Addis Ababa University

College of Health Sciences

School of Pharmacy

Evaluation of Analgesic and Anti -inflammatory activity of *Moringa stenopetala* Bak.(Moringaceae) in mice.

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A Thesis Submitted to the Department of Pharmacology and Clinical Pharmacy presented in fulfilment of the requirements for the Degree of Master of Science in Pharmacology.

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School of Pharmacy

This is to certify that the thesis prepared by Helen Geremew, entitled evaluating analgesic and anti-inflammatory activity of *Moringa stenopetala* and submitted in partial fulfilment of the requirements for the degree of masters of Science complies with regulations of the university and meets the accepted standards with originality and quality.

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Evaluation of analgesic and anti- inflammatory activity of *Moringa stenoptala* Bak. (Moringaceae) in mice.

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Abstract

Moringa stenopetala Bak F.is commonly used in folk medicine as a single drug remedy to treat various aliments like; hypertension, headache, stomach disorders, asthma, diabetes and malaria. However, the use of the herb for treatment of pain and inflammation has not been scientifically investigated. Thus, in this experiment 80% methanol crude extract of the leaves of *M.stenopetala* was evaluated for its analgesic and anti-inflammatory properties using established animal models. Swiss albino mice of either sex were randomly divided into five groups of six mice per group. The negative control group was orally given 0.5 ml of distilled water. The positive control received standard drug (morphine 10 mg//kg, Acetyl salicylic acid 81mg/kg, Indomethacin 25mg/kg).The rest of the groups were treated with 80 % methanol extract of *M. Stenopetala* at doses of 200, 400 and 600 mg/kg.Animals were then subjected to a battery of tests including hot plate, tail flick, acetic acid induced writhing, and carrageenan induced paw oedema tests. *M. stenopetala* extract showed a dose-dependent significant reduction of pain in analgesia models ($p<0.001$) with 600 mg/kg dose producing the highest reduction. The extract significantly reduced carrageenan induced inflammation in a dose independent manner, in which the highest reduction of inflammation was observed at 400mg/kg. The data collectively indicate that 80% methanol extract of *M. Stenopetala* leaves have potential analgesic and anti-inflammatory activities against stimuli in the tested animals and can be recommended for further studies.

Key words: *M. stenopetala, analgesic activity, anti- inflammatory activity*

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List of acronyms

5-HT	5-hydroxy tryptamin
AA	Arachidonic Acid
ASA	Acetyl Salicylic Acid
COX	Cyclooxygenase
GABA	Gama Amino Butyric Acid
IASP,	International Association for the Study of Pain
LTs	Leukotriene
NE	Norepinephrine
NO	Nitrogen Oxide
NSAIDS	Non -steroidal Anti- inflammatory drugs
PAG	Periaqudectalgray
PGs	Prostaglandins
ROs	Reactive oxygen species
(TCA)	Tricyclic Antidepressants
TLC	Thin Layer Chromatography
TXs,	Thromboxane

1.Introduction

1.1.Overview of pain

Pain is a multidimensional sensory experience that is intrinsically unpleasant and associated with hurting and soreness. Although it is essentially a sensation, pain has strong cognitive and emotional components. It is linked to or described in terms of suffering. It is also associated with avoidance motor reflexes and alterations in autonomic output. All of these traits are inextricably linked in the experience of pain (Flor, 2000).As a result, the International Association for the Study of Pain (IASP) defined pain as a sensory and emotional experience associated with real or potential injuries, or described in terms of such injuries, which means the perception of pain and its threshold are the result of complex interactions between sensory, emotional, and behavioural factors (Cole, 2002).

Pain is a subjective experience, which cannot be easily measured. It requires consciousness. Describing pain as an 'experience 'separates pain from nociception'. Nociception is the neural process involving the transduction and transmission of a noxious stimulus to the brain via a pain pathway. Pain is the result of a complex interplay between signalling systems, modulation from higher centres and the unique perception of the individual (Steeds, 2009).

1.1.1. Pain pathway

The propagation of pain is initiated with the activation of physiological receptors, called nociceptors. The cell bodies of nociceptors, like those of most other primary afferent neurons, reside in one of three locations (Zeilhofer, 2005): (*a*) dorsal root ganglia, which innervate the trunk, limbs, and viscera and project centrally to the spinal cord dorsal horn; (*b*) trigeminal ganglia, which innervate the head, oral cavity, and neck and project centrally to the brain stem trigeminal nucleus; and (*c*) nodose ganglia, whose peripheral terminals innervate visceral tissues and whose central terminals project to the floor of the fourth ventricle(De León-Casasol, 2007).

The nociceptive signals generated by nociceptor activation are transmitted to the central nervous system by their associated afferent axons (A-delta fibers, which are myelinated fibers that conduct rapidly and C-fibers, which are non-myelinated and

conduct slowly) which correspond to the subclasses of nociceptors(Steeds, 2009).With such form of organization a complex set of pathways transmit pain messages from the periphery to the central nervous system, where control occurs from higher centres. Ascending spinothalamic and spinoreticular tracts convey discriminative and affective aspects of pain up to the brain, where pain signals are processed by the thalamus and sent to the cortex. Descending tracts, via the midbrain periaqueductal grey and nucleus raphe Magnus, have a role in pain modulation (Juliusand Basbaum, 2001).

1.1.2. Classification of pain

Pain is generally classified according to its location, duration, frequency, underlying cause, and intensity. Classification of pain is thus complicated and can be a source of confusion for many clinicians. The duration of the pain process is the most obvious distinction that can be made when classifying pain (Cole, 2002).

i. Acute Pain

Acute clinical pain typically arises from soft tissue trauma or inflammation, which has a sudden onset and a foreseeable end. It plays a biologically adaptive role by facilitating tissue repair and healing. This is achieved by hyper sensitizing the injured area (primary hyperalgesia) as well as the surrounding tissues (secondary hyperalgesia) to all types of stimuli such that contact with any external stimulus is avoided and the reparative process can precede undisturbed (Macintyre *et al.,* 2010).

ii. Chronic Pain

Chronic pain persists beyond the expected time frame for a given disease process or injury and has been arbitrarily defined as having a duration greater than 3 to 6 months. Such pain may arise as a result of sustained noxious input such as on-going inflammation, or it may be autonomous, with no temporal relation to the inciting cause. It is maladaptive and offers no useful biologic function or survival advantage, with the nervous system itself actually becoming the focus of the pathology and contributing to patient morbidity (Flor, 2000).

Chronic pain can make the nervous system become more sensitive to pain over time. For example, due to chronic pain repeatedly stimulating the nerve fibres and cells that

detect, send, and received pain signals that stimulation can not only change the structure of the nerve fibres and cells but also can make them more active and thus increase pain transmission to the spinal cord and brain. Leading to pain that may be a result of stimulation that ordinarily would not be painful or painful stimuli being felt much more strongly and severely (Herdon *et al*., 2008).

