). Plants act as a source and template of new antimicrobial drugs (Pretorious *et al.,* 2003), for instance, *Kalanchoe crenata (KC)* plant is being utilized by the natives in Cameroon to treat coughs, dysentery and wounds (Nguelefack *et al.,* 2003). Studies have shown that *KC* extracts have antimicrobial activity (Vinit *et al.,* 2012), which strongly supports its' use

by herbalists because it has bioflavonoids, tannins, alkaloids and terpenes which are believed to be responsible for the observed anti-microbial activity (Ruma *et al.,* 2015).

2.2.3 Anti-inflammatory and analgesic

Tissues respond physically to injuries leading to accumulation of blood cells and plasmatic fluids to form inflammation (Vino *et al.,* 2011). Plants have phytochemicals, especially steroids, alkaloids, flavanoids and terpenoids which could be the reasons behind their use as anti-inflammatory and as analgesics (Tajuddin *et al.,* 2011). Flavanoids are known to inhibit production of enzyme prostaglandin synthase which are pain mediators and are responsible for inflammation hence their inhibition results to analgesic and anti-inflammatory effect (Moein & Moein, 2010). Analgesic herbs are used to relieve various aches, for example, headaches, toothaches, lower back pain and neuralgia (Rupa *et al.,* 2012). Some of the plants that have been tested for their analgesic activity include: *Phyllanthus lawii (PL), Diallium guineense (DG), Jathropha gossypiofolia linn (JGL), Pangamia pinnata linn (PPL)* and *Papaver somniferrum (PS)* as discussed below.

2.2.3.1 *Phyllanthus lawii* (*PL*)

It is a glabrous shrub which grows along river banks and it belongs to *Euphorbiaceae* family. It is taken with sugar – candy orally for 7 days to treat stomach, brain and lung cancers. The extracts from *PL* are dominated by pentacyclic triterpernoids which possess analgesic activity which are reported to be inhibitors of NF-$_K$b signaling (Salminen, 2009). The methanolic extract of *PL* inhibited the acetic acid induced pain with potency compared with aspirin (Kondangala *et al.,* 2011).

2.2.3.2 *Dialium guineense* (*DG*)

It is a small tree or shrub with dense crown and hanging leaves. *DG* belongs to family *Fabiceae*. Its bark is grayish, smooth and slashes reddish sometimes releasing a red gum. The seeds are sweetly edible and it is found in savannah forests. Different parts of the tree are used by indigenous people from Nigeria for treating various ailments. For example, barks are used to treat pain, leaves used for fever, prenatal pains and edema while the fruits are used for diarrhea (Gideon *et al.,* 2013). It is also used to treat fever and headache. The analgesic activity may be acting through inhibition of prostaglandin pathway or through a peripheral pain mechanism (Ezeja *et al.*, 2011).

2.2.3.3 *Jatropha gossypiofolia linn* (*JPL*)

The plant belongs to *Euphorbiaceae* family. It grows wildly as bushy gregarious shrub. It is known to possess anticancer, hepatoprotective and pesticidal activity. The leaves from *JPL* are used to bath wounds, to relieve stomachache, venereal diseases and as blood purifiers. Stem sap is believed to stop bleeding and itching of cuts and scratches (Taofeeq *et al.,* 2005). The roots are used against leprosy and as antidote in snake bite and in urinary complains. Flavanoids from leaves are vitexin, isotexin and pigenin. The bark contains the alkaloid jatrophine while lignin jatroiden is found in stem. Its latex yielded two cyclic octapeptides namely glycosine, A and B. Aerial parts contain a lignin and gossy piline. The methanolic extract of aerial parts of *JPL* produces significant analgesic and anti-inflammatory activities in dose dependent manner (Panda *et al.*, 2009).

2.2.3.4 *Pangamia pinnata linn (PPL)*

It is a member of *leguminoseae* family. It is a medium sized evergreen tree found in coastal regions in India. The dry leaves of *PPL* are used by herbalists for the treatment of diarrhea, diabetes and inflammatory disorders (Arote & Yeole, 2010). The ethanolic extract of *PPL* leaves exhibits analgesic activity at dose level of 250 and 500 gm/kg body weight (Rahul *et al.*, 2011).

2.2.3.5 *Papaver somniferum (PS)*

It is popularly known as Opium Poppy or Keshi. It has been used as an analgesic since ancient times. It has various alkaloids which include codeine and paparevine apart from alkaloid morphine which is the main analgesic constituent first isolated in 1905 (Avantika, *et al.*, 2012).

2.3 Botanical background of the Euphorbiaceae family

The *Euphorbiaceae* family is the largest family of Angiosperms with 300 genera and 500 species for example, *Euphorbia* (Adedapo *et al.*, 2005), *Euphorbia polycantha* (Gihan, A., & Abdalla, 2015), *Euphorbia heliscopia* (Bashis *et al.*, 2001) *and Euphorbia thymifolia* (Prashant & Shital, 2013). They are often poisonous, prostate, and erect or scan dent annual biennial or perennial herbs, shrubs or trees, succulent or not spiny, sometime, with phylloclade, with or without a milky latex or colored sap. The genus *Euphorbia* is the largest genera with flowering plants, having about 2000 species. It is cosmopolitan restricted to tropical, subtropical and warm temperature regions, monocious herbs, shrubs or trees often succulent. They possess milky latex and with a simple indumentums (Muhammad *et al.*, 2012). The leaves are made of three types; lower,

median and upper. Leaves are ray-whorled or opposite free or connate. Most leaves are sessile, have shortly petiolate, stipulate or not, simple or toothed, penni or palm nerved (Ali *et al.*, 2014). About 2000 species of genus *Euphorbia* are known in the family of *Euphorbiaceae*. Most of the known species contain latex and a distinctive flower structure. A good percentage of the ones that originate from Africa and Madagascar are tender (Julius & Patrick, 2011).

2.4 Secondary metabolites from euphorbia genera

2.4.1 Terpenoids

The terpenoids (isoprenoids) are a large and diverse class of naturally occurring organic chemicals similar to terpenes, derived from five-carbon isoprene units assembled and modified in thousands of ways. Plant terpenoids are used extensively for their aromatic and herbal therapeutic remedies most of which are under investigation for antibacterial, antineoplastic, and other pharmaceutical functions (Belonwu *et al.*, 2013).

The diterpernoids in genus *Euphorbia* are well-known in irritating skin and tumor -promotion. These terpenoids include; jatrophane, lathyrane, myrsinane, tigliane and sesquiterpenoids. The constituents have been extracted and isolated from different parts of the plants i.e. leaves, aerial parts, milky latex, roots and seeds. Major effort has been directed to *Euphorbia* diterpenes due to their varied structures and therapeutical significance (Samir *et al.*, 2002); Macro cyclic diterpenes from *Euphorbia* species are said to have cytotoxic, antitumor, antibacterial, anti-inflammatory and anti-HIV activities. Their biological action comprises anti proliferative, modulability of multi-drug

resistance, antiviral, anti-diarrheal, molluscicidal, antifeedant, antimicrobial and antipyretic- analgesic actions (Ozbilgin & Saltan, 2012).

2.4.2 Alkaloids

Alkaloids are a group of naturally occurring chemical compounds that contain mostly basic nitrogen atoms in addition to conventional carbon and hydrogen atoms. Alkaloids have a wide range of pharmacological activities including antimalarial (e.g. quinine), antiasthma (e.g. ephedrine), anticancer (e.g. homoharringtonine), cholinomimetic (e.g. galantamine), vasodilators (e.g. vincamine), antiarrhythmic (e.g. quinidine), analgesic (e.g. morphine), antibacterial (e.g. chelerythrine), and antihyperglycemic activities (e.g. piperine). Many have found use in traditional or modern medicine, or as starting points for drug discovery. Other alkaloids possess psychotropic (e.g. psilocin) and stimulant activities (e.g. cocaine, caffeine, nicotine), and have been used in entheogenic rituals or as recreational drugs (Klepstad *et al.*, 2003).

After the use of herbs for a long period, the isolation of active principles like alkaloids in 19thCentury, marked a new era in the use of herbs and the start of plant medicine research in modern diseases (Ameyaw & Duker –Eshun, 2009). Alkaloids have grown to be the largest group of natural products because it is believed to display a diversity of structure unmatched by other group of natural products. They show great variety in their botanical and biochemical origin (Matma *et al.*, 2013). Alkaloids are organic bases where many are poisonous, others are addictive such as cocaine and some are used clinically, for example, morphine (Sarah, 2004).

2.4.3 Tannins

Tannin (tannoid) is an astringent, polyphenol compound that binds to and precipitates proteins and various other organic compounds including amino acids and alkaloids. The tannin compounds are widely distributed in many species of plants, where they play a role in protection from predation, and perhaps also as pesticides, and in plant growth regulation (Belonwu *et al.*, 2013).

Various tannins have been discovered in the family *Euphorbia spp.* The *E. ocellata* Durand and Hilgard of the Pacific coast is used as an antidote for snake bites, and is said to contain 2.82% of gallo-tannic acid. Tanins were also isolated from *Euphorbia prostrata* and identified as gallic acid, corilagin, geranin, tellimagradin I, II, rugosin A, rugosin E, rugosin D and rugosin G on the basis of physicochemical and spectroscopic methods (Abdul, *et al.*, 2014). Chemical investigation of *Euphorbia thymifolia* also led to the isolation and characterization of a new hydrolysable tannin named isomallotinic acid (Maoulainine *et al.*, 2012). Gvazava and Alaniya (2005) also isolated three tanins known as glareins A-C from *Euphorbia glareosa* leaves. The following hydrolysable tannins have also been found in *E. helioscopia*; helioscopin A and B (41 & 42), Helioscopinin A and B, glarein, punicafolin, elaeocarpusin, furosin, mallotusinin, carpinusin (Maoulainine *et al.*, 2012; Park *et al.*, 2001).

2.4.4 Flavanoids

Flavanoids (or bioflavonoid) are a class of plants' secondary metabolites and are classified into six major groups based on their molecular structures, namely: flavones (**4**), flavonols (**5**), flavanones (**6**), flavon-3-ols (**7**), isoflavones (**8**), flavanonol (**9**) (Figure 2.2) (Shashank & Abhay, 2013).

Figure 2.2: Major groups of flavanoids.

Flavanoids are very important for humans and animals in general because they serve as anti-carcinogenic compounds (Shashank & Abhay, 2013).

Several flavanoids have been discovered in the *Euphorbia spp* (Figure 2.3). Flavones such as Luteolin (**10**), Apigenin (**11**) and Chrysin (**12**), have been discovered in *E. ocellata.* Additionally, flavanols such as Quercetin (**13**), Kaempferol (**14**) and Galangin (**15**) were discovered by Albert Szent Gyorgi in 1930 (Lakhanpal P & Rai, 2007). Only two flavanones have been reported in *Euphoria Spp.* These are Hesperetin (**16**) and Narigenin (**17**). A novel flavanone, Taxifolin (**18**) in *E. glareosa* leaves was reported recently (Amir *et al.*, 2015). They additionally discovered two novel isoflavones namely Genistein (**19**) and Daidzein (**20**). Catechin (**21**) and Epicatechin (**22**) are hydrolysable flavan-3-ols found in *E. helioscopia* (Shashank & Abhay, 2013).

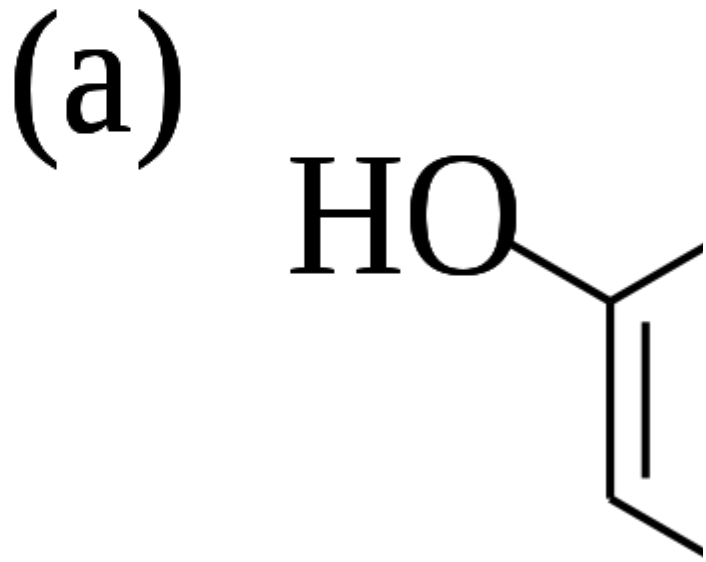


Figure 2.3: Flavanoids from Euphorbiaceae family: (a) Flavonols, (b) Flavanones, (c) Flavanonol, (d) Flavanones, (e) Isoflavones (Shashank & Abhay, 2013).

2.4.5. Steroids

Steroids can be synthesized or occur naturally in living organisms (Carl & Joanne, 2002 ; Yadav *et al.,* 2011; Taves *et al.,* 2011). They have various biological activities (Anna, 2006) for example; in both animals and plants, convergent uses of polyhydroxylated steroids to coordinate overall development have been demonstrated. In plants and animals, steroids control a certain biological response which alters patterns of gene expression in target cells (Elizabeth & Mark, 2009). In addition, various steroids are produced by plants for various reasons, i.e. physiological, allelochemical substances related to animal hormones and plant – allelochemicals for protection (Laurence *et al.,* 2001). Plants synthesize steroids to fight insects or scare them away (Christopher *et al.,* 2002). Previous experiments applied plant steroid hormones and monitored their responses. This showed effects on the stem elongation, micro-tabule orientation and leaf unrolling (Gerard & Takao, 2001). A research on dwarf mutants in plants indicated that dwarf mutation can be corrected by applying brassinosteroid (Tomoaki *et al.,* 2012).

Steroids in *Lageneria siceraria* possess cardio protective (Kirtikar & Basu, 2006; Yash, *et al.,* 2014) and anti-inflammatory properties (Gangwal *et al.,* 2010). Besides, research has indicated that combination of flavonols and steroids improve pollen germination of some plant species (Manoel *et al.,* 2011). A few steroids have been found in *E. prostrata* plant and they include; stigmasterol, campesterol and cholesterol (Syed, *et al.,* 2013).

