Evaluation of analgesic and anti-inflammatory activity of solvent fractions of leaves of Moringa stenopetala *(*Bak.)Cuf. (Moringaceae) in Swiss Albino Mice.

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A Thesis submitted to the Department of Pharmacology and Clinical Pharmacy, School of Pharmacy, College of Health Sciences in partial fulfillment of the requirement for the Degree of Master of Science in Pharmacology.

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This is to certify that the thesis prepared by Yohannes Tamrat, entitled "Evaluation of the analgesic and anti-inflammatory activities of the solvent fractions of leaf extracts of *Moringa stenopetala* Bak. (Moringaceae) in Swiss albino mice" and submitted in partial fulfillment for the requirements of the Degree of Master of Science in Pharmacology complies with the regulations of the University and meets the accepted standards with respect to originality and quality.

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Abstract

Evaluation of analgesic and anti-inflammatory activities of solvent fractions of the leaves of *Moringa stenopetala Bak.f.* in Swiss albino mice;

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Many people still experience pain and inflammation regardless of the available drugs for treatments. In addition, the available drugs have many side effects, which necessitated a quest for new drugs from several sources with medicinal plants being one of them. This study evaluated the analgesic and anti- inflammatory activity of the solvent fractions of *Moringa stenopetala*. Successive soxhlet and maceration were used as methods of extractions and Chloroform, methanol and water were used as solvents. Radiant tail flick latency, acetic acid induced writhing and carrageenan induced paw edema models were used to assess the analgesic and anti-inflammatory activities. The test groups received different doses of the three fractions 100 mg/kg, 200 mg/kg and 400 mg/kg. The positive control groups received either Morphine 20 mg/kg or Aspirin at 100 mg/kg or 150 mg/kg. The control groups received the vehicles distilled water or 2% Tween80, 10 ml/kg. In all models the chloroform fraction had protections only at a dose of 400 mg/kg. But, the methanol and aqueous fraction at all doses have shown a significant central and peripheral analgesic and anti-inflammatory activity having comparable effects with standards. Phytochemical screening test revealed differential distribution of secondary metabolites amongst the fractions and the metabolites identified either alone or in combination appeared to be responsible for the observed effect. This study showed the

aqueous fraction being the most active fraction followed by the methanol and then chloroform fraction.

Key words: Analgesic activity, anti-inflammatory activity, solvent fractions, *Moringa stenopetala*

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List of Abbreviations

AA	Arachidonic Acid
APAP	Acetaminophen
CNS	Central Nervous System
COX	Cyclooxygenase
DW	Distilled Water
EPHARM	Ethiopian Pharmaceuticals Manufacturing
EPHI	Ethiopian Public Health Institute
IL	Interleukin
IASP	International Association for the Study of Pain
MAPK	Mitogen Activated Protein Kinase
NO	Nitric Oxide
NSAIDs	Non-Steroidal Anti-Inflammatory Drugs
NF-κB	Nuclear Factor-κappa B
OECD	Organization for Economic Cooperation and Development
OTC	Over The Counter
PG	Prostaglandins
SEM	Standard Error of the Mean
TLR	Toll-Like Receptors
TNF α	Tumor Necrosis Factor Alpha
WHO	World Health Organization

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1. Introduction

1.1. Pain and its management

1.1.1. Definition and epidemiology of pain

The International Association for the Study of Pain (IASP) defines pain as "an unpleasant sensory and emotional experience associated with an actual or a potential tissue damage, or described in terms of such damage" (IASP, 2015). Pain is always subjective; each individual learns the application of the word through experiences related to injury in early life. Accordingly, pain is the experience that we associate with actual or potential tissue damage (IASP, 2015). It varies from person to person and in the same person from time to time. There may be a strong emotional component contributing to the pain experiences; but that does not mean that the suffering is less important (Rajagopal, 2006). It is also a specific enteroceptive sensation; as it can be perceived as arising from a particular portion of the body, its temporal properties can be detailed, it can be differentiated qualitatively and it involves dedicated subsets of peripheral and central neurons (Craig and Sorkin, 2001).

Pain is the most common reason a patient sees a physician. For most patients, it is of short duration and quickly forgotten. Unfortunately, for some, the pain does not pass but becomes a continuous burden and an unrelenting suffering (Debon *et al.*, 2013). Common chronic pain conditions affect approximately 100 million U.S. adults at a cost of $560-635 billion annually in direct medical treatment costs and lost productivity (Committee on Advancing Pain Research, Care, and Education, 2011). Pain, especially when chronic, markedly decreases individuals' health status and quality of life and can detrimentally affect the families of patients. It often interferes with every day work activities (Breivik

et al., 2013). In addition, the presence of a long lasting pain syndrome is a leading risk factor for suicide (Jamison and Edwards, 2012). It is thus, a serious and costly public health problem (Holtman *et al.*, 2010).

1.1.2. **Nociceptive transmission**

Nociception is the encoding and processing of noxious stimuli in the nervous system that can be measured with electrophysiological techniques (Schaible, 2006). Primary afferent sensory nerve fibers with cell bodies in the dorsal root ganglia (DRG) innervate all tissues of the body. Among are the small myelinated (Aδ) and the unmyelinated (C) sensory fibers are those that respond selectively to noxious or potentially damaging stimuli, called nociceptors (Craig and Sorkin, 2001). Mechanical, chemical, or thermal nociceptive stimulation will recruit peripheral nociceptors that conduct the nociceptive signal in the primary somatosensory neuron to the dorsal horn of the spinal cord (Marchand, 2008).

In the dorsal horn, the primary neuron will make a synaptic contact with the secondary or projection neuron. Secondary neurons from the spinothalamic (lateral) and spinoreticular (medial) tracts will immediately cross in the spinal cord and send afferent projections to higher centers. A large proportion of afferents will make a second synapse in the lateral and medial nuclei of the thalamus, which subsequently make synaptic contact with tertiary neurons. Tertiary neurons from the thalamus send afferents to the primary and secondary somatosensory cortices and project to limbic structures (Marchand, 2008).

Nociceptive primary afferent fibers communicate with second-order dorsal horn cells by release of neurotransmitters, neuromodulators and trophic agents (Craig and Sorkin, 2001). Descending tracts reduce or facilitate the spinal nociceptive processing. The

descending tracts are formed by pathways that originate from brain stem nuclei (particularly the periaqueductal grey (PAG) and the rostral ventromedial medulla (RVM)) and descend in the dorsolateral funiculus of the spinal cord. Descending inhibition is part of an intrinsic anti nociceptive system (Fields and Basbaum, 1999).

1.1.3. **Classification of pain**

Pain can be categorized according to several variables with the general classification being based on its location, duration, frequency, underlying cause and intensity. Classification of pain is thus complicated and can be a source of confusion for many clinicians. Duration of the pain process is the most obvious distinction (Cole, 2002) for classifying pain as acute and chronic (Oertel and Lötsch, 2013).

Acute pain

Acute pain has a sudden onset, felt immediately following injury, is severe in intensity, and is usually short-lasting (less than 30 days). It arises as a result of tissue injury stimulating nociceptors and generally disappears when the injury heals (WHO, 2012).

Acute pain develops when a stimulus, such as pressure, heat or inflammation, is presented to the body (Lee *et al*., 2011). It has the primary biological function of warning an organism of impending or immediate threat of tissue damage (Helms and Barone, 2008; Segall *et al*., 2012).

Chronic pain

Chronic pain is defined as a pain which persists a month beyond the usual course of an acute disease or a reasonable time for an injury to heal, or is associated with a chronic

pathological process which causes continuous pain, or pain which recurs at intervals for months or years (Rajagopal, 2006).

Due to the apparent changes in neurophysiology, it can be considered as a pathological condition itself (Lamont *et al.*, 2000; Australian Physiotherapy Association, 2012). Chronic pain may result from a chronic disease and may then actually result from persistent nociceptive processes (Schaible, 2006).