1.2. Overview of inflammation

Inflammation is an orchestrated biological process, induced by microbial infection (like bacteria, viruses, fungi, parasites) or tissue injury. It may also be caused by an antigen-antibody reaction, mechanical trauma, organic and inorganic poisons and foreign bodies. A major trigger of inflammation is the recognition of microbes by specific receptors of the innate immune system, which play a crucial role in the induction of early signals initiating and establishing the inflammatory setting (Lafuente *et al.,* 2009). Signs of inflammation include redness, swelling, heat, pain, and loss of function. These signs of inflammation are a consequence of increased blood flow and capillary permeability, vasodilatation, release of soluble mediators, extravasation of fluids, influx of phagocytic cells, and tissue damage (Punchard*et al*.,2004).

Inflammation could be either acute or chronic. The acute inflammatory response involves a complex series of events, including dilatation of arterioles, venules, and capillaries with increased vascular permeability, exudation of fluids including plasma proteins, and leukocyte migration into the inflammatory sites. Chronic inflammation on the other hand, is defined as prolonged process in which tissue destruction and inflammation occur at the same time (Maaloufet *al*., 2010).

The inflammatory response is a natural defense mechanism that is triggered whenever body tissues are damaged in any way. Most of the body defense elements are located in the blood and inflammation is a means by which body defense cells and defense chemicals leave the blood and enter the tissue around the injured or infected site (Wahl *et al.,* 2001). Although at first, edema may seem to be detrimental to the body, it is not. The entry of protein-rich fluids into the tissue spaces has the following advantages:(i) helps to dilute harmful substances, which may be present ;(ii) brings in large quantities of oxygen and nutrients necessary for the repair

process; and (iii) allows the entry of clotting proteins which form a gel like fibrin mesh in the tissue space that effectively isolates the injured area and prevents the spread of bacteria and other harmful agents into the adjacent tissues. It also forms scaffolding for permanent repair. Thus, the ideal inflammatory response is rapid and destructive, yet specific and self-limiting (Khan and Khan, 2010).

1.2.1. Phases of inflammation

Inflammation can be divided into several phases. The earliest gross event of an inflammatory response is temporary vasoconstriction, i.e. narrowing of blood vessels caused by contraction of smooth muscle in the vessel walls, which can be seen as blanching (whitening) of the skin. This is followed by several phases that occur over minutes, hours and days later, outlined below (Puncard*et al*., 2004).

The acute vascular response follows within seconds of the tissue injury and last for some minutes. This results from vasodilation and increased capillary permeability due to alterations in the vascular endothelium, which leads to increased blood flow (*hyperaemia*) that causes redness (*erythema*) and the entry of fluid into the tissues (*oedema*) (Kilicarslan*et al.,* 2013). This phase of the inflammatory response can be demonstrated by scratching the skin with a finger-nail. The "wheal and flare reaction" that occurs is composed of (a) initial blanching of the skin due to vasoconstriction, (b) the subsequent rapid appearance of a thin red line when the capillaries dilate; (c) a flush in the immediate area, generally within a minute, as the arterioles dilate; and (d) a wheal, or swollen area that appears within a few minutes as fluid leaks from the capillaries. It is usually terminates after ten minutes (Wahl *et al.,* 2001).

If there has been sufficient damage to the tissues, or if infection has occurred, the acute cellular response takes place over the next few hours. The hallmark of this phase is the appearance of granulocytes, particularly neutrophils, in the tissues. These cells first attach themselves to the endothelial cells within the blood vessels (margination) and then cross into the surrounding tissue (diapedesis) (Lafuente *et al.,* 2009). During this phase erythrocytes may also leak into the tissues and a haemorrhage can occur (e.g. a blood blister). If the vessel is damage, fibrinogen and fibronectin are deposited at the site of injury, platelets aggregate and become activated, and the red cells stack together in what are called "rouleau" to help stop

bleeding and aid clot formation. The dead and dying cells contribute to pus formation (Silva and Menezes , 2011).

If the damage is sufficiently severe, a chronic cellular response may follow over the next few days. A characteristic of this phase of inflammation is the appearance of a mononuclear cell infiltrate composed of macrophages and lymphocytes. The macrophages are involved in microbial killing, in clearing up cellular and tissue debris, and they also seem to be very important in remodelling the tissues (Khan and Khan, 2010).

Over the next few weeks, resolution may occur, meaning that the normal tissue architecture is restored. Blood clots are removed by fibrinolysis, and if it is not possible to return the tissue to its original form, scarring results from in-filling with fibroblasts, collagen, and new endothelial cells. Generally, by this time, any infection will have been overcome (Silva and Menezes, 2011). However, if it has not been possible to destroy the infectious agents or to remove all of the products that have accumulated at the site completely, they are walled off from the surrounding tissue in granulomatous tissue. A granuloma is formed when macrophages and lymphocytes accumulate around material that has not been eliminated, together with epitheloid cells and gigantic cells (perhaps derived from macrophages) that appear later, to form a ball of cell (King, 2012).

1.2.2. Mediators of inflammation

The spread of inflammatory response following injury to a small area of tissue suggests that chemical substances are released from injured tissues, spreading outwards into uninjured areas. These chemicals, called endogenous chemical mediators, includes; histamine, serotonin, leukotriene, prostaglandins and bradykinin, which cause vasodilatation, migration of neutrophils, chemotaxis, and increased vascular permeability (Meeran and Messent , 2001).

Histamine is the best-known chemical mediator in acute inflammation. It causes vascular dilatation and the immediate transient phase of increased vascular permeability. It is stored in mast cells, basophils and eosinophil, and platelets. Histamine released from these sites is stimulated by complement factors, and by lysosomal proteins released from neutrophils, including cationic proteins, which may

increase vascular permeability, and neutral proteases, which may activate the complement system(Lawrence*et al.,* 2002).Prostaglandins on the other hand are group of long-chain fatty acids derived from arachidonic acid and synthesized by many cell types. Some prostaglandins potentiate the increase in vascular permeability caused by other compounds and others modulate platelet aggregation. Part of the anti-inflammatory activity of drugs such as the non-steroidal anti-inflammatory drugs is attributable to inhibition of the enzymes involved in prostaglandin synthesis (Prisk and Huard, 2003).