2.5 Euphorbia prostrata

2.5.1 Physical characteristics

It is an annual herb about 15-19 cm. Its stems are flattened and glabrous beneath, red, sometimes green or yellow green. The leaves are opposite, stipules long triangular, which

are easily fallen. Margins are entire or irregularly finely serrulate, apex rounded. The male flowers are many and usually shorter than cup while the female flowers are pedicellate, exerted from inloucre, ovary is sparsely pubescent on angles, styles nearly connate at base. The flower parts are difficult to discern without magnification. Seeds are yellow ovoid-tetragonal, each side with 6 to 7 transverse furrows (Syed *et al.*, 2013). It is a prolific seed producer and so often, hundreds of seeds will germinate at the same time. Spurge develops a deep taproot, the weed could fairly be hand-weeded. It has no taste or odor (Serkan & Giilcin, 2012).

Figure 2.4: *Euphorbia prostrata* plant

2.5.2 Known Compounds from *Euphorbia prostrata*

A few compounds have been isolated and identified in this plant. Among them are; leuteolin (**10**), diosmin (**23**), neptin (**24**), hespiridin (**25**) and epicatechin (**26**), as shown in Figures 2.3 & 2.5. Diosmin(**23**) and hespiridin (**25**) have been shown to be effective

and safe in treatment of varicose veins and hemorrhoids (Thorne Research, 2004). Other studies have shown that the extracts can be used in wound healing and as an anti-hemorrhoidal (Ashwin *et al.,* 2015).

Figure 2.5: Some known compounds from *Euphorbia prostrata* (Ashwin *et al.,* 2015)

2.5.2 Bioactivity of *Euphorbia prostrata*

Therapeutic studies of *E. prostrata* have confirmed that it exhibits a broad range of biological effect and it is very important for future development of drugs.

2.5.2.1 Antioxidative

Ramhat, *et al.,* (2012) found out that the methanolic extract of *E. prostrata* possesses scavenging properties and scavenged 1-1 diphenyl-2- picryl-hydrazyl (DPPH). This is likely due to presence of phenolic species which are present in the *E. prostrata* plant. They inhibit free radicals that cause oxidative stress (Duenas *et al.,* 2006).

2.5.2.2 Antibacterial

E. prostrata exhibited significant antimicrobial activity when tested against *Escherichia coli*, *Micrococcus luteus*, *Staphylococcus aureus* and *Pseudomonas aeruginosa* (Ramhat, *et al.,* 2012).

2.5.2.3 Efficacy of *E. prostrata* in early stages of symptomatic hemorrhoids.

Gupta (2011) in his study found that *E. prostrata* can intervene effectively in initial stages of hemorrhoids. Elagic acid in *E. prostrata* is known to suppress histamine release which is mediated by histamine liberators (Sumboonnanonda & Lertsithichai, 2004). Also flavanoids in *E. prostrata* plant are known to decrease the edema (Christopher *et al.,* 2014).

2.5.2.4 Irritant effect

Chloroform extract of *E. prostrata* exhibited irritancy on rabbit's ear. The presence of –OH, –C=C– or –COOH group in the plant is responsible for the irritancy causing inflammation hence hurting the epidermis (Syed *et al.,* 2013).

2.6 Pain and inflammatory mechanism

Nervous system has a lot of responses depending on different conditions (Streeter, Gerbarg *et al.*, 2012). A constant activation of nociceptor in response to noxious stimulus cause nociceptive pain (Coda & Bonica, 2001). Correspondence between pain perception and stimulus intensity is close and pain sensation indicates tissue injury (Janel, *et al.*, 2010). When the tissue is injured, pronociceptive mediators (peripheral sensitization) that sensitize peripheral nerve terminals are released (Nasreen *et al.*, 2015), leading to phenotypic alterations (central sensitization) of the sensory neurons and rise in excitably of spinal cord dorsal horn neurons (Scholz & Woolf, 2002). Central sensitization is associated with persistent pain and the spread of pain on injured tissues (Tsuda *et al.*, 2006). Due to mal- adaptive changes, it has been noted that established pain is difficult to suppress than acute that take place in the central nervous systems (Janel *et al.*, 2010). Nociceptors are sensory receptors responsible for the detection of noxious stimuli and transforming them into electrical signals which are conducted to the central nervous system. The nociceptors are distributed throughout the body and can be stimulated by thermal, chemical or mechanical stimuli (Field & Cox, 2006). Some chemicals are released within the human and animal bodies and these chemicals stimulate the nociceptor. These chemicals include bradykinin, serotonin, prostaglandins and cytokine which are released from injured tissues. Inhibition of these chemicals lowers the stimulus of the nociceptor hence less pain (Woolf, 2004).

2.7 Pharmacotherapy of pain

Synthetic drugs used in the management of pain are many and some of them have side–effects because they are not specific enough on their actions and interact with receptors

other than the one of interest. Example of these drugs include morphine **(27)**, Declofenac **(28)** and indomethacin (indocid) **(29)** (Bowman, 2006).

2.7.1 Morphine

Morphine (**27**) is one of the narcotic analgesic drugs occurring naturally as phenanthrene alkalaoid derived from opium. Chemically, it is designated as 7,8-dihydro-4,5-epoxy-17-methylmorphinsn-3,6 diol sulfate pentahydrate (Guillaume, 2012; Nations, 2013). Morphine exerts its pharmacological effects on central nervous system and gastro intestinal tract. Primary therapeutic value of morphine is analgesic and sedation by increasing patient's tolerance to pain and decreases perception of suffering (Julien, 2001). Opiates are ideal for treatment of deep chronic pain and they work on central nervous system (Andrew *et al.,* 2008).

Morphine is associated with side effects such as vomiting, nausea, constipation, difficulty in urination, hypotension & fainting (Donald, 2014). It induces the release of histamine which leads to dialation of cutanious blood vessels resulting to flushing of the neck and sweating (Goldstein, 2001). Analgesic effect of morphine occurs within 5-20 minutes and rise to peak analgesia after 60 minutes after single intramuscular injection (Hodgson, 2001). Duration of analgesia after single dose administration lasts for 3 to 4 hours. Opioid

analgesics like morphine induce tolerance to their effects, hence shorter duration of analgesia following subsequent doses (Edward, 2013).

2.7.2 Declofenac

Declofenac (**28**) is an oral or inject able drug which belongs to Non-Steroidal Anti-Inflammatory Drugs (NSAID) (Jean, *et al.*, 2012; Tong *et al.*, 2012). It is among the first – line drug of choice for treatment of intensive pain like back pain, migraine attacks, control of pain in dental and minor surgeries (Studies National Institute of Clinical, 2011).

Declofenac exhibits pronounced anti- inflammatory, anti-pyretic and analgesic properties. Its mechanism of action is inhibition of prostaglandins which play major role in pain, fever and inflammation. Clinical studies have showed that Declofenac relieve pain and reduce bleeding in dysmorrhea. Half-life of Declofenac is 4 – 6 hours after administration (Krum *et al.*, 2012). Declofenac is contraindicated to patients with asthma, peptic ulcers, gastro-intestinal bleeding and renal failure (Forrest, *et al.*, 2006). The un-desirable side effects of Declofenac are drowsiness, fatigue and visual disturbances (Coxib and traditional NSAIDS Trialists Collaboration, 2013).

2.7.3 Indomethacin

It is an NSAID drug which is a good rodenticide. It possesses antipyretic and analgesic properties (Hemamalini *et al.,* 2010). Indomethacin is potent inhibitor of prostaglandin synthase hence its mode of action is due to decrease in peripheral tissues (Vishal *et al.,* 2013). It does not alter progressive course of the underlying disease, it reliefs symptoms only. Peak plasma concentrations are about 2 hours. It is eliminated by renal and billary excretion (Vishal *et al.,* 2013).

2.8 Evaluation of analgesic activity

There are three major models to test analgesic activity of a drug, namely; acetic acid induced writhing, hot plate method and tail immersion test.

2.8.1 Acetic acid induced writhing

Writhing could be a stretch, hind legs extension, abdomen contraction so that the abdomen touches the floor or twisting the trunk. Rats or mice are used in this model. Animals are fasted for 12 hours before the assay. The animals are then divided into 5 groups or 3 groups, depending on the doses they will receive. A solution of acetic acid (1 % v/v) in distilled water and a positive control drug is prepared. The animals are given the drug by gastric gavage. After 1 hour administration of the drug and extracts, the animals are then given a solution of acetic acid intraperitoneally (1mL/ kg body weight)

to induce pain, writhes or constrictions. Number of writhes observed in each animal is counted for 30 minutes and recorded. Protection percentage against abdominal writhing is used to assess the extent of analgesia (Ezeja *et al.,* 2011).

$$Protection\ percentage = \frac{Mean\ control - Mean\ treated\ group}{Mean\ of\ control\ group} \times 100$$

$$Protection\ percentage = \frac{Mean\ control\ group - Mean\ percentage\ group}{Mean\ control\ group}\ x\ 100$$

……. (Equation 1)

Table 2.1: Acetic acid- induced writhing Reflex in rats/ mice

Group	Treatment mg/kg	Mean Number of writhing	Protection %
A	Tween solution		
B	Positive control drug		
C	Extract 250 mg/kg		
D	Extract 500 mg/kg		
E	Extract 1000 mg/kg		

This method is used to assess analgesic drugs that act via peripheral pain mechanism. Acetic acid- induced writhing model arise in feeling of pain by triggering inflammatory response leading to release of arachidonic acid tissue (Ezeja *et al.,* 2011).

2.8.2 Hot plate method

This is where the paws of mice/rats are subjected to heat since they are very sensitive to heat at temperatures 55°C ± 1°C. The animals respond by jumping, withdrawal or licking of the paws. The hot plate can be a glass surface or a copper plate. Animals are placed on

the hot plate and the time they take for them to lick or jump is recorded by use of stop watch. Animals are fasted for 12 hours before the experiment up to the end. The animals are placed on the hot plate and the pain reaction time is recorded (PRT) before positive and test drugs are given orally. After 30 minutes of drug administration, animals are placed on the hot plate and observations made and PRT is recorded. This is done for the time intervals of 60, 90, 120 and 180 minutes and a table is tabulated as follows:

Table 2.2: Hot plate induced pain

Group	Treatment mg/kg	Mean pain reaction time in seconds						
		0 min	**30 min**	**60 min**	**90 min**	**120 min**	**150 min**	**180 min**
A	Tween solution							
B	Positive control drug							
C	Extract 250 mg/kg							
D	Extract 500 mg/kg							
E	Extract 1000 mg/kg							

Hot plate method is used to test analgesic drugs that act centrally by inhibiting or minimizing the release of prostaglandins which are pain mediators (Ezeja *et al.*, 2011).

2.8.3 Tail immersion test

Tail immersion model employs the same mechanism as hot plate method. Animal's tail of about 5 cm is immersed in hot water with temperatures of 50°C± 5°C and the time taken for it to withdraw its tail from hot water is recorded. The reaction time (Pain Reaction Time) is determined before and after oral administration of the test and standard drug.

Table 2.3: Tail immersion response

Group	**Treatment mg/kg**	**Mean pain reaction time in seconds**						
		0 min	**30 min**	**60 min**	**90 min**	**120 min**	**150 min**	**180 min**
A	Tween solution							
B	Positive control drug							
C	Extract 250 mg/kg							
D	Extract 500 mg/kg							
E	Extract 1000 mg/kg							

Tail immersion method is used to test analgesic that act centrally by inhibiting the synthesis of prostaglandins synthase enzymes which are pain mediators (Panda, *et al.*, 2009). Tail immersion method was employed in this study.

CHAPTER THREE

MATERIALS AND METHODS

3.1. Materials

3.1.1 Reagents / Chemicals

All the reagents used were purchased from Chemo-quip Kenya and Gelsup laboratories suppliers, Kenya. They were all of analytical grade (AG).

Solvents used were hexane, ethyl acetate, acetone, dichloromethane and chloroform. Chemicals used were, sulphuric acid, glacial acetic acid, bismuth, ferric chloride, potassium iodide and hydrochloric acid.

3.1.2 Equipment

The following equipment were used for sample analysis.

GC-MS (Turbo mass model 20141128), FT-IR (Perkin- Elmer, Beaconsfield, (UK Spectrum one FT-IR spectrometer) and UV-Vis (Shimadzu, UK).

3.1.3 Animals

Albino rats weighing 150-250 gm of either sex were obtained from University of Eldoret animal house after obtaining permission from Kenya Society for the Protection and Care for Animals (KSPCA). The rats were brought to the laboratory and maintained in cages at 25±1^{o}C and fed for two days. They were then starved for 12 hours and were given clean water prior to the experiment according to (Shashank & Abhay, 2013).

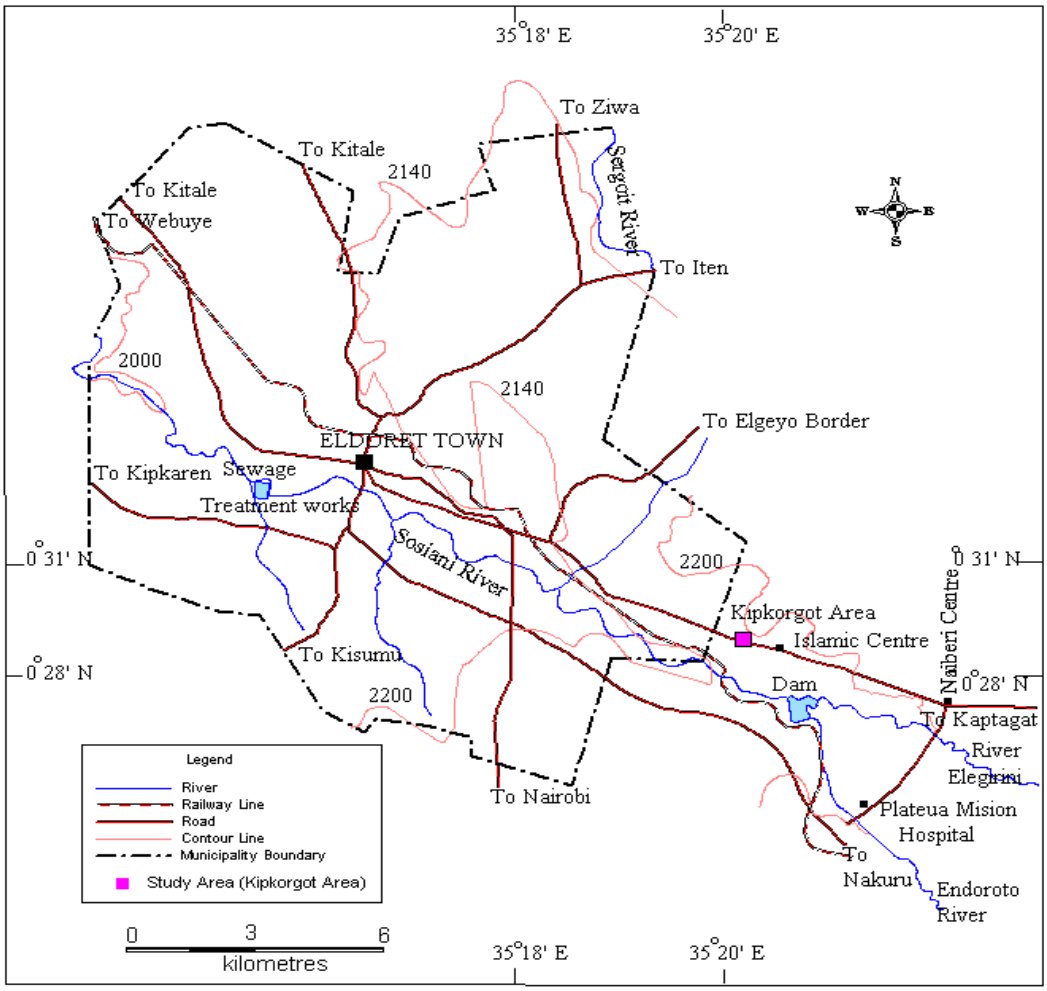


Figure 3.1: Map showing the site where the plant material was collected.