1.1.4. **Management of pain**

Unrelieved acute pain can cause chronic pain, and long-standing pain can cause anatomical and even genetic changes in the nervous system (Rajagopal, 2006). Management of pain should be tailored to the individual patient on the basis of pain type(s), the causative disease(s), and psychosocial aspects. Evidence-based symptomatic pharmacotherapy is the mainstay of the treatment of pain, and it should be titrated individually according to the efficacy and possible contraindications or side effects (Attal *et al.*, 2006).

Non-pharmacological measures of pain management may be helpful in pain management including the application of heat or cold, massage, therapeutic touch, guided imagery, and relaxation techniques (Helms and Barone, 2008).

Analgesic drugs are mainly divided into two classes: opiate receptor agonists and non-steroidal anti-inflammatory drugs (NSAIDs) (Ye *et al.*, 2012) or opioids or non-opioids (Vittalrao *et al.*, 2011). Other currently available analgesic drugs include antidepressants, anticonvulsants (Holtman *et al.*, 2010), sodium (Na^+) channel blockers, Glutamate receptors antagonists (Gao and Ji, 2010).

Non-steroidal anti-inflammatory drugs (NSAIDs)

This group of drugs remains the mainstay as a potent analgesic and anti-inflammatory agents (Viljoen *et al.*, 2012). They are often referred to as peripherally acting non-opioid analgesics. All of NSAIDs appear to share at least one common mechanism, namely inhibition of cyclo-oxygenase (COX) enzyme(s) which leads to a decrease in the synthesis of various prostaglandins and thromboxanes (Modi *et al.*, 2012). Depending on their mechanism of action, NSAIDs are broadly divided into two major classes as nonselective COX inhibitors and selective COX-2 inhibitors (Vane *et al.,* 1998).

There are many potentially significant adverse events that may occur with prolonged use of NSAIDs. Patients may develop gastrointestinal (dyspepsia, bleeding, and peptic ulcer formation through inhibition of protective prostaglandin formation), hematologic (platelet inhibition due to inhibition of thromboxane synthesis (Niemi *et al.*, 1997) and renal dysfunction (Munir *et al.*, 2007).

Acetaminophen

Acetaminophen or paracetamol, is a very popular and among commonly used analgesics (Rezende *et al.*, 2008) for the management of acute and chronic pain. It is generally well-tolerated with demonstrated analgesia (Anderson and Holford, 2008). It is generally accepted that the two systemic effects of paracetamol of therapeutic significance are analgesia and anti-pyresis, while its anti-inflammatory and anti-rheumatic activities are negligible (Bruton *et al.*, 2006).

Multiple targets have been mechanisms of action proposed for its mechanism, including COX-1, COX-2, a putative COX-3 isozyme (Rezende *et al*., 2008; Raffa *et al*., 2010),

reinforcement of descending inhibitory serotonergic pain pathways (Pickering *et al.*, 2008), inhibition of the L-arginine-nitric oxide (NO) pathway (Bujalska, 2004), mediated through substance P or N-methyl-D-aspartate (NMDA) (Hunskaar *et al.*, 1985) and active paracetamol metabolites have effect on cannabinoid (CB) receptors (Ottani *et al.*, 2006).

Its use is closely being monitored by FDA in terms of inducing adverse skin reactions (J"èwiak-benista and Nowak, 2014) and also severe liver impairment after paracetamol overdose is also documented (Toms *et al.*, 2008).

Opioids

Opioid analgesics are widely accepted for first-line treatment of severe acute pain and chronic pain (Fine, 2012).

Opioid receptors (μ, δ, κ) are G protein-coupled receptors distributed throughout the central nervous system. Activation of opioid receptors by endogenous or synthetic opioids results in closing of the voltage sensitive calcium channels, K^+ (potassium) efflux leading to hyperpolarization; and inhibition of adenylyl cyclase to produce cAMP. This results in reduced neuronal excitability and a reduction in transmission of nerve impulses and release of excitatory neurotransmitters (McDonald & Lambert, 2008).

When used over a protracted period of time, prescription opioid abuse may become a concern, especially in patients with a prior history of a substance use disorder (Ives *et al.*, 2006). Major side effects of opiates include physical dependence, tolerance, respiratory depression and constipation (Köksal *et al.*, 2007).

1.2. Inflammation and its management

1.2.1. Overview of inflammation

It is a pervasive form of defense that is broadly defined as a nonspecific response to tissue malfunction and is employed by both innate and adaptive immune systems to combat pathogenic intruders (Noah *et al.*, 2012). Based on visual observation, it is characterized by the cardinal signs of: sensation of heat, redness, swelling, pain and loss of function (Punchard *et al.*, 2004).

The primary functions of inflammation are to rapidly destroy or isolate the underlying source of the disturbance, remove damaged tissue, and then restore tissue homeostasis (Medzhitov, 2008). Although the inflammatory response is essential for host defense, it is very much a double-edged sword (Wu *et al.*, 2013). Because, inflammation itself can damage otherwise healthy cells which could then further stimulate inflammation. This runaway inflammation can lead to an organ failure and/or death (Kumar *et al.*, 2004).

Inflammation is a major feature of many diseases (Shu *et al.*, 2013). Inflammatory reactions are not only the response of living tissues to injury and infection, but also are relevant to disease developments, such as asthma, multiple sclerosis, colitis, inflammatory bowel disease and atherosclerosis (Das *et al.*, 2012).

1.2.2. The inflammatory response

The inflammatory response is a complex process that includes activation of white blood cells, the release of immune system chemicals such as complements and cytokines, and the production and release of inflammatory mediators and prostaglandins (Cotran *et al.*, 2001). At its basic level, it is a tissue-destroying process that involves the recruitment of

blood-derived products, such as plasma proteins, fluid, and leukocytes, into perturbed tissue (Noah *et al.*, 2012).

Alarmins or damage-associated molecular patterns (DAMPs) are endogenous molecules that signal damage or necrosis and are also recognized by the innate immune system. Toll like receptors (TLRs), which are membrane-spanning pattern-recognition receptors, recognize molecular patterns; represent a germ line encoded non self-recognition system that is hardwired to trigger inflammation (Lawrence, 2009; Shelton & Miller, 2010).

Once recognition of ligands occurs, TLRs activate common signaling pathways; NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) and interferon regulatory factors. Activation of TLRs also activate also mitogen-activated protein kinase (MAPK) cascade (Shelton and Miller, 2010; Slavich and Irwin, 2014). The two principal transcription factors in turn, drive the expression of pro-inflammatory immune response genes such as tumor necrosis factor alpha (TNF-α) and interleukin (IL-1) that produce cytokines (Slavich & Irwin, 2014). They also result in an increased expression of chemokines and other inflammatory mediators (Shelton & Miller, 2010). Cytokines are classified either pro-inflammatory or anti-inflammatory, depending on the way they influence inflammation (Viljoen *et al.*, 2012). Many of these cytokines are pro-inflammatory (e.g., IL-1α/β, IL-6, IL-18, TNF-α) (Shelton & Miller, 2010; Viljoen *et al.*, 2012). IL-1 activation of IL-1 receptors induces the transcription of other pro-inflammatory (IL-6 and TNF-α, which can also increase IL-1 expression) and anti-inflammatory (IL-10) cytokines. TNF-α also induce secretion of other inflammatory cytokines (Shelton & Miller, 2010; Ma *et al.*, 2013). The inflammatory cytokines also increase vascular permeability. Due to these reasons, they allow immune cells to leave

the blood vessels and migrate to tissues. This process of redistributing cells of the innate immune is aided by chemokines (Slavich & Irwin, 2014).

1.2.3. **Types of inflammation**

Acute inflammation is an immediate and early response to an injurious agent and it is relatively of short duration, lasting for minutes, several hours or few days. It is characterized by exudation of fluids and plasma proteins and the emigration of predominantly neutrophilic leucocytes to the site of injury (Bezabeh *et al.*, 2004). Acute inflammation has a limited beneficial response, particularly during infectious challenge (Gabay, 2006).