1.3. Management of pain

The human body itself produces many substances that influence the way in which "pain" messages are sent from the periphery to the brain and also the way in which the brain responds to these messages. These substances include endogenous peptides and other chemical messengers such as noradrenalin; serotonin (5-HT) and GABA (Konerue*t al*., 2009).Medications (drugs) on the other hand are external substances that can be used to supplement the body's inbuilt pain killing systems. Often medications either mimic or influence the level of the body's own painkillers. Use of medication can have the advantage of more profound pain relief (at least for a time being), but this is often offset by the disadvantage of side effects. The use of morphine-like drugs can have the additional disadvantage of suppressing the body's own endorphin levels (Attal, 2001).

1.3.1. Drugs acting on the opioidsystem

The opioid system is one of the main systems engaged in strongly conserved evolutionary mechanisms like pain perception and modulation, reward, addiction and fear behaviours (Lesniak and Lipkowski, 2011). Opioid peptides that are produced in the body include: endorphins, enkephalins, dynorphins and endomorphins. Each family is derived from a distinct precursor protein and has a characteristic anatomical distribution (Jordan and Devi, 1998).

Opioids by acting on pre-synaptic terminal of primary nociceptive afferents decrease the release of excitatory neurotransmitters from nociceptive neurons. A postsynaptic action is demonstrated by the ability of opioids to block excitation of dorsal horn

neurons directly evoked by glutamate, reflecting a direct activation of dorsal horn projection neurons (Fields, 2007). In periaqueductal grey matter they block the release of GABA from tonically active systems that otherwise regulate the projections to the medulla. They also facilitate the release of non -opioid neurotransmitters in the spinal cord, especially serotonin (5-HT) and norepinephrine (NE),which directly inhibit the release of transmitters from the incoming nociceptive afferent signal, and the second– order pain transmission. Peripherally, endogenous opioid peptides released from immune cells, which in the presence of inflammatory mediators migrate to inflamed tissue in a process referred to as "homing" a centrally-mediated mechanism (Gentilucci, 2004).

The opioid drugs are all different in the way they affect receptors. This means that some opioids may be more effective than others for treating specific pain mechanisms. It also means that the side effect profile varies. Commonly used opioids include codeine, morphine,pethidine, fentanyl, oxycodone, hydromorphone, methadone and buprenorphine (Brain and Cox,2006).They are usually very effective in treating acute nociceptive (tissue damage) pain. They can often be helpful in treating acute neuropathic (nerve injury) pain. In managing persistent pain opioids are much less effective but there can be a role in a selected sub-group of people with either nociceptive or neuropathic pain. Generally the pain reduction achieved is much less in persistent pain and the tolerance problem means further reduction in benefit over time (Mcnallyand Akil,2002).

1.3.2. Drugs acting on other systems

Antidepressant drugs act by altering the levels of specific chemicals in the brain. Noradrenalin and serotonin are the substances primarily involved and brain levels impact both mood and pain. While both substances have an effect on mood, noradrenalin appears to be more important in pain pathways. Increased brain noradrenalin can damp down transmission of pain messages. Tricyclic antidepressants (TCA) act by blocking reuptake of both noradrenalin and serotonin once they have been released at their site of action. This increases the effective levels of both substances in the brain (Long, 2011).

Anticonvulsants are a family of drugs that reduce excessive electrical activity in the brain and thereby stop seizures. The mechanism of neuropathic pain also involves increased electrical activity. This can occur in the peripheral nerves, spinal cord and brain. Some of the drugs used to treat epilepsy can therefore also be effective in neuropathic pain. Anticonvulsants act by a number of different mechanisms to reduce the excessive electrical activity. They can block the sodium or calcium channels in nerves that are part of the electrical transmission system. Some agents increase brain levels of inhibitory messengers such as GABA (Page, 1996).

Local anaesthetics are valued for the ability to prevent membrane depolarization of nerve cells. They prevent depolarization of nerve cells by binding to cell membrane sodium channels and inhibiting the passage of sodium ions. The sodium channel is most susceptible to local anaesthetic binding in the open state, so frequently stimulated nerves tend to be more easily blocked. Differential blockade to achieve pain and temperature block (A-δ, Cfibres) while minimizing motor block (A-α fibres) can be achieved by using certain local anaesthetics and delivering specific concentrations to the nerve(Attal *et al*., 2010).

1.4. Management of inflammation

There are more drugs designed to fight inflammation in the human body than any other single category of drugs. Anti-inflammatory agents stop or disrupt inflammation by suppressing or altering the chemical signals associated with the inflammatory response (Dinarello, 2010).

1.4.1. Non-steroidal anti -inflammatory drugs

Non-steroidal anti-inflammatory drugs (NSAIDs) mediate their action by inhibiting both cyclooxygenase (COX-1 and -2) include; Aspirin, Ibuprofen and Indomethacin. Selective COX-2 inhibitors include the Coxibs (e.g., Celecoxib) and Meloxicam. NSAIDs are mainly indicated for mild to moderate pain and rheumatoid arthritis. Other indications include osteoarthritis, soft-tissue injury, renal colic, postoperative pain, and dental procedures (Vanegas *et al.,* 2010). The main mechanism of action of these drugs is believed to be the inhibition of the COX enzymes (COX-1 and COX-2) and consecutively the conversion of AA to PGs (Vane, 1996).

1.4.2. Glucocorticoids

Glucocorticoids (corticosteroids or steroids) are the most effective anti-inflammatory agents available for many inflammatory and immune diseases including, asthma, rheumatoid arthritis, inflammatory bowel disease, and other autoimmune diseases. Glucocorticoids are able to bind with the cortisol receptors and trigger various biological effects (Singh *et al*., 2004). Although there are several mechanisms by which glucocorticoids reduce inflammation, a major one could be to reduce expression of cytokine-induced genes. Glucocorticoids inhibit the expression of pro-inflammatory cytokines. They also inhibit the generation of inflammatory mediators PGs, TXs, LTs and NO by suppression of gene expression of PLA2, COX-2, and iNOS. Further they alter recruitment and activation of inflammatory cells such as, monocytes, macrophages, eosinophils or lymphocytes (Rhen and Cidlowski, 2005).

1.5. Herbs with anti-inflammatory and analgesic activity

Unlike modern allopathic drugs which are single active components that target one specific pathway, herbal medicines work in a way that depends on an orchestral approach. A plant contains a multitude of different molecules that act synergistically on targeted elements of the complex cellular pathway (Abebe and Hagos, 1991). Medicinal plants have been source of wide variety of biologically active compounds for many centuries and used extensively as crude material or as pure compounds for treating various disease conditions (Anilkumar, 2010).The uses of herbal medicines have become popular due to toxicity and side-effects of allopathic medicines. Medicinal plants play an important role in the development of potent therapeutic agents. Purified natural compounds from plants can serve as template for the synthesis of new generation drugs with low toxicity and higher therapeutic value (Kumar *et al.,* 2013).