3.2 Methods

3.2.1 Summary of experimental procedures

The schematic representation of the procedure followed in extraction, isolation, characterization of crude extract and purified fraction and bioassay in this study is as shown in Figure 3.2.

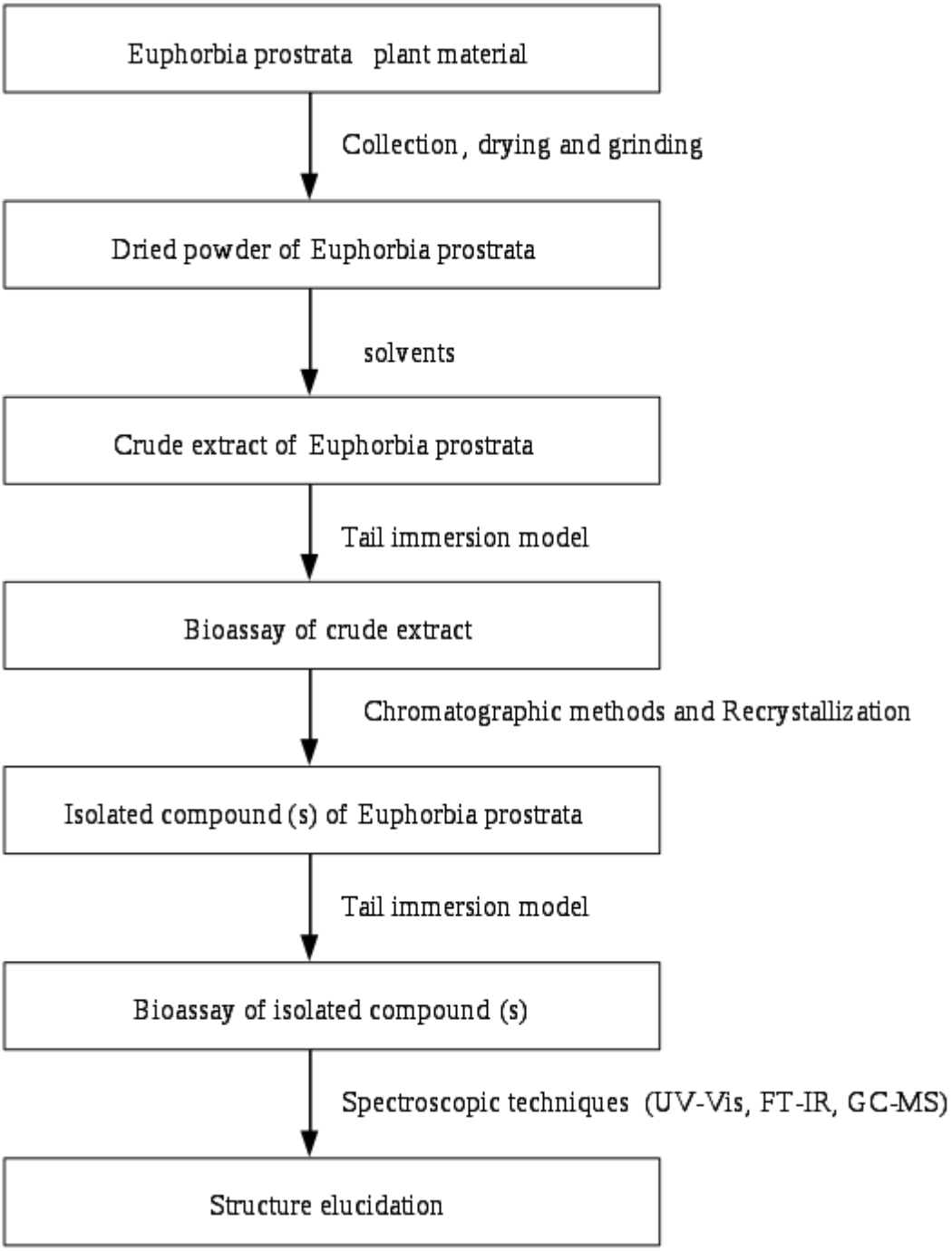


Figure 3.2 Schematic representation of procedure followed in extraction, isolation, Characterization of crude extract and isolated compounds from *E. prostrata*.

3.2.2 Plant collection and identification

The plant material was collected from Kipkorgot area along Eldoret-Ravine Road, Uasin-Gishu County, as shown in Figure 3.1. The geographical coordinates of Kipkorgot area are: 0° 31° 28’ North, 0° 35° 18’ East. Fresh green plant were picked and taxonomically identified at the Department of Botany, School of Biological and Physical Sciences, Moi University. The plant specimen was deposited appropriately in Moi University herbarium and was assigned accession number MU/BL/0050/2015.

3.2.3 Sample processing

All parts of the plant were washed and weighed before air drying for 8 weeks to remove water completely. Its weight was taken again after grinding into fine powder so as to increase the surface area of the sample and enhance the contact between the solvent and the sample.

3.2.4 Extraction

Serial extraction was done by soaking 3.9 kg of powdered plant in three solvents (hexane, ethyl acetate and acetone) of 10 litres each respectively for 48 hours. Filtration was done using cotton wool followed filter paper. The extracts were concentrated using vacuum rotary evaporator.

Percentage yield of *E. prostrata* extracts was determined by using Equation 2 (Anokwuru *et al.*, 2011).

Percentage yield = $\frac{W_2 - W_1}{W_0} \times 100$ …….. (Equation 2)

W_0: - The weight of the initial dried sample.

W_1: - The weight of the container alone

W_2: - Weight of the extract and container

3.2.5 Isolation

Active crude extracts were subjected to column chromatography. For each crude extract, 30 g was absorbed in 30 g of silica gel and loaded into the column packed with 200 g of silica gel. The column was eluted using increasing gradient of hexane in DCM starting with the ratio 8:2 to 2:8, followed by DCM with EtOAc solvent system in the ratio of 9:1 and finally EtOAc with methanol in the ratio of 9:1. Semi pure fractions were concentrated using rotary evaporator and re- packed in analytical columns. The analytical columns were eluted using increasing gradient of hexane and DCM (9:1) followed by DCM- EtOAc (9:1 v/v) and finally MetOH and EtOAc solvent system (1:1 v/v). All fractions were pooled according to their TLC profiles and re-crystallized.

3.2.6 Re-crystallization

The semi-pure fraction weighing 50 mg was dissolved in hexane and water was added thereafter to form a semihydrate which is insoluble in hexane and forms a precipitate. The slurry was filtered to remove the crystals from the solution. Cold hexane was used to wash the crystals (Chiang, 2006).

3.2.7 Sample analysis

All the crude extracts were subjected to IR spectroscopy after completely drying them at 30 °C. Dried crude extract was mixed with KBr (IR- grade) at a ratio of 1:100 and pressed to a pellet. The pellet was put into the sample holder of Perkin Elmer Spectrophotometer and operated in the range of 4000-650 cm^{-1}. The obtained spectral data was used to identify functional groups present in the crude extracts of *E. prostrata* from the serial extraction.

The isolated active fraction was subjected to spectroscopic analysis (UV-Vis, FT-IR and GC-MS) and compared with the spectral data reported in literature.

For the UV- visible Spectrophotometer, dry fraction weighing 5 mg of the fraction was mixed with 2 mL of distilled water to dissolve it and was placed in a cuvette. The samples were scanned at the range of 200-800 nm to detect its characteristic wavelength.

FT-IR results were recorded in the solid state as KBr pellet, dispersion was done using Perkin- Elmer, Beaconsfield, UK.

The GC-MS analysis was performed on a Turbo mass model 20141128. Oven initial temperature was 200 °C for 1 min, ramp 15 °C/minute to 300 °C. Carrier gas was He, solvent delay was 2.10 min, transfer temperature was 200 °C, source temperature was 180 °C, and scan was 45 to 800 Da, column measurements were 30.0 m by 250 µm. Identification was based on sample condition data and electron impact –mass spectra (EI-MS).

The GC indicates the relative concentrations of various compounds which are eluted at different retention time. Mass spectrometer analyzes eluted compounds at different times identifying the structure and nature of the compounds (Chandana *et al.*, 2013).

3.2.8 Phytochemical screening

Phytochemical screening of the crude extracts and fractions was done for qualitative purposes using standard procedures according to Uddin & Rauf (2012).

Tannins test: To test for tannins, each crude extract weighing 0.2 g was mixed with 3 mL of water, heated on water bath and filtered. Thereafter, three drops of ferric chloride ($FeCl_3$) solution were added to the filtrate, a dark green colour showed a positive test for tannins.

Anthraquinones test: Test for anthraquinones was done by boiling 1g of each crude extract with 5 mL of 10% HCl for a few minutes on water bath. It was then filtered and allowed to cool. $CHCl_3$ of 5 mL was added to the filtrate and thereafter 4 drops of 10% ammonia was added to the mixture and heated. Appearance of rose- pink colour indicated the presence of anthraquinones.

Flavanoids test: Test for flavanoids was done by dissolving 0.5 g of each crude extract in 5 mL of 10% NaOH and 3mL of 2 M HCl. Decolourization of a yellow solution indicated the presence of flavanoids.

Steroids test (Liebermann bur chard reaction)**:** To test for steroids, 2 mL of acetic anhydride ($(CH_3CO)_2O$) was added to 1 g of each crude extract and followed by 2 mL concentrated H_2SO_4. Change of colour from violet to blue or green or red, indicated the presence of steroids.

Terpenoids test: (Salkowishki Test): Terpenoids test was done by mixing 0.5 g of each crude extracts with 2 mL of chloroform ($CHCl_3$) and 3 mL of conc. H_2SO_4 leading to the formation of a layer. An interface which is reddish in colour indicated the presence of terpenoids.

Diterpenes test: Diterpenes were tested by dissolving 2 g of each crude extracts in water then 3 drops of copper acetate (Cu $(CH_3COO)_2$) solution was added. A change of colour from blue to emerald green indicated the presence of diterpenes.

Alkaloids test: Alkaloids were tested by warming 2 g of each crude extract with 5 mL of 2% H_2SO_4 for 2-3 minutes. It was then filtered and 2 drops of Dragendrof's reagent was added. Formation of orange precipitate indicated the presence of alkaloids (Muhammad *et al.,* 2012).

3.3 Bioassay

The Bioassay was conducted using Tail Immersion Model. Albino rats weighing 150 g-200 g were randomly divided into five (5) groups of three (3) rats each, all fasted for 12 hours with clean drinking water provided. The rats were subjected to tail immersion before pre-treatment to confirm sensitivity to pain. A length of about 4 to 5 cm of each rat's tail was dipped into a water bath containing warm water maintained at a temperature of 50 ± 5 °C and the time taken for the mice to flick its tail or withdraw it from the warm water was noted. The behavior was regarded to be pain reaction time (PRT) which was recorded for all the rats at intervals of 30 minutes up to 180 minutes. The cut off time was set at 15 seconds to avoid tissue damage. Analgesic activity was evaluated from the registered PRT's with the higher the PRT value being the higher the analgesic activity.

The procedure in the tail immersion test was based on the fact that analgesic drugs selectively prolongs the PRT of the typical tail withdrawal reflex in mice/rats since they are very sensitive to temperatures between 50±5 °C. In this model, increase in pain reaction time (PRT) indicated the level of analgesia of the extract.

3.3.1 Experimental design for bioassay.

The Tail Immersion model was done according to (Prabhavathi *et al.*, 2012 ; Debasis, 2012) with a few modification.

*(**KEY**: HE:- Hexane extract, EE:- Ethyl acetate extract, AE:- Acetone extract)*

Group 1: Served as a negative control and received 20 tween solutions orally.

Group 2: Served as a positive control and received Declofenac suspension in distilled water at a dose of 10 mg/kg bodyweight orally.

Group 3: Served as test and received *E. prostrata (*HE, EE, &AE) extracts, 200 mg/kg body weight orally.

Group 4: Served as test and received *E. prostrata (*HE, EE, &AE) extracts, 500 mg/kg body weight orally.

Group 5: Served as test and received *E. prostrata (*HE, EE, &AE) extracts, 1000 mg/kg body weight orally.

Analgesic activity of fractions was done in the same way as explained in crude extracts but a single dose was used due to low yield. The experimental design for the fractions was as follows:

Group 1: Served as a negative control and received 20 tween solutions orally.

Group 2: Served as a positive control and received Declofenac suspension in distilled water at a dose of 10 mg/kg bodyweight orally.

Group 3: Served as test and received *E. prostrata* fractions from ethyl acetate and hexane 10 mg/kg body weight orally.

3.4 Statistical Analysis

All data are presented as mean ± SD of the mean. Differences between experimental groups were analyzed by student t- test. Difference between groups were considered significant at $p<0.05$.

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1 Sample

Moisture and volatile content of plants depends on the environment and weather conditions (Paini *et al.*, 2014). Weight lost results is indicated in Table 4.1. It was observed that the plant lost about 70% weight of moisture and other volatile compounds.

Table 4.1: Weight of the collected *E. prostrata* plant

Plant species	Fresh weight	Dry weight	Lost weight of water and volatile compounds
E. prostrata	12.92kg	3.90kg	9.02kg

4.2 Extraction and percentage yield

Successive solvents extraction of crude extracts from *E. prostrata* was carried out (Figure 4.1). Both non-polar and polar solvents were used where hexane was non-polar solvent, ethyl acetate was of medium polarity and acetone slightly polar than ethyl acetate. The yield obtained from the three solvents using equation 2 showed that the components with intermediate polarity were in highest yield (5.80 %). The components having less polarity were the next in the yield (1.03%) extracted with hexane, and polar components were extracted by acetone were the least (0.08%) (Table 4.2).