Chronic inflammation can be defined as a prolonged inflammatory process (weeks or months) where an active inflammation, tissue destruction and attempts at repair are proceeding simultaneously (Bezabeh *et al.*, 2004).

One hallmark of acute inflammation is that initially the leucocyte infiltrate is mostly neutrophilic, but after 24 to 48 hours monocytic cells predominate. In contrast, chronic inflammation is histologically associated with the presence of mononuclear cells, such as macrophages and lymphocytes (Gabay, 2006).

1.2.4. **Management of inflammation**

Corticosteroids

Corticosteroids (CS) are among the most widely used drugs and are effective in many inflammatory and immune diseases (Barnes, 2006). Corticosteroids therapy affects endogenous corticosteroid production and has a suppressive effect on Hypothalamo-Pituitary Adrenal (HPA) axis. The actions of all CS are mediated by interaction of hormone with CS receptor, which regulates gene transcription. They continue to act

inside the cell even after their disappearance from the circulation, as the events initiated and the products of these events (such as specific proteins) may be present even after disappearance of CS from the circulation (Gupta and Bhatia, 2008).

They are potent inhibitors of T cell activation and cytokine secretion (Farrell and Kelleher, 2003). The glucocorticoid-receptor complex can either induce key anti-inflammatory genes that encode anti-inflammatory molecules or selectively repress specific inflammatory genes that encode cytokines, chemokine, adhesion molecules, inflammation associated enzymes, lipid mediators of inflammation and receptors (Derendorf and Meltzer, 2008). Their use, however, requires close monitoring because their side effects are widespread to every organ of the body (Viljoen *et al.*, 2012).

1.3. Herbal analgesics and anti-inflammatory agents used in Ethiopia

There are 6500 species of higher plants in Ethiopia making the country one of the most diverse floristic regions in the world (Bekele, 2007).

Many plants are used as an analgesic and/or anti-inflammatory agents in traditional medicine practice in Ethiopia. Some of these plants include: *Allium sativum* (Giday, 2001), *Zingiber officinale* (Yirga, 2010), *Moringa stenopetala* (Teklehaymanot and Giday, 2010), *Taverniera abyssinica and Nigella sativum* (Yirga, 2010; Wabe *et al.*, 2011), *Albuca abyssinica* (Mesfin *et al*., 2013), *Ruta chalepensis* (Mesfin *et al*., 2009; Bekele and Reddy, 2015), *Zehneria scabra* (Akele, 2012) *and Lepidium sativum* (Bekele and Reddy, 2015)

1.4. The Experimental plant

Moringa stenopetala, belonging to Moringaceae, is a native tree in arid and semi-arid regions in the southern Rift Valley of Ethiopia (Jiru *et al.*, 2006). It is also reported to

occur in Djibouti, Uganda and Sudan (Ghebreselassie *et al.*, 2011). It is known by different vernacular names as "Shiferaw" (Amharic), "Halako" (Gamo & Wollayita)," Shelchada" (Konso), and "Cabbage tree" (English) (Musa *et al.*, 2015).

The local farmers use the species as one of the major arable tree inter-crop in the multi storey system especially by the Konso people in Gamo Gofa (Jiru *et al.*, 2006). It is a fast–growing tree with 6-12 m tall, white to pale gray smooth bark, alternate multi-pinnate leaves and long taproots with few lateral roots (Musa *et al.*, 2015). The tree is resistant to both insects and pests character and one Moringa tree can support a large family for many years (Abuye *et al.*, 2003). The leaves are one of the best vegetable foods that can be found in the locality. In fact, all parts of the tree except the wood are edible, providing a highly nutritious food for both humans and animals (Padayachee and Baijnath, 2012).

Moringa stenopetala has a high content of antioxidants and is also rich in protein, calcium and iron (Yang *et al.*, 2006; Carlsen *et al.*, 2010.) and has shown in protection against liver fibrosis (Glærum, 2012). There are claims that the leaves and roots, steeped in water are used to treat stomach disorders, asthma and diabetes (Mekonen *et al.*, 1996). The leaves, boiled in water, can treat and cure headache, malaria, hypertension and stomach pain (Teklehaymanot and Giday, 2010; Seid, 2013) and used as medicine in areas where visceral leishmaniasis prevail (Mekonen *et al.*, 1998), for remedy in vomiting (Mesfin *et al.*, 2009), and also active against Trypanosoma congolense (Kifleyohannes *et al.*, 2014).

Bioassay guided fractionation revealed that both the leaves and seeds of the plant are endowed with antimicrobial activity against *Staphylococcus aureus*, *Salmonella typhi*,

Shigella and Candida (Mekonen *et al.*, 1998). *Moringa stenopetala* could be used for prevention/treatment of hyperglycemia and hyperlipidemia (Toma *et al.*, 2014). It was shown to reduce serum glucose and cholesterol level (Ghebreselassie *et al.*, 2011). And also has shown analgesic and anti-inflammatory activity (Geremew *et al.*, 2015).

Numerous moringa species including *Moringa concanensis* (Jayabharathi and Chitra, 2011), *Moringa olifera* (Kumbhare and Sivakumar, 2011) and *Moringa pergerina* (Goyale *et al.*, 2006) have shown analgesic and anti-inflammatory activities.

1.5. Rationale for the study

Due to extensive use of analgesic and anti-inflammatory agents, the toxicity and untoward effects do occur especially when therapy of pain and inflammation involves the use of higher doses for longer periods. Although there is an increment of knowledge and developments in technological resources regarding pain and inflammation, many patients still experience pain (Nash *et al.*, 1999). This results in a reduction of living quality and functional situation of the patients, increase in the fatigue levels and impairments in daily life activities in working capacity and social interactions (Allard *et al.*, 2001; Kim *et al.*, 2004).

Plants are one of the most important sources of medicines (Hemamalini *et al.*, 2010). About 80% of the total population of Ethiopia depends on traditional medicine to treat different types of human ailments (Bekele, 2007). Many medicines of plant origin had been used since ages without any severe adverse effects. It is therefore, essential that efforts should be made to introduce new medicinal plants to develop more effective and cheaper drugs. Plants represent a large natural source of useful compounds that might serve as a lead for the development of novel drugs (Vittalrao *et al.*, 2011).

A previous study done by Germew *et al* (2015) attempted to show the analgesic and anti-inflammatory activities of leaf extracts in mice. This study will attempt to ascertain in which fraction(s) the constituents responsible for analgesic and anti-inflammatory activities are concentrated so as to provide a clue about the nature of the phytochemical constituents responsible for its actions. In addition, this study may serve as baseline information for further investigation and identification of the specific agents responsible for the analgesic and anti-inflammatory activity of the plant in an effort to contribute to the discovery of new drugs with high activity and low toxicity.

2. Objectives

2.1.General objective

- To evaluate the analgesic and anti-inflammatory activities of the solvent fractions of the leaves of *Moringa stenopetala* in Swiss albino mice.

2.2.Specific objectives

- To assess acute toxicity of solvent fractions of the leaves of *Moringa stenopetala*,
- To evaluate the central analgesic effect of solvent fractions of the leaves of M*oringa stenopetala* in vivo using radiant tail-flick latency method,
- To evaluate the peripheral analgesic effect of solvent fractions of the leaves of *Moringa stenopetala* in vivo using acetic acid induced writhing method,
- To evaluate the anti-inflammatory effect of solvent fractions of the leaves of *Moringa stenopetala* in vivo using carrageenan induced paw edema and,
- To determine the phytochemical constituents of the solvent fractions of the leaves of *Moringa stenopetala*.

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3. Material and Methods

3.1.Drugs and chemicals

Aspirin and morphine, as a powder form, and distilled water were obtained from Ethiopian Pharmaceuticals Manufacturing (EPHARM). Carrageenan (Sigma Aldrich, Germany) was obtained from Ethiopian Public Health Institute (EPHI). Tween 80% (Uni-chem Chemical Reagents, India), Normal saline (Fresenius Kabi, India), Glacial acetic acid, Chloroform and Methanol (Carlo Erba group reagents, Italy) were purchased from their respective vendors. Acetic anhydride and Mayer's reagent (May and Baker LTD Dagenham, England), and Dragendroff's reagent and sulfuric acid (Fisher Scientific, UK) were obtained from the Department of Pharmaceutical Chemistry and Pharmacognosy, School of Pharmacy, Addis Ababa University.