The research into plants with alleged folkloric use as pain relievers and anti-inflammatory agents is definitely a fruitful and logical research strategy in the search for new analgesic and anti-inflammatory drugs(Abebe and Hagos,1991).Some of the important plants which are found effective as anti-inflammatory include; Aloe, Chickweed, Ginger, Licorice, Meadowsweet, St.Johnswort, Turmeric, and Willow(Chrubasik*et al*., 2007).Herbs or constituents with analgesic activity include

are Aconite, Belladonna, Henbane, Hops, Lobelia, Marijuana, Tea, Oats, Passionflower, St. Johnswort and Wild lettuce(Kanaria*et al.,*2007).

1.6.*Moringa stenopetala*

The family Moringaceae is a monotypic family of single genera with around 33 species of which 4 are accepted, 4 are synonym and 25 are unassessed. Out of these, 13 species are documented (Padayachee and Baijnath, 2012).*M. stenopetala,* a smooth barked deciduous tropical plant, itis endemic to east Africa mainly present in Kenya and Ethiopia. It is also reported to occur in Djibouti, Uganda and Sudan (Ghebreselassie*et al*., 2011).It is a traditional medicine and nutritional plant and widely distributed in the south western(Arbaminch, konso, Negelle and WellaytaSodo)part of Ethiopia at an altitude range of about 1100 to 1600 meters. *Moringastenopetala*is commonly called *Shiferaw or aleko* among local communities (Seid, 2013).

Preliminary phytochemical study of the extract revealed the presence of, flavonoids, tannins, alkaloids, saponins, polyphenols, and glycosides (Arora*et al*., 2013).The active constituents in the leaves *of M.stenopetala*are glucosinolates, benzyl glucosinolate, and benzylisocyanate. Phenol carboxylic acids and fatty acids including oleic acid (60 to 70%), palmitic acid (3-12%) stearic acid (3-12%) as well as eicosanoic acid and lignoceric acid in addition to mustard oil are other constituents (Toma*et al.,* 2012).

There are claims that the leaves and roots, steeped in water, are used to treat malaria, hypertension,headache, stomach disorders, asthma and diabetes(Mekonen*et al.,* 1996). *M. stenopetala*is also used as a herbal medicine in areas where visceral leishmaniasis or kala-azar (caused by the *Leishmania* parasite) prevail. Bioassay guided fractionation revealed that both the leaves and seeds of the plant are endowed with antimicrobial activity against *Staphylococcus aureus, Salmonellatyphi, Shigella* and *Candida* (Mekonen *et al*., 1998).

Numerous moringa species including *Moringa concanensis (*Jayabharathi and Chitra, 2011)*,Moringa olifera* (Kumbhare and Sivakumar, 2011),and *Moringa pergerina* have been evaluated for their analgesic, antipyretic and anti-inflammatory activity (Goyale *etal*., 2006). However, no studies have so far been carried out on *M.*

stenopetala, although the plant is claimed to be used for its analgesic activity by Ethiopian traditional healers (Seid, 2013).). The present study therefore attempts to prove scientifically the traditional claim of this plant in Ethiopia.

A B

Fig 1. Photograph of *Moringa stenopetala* A)leaves B) seeds

2. Objective

2.1. General Objective

- To investigate the possible analgesic and anti-inflammatory effects of 80% methanol extract of the leaves of *M. stenopetala* in animal models of analgesia and inflammation.

2.2. Specific Objectives

- To explore whether the plant possesses central mediated analgesic activity using hot plate method and tail flick method
- To assess the peripheral analgesic activity of the plant using acetic acid induced writhing test.
- To evaluate anti-inflammatory activity of the extract using carrageenan induced paw oedema model.

3. Materials and Methods

3.1 Drugs, reagents and chemicals

Methanol (Cheshire, UK) for the extraction, morphine(Ethiopian Pharmaceutical Manufacturing),acetyl salicylic acid ASA(Julphar PharmaceuticalsEthiopia) and Indomethacin (Cadilla Pharmaceuticals, Ethiopia).Distilled water (DW) (Ethiopian Pharmaceuticals Manufacturing) as a vehicle, normal saline (NS) and Tween 80 (TW80)(BDH Chemical Reagents, England).Glacial Acetic acid (Loba Chemicals), and carrageenan(Sigma Chemicals Co., St Louis, USA). Hexane (Laboratory Fine Chemicals Pvt Ltd India), ethyl acetate(BDH chemicals Ltd Poole England) and chloroform(Research Lab Chemical Industries India) were obtained and used as received.

3.2 Collection of plant material

The fresh leaves of the *M .stenopetala* were procured from local market(shiro-meda), and it was identified and authenticated by a taxonomist (Melaku Wendaferash) and was deposited with a collection number (H011) at the National Herbarium, College of Natural Sciences, Addis Ababa University for future reference.

3.3 Experimental Animals

A total of 120 healthy Swiss albino mice of either sex having weights ranging from 20 to 35g, and six to eight weeks of age were either procured from Ethiopian Health and Nutrition Research Institute (EHNRI) or bred in the animal house of the School of Pharmacy, Addis Ababa University were used for the experiment. All animals were fed with commercial pellets and had free access to water *ad libitum*. Animals were fasted overnight and weighed before each experiment.In addition the animals were acclimatized for one week before commencement of the experiment. The care and handling of animals was according to accepted international guidelines (OECD, 2008).

3.4. Extraction

The plant was cleaned, shade dried and milled into fine powder. Accurately weighed powder was then taken and macerated with 80%methanol (1:2) for 24 h. The contents were frequently shaken during the first 6 h and allowed to remain with solvent for 18 h. After 24 h, the extract was filtered with cotton and then by suction filtration apparatus (Oakton, U.S.A) using Whatman filter paper (No. 1). The marc was re-macerated twice using the same volume of solvent to exhaustively extract the plant material. The filtrates were recombined and concentrated on rota vapour (Büchi, Switzerland)at 40◦C under reduced pressure. The resulting concentrate was freeze-dried in a lyophilizer(Delvac, India).The dried residue was then weighed to calculate the yield and it was found to be 11.93%.

3.5. Grouping and dose selection

Mice were randomly divided into five groups with each group consisting of six mice. Group I served as a negative control were administered with 0.5ml of distilled water. Groups II, III and IV were given 200, 400 and 600 mg/kg of the extract, respectively. Group V received standard drug (i.e. 10mg/kg of morphine for hot plate and tail flick test, 81 mg/kg of ASA for writhing test and 25 mg/kg of indomethacin for carrageenan induced paw model. Administration of all agents was performed via an oral route using gavage. Dose selection was carried out based on initial experiments.