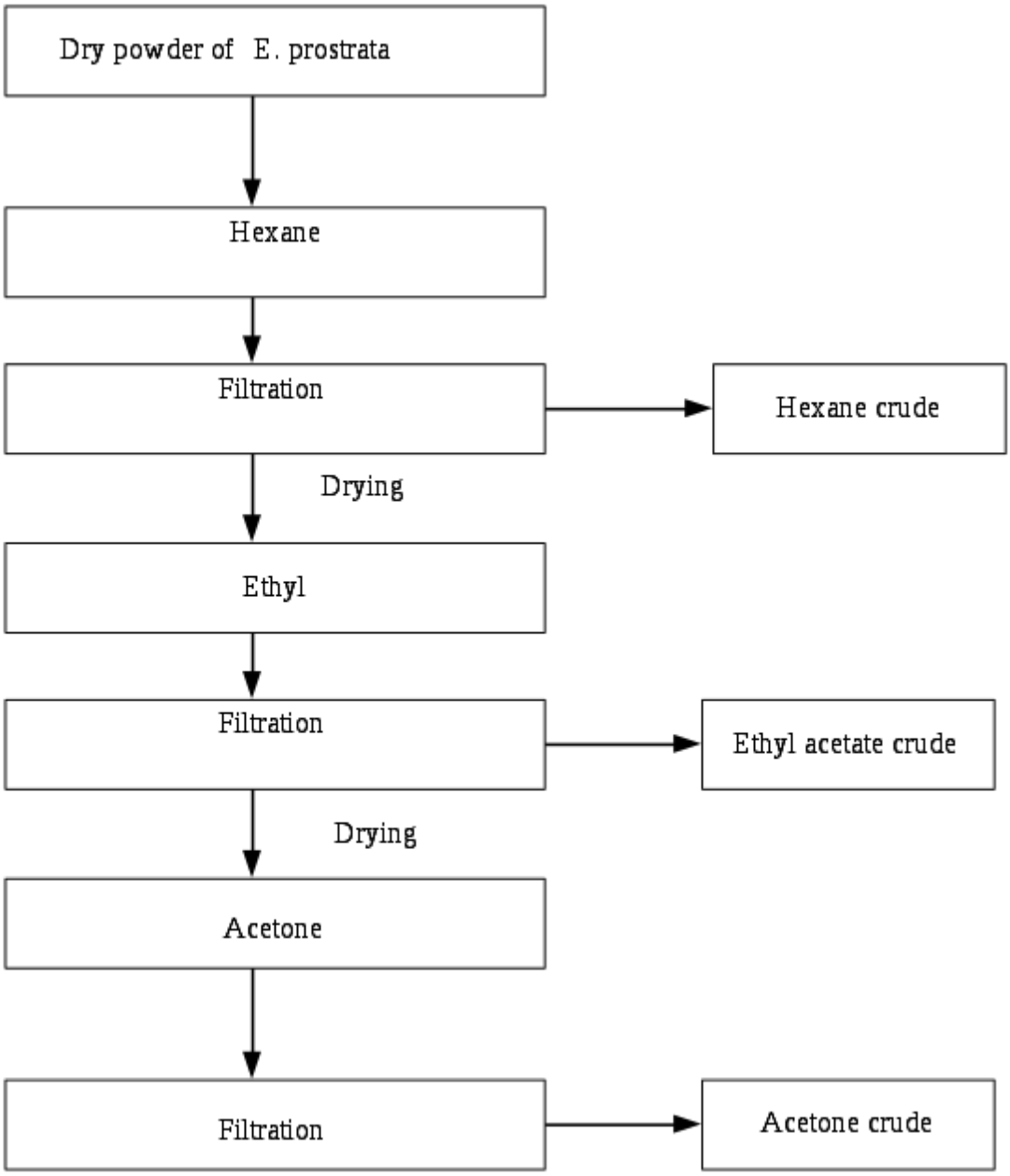


Figure 4.1: Schematic presentation of serial extraction.

Table 4.2: Percentage yield of the *E. prostrata* plant

Plant species	Hexane	Ethyl acetate	Acetone
E. prostrata	1.03%	5.80%	0.80%

The acetone's percentage yield was very low because ethyl acetate and acetone are close in polarity (acetone 5.1 > ethyl acetate 4.4), most of the mid- polar compounds had been extracted by ethyl acetate (Annwar *et al.*, 2010).

4.3 Isolation

Using the bioassay data (Table 4.6 to 4.7) only two crude extracts (hexane and ethyl acetate) were subjected to isolation. This is because, acetone's crude extract showed low analgesic activity (Table 4.8 and Figure 4.10) compared to hexane and ethyl acetate.

4.3.1 Hexane crude extract

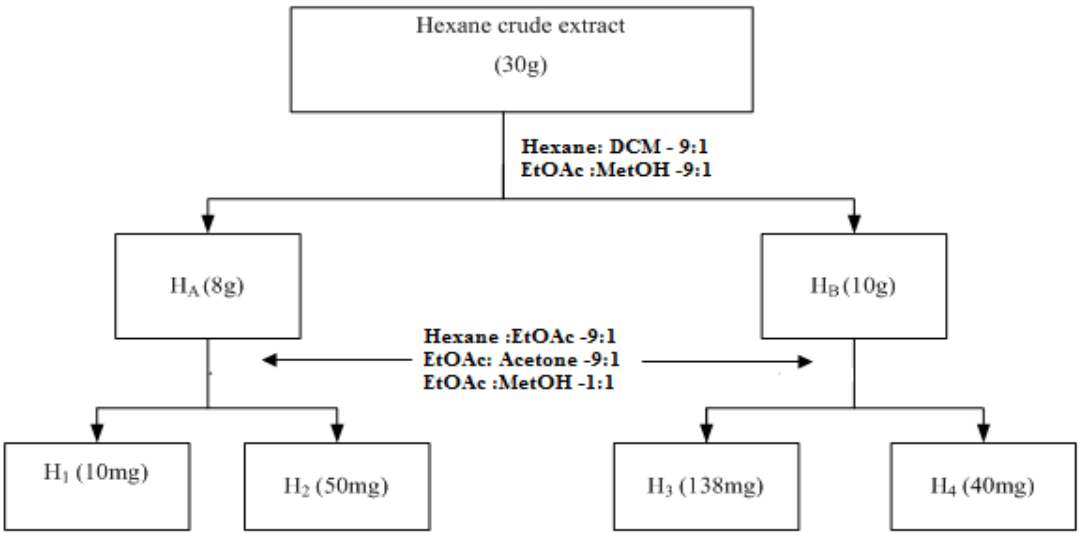


Figure 4.2: A flow chart showing the isolation of compounds from hexane crude extract.

Hexane crude extract was further subjected to column chromatographic analysis to isolate the active analgesic compounds. It gave two semi pure fractions (H_A and H_B) (Figure 4.2). The semi-pure fraction H_A (8g), on column chromatography, gave two fractions; of

the two fractions, the first one gave one spot of retention factor (R_f) value of 0.40 on TLC plates. This fraction was labeled H_1 (orange in colour). The second fraction had one spot of R_f value of 0.65 and it was labeled H_2 (yellow in colour). The semi-pure fraction H_B, after column chromatography, gave two fractions. The first fraction on TLC plate gave one spot with R_f value of 0.71. It was concentrated and labeled H_3 (purple in colour). The second fraction from H_B gave one spot of R_f value of 0.30 on TLC. This fraction was labeled H_4 (cream in colour).

4.3.2 Ethyl acetate crude extract

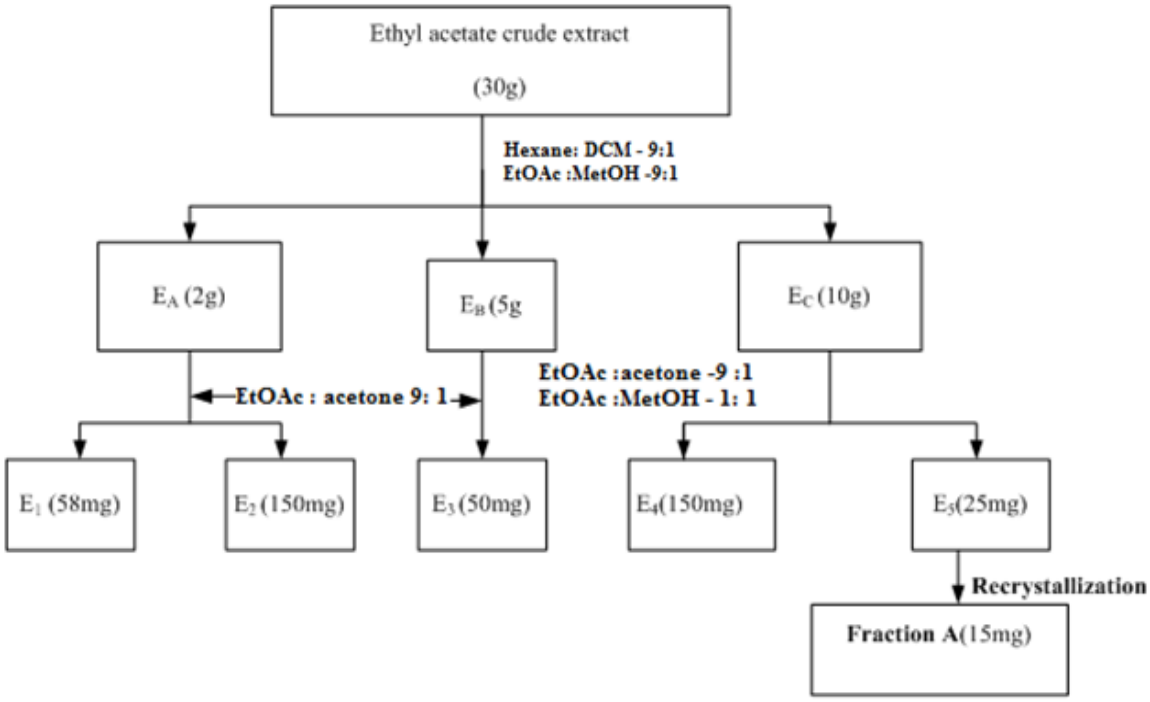


Figure 4.3: A flow chart showing the isolation of compounds from ethyl acetate crude extract.

Ethyl acetate crude extract was further subjected to column chromatographic analysis to isolate the active analgesic compounds (Figure 4.3). It gave four semi pure fractions (E_A,

E_B and E_C) and they were further isolated. Fraction E_A gave two fractions on column chromatography. The first fraction had one spots on TLC plates with R_f value of 0.45 and the other fraction on TLC plate had the R_f of 0.65. The two were labeled E_1 (cream in colour) and E_2 (yellow in colour). Semi-pure fraction E_B produced one fraction which on TLC gave one spot (R_f. value of 0.56). This spot was labeled E_3 (purple in colour). The semi- pure fraction E_C had two fractions on column chromatography. Their R_f values on TLC plate were 0.44 and 0.54. The first one was labeled E_4 (cream in colour) and E_5 (yellow in colour) respectively.

4.4 Phytochemical screening

A good number of phytochemical studies on the *E. prostrata* plant have shown the presence of important phytochemicals the main ones being flavanoids, terpenoids, tannins and steroids. In this study, phytochemical screening of the *E. prostrata* crude extracts from the three solvents indicated the presence of the important metabolites as indicated in Table 4.3. From the data, ethyl acetate gave more phytochemical compounds from *E. prostrata* plant namely, flavanoids, tannins, terpenoids, diterpenes and steroids which was in agreement with studies done by (Ramhat *et al.*, 2012). Hexane was able to extract diterpenes, terpenoids and flavanoids while acetone extracted flavanoids only. This was in agreement with work done by (Muhammad *et al.*, 2012). *Euphorbia* species can have different constituents depending on its environment due to survival adaptations as found out by (Julius & Patrick, 2011). It was also evident that intermediate polar solvents could extract more bioactive compounds unlike non-polar solvents which conform to work done by Shashank & Abhay (2013).

Table 4.3: Phytochemical screening of hexane, ethyl acetate and acetone crude extracts

Chemicals	Hexane extract	Ethyl acetate	Acetone
Alkaloids	-	-	-
Anthraquinones	-	-	-
Diterpenes	+	+	-
Flavanoids	+	+	+
Steroids	_	+	
Tannins	_	+	-
Terpenoids	+	+	-

+: present, - : absent

Owing to the fact that ethyl acetate being of slightly higher polarity than hexane, it could extract steroids, tannins, terpenoids and flavanoids in agreement to previous studies (Kumar, *et al.*, 2009; Paini *et al.*, 2014) work. Phytochemical screening of fractions indicated that fractions (H_1 & E_1), (H_2& E_2), and E_5 (A- after recrystalization) were flavanoids, terpenoids and steroid respectively as shown in Table 4.4.

Table 4.4: Phytochemical screening of fractions

Fraction	Steroid	Flavonoid	Terpenoid
H_1	-	+	-
H_2	-	-	+
E_1	-	+	-
E_2	-	-	+
E_5	+	-	-
A	+	-	-

Key: H: Fractions from hexane crude extract, E: Fractions from ethyl acetate crude extracts

4. 5 Spectroscopic analysis

4.5.1 Crude extract

IR aids in identification of chemical constituents and structure elucidation of compounds (Deepa *et al.*, 2014). The IR analysis of the crude extract was performed to identify the functional groups which are present in *E. prostrata* plant using peak values in infrared radiation region.