3.2.Plant material

The leaves of *Moringa stenopetala* were collected from Wolayta town in southern central Ethiopia, SNNPR, 313 km from Addis Ababa, in February 2015. The collected plant specimen was identified and authenticated and a voucher specimen of the plant (ms001/2015) was deposited for future references at the National Herbarium of College of Natural and Computational science, Addis Ababa University. The leaves were washed gently by rinsing on running water to remove dust particles, and air-dried under shade. The leaves were then pulverized to a coarse powder using mortar and pestle.

3.3.Experimental animals

Healthy Swiss albino mice, weighing 25–35 g and aged 6–8 weeks were used in the experiment. Male mice were used in the main study, whereas female mice were used in

the acute toxicity study. Animals were purchased and/or obtained from the animal house of EPHI, Addis Ababa, Ethiopia and from the animal house of School of Pharmacy, Addis Ababa University. Mice were kept in plastic cages at room temperature and on a 12 h light/dark cycle with access to standard laboratory pellet food and water *ad libitum*. They were acclimatized for a week to the laboratory condition before commencement of the experiment. All studies were conducted in accordance with international guidelines (Institute for Laboratory Animal Research, 1996; OECD, 2008).

3.4.Preparation of solvent fractions

Successive soxhlet and maceration techniques were used for the extraction of the plant material. The powdered leaf was placed in the extraction chamber of the soxhlet apparatus. For each 50 gram of plant powder 300 ml of solvent was used. The leaf powder was subjected to successive soxhlet extraction with two solvents of different polarity (chloroform and absolute methanol).

The first extracting solvent (chloroform) in the flask was heated until clear liquid contents of the chamber siphoned into the solvent flask. The chloroform fraction was then filtered through filter paper (Whatman No.1) and concentrated and the solvent removed using rotary evaporator (Buchi Rota vapor, Switzerland) under reduced pressure set at 40^{o}C followed by drying using oven at room temperature. The marc was then collected and dried at room temperature to remove chloroform. And then, it was extracted using absolute methanol following the same procedure. Then, the marc of absolute methanol fraction was collected and dried at room temperature to remove the methanol.

Finally, the dried marc was cold macerated in an Erlenmeyer flask with distilled water and allowed to stand at room temperature for a period of 72 hours with occasional

shaking using mini orbital shaker (Stuart, United Kingdom). It was then filtered through gauze. The residue was re-macerated two times for a total of six days in order to obtain better yield. The filtrates from the three batches were combined together and left over in a deep freezer. The filtrate was freeze dried in a lyophilizer (Operon, Korea vacuum limited, Korea) to remove water.

After drying, percentage yield of all fractions was determined using the formula below (Patil and Gaikwad, 2011). The yields of chloroform, methanol and aqueous fractions were 4.5%, 7.8% and 6.4%, respectively.

$$\text{Percentage yield of the extract} = \frac{W1}{W2} \times 100$$

Where, W1= Net weight of yield in grams after extraction and

W2= Total weight of leaf powder in grams taken for extraction

The fractions were then stored in a deep freezer (-20 ^{o}C) until used in the experiment.

3.5.Acute toxicity test

Acute oral toxicity test for the leaf fractions of *Moringa stenopetala* was carried out as per the Organization for Economic Co-operation and Development (OECD) guidelines for Testing of Chemicals 425:2008. Fasted female mice were used for this limit test sequentially. Three female Swiss albino mice were used as a pilot study and fasted for 4 hours prior to the experiment and 2 hours after the experiment. The mice were administered with a single dose 2000 mg/kg of each chloroform, absolute methanol and aqueous fractions orally using oral gavage. Since no death was observed within 24 hours, additional four mice were used for each of the fractions, and administered the same dose of fractions. The animals were observed continuously for 4 h with 30 min interval during

the experiment and then for 14 consecutive days with an interval of 24 hours for the general signs and symptoms of toxicity, food and water intake and mortality (OECD, 2008).

3.6.Animal grouping and dosing

Male mice were randomly divided into five groups (negative control, positive control and three test groups) comprising of six animals in each group to perform the analgesic and anti-inflammatory activity test. The first group was assigned as a control and received the vehicle (2% Tween 80 or distilled water) orally at a volume of 10 ml/kg. The second group was assigned as a positive control and administered with standard drugs 20 mg/kg morphine for a radiant tail-flick method (Matsumoto *et al.*, 2004). Aspirin at a dose of 150 mg/kg was administered for acetic acid induced writhing (Owoyele *et al.*, 2001) and 100 mg/kg for carrageenan induced paw edema model (Khan *et al.*, 2011). The rest three groups were given different doses (100 mg/kg, 200 mg/kg and 400 mg/kg) of the three fractions. Dose selection was made based on the results of acute toxicity test and pilot experiments. All administrations were carried orally using an oral gavage.

3.7.Analgesic activity

3.7.1. Radiant heat tail-flick method

The method of D'Amour and Smith (1941) with slight modification was used to study the analgesic activity of *Moringa stenopetala*. Before and following administration of the agents as per their grouping, heat stress was applied to tails of mice and the change in sensitivity was measured using Analgesiometer (Techno Type: Mark-IB, SL.No; 720121). The current intensity passing through the naked nichrome wire was maintained at 5 amperes (Das *et al.*, 2012). The distance between the heat source and the tail skin

was 1.5 cm. The cut-off time of 10 sec was used to prevent tissue damage (Matsumoto *et al.*, 2004).

Observations were made at an interval of 30, 60, 90 and 120 min after and at initial reading prior to drug administration (pre-treatment) (Das *et al.*, 2012).

Anti-nociception in tail-flick was quantified as the maximum possible effect (MPE) using the following formula (Torres, 2001):

$$\text{MPE \%} = \frac{\text{Post treatment latency} - \text{Pre treatment latency}}{\text{Cut off time } 10 - \text{Pretreatment latency}} \times 100$$

3.7.2. Acetic acid induced writhing method

The method of Koster *et al* (1959) was used. Mice were injected 0.6% acetic acid solution (10 ml/kg) intraperitoneally (i.p) one hour after administration according to the respective groups as described above in grouping and dosing section (Owoyele *et al.*, 2001).

A number of writhes (which consists of a contraction of the abdominal muscle together with a stretching of the hind limbs) (Jayanthi and Jyoti, 2012) was counted for 20 min after a latency period of 5 min (Cavalcante-Silva *et al.*, 2012) to assess the analgesic activity of various groups, and it was expressed as the percentage inhibition between control group and different fractions of *Moringa stenopetala* treated group mice (Jayanthi and Jyoti, 2012).

The percent inhibition was calculated using the formula described below (Hernández-Ortega *et al.*, 2012):-

$$\frac{\text{Mean number of writhes control} - \text{Mean number of writhes (treated)}}{\text{Mean number of writhes control}} \times 100$$

3.8.Anti-inflammatory activity

3.8.1. Carrageenan induced mice paw edema

The anti-inflammatory activity in mice was determined according to the method of Winter *et al* (1962) with slight modification. Acute inflammation was produced by injection of carrageenan (1% w/v carrageenan in normal saline) in the right hind paw of the mice (Deng *et al.*, 2011). Carrageenan was injected one hour after oral administration of the extract, the standard drug or the vehicle. The inflammation was quantitated in terms of ml i.e., displacement of water by edema using a digital plethysmometer (Ugo Basile Company: Cat No 7140, Italy) (Liao *et al.*, 2012) at time 0, 1, 2, 3, 4 and 5 h after carrageenan injection (Sharma *et al.*, 2010) The percent inhibition of edema was calculated in comparison to the animals in the control group (Saini & Singhal, 2012) and was calculated using the following formula (Olukunle *et al.*, 2011):-

$$\%\ \text{Inhibition of paw edema} = \frac{\text{Vt} - \text{Vo}\ \ \text{control} - (\text{Vt} - \text{Vo})\text{Treated}}{\text{Vt} - \text{Vo}\ \ \text{control}} \times 100$$

Where:-Vt: is the right hind paw thickness volume (in ml) at time t,

Vo: is the right hind paw thickness volume (in ml) before carrageenan injection,

Vt –Vo: control and treated is edema or paw size after carrageenan injection to control and drug treated groups respectively

3.9. Preliminary phytochemical screening

Phytochemical screening tests were carried out on each of the chloroform, absolute methanol and aqueous fractions of the *Moringa stenopetala* (Ayoola *et al.*, 2008; Sasidharan, 2011) as described below.