3.6. Hot plate method

Following administration according to respective grouping mice were placed on a hot plate maintained at 55 ± 1 °C. Latency of nociceptive response such as licking, flicking of a hind limb or jumping was measured (Le bars *et al*., 2001).Measurements were performed at time 0 before and 30, 60, 90 and 120 min after drug administration, with a cut-off time of 15 s to avoid lesions to the animals' paws. Maximum Possible Analgesia (%MPA), was calculated for each group as shown below (Debebe *et al*., 2007).

$$(\%\text{MPA}) = \frac{\text{latency test} - \text{latency pre drug} \times 100}{\text{Cut off} - \text{latency pre drug}}$$

3.7. Tail-flick method

Tail-flick latency was assessed by the analgesiometer (India). The strength of the current passing through the naked nichrome wire was kept constant at 5 ampere. The distance between heat source and the tail was 1.5 cm and the application site of the heat on the tail was maintained within 2 cm, measured from the root of the tail. Cut-off reaction time was 10 sec to avoid any tissue injury during the process. The time taken by mice to withdraw (flick) the tail was taken as the reaction time (Milind and Monu, 2013). The animals were subjected to the same test procedure at 0 before and 30, 60, 90, and 120 min after administration of treatment as described in the grouping and dosing section. Tail flick latencies were converted into the percent maximal possible effect (%MPE) using the formula shown below (Torres *et al.,* 2001).

$$\%MPE = \frac{\text{(post-drug latency- pre-drug latency) x 100}}{\text{(cut off time -pre-drug latency)}}$$

3.8. Acetic acid-induced writhing test

Writhing syndrome was elicited by intra-peritoneal injection of acetic acid (0.15ml/10g of 0.6% solution) and numbers of writhes displayed from 5 to 20 min were recorded (Shanmugasundaram and Venkataraman2005).The extracts and reference standard ASA were administered in their respective doses 30 min prior to the test and percentage inhibition of writhing was calculated as follows; (Das*et al*., 2013).

$$\%\text{Inhibition} = \frac{\text{Mean number of writhes (control)} - \text{Mean number of writhes(test)}}{\text{Mean number of writhes (control)}} \text{X } 100$$

3.9.Carrageenan induced paw oedema test

Following one hour after administration of vehicle, extract, standardacute inflammation was produced by injection of carrageenan (0.05 ml of 1% w/v suspension), in the right hind paw of the mice. Inflammation was quantitated in terms of volume i.e. displacement of water by oedema using a digital

plethysmometer0h before and 1,2, 3, 4, and 5h after carrageenan injection (Juhas*et al.*, 2008). Indomethacin was used as standard drug which has anti-inflammatory activity at both early and late phase of inflammation.T he percentage inhibition of oedema was calculated for each group with respect to its vehicle-treated control group using the following relationship ;-(Das *et al.*, 2010)

$$\text{Percentage inhibition of paw} = \frac{(Vt-V0)\text{control} - (Vt-V0)\text{treated}}{(Vt-V0)\text{control}}$$

Where, VO=paw volume before drug administration

Vt = Paw volume after drug administration

3.10. Statistical analysis

The experimental results obtained are expressed as mean ± standard error of mean (SEM).The data was analysed by one-way analysis of variance (ANOVA) and differences between samples were determined by Tukey multiple comparison post hoc test using SPSS (Statistical program for Social Sciences)version 16.0 .The level of significance was set at $p<0.05$.

4. Results

4.1. Analgesic activity

4.1.1. Hot plate model

In the hot-plate method both the extract and morphine prolonged the reaction time significantly ($p<0.001$) as compared to negative control throughout the observation period (Table 1).Prolongation of reaction time produced by 200 mg/kg of the extract was significantly lower ($p<0.001$) compared to morphine at all-time points. However, with 400 mg/kg and 600 mg/kg significant difference was noted at 30 & 60 and 30and 120 min, respectively, in relation to the standard drug. Comparing different doses of the extract revealed that there is positive relation -ship between reaction time and increase dose of the extract in which, protection against thermal stimuli with 600 mg /kg was significant compared to all doses of the extract.

Table 1.Effect of 80% methanol extracts of *M. steneoptala* on hot plate test

Group	Latency (sec) ±SEM				
	0	30	60	90	120
CON	3.33±0.42	5.00±0.68	3.33±0.42	3.00±0.36	2.50±0.22
200 mg/kg	3.83±0.40	7.33±0.49 $a^1b^3c^3$	7.33±0.33a^3 b^3c^3	7.33±0.33a^3 b^3c^3	6.83±0.30 $a^3b^3c^3$
400mg/kg	3.16±0.16	9.83±0.47 $a^3b^3c^3$	11.33±0.55 a^3 b^1c^3	11.00±0.57 a^3 c^2	9.50±0.42 a^3c^2
600mg/kg	3.00±0.44	11.00±0.6 8a^3b^2	14.16±0.30 a^3	13.66±0.49 a^3	12.00±0.3 6 a^3b^2
MO10 mg/kg	3.33±0.21	14.50±0.2 2a^3 c^2	13.00±0.25 a^3	12.00±0.36 a^3	9.60±0.60 a^3c^2

Data represent mean ± S.E.M (n = 6) ;[1]$p<0.05$, [2]$p<0.01$, [3]$p<0.001$; [a] relative to control [b]: relative to standard, [c] relative to 600mg/kg CON, control and received distilled water MO, morphine.

Fig 3 depicts percentage increase in reaction time across the observation period. Time to peak activity was longer for the extract (60 min) than for the standard drug (30 min). Morphine's protection waned across time. By contrast, the change for 200 mg/kg somewhat remained constant with increase in observation time. The trend with 400 mg/kg and 600 mg/kg was similar, with the latter exhibiting an effect of higher magnitude.