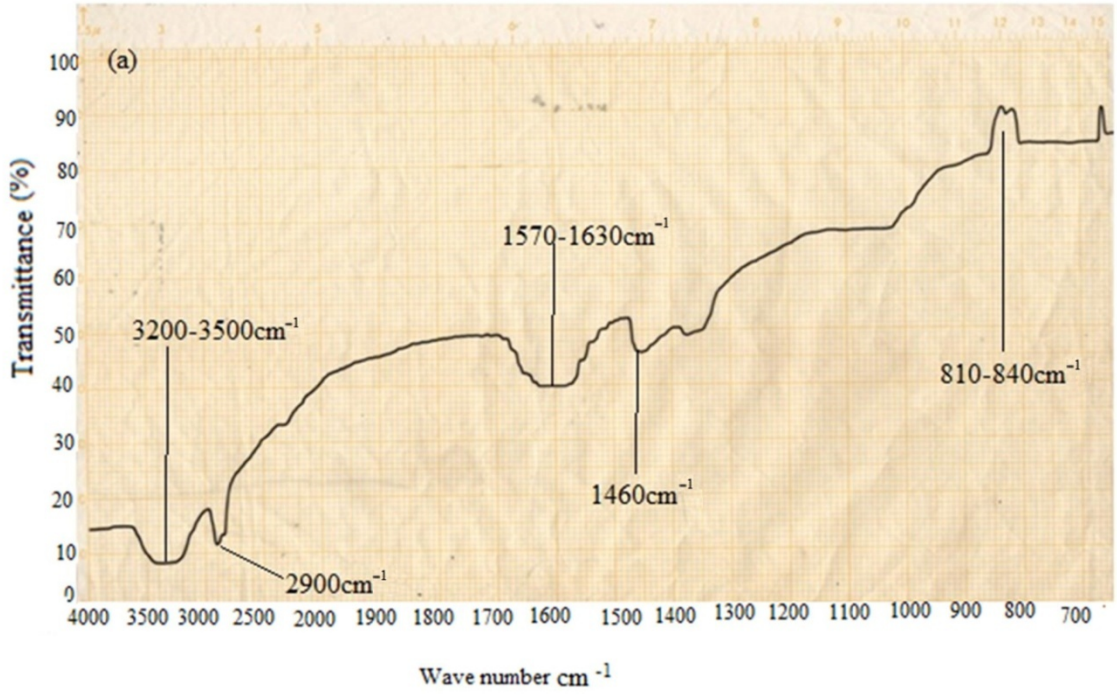


Figure 4.4 (a): IR spectrum for hexane crude extract.

For hexane crude extract (Figure 4.4 a), major bands were observed at 3200-3500 cm^{-1}, 2900 cm^{-1}, 1570-1630 cm^{-1}, 1460 cm^{-1} and 810-840 cm^{-1}. The broad peak at 3200-3500 cm^{-1} indicates the O-H stretch which could be due to presence of phenols and alcohols. The small peak at 2900 cm^{-1} corresponds to C-H stretch suggesting the presence of alkanes. The broad peak at 1570-1630 cm^{-1} is assigned to N-H bend which suggests the presence of amides. The band at 1460 cm^{-1} indicates the presence of C=C stretch

usually shown by aromatic compounds. There was a band at 1335 cm^{-1} suggesting the presence of aromatic nitro compounds. The band at 810-840 cm^{-1} could be due to presence of C-H stretch for aromatic compounds.

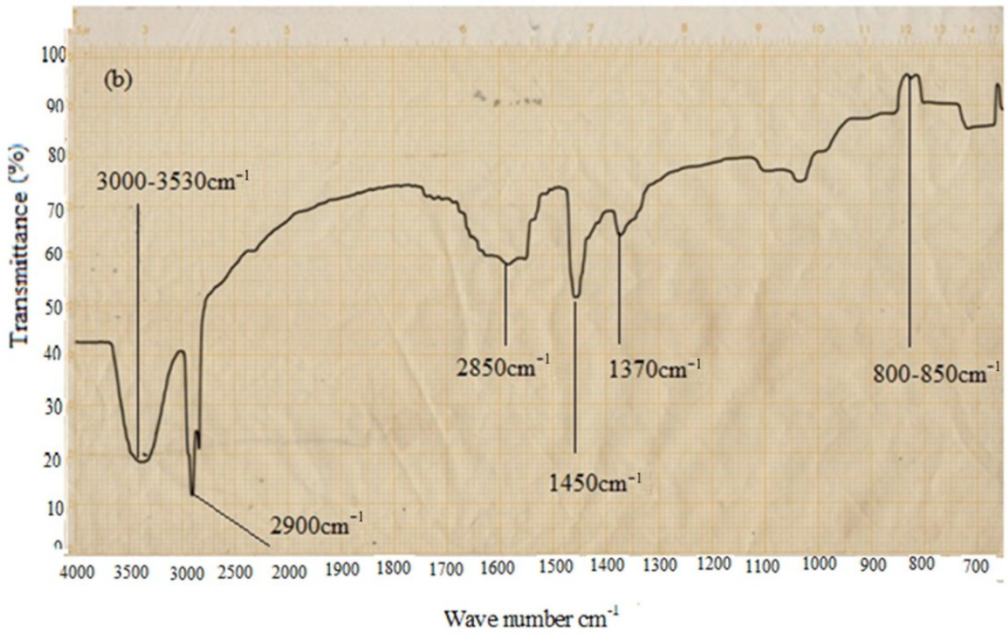


Figure 4.4 (b): IR spectrum for ethyl acetate crude extract.

Ethyl acetate crude extract (Figure 4.4(b)), major bands occurred at 3000 -3530cm^{-1}, 2900 cm^{-1}, 2850 cm^{-1}, 1450 cm^{-1}, 1370 cm^{-1} and 800-850 cm^{-1}. The broad band at 3000-3530 cm^{-1} was probably due to the presence of O-H stretch which could imply that phenols or alcohols are present. The band at 2900 cm^{-1} and 2850 cm^{-1} suggests the presence of C-H stretch for alkanes. The band at 1450 cm^{-1} which is small suggests the presence of C=C ring stretch for aromatic compounds. There was a very small peak at 1370 cm^{-1} which could be due to the presence of N-O (asymmetric stretch) for nitro compounds. The last band of 810-840 cm^{-1} suggests the presence of C-H bend for aromatic compounds.

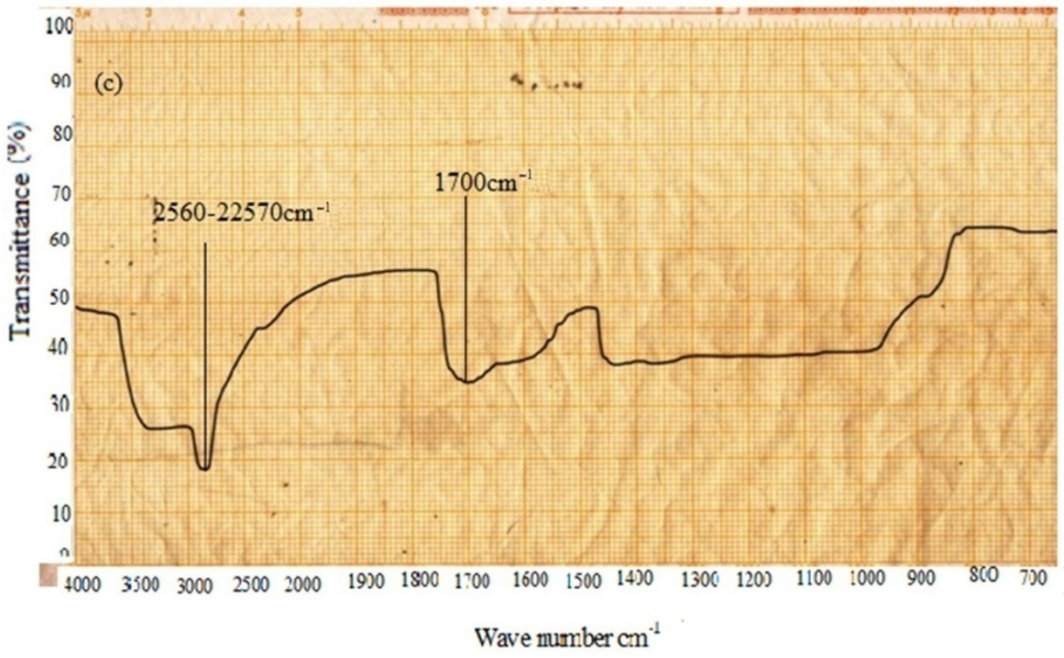


Figure 4.4 (c): IR spectrum for acetone crude extract.

Acetone crude extract, when subjected to IR spectroscopy, two broad bands were observed (Figure 4.4 c) at region of 2560-2570 cm^{-1} suggesting the presence of O-H stretch for carboxylic acid, and a very small and broad band at 1700 cm^{-1} which could be due to the presence of C=O stretch for ketones.

In the functional groups observed, OH group was found to be present uniformly in all the crude extracts. Hydroxyl group possess high ability of forming hydrogen bonding, hence presence of OH group indicates the higher potential of the extracts towards inhibitory activity against many health problems like antimicrobial, and antioxidants as pointed out by (Ashokkumar & Ramaswamy, 2013).

4.5.2 Fractions

From bioassay which will be explained at the end of this chapter (Section 4.6), semi pure fraction E_5 had higher analgesic activity. It was then re-crystalized to give fraction A.

Electronic Impact Mass Spectrum (EIMS) of fraction A is shown in Figure 4.6 i-vi. GC-MS gave molecular ion peaks at m/z 412, 414 and 426 at retention time of 12.6, 13.04 and 13.21 respectively.

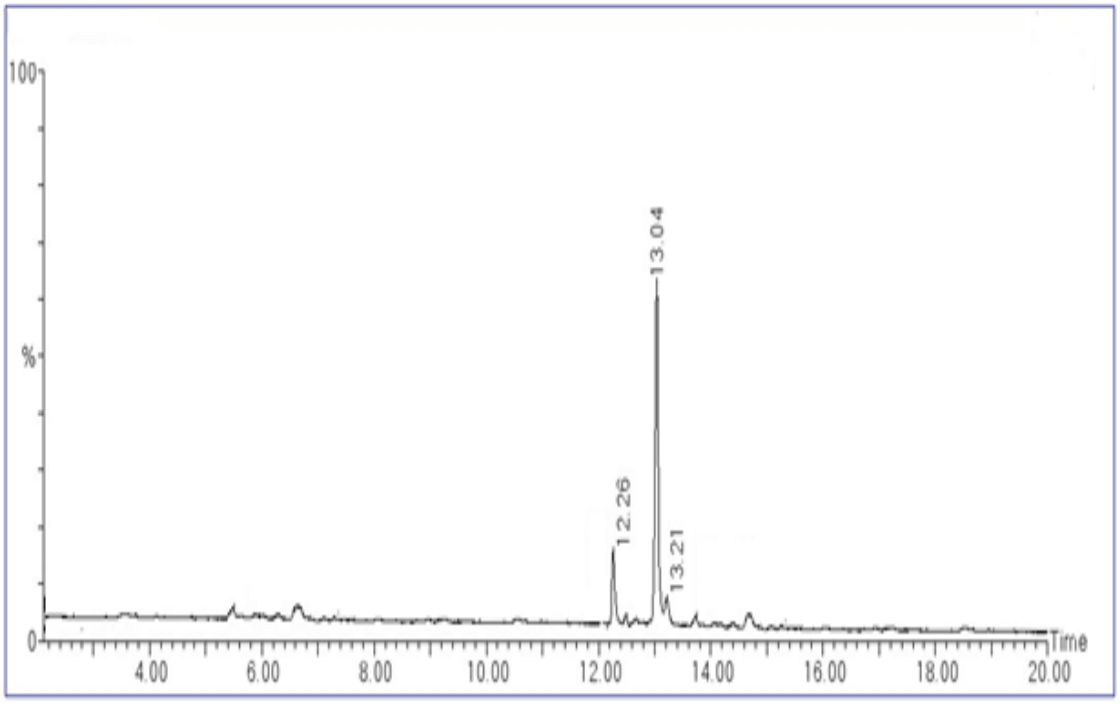


Figure 4.5 (a): The GC chromatogram of fraction A of *E. prostrata* extracts showing major peaks at retention time 12.26, 13.04 and 13.21**.**

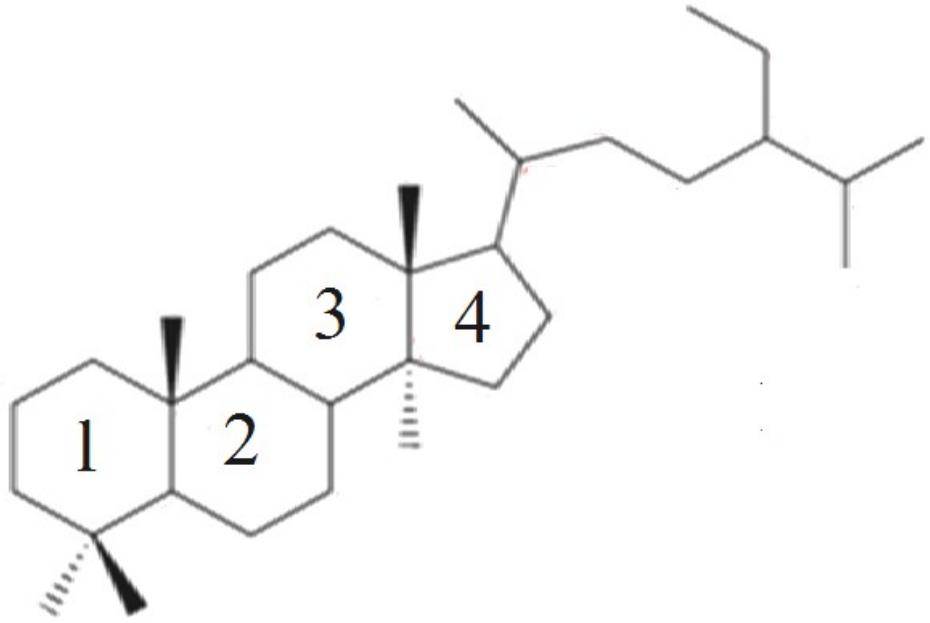


Figure 4.5 (b); Skeleton structure of steroid (Bulama *et al.*, 2015).

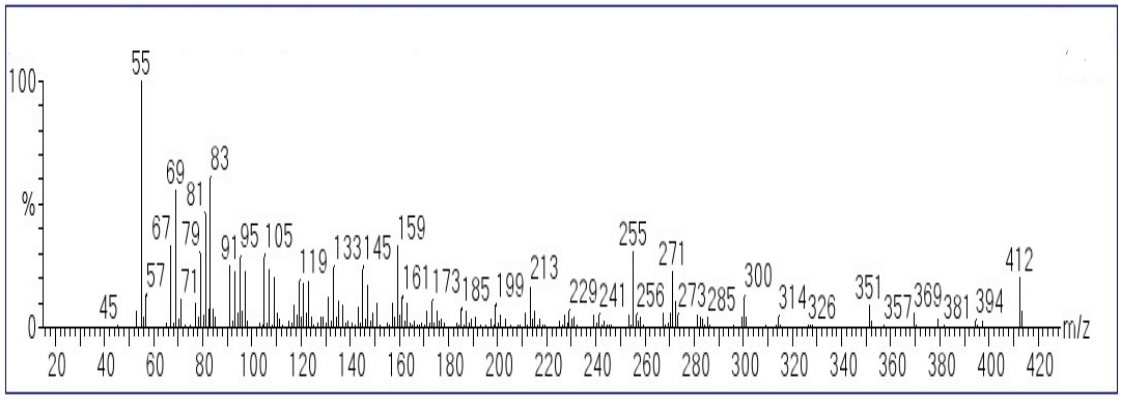


Figure 4.6: (i) MS spectra of peaks at retention time 12.26.