Test for Saponins

To 0.5 g of each fraction, 5 ml of distilled water was added in a test tube. Then, the solution was shaken vigorously and observed for a stable persistent froth. Formation of froth indicates the presence of saponins.

Test for terpenoids (Salkowski test)

To 0.5 g of each solvent fraction of *Moringa stenopetala* leaves, 2 ml of chloroform was added. Then, 3ml concentrated sulfuric acid was carefully added to form a layer. A reddish brown coloration of the interface indicates the presence of terpenoids.

Test for tannins

About 0.5 g of each fraction was boiled in 10 ml of water in a test tube and then filtered. A few drops of 0.1% ferric chloride were added. A brownish green or a blue-black precipitate indicated the presence of tannins.

Test for cardiac glycosides (Keller-Killiani test)

To 0.5 g of each fraction diluted to 5 ml in water was added 2 ml of glacial acetic acid containing one drop of ferric chloride solution. This was under laid with 1 ml of concentrated sulfuric acid. A brown ring at the interface indicated the presence of a deoxysugar characteristic of cardenolides. A violet ring may appear below the brown ring, while in the acetic acid layer a greenish ring may form just above the brown ring and gradually spread throughout this layer.

Test for alkaloids

0.5 g of each fraction was diluted to 10 ml with acid alcohol, boiled, and filtered. To 5 ml of the filtrate, 2 ml of dilute ammonia and 5ml of chloroform was added and shaken

gently to extract the alkaloidal base. The chloroform layer was extracted with 10 ml of acetic acid. This was divided into two portions. Mayer's reagent was added to one portion and Draggendorff's reagent to the other. The formation of a cream (with Mayer's reagent) or reddish brown precipitate (with Draggendorff's reagent) was regarded as positive for the presence of alkaloids.

Test for flavonoids

About 10 ml of ethyl acetate was added to 0.2 gram of each fraction and heated on a water bath for 3 min. The mixture was cooled and filtered. Then, about 4 ml of the filtrate was taken and shaken with 1 ml of dilute ammonia solution. The layers were allowed to separate and the yellow color in the ammoniacal layer indicated the presence of flavonoids.

Test for steroids

2 ml of acetic anhydride was added to 0.5 g fraction of each sample with 2 ml sulfuric acid. The color changed from violet to blue or green in some samples indicating the presence of steroids.

Test for antraquinone

100 mg of each fraction was shaken vigorously with 10 ml benzene and filtered with filter paper and the filtrate was then treated with five ml of 10% ammonia solution. The aqueous (ammonia) phase was inspected for pink, red or violet color.

3.10. Statistical Analysis

All values are expressed as mean ± standard error of the mean (SEM) and data was analyzed by one-way analysis of variance (ANOVA) using statistical package for social

science (SPSS) followed by Tukey post-hoc test. Linear regression was used where appropriate. The analysis was performed with 95% confidence interval and the value, $p<0.05$, is considered as statistically significant.

4. Results

4.1.Acute toxicity

Mice used in the acute toxicity study were observed for the first four hours continuously during the study and for the next 14 days to see if any of the fractions of the plant had toxicity. The LD_{50} of all the three fractions of the plant were estimated to be above 2000 mg/kg as neither of the fractions had caused any visible signs of toxicity nor gross behavioral changes and mortality within 24 h as well as in the next 14 days.

4.2. Analgesic activity

4.2.1. Radiant tail flick latency

Chloroform and Methanol fractions

The radiant tail flick latency effect and maximum possible protection of chloroform and methanol fractions of *Moringa stenopetala* is presented in Table 1. Chloroform fraction at 400 mg/kg and all doses of methanol fraction had shown a considerable analgesic activity in comparison with the control ($p<0.001$) at all-time points. But the chloroform fraction at a dose of 100mg/kg had no significant protection. However, the 200mg/kg of this fraction had shown a delayed onset of action starting at 90 min.

When compared in terms of maximum protection; except the chloroform fraction at a dose of 200 mg/kg (22.3%) and methanol fraction at a dose of 100 mg/kg (27.0%), which had shown their maximum protection at 120 min, the rest of the doses of the two fractions had their maximum protections at 90 min. Morphine had its maximum protection at 90 min (90.3%). The maximum observed protections were: chloroform 400 mg/kg (41.2 %), methanol 200 mg/kg (33.4%) and methanol 400 mg/kg (46.6%). The

standard Morphine in 2% Tween 80 had shown a better analgesic activity ($p<0.001$) than the control and the different doses of the two fractions at all-time points.

Table 1: Effects of chloroform and methanol fractions on radiant tail flick latency test

	Latency (sec) and Maximum possible protection (%)								
Group	0 min	30 min	%	60 min	%	90 min	%	120 min	%
TE	2.05±0.13	2.12±0.08	--	2.52±0.14	--	2.80±0.16	--	2.66±0.18	--
MO	2.22±0.04	8.25±0.08^{a3h3}	77.5	8.92±0.05^{a3h3}	86.1	9.25±0.04^{a3h3}	90.3	8.56±0.06^{a3h3}	81.5
CF 100	2.15±0.05	2.26±0.06	1.4	2.54±0.14	5.0	2.99±0.11	10.6	2.64±0.1	6.3
CF 200	2.13±0.06	2.35±0.04	2.8	2.88±0.03	9.2	3.53±0.09^{a3}	17.9	3.88±0.07^{h3a3}	22.3
CF 400	2.2±0.04	2.78±0.07^{a3}	7.5	3.40±0.09^{h2a3}	15.4	5.41±0.09^{g2a3h3}	41.2	4.04±0.07^{a3}	23.6
MF 100	2.16±0.05	2.60±0.03^{a3}	5.6	3.29±0.06^{h3a3}	14.4	3.58±0.09^{e3a3}	18.2	4.27±0.07^{a3}	27.0
MF 200	2.23±0.05	2.76±0.07^{a3}	6.9	3.70±0.08^{a3h3}	19.0	4.83±0.07^{e2a3}	33.4	4.15±0.07^{a3}	24.6
MF 400	2.23±0.06	2.78±0.05^{a3}	7.1	3.86±0.04^{a3f3g3}	21.0	5.84±0.06^{e1a3g3}	46.6	5.41±0.1^{e3a3g3}	41.0

Values are expressed as Mean ± S.E.M (n=6); analysis was performed with One-Way ANOVA followed by Tukey post hoc test; [a] against control, [b] against the standard drug, [c] against CF100, [d] against CF200, [e] against CF400,[f] against MF 100, [g] against MF 200, [h] against MF 400. [1]P<0.05, [2]P<0.01, [3]P<0.001; where TE: Tween Eighty, MO: Morphine, CF and MF refers to chloroform and methanol fractions of *Moringa stenopetala* respectively ; control received 2% Tween 80 where as standard received Morphine orally; Numbers refer to doses in mg/kg.

Aqueous fraction (AF)

The effect of the aqueous fraction of the plant on a radiant tail flick latency and maximum possible protection is shown in Table 2.

When comparing the aqueous doses with each other, there was a significant difference: at 30 min ($p<0.05$) and at 90 and 120 min ($p<0.001$) between 100mg/kg and 200mg/kg. The 200mg/kg and 400mg/kg doses had a comparable effect with morphine at 90 and 120 min. The 400 mg/kg dose had shown a significant difference at 30 and 60 min from 200mg/kg ($p<0.001$) and at all-time points from 100mg/kg ($p<0.001$).