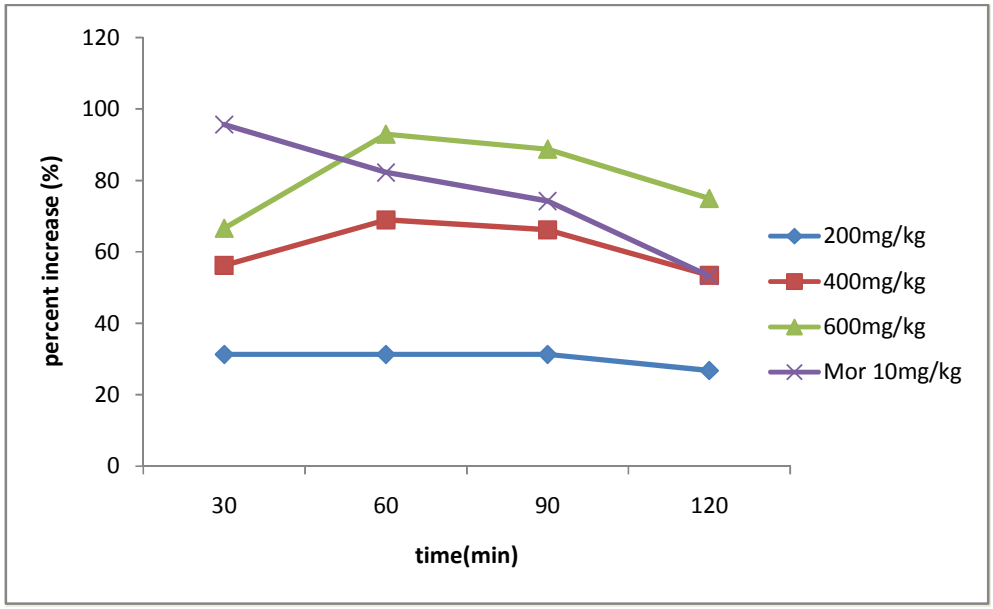

Fig 2.Percent increase in reaction time with the leaf extract of *M. stenoplata* in hot plate paradigm .Data represent mean± SEM (n=6). Doses are for the extract and Mor is for morphine

4.1.2.Tail flick test

In this test, a significant reduction($p<0.01$or $p<0.001$) of painful sensation after application of thermal radiation to the tail was observed following oral administration of the extract and morphine compared to negative control(Table 2). This time morphine was found to be significantly superior only to 200 mg/kg dose of the extract, as there was no apparent change observed between the standard and the other doses at all-time points. Unlike the hot plate test, no significant difference was observed amongst the different doses of the extract.

Table 2.Effect of 80% methanol leaf extract of *M. steneoplata* on tail flick model in mice.

Group	Latency(sec)±SEM				
	0	30	60	90	120
CON	1.66±0.21	5.83±0.30	4.83±0.54	2.66±0.21	2.16±0.16
200 mg/kg	2.33±0.33	8.00±0.00a^3b^3	7.33±0.33a^2b^3	6.50±0.22 a^3b^3	6.16±0.16 a^3b^3
400 mg/kg	4.00±0.51	9.83±0.16a^3	9.00±0.44 a^3	8.83±0.40 a^3	7.66±0.33 a^3
600mg/kg	2.33±0.33	10.00±0.00a^3	9.50±0.22 a^3	8.33±0.21 a^3	8.00±0.25 a^3
MO10 mg/kg	1.33±0.21	10.00±0.00a^3	8.83±0.33a^3	8.53±0.40 a^3	8.33±0.33 a^3

Data represent mean ± S.E.M (n = 6) ;[1]p<0.05, [2]p<0.01, [3]p<0.001; [a] :relativeto control [b]: relative to standard, [c] relative to 600mg/kg CON, control and received distilled water MO, morphine.

Unlike the hot plate test, peak activity for the extract and morphine was achieved at 30 min in the tail flick test (Fig 4). Effect started to decline thereafter to a different extent, with some overlap observed between 200 and 400 mg/kg doses (Fig 4). Even though the activity of morphine declined with time it displayed maximum analgesic activity throughout the study period.

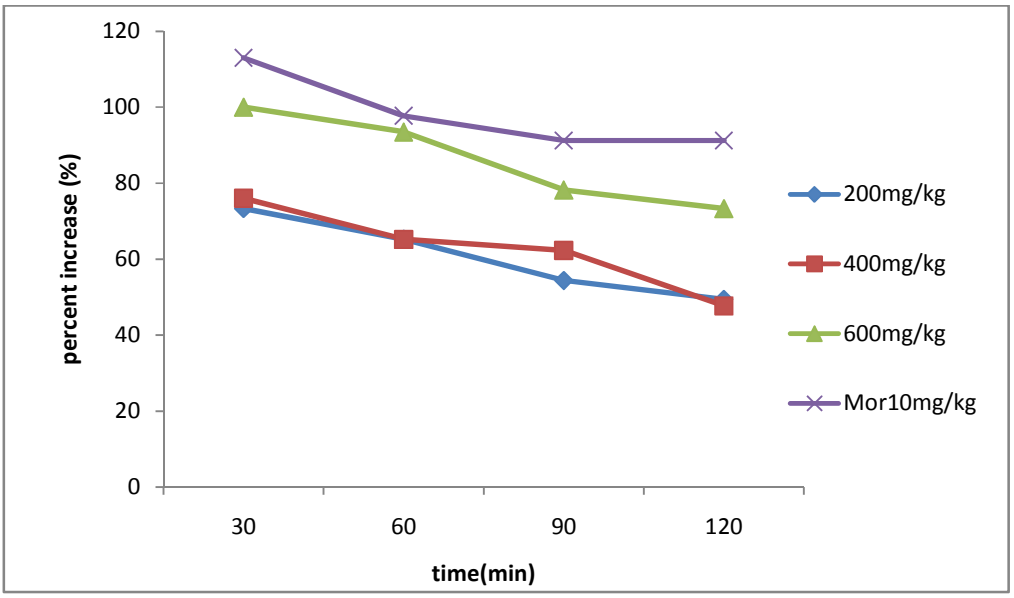

Fig 3.Percent increase in reaction time with the leaf extract of *M .stenoplata* in tail flick test. Data represent mean± SEM (n=6). Doses are for the extract and Mor is for morphine.

4.1.3.Writhing test

While studying peripheral effect of the extract using writhing test, it was observed that mice treated with 80% methanol extract of *M. stenopetala* showed a significant protection ($p< 0.001$) against acetic acid induced writhing compared to negative control group (Table 3).Though ASA produced a significantly greater protection than controls and 200 mg/kg of the extract, no detectable changes were observed when compared to the other two doses. In fact percent inhibition observed with 600 mg/kg and ASA was comparable.

Table 3. Effect of 80% methanol leaf extract of methanol extracts of *M. steneoplata* on writhing test in mice.

Group	No of writhing ±SEM	%inhibition
CON	16.83 ± 2.20	
200mg.kg	9.20 ± 0.40a[2]b [2]	45.33
400mg/kg	4.50 ± 1.10a[3]	73.30
600mg/kg	2.50 ± 0.56a[3]	85.14
ASA 81mg/kg	2.10 ± 0.75a[3]	87.50

The Data represent mean± S.E.M (n = 6) ;[1]p<0.05, [2]p<0.01, [3]p<0.001; [a] :relative to control [b]: relative to standard CON, control and received distilled water ASA is for acetyl salicylic acid

4.2. Anti -inflammatory activity

4.2.1. Carrageenan induced paw model

Sub-planar injection of carrageenan produced an increase in paw oedema, with maximum increase observed 1-2 h following administration. All doses of the plant extract and that of standard drug showed statistically significant inhibitory effect (p<0.01 or p<0.001) on mean increase in paw volume at all-time intervals than negative control group (Table 4).