General structure of steroid is made up of four rings (Figure 4.5) and the fragmentation usually occur within rings and the side chain(Bulama *et al.*, 2015). Molecular ion peaks at m/z 412 showed other ion peaks at m/z 255, 271, 273 and 369 at retention time of 12.26 (see Figure 4.6 i). Ion peak at m/z 255 is due to loss of water molecule and side

chain [M- Side chain- H_2O] $^+$. Ion peak at m/z 273 occurs due to the loss of the side chain [M-side chain]$^+$. There is further loss of two hydrogens, one from hydroxyl group and the other one from the first ring leading to ion peak at m/z 271 [M - side chain – 2H] $^+$ (Rowshanul *et al.,* 2007). Ion peak at m/z 369 is due to the loss of propyl group at the side chain [M - CH_3 CH_2 CH_3] $^+$ (See Figure 4.6 ii). The m/z values are in agreement for the structure of sitosterol similar with the data in the literature of stigmasterol and sitosterol (Chiang, 2006).

Figure 4.6 (ii): Fragmentation of m/z 412.

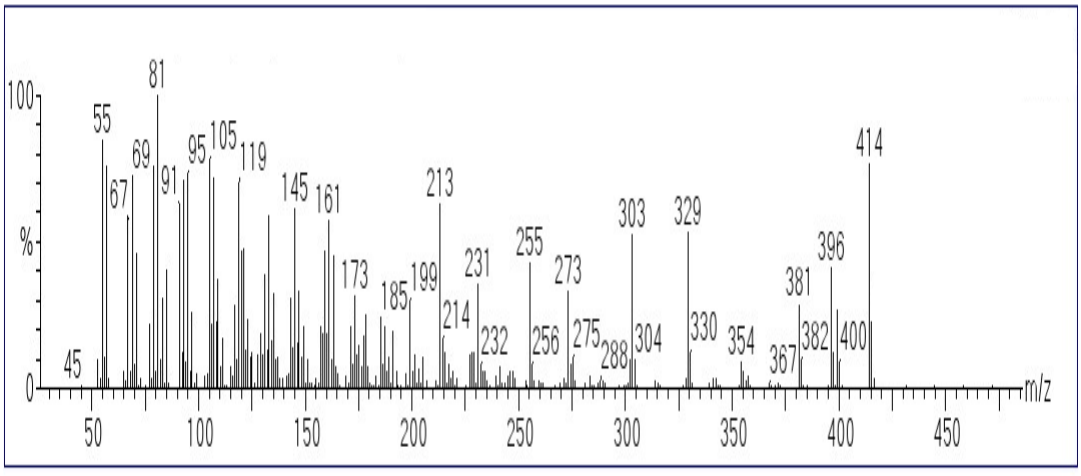


Figure 4.6 (iii): MS spectrum of peaks at retention time 13.04.

Molecular ion peak at m/z 414 has ion peaks observed at m/z 273, 303, 329, 381and 396 at retention time of 13.04 (see Figure 4.6 iii). The ion peak at m/z 273 is due to the loss of side chain [M-side chain] $^{+}$. Ion peak at m/z 396 is due to the loss of water $[M-H_2O]^{+}$. There is a further loss of CH_3, resulting to m/z 381 $[M-H_2O-CH_3]^{+}$. Fragmentation occurs at the first ring and another one between the first ring and the second ring. When it occurs in the first ring, the result is the loss of $[M-C_{24}H_{41}]^{+}$ hence m/z 329. When fragmentation occurs in the first and the second ring, there is a loss of $[M-C_{22}H_{39}]^{+}$ giving rise to ion peak at m/z 303 (See Figure 4.6 iv).

Figure 4.6 (iv): Fragmentation of m/z 414.

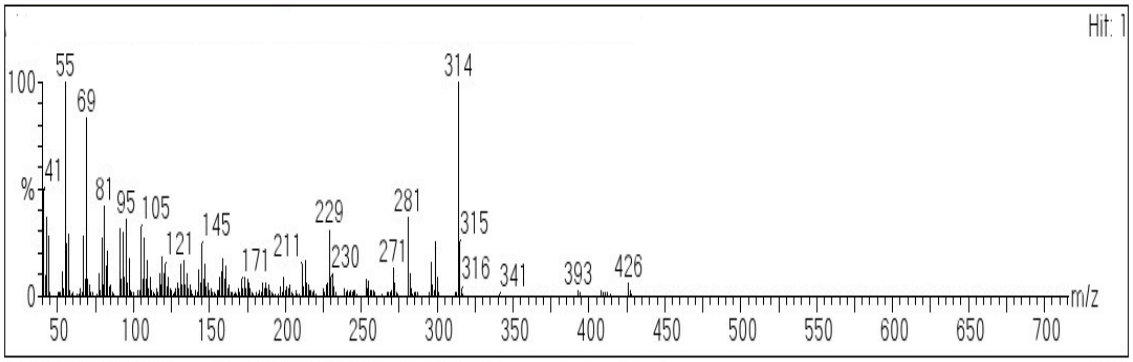


Figure 4.6 (v): MS spectrum of peaks at retention time 13.21.

The molecular ion peak at m/z 426 showed major ion peaks at m/z 314 and 315 (see Figure 4.6 v). Peaks observed at 314 were due to fragmentation at the first and second ring [M- $C_7H_{12}O$] $^+$. Another fragmentation occur at the side chain, hence ion peak at m/z 315 [M –C_8H_{15}] $^+$ (see Figure 4.6 (vi).

Fig 4.6 (vi): Fragmentation of m/z 426.

Important fragmentation of sterols occurs when the sterols rings and side chain fragment. In his study (Chiang, 2006), stigmasterol was eluted first then followed by sitosterol at 19.37 and 20.36 retention time respectively. The current study also showed the same sequence but at different retention times i.e 12.26 and 13.04 for stigmasterol and

sitosterol respectively. Important fragments for stigmasterol are at m/z 300, 303, 271, 273 (Sarunya *et al.*, 2006).

The FT-IR spectrum of fraction A is shown in Figure 4.7 which displays a peak at 3480-3150 cm^{-1} for OH group (alcohols) and at 2800 cm^{-1} for C-H stretching (aliphatic). It further shows a sharp peak at 1650 cm^{-1} for C=C and 1400 cm^{-1} bending for $(CH_2)_n$. Finally it displays a medium peak at 1050 cm^{-1} for cyclo alkane and 870 cm^{-1} for C-H vibration for the unsaturated part. Important absorption peaks of steroids are usually the OH group for alcohols, C-H stretch for alkanes, C=C for olefinic group and C-H bending for cycloalkanes (Veena and Manu , 2013) shown by the FT-IR of fraction A. These absorption frequencies are similar to absorption frequencies observed for steroid β-sitosterol published by Bulama *et al.,* (2015) ; Rowsganul *et al.,* (2007).

Under UV-Vis, fraction A had a strong absorption at λ max =256 nm (Appendix 1). Melting point by use Stuart Melting point apparatus was 163 °C. Phytochemical screening of fraction A (Liebermann burchard reaction) was positive for steroids (Bulama *et al.*, 2015).

From the chemical, physical, and spectral evidences compared with the ones in literature gives the possible structures in fraction A having a steroidal nucleus mixture with retention time at 12.26, 13.04 and 13.21 which are stigmasterol (**30**), sitosterol (**31**) and cholest-5-en-3-ol, 24 propylidene (Cytellin) (**32**) respectively (see Table 4.5).

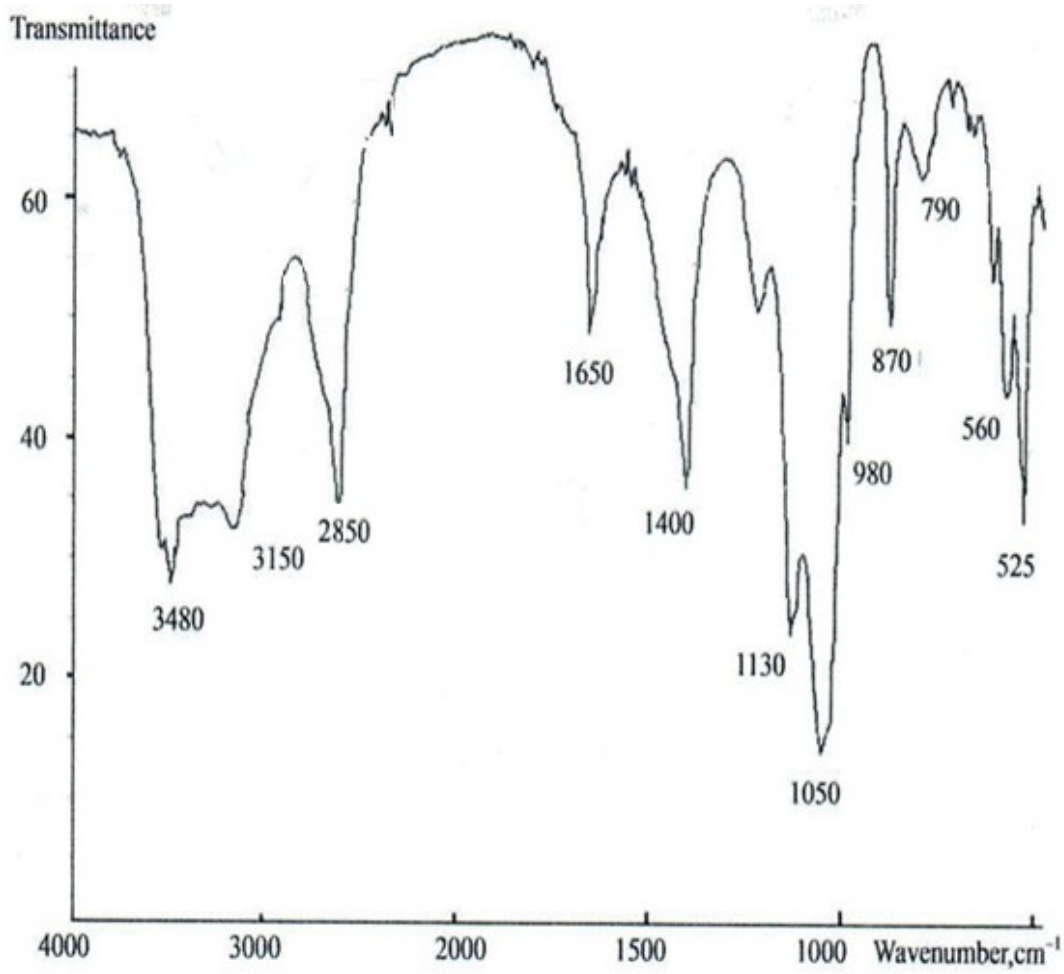


Figure 4.7: FT-IR spectrum of the active fraction from *E. prostrata* plant

Table 4.5: Data and proposed names of the active fraction from *E. prostrata*

RT (min)	Proposed name	Possible molecular structures	EIMS
12.26	Stigmasterol		412[M^+]255, 271, 273 and 369
13.04	Sitosterol		414[M+] 273, 303, 329, and 381, 396
13.21	Cholest-5-en-3-ol,24 propylidene (Cytellin)		426[M+] 281, 314 and 315

RT: - Retention time in minutes, **EIMS:** - Electron impact ionization mass spectra, M^+:- Molecular ion.

4.6 Bioassay

4.6.1 Bioassay using hexane extract

An assay was done for the different concentrations of the *E. prostrata* crude extract obtained using hexane solvent (Table 4.6).

Table 4.6: Mean response time for the five treatments of the rats with hexane crude extract

Treatment	Pain Reaction Time in seconds						
	0 min	**30 min**	**60 min**	**90 min**	**120 min**	**150 min**	**180 min**
a) Tween solution	3.2	3.1	3.1	3.1	3.0	3.2	3.0
b) Declo 10mg/kg	3.6	6.0	8.2	6.1	6.0	4.1	4.0
c) HE 250mg/kg	3.4	3.6	4.0	4.2	4.0	3.1	3.0
d) HE 500 mg/kg	3.0	3.9	4.2	4.4	4.4	4.1	3.4
e) HE 1000mg/kg	3.0	5.1	10.0	9.0	5.0	5.0	4.9

Key: HE: Hexane crude extract, Declo: Declofenac.

HE exhbited significance analgesic activity more than negative control (tween solution) (Table 4.6) . At the dose of 250 mg/kg and 500 mg/kg, it is observed that the analgesic activity was lower than that of positive control (Declofenac) as also observed by Ezeja *et al.*, (2011); Serkan and Giilcin, (2012).

4.6.2 Bioassay using ethyl acetate crude extract

A second assay was done for the different concentrations of the *E. prostrata* crude extract obtained using ethyl acetate solvent.

In Table 4.7 it is seen that all the doses possessed significant analgesic activity more than the negative control. At the dose of 1000 mg/kg, recorded PRT was higher than positive control.

Table 4.7: Mean response time for the five treatments of the rats with ethyl acetate crude extract

	Pain Reaction Time in seconds						
Treatment	**0 min**	**30 min**	**60 min**	**90 min**	**120 min**	**150 min**	**180 min**
a) Tween solution	3.4	3.1	3.3	3.2	3.1	3.1	3.2
b) Declo 10 mg/kg	3	6	8	6.6	6.5	5.3	4
c) EE 250 mg/kg	3.4	4.3	4.3	4.4	4.2	4	3.3
d) EE 500 mg/kg	3.3	4.9	5	4.4	4.1	4	3.8
e) EE1000 mg/kg	3	8.1	10.4	10.2	10.2	9.1	8.3

Key: *EE: Ethyl acetate crude extract, Declo: Declofenac.*

4.6.3 Bioassay using acetone extract.

It is clear from Table 4.8 that analgesic activity at the dose of 250 mg/kg was similar to that of tween solution. This is because the extractive values of acetone were very low. At the dose of 500 mg/kg, slight increase was observed.