As shown in Table 2: the maximum protection was observed at 120 minutes (44.8%, 70.1% and 73.6% for AF 100mg/kg, AF 200mg/kg and 400mg/kg respectively) and the standard morphine showed a 90.2% protection which was observed as early as 30 minutes.

When comparing all of the three fractions, the aqueous fraction had shown a better central analgesic activity, by having a maximum possible protection of 73.6% than the chloroform and methanol fractions which had shown maximum protections of 41.2% and 46.6%respectively.

Table 2: Effects of Aqueous fraction on radiant tail flick latency test

	Latency (sec) and Maximum Possible Protection (%)								
Groups	0 min	30 min	%	60 min	%	90 min	%	120 min	%
DW	2.18±0.034	2.10±0.1	--	2.18±0.2	--	2.26±0.2	--	2.15±0.1	--
MO	2.23±0.034	9.23±0.09^{a3d3}	90.2	8.87±0.21^{a3d3}	85.4	7.44±0.09^{a3d3}	67.0	7.05±0.06^{a3}	62.1
AF 100	2.23±0.052	3.00±0.08^{a3b3d1}	10.0	3.67±0.17^{e3b3a3}	18.6	4.49±0.19^{e3a3d3}	29.2	5.71±0.09^{a3b3d3e3}	44.8
AF 200	2.33±0.049	3.18±0.11^{a3e3}	11.1	3.87±0.07^{a3b3e3}	29.2	5.01±0.11^{a3b3e3}	34.9	7.7±0.22^{a3c3}	70.1
AF 400	2.13±0.044	3.61±0.1^{a3b3c3d3}	18.9	4.79±0.09^{a3b3c3d3}	44.8	6.26±0.05^{a3c3d3}	70.1	7.71±0.27^{a3c3}	73.6

Values are expressed as Mean ± S.E.M (n=6); analysis was performed with One-Way ANOVA followed by Tukey post hoc test; [a] against the control, [b] against the standard drug, [c] against AF100, [d] against AF200, [e] against AF400, [1]P<0.05, [2]P<0.01, [3]P<0.001; AF refers to aqueous fraction of *Moringa stenopetala*, MO: Morphine; and DW stands for Distilled Water. Control received distilled water (10ml/kg), whereas standard received Morphine (20mg/kg) orally; Numbers refers to doses in mg/kg.

4.2.2. Acetic acid writhing test

Methanol and Chloroform fractions

Peripheral anti nociceptive activity was measured as a reduction in the number of writhes. In this test, the methanol fraction at all doses had shown a better reduction in the number of writhes in mice when compared to the control ($p<0.001$) but the chloroform fraction had shown a protection only at a dose of 400 mg/kg ($p<0.01$) in comparison with the control.

There was no significant difference among the standard and the methanol fractions at all doses. Nevertheless, the highest dose of Methanol fraction had a significant difference from the lowest dose of this fraction ($p< 0.05$).

The percentage inhibitions for the chloroform extract were 4.4%, 9.3% and 14.9% at 100 mg/kg, 200 mg/kg and 400 mg/kg respectively. And for the Methanol fraction it was 42.5%, 47.7%, and 53.5% at 100 mg/kg, 200 mg/kg and 400 mg/kg respectively, which was comparable with the standard Aspirin (50.4%).

Table 3: Effects of chloroform and methanol fractions in acetic acid induced writhing test

Group	Mean No. of writhing ± S.E.M	% Inhibition
TE	57.0±2.89	--
ASA 150	28.3±0.88^{a3}	50.4
CF 100	54.5±1.18	4.4
CF 200	51.7±0.76	9.3
CF 400	48.5±0.67^{a2b3f3}	14.9
MF 100	32.8±0.60^{a3h1}	42.5
MF 200	29.8±0.60^{a3}	47.7
MF 400	26.5±0.67^{a3}	53.5

Values are expressed as Mean ± S.E.M (n=6); analysis was performed with One-Way ANOVA followed by Tukey post hoc test; [a] against the control, [b] against the standard drug, [c] against CF100, [d] against CF200, [e] against CF400,[f] against MF 100, [g] against MF 200, [h] against MF 400. [1]P<0.05, [2]P<0.01, [3]P<0.001; TE: Tween 80, ASA: Aspirin, CF and MF refers to chloroform and methanol fractions of M*oringa stenopetala* respectively ; control received 2% tween 80 (10ml/kg) and standard received Aspirin(150 mg/kg) orally; Numbers refer to doses in mg/kg.

Aqueous fraction

All the doses of the aqueous fraction had shown a significant reduction in writhing ($p<0.001$) in comparison with control. There was no significant difference between the doses and the standard. But, the highest dose had shown a significant difference from the lowest dose ($p<0.05$) in protection from writhing.

The percentage inhibition for this fraction was 47.0%, 51.5% and 58.0% at 100 mg/kg, 200 mg/kg and 400 mg/kg respectively as shown in Table 4, which is comparable with the standard 48.8%.

Table 4: Effects of aqueous fraction in acetic acid induced writhing test

Group	Mean No. of writhing ± S.E.M	% Inhibition
DW	55.3±2.47	--
ASA 150	28.3 ± 0.88^{a3}	48.8
AF 100	29.3 ± 0.56^{a3e1}	47.0
AF 200	26.8 ± 0.70^{a3}	51.5
AF 400	23.2 ± 0.95^{a3}	58.0

Values are expressed as Mean ± S.E.M (n=6); analysis was performed with One-Way ANOVA followed by Tukey post hoc test; [a] against control, [b] against the standard drug, [c] against AF100, [d] against AF200, [e] against AF400, [1]P<0.05, [2]P<0.01, [3]P<0.001; DW: Distilled Water, ASA: Aspirin, AF refers to aqueous fraction of *Moringa stenopetala;* control received distilled water (10ml/kg) where as standard received Aspirin (150 mg/kg) orally; numbers refer to dose in mg/kg.

When comparing the three fractions, the aqueous fraction at the highest dose (400mg/kg), had the best protection (58.0%) than the highest doses of chloroform (14.9%) and methanol fractions (53.5%). The methanol and aqueous fractions were found to be dose dependent (R^2= 0.9992 for MF and R^2= 0.9893 for AF).

4.3.Anti-inflammatory activity

4.3.1. Carrageenan- induced paw edema

Methanol and Chloroform fractions

The highest dose of chloroform fraction had shown a significant activity ($p<0.05$ at 2^{nd} and 3^{rd} hours, $p<0.01$ at 4^{th} and $p<0.001$ at 5^{th} hour) showing an increase in protection from edema with time. But, the lowest and the middle dose of chloroform fraction had shown no significant inhibition of carrageenan induced paw edema.

Except at a dose of 200mg/kg at 3^{rd} hour ($p<0.01$), all doses of methanol fraction had shown a great significant reduction from an increase in paw volume ($p<0.001$) as compared with the control (2% Tween 80) starting from the second hour after carrageenan injection. And there was no significant difference between the standard and the highest dose of the methanol fraction at all points except at the 3^{rd} hour ($p<0.05$).

When compared with each other, MF at a dose of 400 mg/kg had shown a significant difference with: CF 400 mg/kg ($p<0.001$ at all-time points, except $p<0.05$ at 3^{rd} hour), MF 100 mg/kg ($p<0.05$ at 2^{nd} & 4^{th}hour, $p<0.01$ at 5^{th}hour) and MF 200mg/kg ($p<0.01$ at 2^{nd} & 4^{th} hours and $p<0.001$ at 5^{th} hour).

The standard Aspirin, showed protections from an increase in paw volume starting from the first hour ($p<0.05$) and ($p<0.001$, rest of time) in comparison with the control and with all doses of the chloroform and methanol fraction.