Table 4. Effect of 80% methanol leaf extract of *M. stenopetala* on carrageenan induced paw model

Group	Mean paw volume ±SEM					
	0hr	1hr	2hr	3hr	4hr	5hr
CON	58.66±3.05	87.33±3.52	86.33±3.60	76.50±4.68	67.83±3.26	65.00±2.64
200mg/kg	44.16±0.47	66.16±1.51a^{3}	59.66±0.88 $a^{3}b^{1}$	54.66±0.76 a^{3}	49.16±1.07 $a^{3}b^{1}$	45.16±0.54$a^{3}b^{1}$
400mg/kg	41.66±0.80	72.00±1.77a^{2}	60.33±2.37 a^{3}	51.00±1.48 $a^{3}b^{2}$	45.33±0.84a^{3} b^{2}	43.33±0.61 $a^{3}b^{2}$
600mg/kg	37.50±1.31	62.83±3.04a^{3}	58.16±2.44$a^{3}b^{1}$	53.66±1.90$a^{3}b^{1}$	49.83±1.57 a^{3} b^{1}	45.00±1.06 $a^{3}b^{2}$
IM 25mg/kg	54.50±1.60	68.33±3.46a^{2}	69.83±1.58 a^{3}	64.16±1.57 a^{1}	57.83±1.60a^{2}	52.16±0.75 a^{3}

Data represent mean± S.E.M (n = 6) ; [1]p<0.05, [2]p<0.01, [3]p<0.001; [a] :relative to control [b]: relative to standard, CON, control and received distilled water IM is for indomethacin

As shown Fig 4maximum and minimum volume reduction was attained at the 5th and 1st h of the study period, respectively, for all doses of the extract and the standard. At the peak of activity (5h) the percentage inhibition for (200,400,600 mg/kg) was 63% 73% and 34%, respectively. The inhibition for the standard was however, 84.22%. Three hours after administration of *M. stenopetala* maximum percentage inhibition against carrageenan induced hind paw oedema was observed at 400 mg/kg dose. Even 200mg/kg of the extract had higher anti-inflammatory activity than 600 mg of the extract throughout the study period.

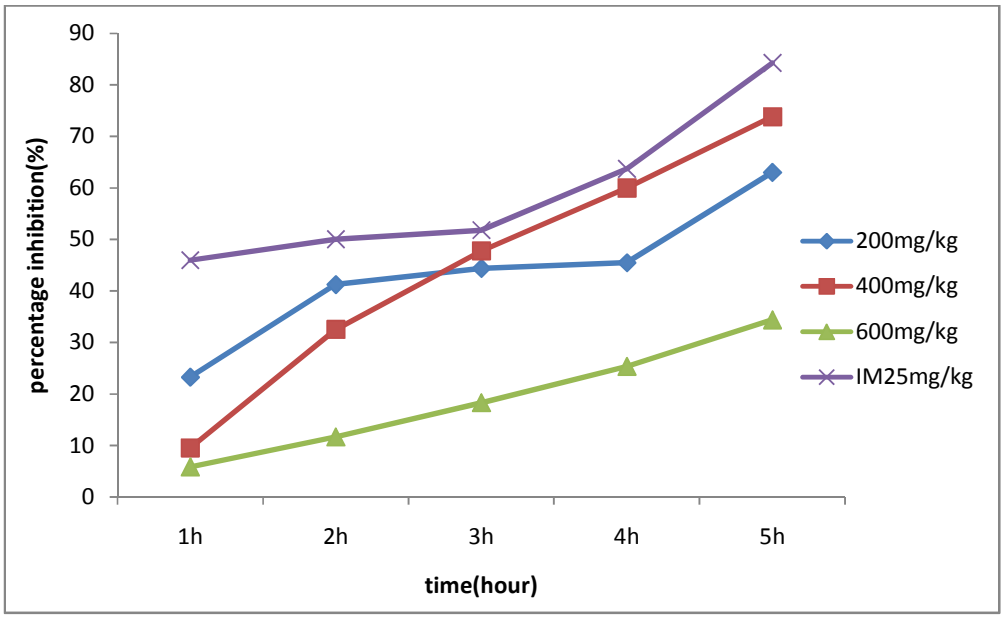

Fig 5.Percent inhibition of oedema with the leaf extract of *M. stenoplata* in carrageenan induced paw oedema model. Data represent mean±SEM (n=6). Doses are for the extract and IM is for indomethacin.

5. Discussion

Considering the costs imposed to the society for pain and inflammation relief treatments, and having the knowledge about the numerous side effects of the available analgesics in the clinic, the need for new analgesic drugs with higher efficacy and fewer side effects seems imperative (Tempon*iet al.,* 2012). As a result searching for medicinal plants which have been widely used in traditional medicine to treat different pain conditions, and also yield novel substances, with anti-inflammatory activity are of especial importance in this regard (Baye,2012).Therefore, *Moringa stenopetala* which was commonly used in Ethiopian folk medicine to treat different ailments and pain conditions was chosen for this study (Ghebreselassie*et al*., 2011).

5.1 Analgesic activity

i. Central analgesic activity

Pain induced by thermal stimulus of the hot plate and thermal radiation of tail flick is specific for centrally mediated activity. They were selected for this study because of several advantages including sensitivity to strong analgesics, limited tissue damage, accuracy of results and they are also less time consuming (Sharma *et al*., 2012).

In the hot plate method, a plate heated at a constant temperature produces two behavioural components that can be measured in terms of their reaction times, namely paw licking and jumping. Both are considered to be supra-spinally integrated responses (Milind and Monu, 2013).As far as analgesic substances are concerned, the paw-licking behaviour is affected only by opioids. On the other hand, the jumping reaction time is increased equally by less powerful analgesics such as acetylsalicylic acid or paracetamol (Le bars*et al.,* 2001).

In this method, duration of time for peak activity was longer for the extract (60 min) than for the standard drug (30 min), this time gap may be due to the time lag between drug entering the central compartment and distribution into the target site or formation of an active metabolites that are endowed with analgesic activity. This might also be the reason for the longer lasting analgesic activity of the extract at doses of 200mg/kg and 600mg/kg throughout the study period as compared to the standard. Better activity of 600 mg/kg of the extract compared to morphine, suggests

that there may be other constituents that contribute for the analgesic activity of the extract in addition to opioid like constituents.

Opioid-like activity of the extract was assessed by using tail flick method. The application of thermal radiation to the tail of mice provokes the withdrawal of the tail by a brief vigorous movement which is integrated spinally. Usually withdrawal time is within 2&10sec. The lengthening of this reaction time by animal seen after administration of a drug is interpreted as analgesic action (Milind and Monu, 2013).