Table 4.8: Mean response time for the five treatments of the rats with acetone crude extract

	Pain Reaction Time in seconds						
Treatment	**0 min**	**30 min**	**60 min**	**90 min**	**120 min**	**150 min**	**180 min**
Tween solution	3.2	3	3.2	3.2	3	3.2	3.2
Declo10 mg/kg	3	6	8.1	6.3	6.4	5.3	4
AE 250 mg/kg	3	3.1	3.4	3.2	3	3	3
AE 500 mg/kg	3.3	3.34	3.54	3.5	3.34	3.3	3.3
AE 1000 mg/kg	3	3.5	4	3.9	3.7	3.5	3.2

Key: *AE: Acetone crude extract, Declo: Declofenac.*

Even at the dose of 1000 mg/kg, the analgesic activity of AE was significantly lower than that of positive control by having optimum PRT of 4 seconds and positive control having 8.1 seconds. This is attributed to the fact that acetone crude extract had less phytochemical than the other crude extracts. Optimum PRT was observed at 60 minutes (Table 4.9 and Figure 4.8) implying that the positive control and the extract's analgesic activity had the same time of activity on the animals.

Table 4.9: Bioassay with crude extracts from the three solvents at 60 minutes

Solvent	**Pain Reaction Time in seconds**		
	250 mg/kg	**500 mg/kg**	**1000 mg/kg**
Hexane	4	4.2	10.2
Ethyl acetate	4.3	5	10.4
Acetone	3.4	3.5	4
Declofenac	8.1	8.1	8.1
Tween solution	3.2	3.2	3.2

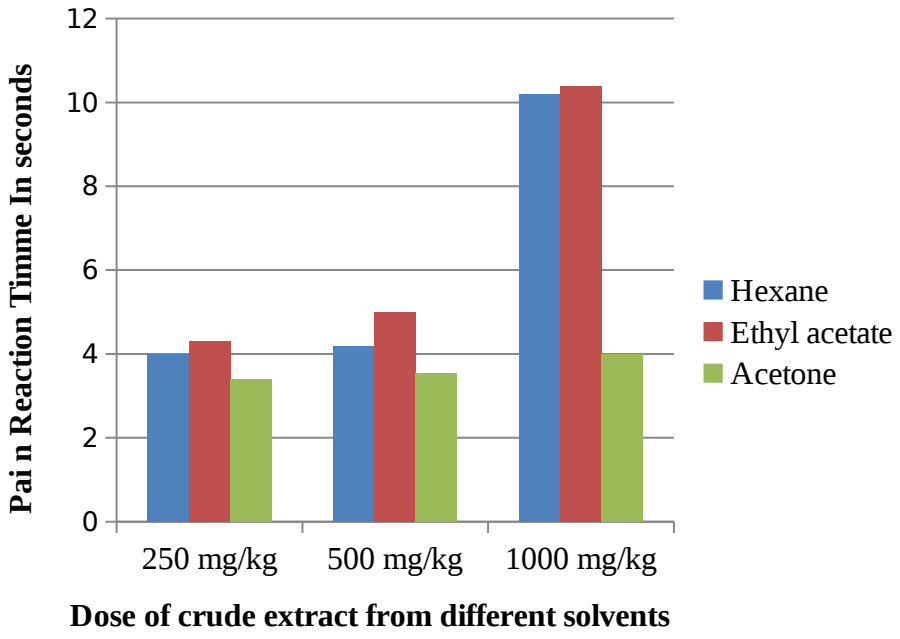


Figure 4.8: Superimposable bar graph of mean for bioassay with crude extracts from the three solvents at 60 minutes.

E. prostrata is an indigenous plant which is available globally in tropical and subtropical parts of the world (Syed *et al.*, 2013). The parts of the plant e.g leaves, flower and roots have been reported to have significant pharmacological activities (Bashir *et al.*, 2012). Despite the fact that *E. prostrata* have wide use in traditional medicine for treatment of many ailments, reports regarding analgesic activity has not been exploited much.

The procedure in the Tail Immersion Model test was based on the fact that analgesic drugs selectively prolongs the reaction time of the typical tail withdrawal reflex in mice/ rats since they are very sensitive to temperatures between 50±5 °C (Debasis, 2011). In this model, increase in pain reaction time (PRT) indicated the level of analgesia of the crude extract. It was found that the ethyl acetate crude extract exhibited better analgesic activity than both the hexane and acetone crude extracts.

It was observed by Ezeja *et al.*, (2011) that at the dose of 1000 mg/Kg, the crude extracts had an increase in PRT significantly *P =0.03* compared to negative control. The results for all the crude extracts showed a significant increase in PRT at the dose of 1000 mg/kg when compared to the other concentrations (250 and 500 mg/kg) (Tables 4.6 – 4.8) indicating that the analgesic effect of the crude extracts are directly proportional to concentration. Acetone crude extract gave very low PRT's. However, the PRT for the ethyl acetate crude extract was far much better as compared to the hexane crude extract (Figures 4.11- 4.13). The active ingredients in this study were of mid polarity because ethyl acetate extracted more phytochemicals than hexane. This was in agreement with Annwar *et al.,* (2013) work who found out that the crude extract from ethyl acetate had higher activity.

The optimum PRT recorded at 60 minutes was 10.4 second for ethyl acetate crude extract and at 120 minutes was 10.2 seconds. However, for ethyl acetate crude extract, the PRT reduces to 4.9 sec after 180 minutes while for hexane crude extract, the PRT reduced to 4.3 sec after 180 minutes. Compared to the positive control (Declofenac), the *E. prostrata* extracts produced better results in terms of the optimum PRT. The optimum PRT for Declofenac was 8.2 sec at 60 minutes while for tween solution; the PRT was 3.3 sec at 60 minutes. This is a clear indication that the crude extracts from *E. prostrata* had a far much better analgesic effect compared to Declofenac, a synthetic analgesic drug.

Bioactive compounds are present in both extract which could have led to the observed increase in PRT of both hexane and ethyl acetate crude extracts. The same activity was observed by Okeniyi *et al.,* (2012) who found out that crude extracts of hexane and ethyl acetate from *Euphorbia heterophylla* (*Euphorbiaceae*) root exhibited antinociceptive

activity. It has also proved that some of the plants in *Euphorbiacea*e family possess analgesic activities (Parveen *et al.*, 2007).

The chemical mediators like prostaglandins, serotonins, histamines, bradykinin etc are involved in the mediation of pain and inflammation. Hexane, ethyl acetate and acetone solvents were used for successive extraction of *E. prostrata*. After preliminary phytochemical screening only the hexane and ethyl acetate crude extracts were chosen for further studies as it contained maximum amount of steroids, tannins, terpenoids and flavanoids. The preliminary phytochemical screening report shows that the active principles responsible for biological activity are present. Analysis of tables and figures shows that the activities of hexane and ethyl acetate crude extracts are nearer to standard and can be chosen as primary analgesic and anti-inflammatory.

4.6.4 Bioassay of fractions

After column chromatography, hexane crude extract gave four fractions while ethyl acetate gave five fractions under different solvent gradients. H_1-H_4 from hexane crude extract and E_1–E_5 from ethyl acetate crude extracts (Figure 4.2 and 4.3 respectively).

Semi pure fractions were subjected to bioassay to a certain analgesic activity as previously done for crude extracts but single dose was used because of low yield. The bioassay indicated that 5 fractions were active in analgesic activity which on phytochemical screening revealed that they were fractions with flavanoids, terpenoids and steroids but fraction with steroids had better activity than the rest as shown in Table 4.4 and 4.10.

Results of bioassay of fractions, isolated compound, tween solution and declofenac are shown in Table 4.10

Table 4.10: The bioassay with semi pure fractions and recrystallized fraction A

	Pain Reaction Time in seconds						
Treatment-	**0 min**	**30 min**	**60 min**	**90 min**	**120 min**	**150 min**	**180 min**
E_1 10 mg/kg	3.1	3.3	4.34	3.8	3.7	3.4	3.3
E_2 10 mg/kg	3	3.8	4	3.8	3.4	3.2	3.2
E_5 10 mg/kg	3.1	4.9	6.21	6	5.1	5	4.5
H_1 10 mg/kg	3.2	3.3	4.8	3.5	3.34	3.3	3.3
H_2 10 mg/kg	3	3.8	4.3	3.5	3.3	3.2	3
A 10 mg /kg	3.1	3.1	6	5.5	5	4.8	4.3
Declo 10 mg/kg	3	6	8	6.6	6.5	5.3	4
Tween solution	3.4	3.1	3.3	3.2	3.1	3.1	3.2

Table 4.11: Mean response time for the Bioassay with fractions from hexane crude extracts and controls

	Pain Reaction Time in seconds						
Treatment	**0 min**	**30 min**	**60 min**	**90 min**	**120 min**	**150 min**	**180 min**
H_1 10 mg/kg	3.2	3.3	4.8	3.5	3.34	3.3	3.3
H_2 10 mg/kg	3	3.8	4.3	3.5	3.3	3.2	3
Delco 10 mg/kg	3	6	8	6.6	6.5	5.3	4
Tween solution	3.4	3.1	3.3	3.2	3.1	3.1	3.2

For HE, it is seen that at the dose of 500 mg/kg, its PRT was lower than fraction H_1 but almost the same in activity as fraction H_2. Fraction E_5, PRT was significant at $p < 0.05$ higher than the other compounds.

Table 4.12: Mean response time for the bioassay with fractions from ethyl acetate crude extracts and controls

	Pain Reaction Time in seconds						
Treatment	**0**	**30**	**60**	**90**	**120**	**150**	**180**

	min	min	min	min	min	min	min
E_1 10 mg/kg	3.1	3.3	4.34	3.8	3.7	3.4	3.3
E_2 10 mg/kg	3	3.8	4	3.8	3.4	3.2	3.2
E_5 10 mg/kg	3.1	4.9	6.21	6	5.1	5	4.5
A 10 mg /kg	3.1	3.1	6	5.5	5	4.8	4.3
Delco 10 mg/kg	3	6	8	6.6	6.5	5.3	4
Tween solution	3.4	3.1	3.3	3.2	3.1	3.1	3.2

Compounds from ethyl acetate crude extract had better analgesic activity than the compounds from hexane crude extract (Table 4.11 and Table 4.12). Fraction A had lower analgesic activity than fraction E_5 which could be due to purification hence activity is lost since there is no more synergism.

Table 4.13: Analgesic activity with fraction A and fraction E_5

	Pain Reaction Time in seconds						
Treatment	**0 min**	**30 min**	**60 min**	**90 min**	**120 min**	**150 min**	**180 min**
E_5 10 mg/kg	3.1	4.9	6.21	6	5.1	5	4.5
A 10 mg /kg	3.1	3.1	6	5.5	5	4.8	4.3
Delco 10 mg/kg	3	6	8	6.6	6.5	5.3	4
Tween solution	3.4	3.1	3.3	3.2	3.1	3.1	3.2

The analgesic activities of fractions and isolated compounds were lower than the standard drug but more than teen solution. Fraction E_5 was much higher than the rest by recording the highest PRT at 60 minutes of 6.21 seconds (Table 4.13).

Phytochemical screening indicated the presence of important phytochemicals in the crude extracts and some of the fractions as shown in Table 4.3 and 4.4. The analgesic effect of *E. prostrata* crude extracts and its compounds could be through central mechanism where the compounds inhibit the prostaglandins and endogenous substances that are involved in pain transmission (Debasis, 2011). Terpenoids are known to inhibit $NF_{-k}B$ signals (Kondangala *et al.*, 2011). Flavanoids are believed to possess analgesic activities by inhibiting the enzyme prostaglandins synthetase which is involved in pain perception. This suggests that the flavanoids found in hexane and ethyl acetate crude extract of *E. prostrata* could be responsible for analgesic activity. It is also possible that the extracts act on pain through central mechanism (Aditya *et al.*, 2013). The higher analgesic activity of ethyl acetate witnessed could be associated with more phytochemical it extracted than other solvents.

4.7 Statistical analysis

Using a t- test distribution statistic for a small sample (Equation 4), the claim that PRT mean of ethyl acetate extracts is better than PRT mean of hexane extract is substantiated by $\alpha = 0.10$ level of significance. Compared to positive control (Declofenac), the *E. prostrata* extracts produced better results in terms of the optimum PRT. The optimum PRT for Declofenac had a mean of 8.04 seconds at 60 minutes while for ethyl acetate extract; the PRT at 1000 mg/kg dosage was 10.47 seconds. The standard deviation for

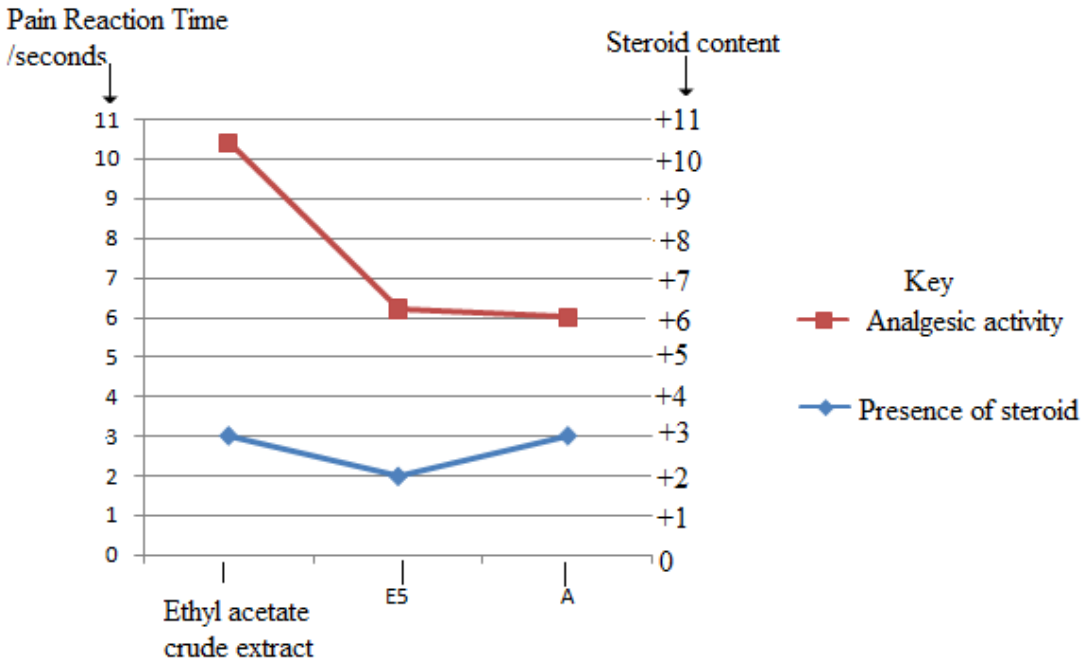
Declofenac and ethyl extract was 0.158 and 0.058 respectively with a t-test value of 24.99. This points to a strong evidence to support the better performance of ethyl acetate extract at $\alpha = 0.05$ level of significance.