Table 5: Effects of the chloroform and methanol fractions of *Moringa stenopetala* on carrageenan induced paw edema test

	Change in paw volume (ml) and percent inhibition (%)										
Group	0 hour	1 hour	%	2 hour	%	3 hour	%	4 hour	%	5 hour	%
TE	0.43±0.01	0.57±0.01	--	0.73±0.01	--	0.76±0.01	--	0.81±0.02	--	0.88±0.04	--
ASA	0.42±0.01	0.51±0.02^{a3}	36.1	0.53±0.01^{a3g3}	63.9	0.58±0.02^{a3h1}	50.8	0.64±0.01^{a3g3}	42.6	0.67±0.01^{a3g3}	45.3
CF 100	0.43±0.01	0.57±0.01	4.5	0.68±0.01	15.1	0.72±0.03	11.4	0.79±0.01	4.8	0.85±0.01	6.3
CF 200	0.44±0.01	0.56±0.01	7.7	0.67±0.01	18.2	0.72±0.02	12.9	0.78±0.02	9.5	0.85±0.01	9.2
CF 400	0.45±0.01	0.58±0.01	2.3	0.67±0.01^{a1h3}	24.4	0.70±0.01^{a1h1}	22.8	0.76±0.01^{a2f1h3}	18.0	0.80±0.01^{a3h3}	20.8
MF 100	0.42±0.01	0.54±0.01	11.3	0.63±0.02^{a3h1}	26.8	0.67±0.01^{a3b3}	24.0	0.71±0.01^{a3h1}	22.8	0.76±0.01^{a3h2}	23.4
MF 200	0.45±0.01	0.57±0.01	3.8	0.64±0.01^{a3h2}	33.0	0.69±0.01^{a2b3}	25.2	0.73±0.01^{a3h2}	25.4	0.78±0.01^{a3h3}	26.3
MF 400	0.44±0.01	0.54±0.02	21.8	0.58±0.01^{a3}	52.2	0.64±0.02^{a3b1}	37.2	0.68±0.03^{a3e3}	36.8	0.71±0.01^{a3}	39.7

Values are expressed as Mean ± S.E.M (n=6); analysis was performed with One-Way ANOVA followed by Tukey post hoc test; [a] against the control, [b] against the standard drug, [c] against CF100, [d] against CF200, [e] against CF400,[f] against MF 100, [g] against MF 200, [h] against MF 400. [1]P<0.05, [2]P<0.01, [3]P<0.001; ; TE: Tween Eighty, ASA: Aspirin, CF and MF refers to chloroform and methanol fractions of *Moringa stenopetala* respectively ; control received 2% Tween 80, 10ml/kg, whereas :ASA, standard received Aspirin 100mg/kg orally; Numbers refer to dose in mg/kg.

As shown in Table 5 above the maximum protection from increase in paw volume was observed at the second hour from all doses and the standard Aspirin (63.9 %) as: 15.1%, 18.2 %, 24.4% for chloroform and 26.8%, 33.0%, 52.2% methanol fraction for the 100mg/kg, 200 mg/kg, 400mg/kg doses respectively.

Aqueous fraction

All doses of the aqueous fraction and that of the standard showed statistically significant inhibitory effect ($p<0.001$) on the mean increase in paw volume starting from the second hour after carrageenan injection than the control group.

There was no significant difference between 100 mg/kg and 200 mg/kg at all point times measurements. There was a significant difference between 100 mg/kg and 400 mg/kg ($p<0.05$) at all-time points, except at the fourth hour where they had shown a comparable inhibition from paw edema.

As it can be depicted from Table 6 the maximum inhibition from an increase in paw volume was observed at the second hour for all doses and the standard Aspirin as 52.1%, 59.9 %, 67.0% and 62.8 % for the 100mg/kg, 200mg/kg, 400mg/kg and the standard Aspirin respectively.

Table 6: Effects of the aqueous fraction of *Moringa stenopetala* on carrageenan induced paw test

	Change in paw volume (ml) and percent inhibition (%)										
Group	0 hour	1 hour	%	2 hour	%	3 hour	%	4 hour	%	5 hour	%
DW	0.43±0.01	0.56±0.01	--	0.71±0.01	--	0.73±0.01		0.80±0.01	--	0.88±0.02	--
ASA	0.42±0.01	0.51±0.02	35.6	0.53±0.01^{a3c1}	62.8	0.58±0.02^{a3}	48.1	0.64±0.01^{a3}	42.4	0.67±0.01^{a3}	45.8
AF 100	0.46±0.02	0.55± 0.02	30.3	0.59±0.01^{a3e1}	52.1	0.62±0.01^{a3e1}	47.4	0.66±0.02^{a3}	45.6	0.72±0.02^{a3e1}	42.0
AF 200	0.44±0.02	0.52±0.01	34.8	0.55±0.01^{a3}	59.9	0.59±0.01^{a3}	51.3	0.63±0.01^{a3}	48.8	0.68±0.01^{a3}	46.9
AF 400	0.43±0.02	0.50±0.01	47.0	0.53±0.01^{a3}	67.0	0.56±0.02^{a3}	60.1	0.60±0.02^{a3}	54.9	0.65±0.01^{a3}	51.8

Values are expressed as Mean ± S.E.M (n=6); analysis was performed with One-Way ANOVA followed by Tukey test; ^{a} against the control, ^{b} against the standard drug, ^{c} against AF100, ^{d} against AF200, ^{e} against AF400, $^{1}P<0.05$, $^{2}P<0.01$, $^{3}P<0.001$; DW: Distilled Water, ASA: Aspirin, AF refers to aqueous fraction of *Moringa stenopetala;* control received distilled water (10ml/kg), whereas ASA (100mg/kg): standard received Aspirin orally; Numbers: doses in mg/kg.

4.4.Preliminary Phytochemical screening

Phytochemical screening test revealed that terpenoids and anthraquinones were exclusively found in methanol fraction. Cardiac glycosides and flavonoids were found only in aqueous fraction. Steroids were exclusively found in chloroform fraction. Amongst all fractions, the methanol fraction appeared to contain more constituents.

Table 7: Preliminary phytochemical screening of solvent fractions of *Moringa stenopetala* leaves

	Fraction		
Constituents	Chloroform fraction	Methanol fractions	Aqueous fraction
Alkaloids	+	+	-
Tannins	-	+	+
Saponins	-	+	+
Terpenoids	-	+	-
Steroids	+	-	-
Flavonoids	-	-	+
Anthraquinones	-	+	-
Cardiac Glycosides	-	-	+

Key: - + Present, - absent

5. Discussion

In the acute toxicity study, all of the three fractions were found to be safe even at a higher dose of 2000mg/kg which is in line with a study by Musa *et al* (2015) where, the butanol fraction of *Moringa stenopetala* was tolerated in rats up to an oral dose of 5000mg/kg. Moreover, the leaves of this plant are eaten as a cabbage in the southern part of Ethiopia (Abuye *et al*., 2003).

The analgesic activities were evaluated using two animal models. The radiant tail flick test, a thermal method (Siddalingappa *et al.*, 2012), was selected to investigate central analgesic activity (Silva *et al*., 2003; Khobragade *et al.*, 2012) and acetic acid-induced writhing response, a chemical method (Marzouk *et al*., 2011), was selected to observe its peripheral analgesic effects (Das *et al.*, 2012).

In tail flick latency model, the lowest dose of chloroform fraction had not shown a significant protection but the middle dose had a delayed onset of action. This might be attributed to the inability of secondary metabolites to reach sufficient concentrations which are responsible for the anti- nociceptive activity. But, the highest dose of chloroform and all doses of methanol and aqueous fraction have shown a significant analgesia in comparison with the control. The analgesic activity of aqueous extract at doses of 200 and 400mg/kg were comparable to that of morphine at 90 and 120 min. There were differences in point times at which peak protection occurred, which may possibly be due to metabolic rate differences and/or nature of phyto constituents' available in the fractions.

So it is possible to assume, this plant had shown a raise in a threshold for pain or alteration in physiological response to pain, central anti-nociceptive activity, which most

likely to be mediated by central or supra-spinal action and suggests a mechanism of action related to activation of opioid receptors or promoted the release of endogenous opio-peptides.