Unlike the hot plate test, in the tail flick test peak activity for the extract and morphine was achieved at 30 min, and there was a constant decline in activity with time. This may be due to the susceptibility of this method to habituation and learning phenomena which result in progressive shortening of response reaction time. There was no significant change in analgesic activity observed between the standard and extract at doses of (400mg/kg and 600mg/kg) throughout the study period. This might be due to the presence of constituents which have opioid-like activity that could possibly interact with opioid receptors even at low doses.

The effect of the extract on tail flick response and hot plate method provides a confirmation of its central effect. *M. stenenoptala* showed a central anti nociceptive activity by increasing the latency to discomfort and may act like centrally active drugs, by activating the periaqueductal grey matter (PAG) to release endogenous peptides (i.e., endorphin or enkephalin). These endogenous peptides descend to spinal cord and function as inhibitors of the pain impulse transmission at the synapse in the dorsal horn.

ii. Peripheral analgesic activity

The writhing test, also less commonly known as abdominal contraction test, is used for screening the peripheral anti-nociceptive activity of different compounds in rodents. Intraperitoneal administration of acetic acid irritates serous membranes and provokes a very stereotyped behaviour in mice. This response is characterized by abdominal contractions accompanied by movements of the hind paws particularly. The writhing response is due to sensitisation of chemosensitive nociceptors by prostagl and insparticularly PGE2 and PGF2 as well as lipoxygenase products. (Le

barset *al.,* 2001).The method was selected because of, its ability to mimic human clinical pain conditions and sensitivity to mild analgesics (Sharma *et al.,* 2012).

In the writhing test, in contrast to the above methods, 200mg /kg of the extract showed significant analgesic activity. This is because the method is reliable to detect anti-nociceptive effects of compounds at doses that may not give response in other methods. At higher doses (400mg/kg and 600mg/kg),the extract had comparable activity with that of ASA indicating an increase in concentration of phytoconstituents that possess analgesic activity with increasing dose. This suggests the binding of the active principles to peripheral receptors in the peritoneum. The effect of the extract may therefore be due either to its action on visceral receptors sensitive to acetic acid, or the inhibition of propagation of painful messages to the central level.

From this study it was observed that extract's anti-nociceptive effects could be partially attributed to the secondary metabolites. Preliminary phytochemical screening of the 80% methanol extract of this plant reported in another study indicates the presence of flavonoids, tannins, alkaloids, saponins, polyphenols and glycosides(Tomaet *al* .,2012). These secondary metabolites have been reported to have different extents of analgesic and anti-inflammatory activities. For instance, alkaloids exert their analgesic action by interfering with the CNS neurotransmitter activity (**R**eanmongkol*et al.,* 2005). And some flavonoids isolated from medicinal plants have shown analgesic activity mainly by inhibiting the key enzymes involved in prostaglandin biosynthesis (Kumbhare and Sivakumar, 2011).It was also reported that morphine-β-6-glucuronide which is a glycosidic metabolite binds to μ1 and μ2 receptors with affinities similar to morphine in mouse brain (Kren and Martínkova, 2001).

5.2. Anti-inflammatory activity

Anti-inflammatory activity of the extract was evaluated using carrageenan-induced hind paw oedema model which involves production of PGs and reactive oxygen species (ROS). The time course of oedema development in carrageenan-induced paw oedema model in mice is generally represented by three distinct phases. The first phase of inflammation occurs between 0 and 1.5 h of carrageenan injection and is partly attributed to trauma of injection and also to histamine, and serotonin

components. The second phase (1.5-2.5 h) is associated with the production of bradykinin and protease. The third phase is mediated by prostaglandin and lysosomes. Maximal vascular response as determined with leukocyte migration to the inflamed area, also reaches its maximum level in the third phase. Prostaglandins play a major role in the development of the third phase of inflammatory reaction which is measured from 2.5 to 6 h post-carrageenan injection (Carey *et al*., 2010)

The highest anti-inflammatory activity of the extract was seen at 400mg/kg and lowest activity at 600mg/kg even lower than 200mg/kg. This may be due, at least in part, to the presence of different chemical components which might interfere with anti- inflammatory activity of the extract. It is possible that these components can vary in kind as well as concentrations. The presence of multiple chemicals in a given extract can also interfere with the pharmacokinetic and pharmacodynamic properties of the individual active ingredients.At 200mg/kg of the extract, a biphasic response was observed. This may be due to opposite effects upon the strength of a particular signal and the target tissue sensitivity to the signal, like other anti-inflammatory drugs i.e. Glucocorticoids (Sapolsky *et al*., 2000).

The effect of the extract was most pronounced at the later stages (5h) of the inflammatory response, which corresponds to the phase of prostaglandin release. The extract was however, less effective at earlier phases (1-2h) indicating that the anti-inflammatory activity is less likely to be attributed to inhibition of histamine and 5HT release.

Furthermore, the anti-inflammatory activity observed could possibly be attributed to the secondary metabolites present in the leaves of *Moringastenoptala*. In previous studies alkaloids, isolated from various plants have shown inhibitory effect on eosinophil recruitment, leukotriene production in the pleural cavities, as well as inhibiting in the production of nitric oxide mediators which result in anti-inflammatory effect (Souto*et al*, 2011). Flavonoids also inhibit inflammatory processes by inhibiting phosphodiesterases which involved in cell activation (Lafuente et al., 2009). In addition, polyphenols exert their anti-inflammatory properties through inhibition of the production of inflammatory cytokines and chemokines and suppressing the activity of cyclooxygenase (COX)) and inducible

nitric oxide synthase (iNOS) and thereby decreasing the production of reactive oxygen and nitrogen species (ROS/RNS) (Santangelo *et al*., 2007).

6. Conclusion

The ability of the extract to suppress abdominal writhes, increase tail flick and hot plate latency, as well as suppressing the carrageenan-induced inflammation confirms the analgesic and anti-inflammatory activities of the extract. The data collectively indicate the 80% methanol extract of *M. stenopetala* possesses analgesic and anti-inflammatory properties, which are probably mediated by both central and peripheral inhibitory mechanisms as well as via inhibition of prostaglandin synthesis. The plant can therefore offer a potential benefit in the management of pain and inflammatory disorders.

7. Suggestion for future work

- Further pharmacological studies with fractions of the extracts should be conducted.
- Further studies should be done to elucidate the mechanism (s) of action and the components responsible for these pharmacological effects.
- Toxicological studies of the extract should be conducted to determine the long term safety profile of the plant.
- Since the plant is also claimed to have activity against, asthma, malaria, stomach disorders and others, pharmacological investigations geared toward these activities are suggested

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