4.8 Trend of the steroid content and analgesic activity

Individual fractions of *E. prostrata* plant exhibited low analgesic activity and it is not a surprise since earlier reports have stipulated that most crude extracts are more potent than purified fractions or compounds (Ali *et al.,* 2014; Ruchi & Deepak, 2012). In this study it was observed that analgesic activity and steroidal content of *E. prostrata* constituents decreased on purification (see Figure 4.9).

Table 4.14: Steroid content and analgesic activity

Treatment	Optimum PRT (60 minutes)	Presence of steroid
A 10 mg /kg	10.4	1(+)
Delco 10 mg/kg	6.21	2(+)
Teen solution	6	3(+)



Key: +: Presence of steroid, **E_5 –** fraction from ethyl acetate crude extract**, A –** recrystallized fraction from fraction **E_5**

Figure 4.9: A Superimposable graph showing the trend of steroid content and analgesic activity.

There is observed proportionality of analgesic activity to presence of steroid implying that the analgesic activity of *E. prostrata* is due to steroid presence. From the crude extract to isolated fraction, the analgesic activity goes down (Table 4.14 and Figure 4.9). This is attributed to the fact that some constituents are removed and there is depression of synergistic effect on purification fraction A. The trend of steroid content decreases but rises a bit which is also due to purification of the fraction A.

CHAPTER FIVE

CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

From the results, crude extract indicated that most components of *E. prostrata* were of mid polarity. Both the hexane and ethyl acetate crude extract showed a significant increase in PRT at the dose of 1000 mg/kg when compared to the other concentrations (250 and 500 mg/kg), indicating that the analgesic effect of the crude increased with increasing concentration. Compared to the positive control (Declofenac), crude extracts

of the *E. prostrata* plant produced better results in terms of optimum PRT. Fractions and isolated compounds exhibited a bit lower analgesic activity hence it is thought to be due to the loss of synergism of the phytochemicals.

Qualitative analysis by IR spectroscopy indicated that the crude extracts of *E. prostrata* had phenolic, alcoholic, aromatic, and nitro functional groups.

Column chromatography of the *E. prostrata* crude extracts led to the isolation of active fraction A which was analyzed by FT-IR, UV-Vis, and GC-MS and identified to have a mixture of stigmasterol, sitosterol and cholest-5-en-3-ol, 24 propylidene.

Bioassay of the fraction A on analgesic effect showed that the fraction has analgesic activity close to that of Declofenac at 10 mg/kg body weight. Moreover, its analgesic effect on treated group of rats was found to be 77% in comparison to the untreated group of rats, hence can be selected as a primary analgesic supplement. Statistically, the difference between Declofenac and fraction A was insignificant at $p<0.05$ indicating that they both had comparable analgesic activity. The *E. prostrata* extract produced no death suggesting that the extract was well tolerated by the rats and that the doses used were safe.

Phytochemical screening showed that *E. prostrata* plant contains flavanoids, terpenoids, triterpenoids and steroids which may account for their part of application in herbal medicine and could lead to development of local pharmaceutical industries.

E. prostrata plant possesses secondary metabolites with analgesic activities which are mediated through central mechanism in mammals. Also, it is concluded that steroids are better extracted by solvents with intermediate polarity.

In general, the study showed that the crude extract and the fractions isolated from *E. prostrata* plant have analgesic activity where the most active ingredients have a steroidal nucleus and the analgesic activity relates to it.

5.2 Recommendations

Since the quantities extracted by the hexane, ethyl acetate and acetone were less than 8%, there is need to investigate the yield by other solvents with the aim of getting higher yields. There is an indication that the plant has many bioactive compounds, more investigation should be done to determine each compound. It is also necessary to synthesize the constituents of the active fraction which was found to have analgesic activity for large scale production to allow further investigations. More study should be done on toxicity test along with physiological parameters like body temperature, pulse rate, respiration rate, feeding behaviour, changes in defecation and urination frequency in order to assess any side effects associated with compounds from this plant. Other pathways for analgesics test should be done to establish other routes of mechanism of action i.e peripheral. This may be a lead for clinical trials and hence, the plant could be used for pain treatment in medical field. Based on the bioassay data, it is recommended that a data based computerised modelling for analgesic activity of extracts be done.

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APPENDICES

Appendix 1: UV spectra of fraction A from *E. prostrata*

APPENDIX 2: Bioassay Data for Hexane Crude Extract:

GROUP A (HE)

Treatment	**Pain Reaction Time (seconds)**									
	0	30	60	90	120	150	180	Total	Mean	*S*

	min	min	min	min	min	min	min			
Teen solution	3.5	3	3.2	3	3.1	3.5	3	22.3	3.18571	0.22678
Delco 10 mg/kg	5	6	8.2	6.3	6	5	5	41.5	5.92857	1.14705
HE 250 mg/kg	3.5	3.9	4	4	4	3.3	3	25.7	3.67143	0.40708
HE 500 mg/kg	3	4.03	4.5	4.6	4.3	4	3	27.43	3.91857	0.66514
HE 1000 mg/kg	3	5	10.1	9.08	5.08	5	5	42.26	6.03714	2.55386

GROUP B (HE)

Treatment	**Pain Reaction Time (seconds)**									
	0 min	30 min	60 min	90 min	120 min	150 min	180 min	Total	Mean	*S*
Teen solution	3	3.1	3	3.1	3.01	3	3	21.21	3.03	0.04796
Delco 10 mg/kg	3	6	8.1	6	6.1	5.3	5	39.5	5.64286	1.52846
HE 250 mg/kg	3.4	3.8	3.9	4	4	3	3	25.1	3.58571	0.44881
HE 500 mg/kg	3	3.9	4	4.4	4.5	4.2	3.2	27.2	3.88571	0.57859
HE 1000 mg/kg	3	5	10.1	10	9.6	5.04	5.03	38.17	5.45286	2.96269

Group C (HE)

Treatment	**Pain Reaction Time (seconds)**									
	0 min	30 min	60 min	90 min	120 min	150 min	180 min	Total	Mean	*S*
Teen solution	3	3.1	3	3.1	3.01	3	3.01	21.22	3.03143	0.04706
Delco 10 mg/kg	3	6.01	8.2	6.03	6	5	5	39.24	5.60571	1.56806
HE 250 mg/kg	3.3	3.09	4	4.2	4	3	3.01	24.6	3.51429	0.53022
HE 500 mg/kg	3	3.9	4	4.3	4.5	4.2	3.9	27.8	3.97143	0.48206
HE 1000 mg/kg	3.02	5.2	10.1	9.09	5	5.04	4.9	42.35	6.05	2.54927

Key: HE: Hexane crude extract, *S*: Standard deviation, Delco : Declofenac

APPENDIX 3: Bioassay data of ethyl acetate crude extract.

GROUP A (EE)

Treatment	**Pain Reaction Time (seconds)**									
	0 min	30 min	60 min	90 min	120 min	150 min	180 min	Total	Mean	*S*
Teen solution	3.5	3.1	3.2	3	3.1	3	3.1	22	3.14286	0.17183
Delco 10 mg/kg	3	6	8.21	6.03	6.2	5	5	39.44	5.63429	1.58055
EE 250 mg/kg	3.5	4	4.01	4.5	4.3	4	3	27.31	3.90143	0.50367
EE 500 mg/kg	3.4	4.8	4.9	5.06	4.03	4.1	4	30.29	4.32714	0.60489
EE 1000 mg/kg	2.9	8	10.5	10.2	10.2	9	8	58.8	8.4	2.64008

GROUP B (EE)

Treatment	**Pain Reaction Time (seconds)**									
	0 min	30 min	60 min	90 min	120 min	150 min	180 min	Total	Mean	*S*
Teen solution	3.4	3.3	3.4	3.3	3.1	3	3	22.5	3.21429	0.17728
Delco 10 mg/kg	3	6	7.9	6.9	6.8	5	5	40.6	5.8	1.62173
EE 250 mg/kg	3.5	4	4.02	4.5	4.3	4	3	27.32	3.90286	0.50404
EE 500 mg/kg	3.3	4.9	5.06	4.08	4.1	4	3.9	29.34	4.19143	0.60463
EE 1000 mg/kg	3	8.1	10.4	10.2	10.2	9	8	58.9	8.41429	2.59

Group C (EE)

Treatment	**Pain Reaction Time (seconds)**									
	0 min	30 min	60 min	90 min	120 min	150 min	180 min	Total	Mean	*S*
Teen solution	3.3	3	3.3	3.2	3	3.3	3.4	22.5	3.21429	0.15736
Delco 10 mg/kg	3	6.02	8	6.8	6.4	5.9	5	41.12	5.87429	1.56524
EE 250 mg/kg	3.3	4.9	5.02	4.08	4.1	4.1	3.8	29.3	4.18571	0.60075
EE 500 mg/kg	3.3	4.9	5.02	4.08	4.1	4.1	3.5	29	4.14286	0.64212
EE 1000 mg/kg	3	8.2	10.5	10.2	10.2	9.3	8.9	60.3	8.61429	2.60859

Key: EE: Ethyl acetate crude extract, *S*: Standard deviation, Delco: Declofenac

APPENDIX 4: BIOASSAY DATA OF ACETONE'S CRUDE EXTRACT

Group A (AE)

Treatment	Pain Reaction Time (seconds)									
	0 min	30 min	60 min	90 min	120 min	150 min	180 min	Total	Mean	*S*
Teen solution	3.2	3	3.2	3.2	3	3.2	3.2	22	3.14286	0.09759
AE 250 mg/Kg	3	3.1	3.4	3.2	3	3	3	21.7	3.1	0.15275
AE 500 mg/kg	3.3	3.34	3.54	3.5	3.34	3.3	3.3	23.62	3.37429	0.10179
AE 1000 mg/kg	3	3.5	4	3.9	3.7	3.5	3.2	24.8	3.54286	0.35989
Declo10 mg/kg	3	6.1	8.4	8	6.3	4	3.2	39	5.57143	2.21413

GROUP B (AE)

Treatment	Pain Reaction Time (seconds)									
	0 min	30 min	60 min	90 min	120 min	150 min	180 min	Total	Mean	*S*
Teen solution	3.2	3	3	3.2	3	3.1	3.1	21.6	3.085714	0.089974
AE 250 mg/Kg	3	3	3.3	3.3	3.2	3	3	21.8	3.114286	0.146385
AE 500 mg/kg	3.3	3.35	3.5	3	3.3	3.2	3.1	22.75	3.25	0.165831
AE 1000 mg/kg	3	3.3	3.9	3.7	3.7	3.3	3.2	24.1	3.442857	0.325869
Declo10 mg/kg	3	6.1	8.4	8	6.3	4	3.2	39	5.571429	2.214132

Group C (AE)

Treatment	Pain Reaction Time (seconds)									
	0 min	30 min	60 min	90 min	120 min	150 min	180 min	Total	Mean	*S*

Teen solution	3	3.1	3	3.2	3.1	3	3.1	21.5	3.071429	0.075593
AE 250 mg/Kg	3.3	3.3	3.5	3.3	3.1	3	3	22.5	3.214286	0.186445
AE 500 mg/kg	3	3.4	3.5	3.4	3.3	3.1	3	22.7	3.242857	0.20702
AE 1000 mg/kg	3.2	3.5	4	3.89	3.7	3.5	3.3	25.09	3.584286	0.295063
Declo10 mg/kg	3	6.1	8.4	8	6.3	4	3.2	39	5.571429	2.214132

Key: AE: Acetone crude extract, *S*: Standard deviation, Delco: Declofenac

APPENDIX 5: Table of Characteristic IR Absorptions

Table of Characteristic IR Absorptions

frequency, cm^{-1}	*bond*	*functional group*
3640–3610 (s, sh)	O–H stretch, free hydroxyl	alcohols, phenols
3500–3200 (s,b)	O–H stretch, H–bonded	alcohols, phenols
3400–3250 (m)	N–H stretch	1°, 2° amines, amides
3300–2500 (m)	O–H stretch	carboxylic acids
3330–3270 (n, s)	–C≡C–H: C–H stretch	alkynes (terminal)
3100–3000 (s)	C–H stretch	aromatics
3100–3000 (m)	=C–H stretch	alkenes
3000–2850 (m)	C–H stretch	alkanes
2830–2695 (m)	H–C=O: C–H stretch	aldehydes
2260–2210 (v)	C≡N stretch	nitriles
2260–2100 (w)	–C≡C– stretch	alkynes
1760–1665 (s)	C=O stretch	carbonyls (general)
1760–1690 (s)	C=O stretch	carboxylic acids
1750–1735 (s)	C=O stretch	esters, saturated aliphatic
1740–1720 (s)	C=O stretch	aldehydes, saturated aliphatic
1730–1715 (s)	C=O stretch	α, β–unsaturated esters
1715 (s)	C=O stretch	ketones, saturated aliphatic
1710–1665 (s)	C=O stretch	α, β–unsaturated aldehydes, ketones
1680–1640 (m)	–C=C– stretch	alkenes
1650–1580 (m)	N–H bend	1° amines
1600–1585 (m)	C–C stretch (in–ring)	aromatics
1550–1475 (s)	N–O asymmetric stretch	nitro compounds
1500–1400 (m)	C–C stretch (in–ring)	aromatics
1470–1450 (m)	C–H bend	alkanes
1370–1350 (m)	C–H rock	alkanes
1360–1290 (m)	N–O symmetric stretch	nitro compounds
1335–1250 (s)	C–N stretch	aromatic amines
1320–1000 (s)	C–O stretch	alcohols, carboxylic acids, esters, ethers
1300–1150 (m)	C–H wag ($–CH_2X$)	alkyl halides
1250–1020 (m)	C–N stretch	aliphatic amines
1000–650 (s)	=C–H bend	alkenes
950–910 (m)	O–H bend	carboxylic acids
910–665 (s, b)	N–H wag	1°, 2° amines
900–675 (s)	C–H "oop"	aromatics
850–550 (m)	C–Cl stretch	alkyl halides
725–720 (m)	C–H rock	alkanes
700–610 (b, s)	–C≡C–H: C–H bend	alkynes
690–515 (m)	C–Br stretch	alkyl halides

m=medium, w=weak, s=strong, n=narrow, b=broad, sh=sharp