In the second model, the acetic acid induced writhing response, is reliable and affords rapid evaluation of the peripheral type of analgesic action. Pain sensation in this writhing method is elicited by triggering localized inflammatory response resulting in release of endogenous substances, free arachidonic acid from tissue phospholipids via cyclooxygenase (COX), prostaglandin biosynthesis and sympathomimetic system. Mediators like PGE_2 and $PGF_{2\alpha}$ levels were increased in the peritoneal fluid of the injected mice (Deraedt *et al.*, 1980; Milind and Monu, 2013). The administration of Aspirin (the standard), a NSAID that inhibits COX-1 and COX-2, inhibited the algogenic action of acetic acid by removing the inflammatory mediators of pain in peripheral tissues.

In this study, fractions of *Moringa stenopetala leaves* had shown a varying degree of protection in response to intra-peritoneal acetic acid administration. Only the higher dose of chloroform had shown a reduction in the writhing response ($p<0.01$), the lowest two doses hadn't shown significant protections, which might be because of lower concentrations of secondary metabolites. And the methanol and aqueous fractions had shown significant reductions in all doses administered ($p<0.001$ for all; which was comparable with the standard Aspirin). When comparing the three fractions, the aqueous fraction at the highest dose (400mg/kg), had the best protection (58.0%) than the highest doses of chloroform (14.9%) and methanol (53.5%).

Since this method is a nonselective method, different agents could reduce the pain induced by acetic acid (Hernández-Ortega *et al.*, 2012). Thus, different drugs such as anti-cholinergics, anti-histaminergics (Atsang *et al*., 2012; Richard *et al*., 2011), local anesthetics, muscle relaxants, anxiolitics, tranquilizers (da Matta *et al*., 2011) and anti-inflammatory substances can produce effects (Islam *et al*., 2010). Based on this fact, it is plausible to assume that other mechanisms beside PG inhibition could also play a role in the analgesic action of the extract.

We can propose from the results depicted, secondary metabolites of semi-polar to polar in nature are responsible for protection from writhing. Since methanol and aqueous fraction had shown greater protections from writhing. The aqueous fraction has got a better anti-nociception activity than methanol fraction, the methanol fraction is better than the chloroform fraction. The difference in rank order of potency could emanate from the differential distribution of the secondary metabolites as given in preliminary phytochemical test (Table 7).

Carrageenan induced paw edema is a widely used model to determine an anti-inflammatory activity of drugs as well as to study the mechanisms involved in inflammation (Posadas *et al.*, 2004). In this experiment, acute inflammation was produced by an injection of carrageenan (1% w/v carrageenan in normal saline) in the right hind paw of the mice (Deng *et al*., 2011). During injection, inflammatory response produced increase in vascular permeability and cellular infiltration leading to edema formation (an increase of paw volume characterized as edema), as a result of extravasation response.

The development of edema in the mice hind paw following the injection of carrageenan has been described as a biphasic, age-weight dependent event in which various mediators operate in sequence to produce the inflammatory response. There are several mediators involved in the inflammation. The initial phase of edema has been attributed to the release of histamine, 5-hydroxytryptamine (5-HT) and bradykinin (Suralkar *et al*., 2008; Mali *et al*., 2013; Samad *et al*., 2013). The second, accelerating phase of swelling has not only been correlated with the elevated production of prostaglandins (Necas and Bartosikova, 2013*)*, but also has been attributed to the induction of inducible cyclooxygenase (COX-2) in the hind paw (Nantel *et al.*, 1999). Local neutrophil infiltration and activation also contribute to this inflammatory response by producing, among other mediators, oxygen- derived free radicals such as superoxide anion (O_2^-) and hydroxyl radicals (OH^-) (Posadas *et al.*, 2004), and also nitric oxide (NO) is one important mediator (Rosa, 1972; Osborne and Coderre, 1999; Posadas *et al*., 2004). The late phase of inflammation can be blocked by the NSAIDs (Handy and Moore, 1998). These two phases are linked with kinin release (Siddalingappa *et al.*, 2011).

The methanol and aqueous fractions had shown a significant inhibition from an increase in paw edema starting from the second hour after carrageenan injection. Since the methanol and aqueous fractions had reduced from paw edema during the late phases (two hours after carrageenan injection), it is possible that the fractions could have inhibitory effect on mediators such as prostaglandins, bradykinin and/or leukotriens or it could have inhibited the synthesis or release of these mediators and/or had free radical scavenging activity (Yang *et al*., 2006; Mali *et al*., 2013; Samad *et al*., 2013). These facts may collectively indicate that the methanol and aqueous fractions of *Moringa stenopetala* may

exert their action also by inhibiting COX, free radical scavenging activity and inhibiting subsequent PG synthesis.

Flavonoids, terpenoids and steroids have been shown to inhibit production of prostaglandins (Awad *et al*., 2004; Das *et al*., 2012). And free radical scavenging activity was reported with flavonoids (Olaleye, 2004; Adedapo *et al*., 2008; Borgi *et al*, 2008; Atsang *et al*., 2012; Hernández-Ortega *et al*., 2012; Kaushik *et al*., 2012). The anti-inflammatory activity of many plants is also related to the presence of saponins (Hernández-Ortega *et al*., 2012), terpenoids (Çadirci *et al*., 2012; Shaheen *et al*., 2013), triterpenes (Adedapo *et al*., 2008), alkaloids (Souto *et al*., 2011), glycosides (Hosseinzadeh and Younesi, 2002) and tannins (Hernández-Ortega *et al*., 2012; Mali *et al*., 2013). Thus, it can be said that the anti-inflammatory action of the fractions observed in carrageenan induced paw edema model could possibly be due to the presence of alkaloids, glycosides, flavonoids, saponins and terpenoids.

Preliminary phytochemical screening performed using thin layer chromatography technique for butanol fraction of *Moringa stenopetala* and its column chromatographic fractions indicated the presence of flavonoids, phenolic compounds and phenolic glycosides (Sileshi *et al*., 2014) and another study indicates the presence of flavonoids, tannins, alkaloids, saponins, polyphenols and glycosides (Toma *et al*., 2012). In addition, the present study had confirmed differential distribution of: terpenoids, anthraquinones, cardiac glycosides, flavonoids, saponins, alkaloids and tannins among fractions.

So, it is possible to assume that the aqueous and methanol fractions and the highest dose of chloroform fractions of *Moringa stenopetala* had shown central and peripheral anti-nociceptive effects. Opioid receptors and the inhibition of the cyclooxygenase enzyme

may mediate these activities and also these fractions had shown activities against acute inflammation. As the preliminary phytochemical results indicated, the anti-nociceptive and anti-inflammatory effects of the fractions may be due to their content of flavonoids, terpenoids, anthraquinones, cardiac glycosides, flavonoids, saponins, alkaloids and/or tannins. So, purification of the aqueous fraction and further definitive studies may reveal the proposed mechanisms and constituents behind the observed effects.

6. Conclusion

The present study attempted to see the analgesic and anti-inflammatory activities of the three fractions of the leaves of *Moringa stenopetala* and revealed that they are safe at high doses and they possessed a varying degree of central, peripheral analgesic and acute anti-inflammatory activities, with the aqueous fraction being the most active fraction followed by the methanol fraction and then chloroform fraction in all the three models. The analgesic and anti-inflammatory activities of the fractions could be attributed to the presence of bioactive agents including flavonoids, tannins, terpenoids, saponins, steroids, glycosides and alkaloids that might have acted separately or synergistically. In addition, the results of the present study suggest that compounds ranging from semi-polar to polar are more likely to be responsible for the observed effect.

7. Recommendations

- Further toxicological studies of sub-acute and chronic toxicities should be done in order to assess the long-term effect of each fraction,
- Further work should be conducted to understand the mechanism (s) of action of the fraction (s) having analgesic and anti-inflammatory action and
- Further studies should be done to isolate, purify and identify pharmacologically active principle(s) responsible for the analgesic and anti- inflammatory activities of the plant.

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