Evaluation of the *in vivo* analgesic and anti-inflammatory activities of 80% methanol extract of *Leonotis ocymifolia (Burm. F.)* Iwarsson leaves

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This is to certify that the thesis prepared by Asnakech Alemu, entitled "Evaluation of the analgesic and anti-inflammatory activities of 80% methanol extract of *Leonotis ocymifolia (Burm.f.)* leaves" and submitted in partial fulfillment of the requirements for 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 the in vivo analgesic and anti-inflammatory activities of 80% methanol extract of *Leonotis ocymifolia (Burm.f.) Iwarsson* leaves

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Pain and inflammation are the most common health problems treated with traditional remedies which mainly comprise medicinal plants. *Leonotis ocymifolia* is one of the medicinal plants used in folkloric medicine of Ethiopia for years to treat various pain and inflammation disorders. However, the plant has not been scientifically evaluated for the claimed activities.

The aim of the present study was to evaluate the anti-inflammatory and analgesic activities of the 80% methanol extract of Leonotis ocymifolia leaves using rodent models. The central and peripheral analgesic activity of the extract was evaluated by using Eddy's hot plate method and acetic acid-induced writhing, respectively. The anti-inflammatory activity of the extract was evaluated by using carrageenan-induced paw edema and cotton pellet granuloma method. The study was carried out in three different dose levels of extracts 100,200 and 400mg/kg orally. The extract did not produce any mortality up to 2000mg/kg. In the hot plate method, the extract at all doses showed a significant ($p<0.001$) dose dependent analgesic effect with latency response of 32.8%, 47.9%, and 62.8% respectively, and inhibition of acetic acid induced writhings in mice was also observed with extract at all dose levels. Maximum anti-inflammatory effect by the 100, 200 and 400 mg/kg of extracts were observed at 6h post-induction, with respective values of 46.3%, 69.13 %, and 75.88%, in carrageenan-induced paw edema and all tested doses of extract significantly inhibited the formation of inflammatory exudates ($p < 0.001$) and granuloma formation ($p < 0.001$). Presence of saponins, alkaloids, flavonoids, tannins, terpenoids and phenols might be responsible for these activities and which are probably mediated via inhibition of various autacoids formation and release. In conclusion, the data obtained from the present study indicates that the extract possessed a significant analgesic and anti-inflammatory activity, upholding the folkloric use of the plant.

Key words: *Leonotis ocymifolia*, analgesic, anti-inflammatory activity, 80% methanol extract and mice.

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ABBREVIATIONS AND ACRONYMS

AA	Arachidonic Acid
AE	Aqueous Extract
AP-1	Activator Protein-1
ASA	Acetyl salicylic acid
ANOVA	Analysis Of Variance
BK	Bradykinin
BP	Back Pain
CAM	Complementary and Traditional Medicine
CNS	Central Nervous System
COX	Cyclooxygenase
DW	Distilled Water
EFMHACA	Ethiopian Food, Medicine and Healthcare Administration and Control Authority
EPHARM	Ethiopian Pharmaceuticals Manufacturing
EPHI	Ethiopian Public Health Institute
GI	Gastrointestinal
IL	Interleukin
IASP	International Association for the Study of Pain
JUSH	Jimma University Specialized Hospital
LBP	Low Back Pain
LD_{50}	Median Lethal Dose
LO	*Leonotis ocymifolia*
LOX	Lipooxygenase
LPS	lipoolysaccharide
LTs	leukotrienes
MAPK	Mitogen-Activated Protein Kinase
MMSH	Murtala Muhammed Specialist Hospital
MO	Morphine

NO	Nitric Oxide
NP	Neuropathic Pain
NSAIDs	Non-Steroidal Anti-Inflammatory Drugs
OECD	Organization of Economic Corporation and Development
P.O	Per-Oral
PNS	Peripheral Nervous System
PGs	Prostaglandins
SEM	Standard Error of the Mean
TNF-α	Tumor Necrosis Factor Alpha
TIRF	Transmucosal immediate-release formulations of fentanyl
TW	2% Tween 80
WHO	World Health Organization
80ME	80% Methanol Extract

1. INTRODUCTION

1.1. Definitions of Pain

Pain is the most common symptom for which patients seek medical attention. The International Association for the Study of Pain defines pain as "an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage" (Neogi, 2013). Pain is a universally understood signal of disease and it is the most common symptom that brings the patient to physician's attention. The function of the pain in sensory system is to protect the body and to maintain the homeostasis (Singal *et al*., 2012; Debasis *et al*., 2011). Pain in human is a multidimensional-complex perception that causes a large burden to individuals and society (Uddin, 2014). Pain is a subjective experience, and its severity can be influenced by many factors including previous experience of pain, cultural background, coping mechanisms, fear, anxiety and depression (Mowat & Johnson, 2013). Due to the subjective component of pain and the problems associated with a correct diagnosis patients are frequently undertreated for acute and chronic situations.

Pain can be either acute or chronic and it is a consequence of complex neurochemical processes in the peripheral and central nervous system (Jayanthi and Jyoti, 2012). Acute pain is a warning that something is not right in the body. Chronic pain is pain that persists beyond the expected time for healing (Fawzi, 2013).

1.2. Pain Classification

Pain is generally classified according to its location, duration; frequency, underlying cause, and intensity (Cole, 2002).The two most commonly used classifications: the pathophysiological mechanism of pain (nociceptive, neuropathic pain and psychogenic pain) and the duration of pain (chronic or acute) (WHO, 2012).

Pathophysiological classification

There are two major types of pain; nociceptive and neuropathic. Clinical distinction between nociceptive and neuropathic pain is useful because the treatment approaches are different. The nociception or neuropathy can be a foundation of importunate pain, involve similar neuronal pathways but considerable physiological differences (Ahmed and Noushad, 2014).

Nociceptive pain: - Pain arises from nociceptors, which are sensitive to noxious stimuli such as heat, cold, vibration, stretch stimuli and chemical substances released from tissues in response to oxygen deprivation, tissue disruption or inflammation (WHO, 2012). There are two types of nociceptive pain: somatic pain is emanating from the skin and deeper tissues such as joints and muscle while pain emanating from the internal organs is referred to as visceral pain. Somatic pain is usually well localized whereas visceral pain is harder to pinpoint. (Fein, 2012).

Neuropathic pain: It can develop after nerve injury, when deleterious changes occur in injured neurons and along nociceptive and descending modulatory pathways in the central nervous system (WHO, 2012). Neuropathic pain is characterized by continuous or intermittent spontaneous pain, typically characterized by patients as burning, aching, or shooting. The pain may be provoked by normally innocuous stimuli (allodynia). Neuropathic pain is also commonly associated with hyperalgesia (increased pain intensity evoked by normally painful stimuli), paresthesia, and dysesthesia (Selph *et al*., 2011).Neuropathic pain can be spontaneous (stimulus-independent or spontaneous pain) or elicited by a stimulus (stimulus-dependent or stimulus-evoked pain) (Cruccu *et al*., 2010).

Psychogenic pain: is related to psychologic abnormalities for example, pain in obsessive compulsive disorders, depression and delusions of parasitosis (Osipovitch and Samuel, 2008). It indicates something "generated" in the psychological domain (Graziottin, 2011).

Classification based on pain duration

It mostly depends on the duration of the pain.

Acute Pain: – pain due to a sudden injury, inflammation, or disease. This usually lasts a short period of time – from seconds to weeks, and usually less than 3-6 months, depending on the type and intensity of the injury (WHO, 2012). Acute pain is encountered in a wide variety of clinical situations, including post-operative patients, victims of trauma, and medical illness (Mowat and Johnson, 2013).

Chronic Pain: – It is continuous or recurrent pain that persists beyond the expected normal time of healing and usually lasts more than 6 months period of time (WHO, 2012). Chronic pain becomes more common for elder people, because health problems that can cause pain, such as osteoarthritis, become more common with advancing age and about 25.3 million U.S. adults (11.2 percent) had pain every day for the previous 3 months and nearly 40 million adults (17.6 percent) had severe pain (Dowell *et al*., 2016).

1.3. Pathophysiology of Pain

The neuronal impulses in fast conducting A delta fibers nociceptors produce the sensation of the sharp, fast pain, while the slower C-fibers nociceptors produce the sensation of the delayed, dull pain (Patel and Kopf, 2010). Transmit mainly mechanical and thermal pain, terminate in the dorsal horns, cross over to the opposite side of the cord and continue upwards to the brain as anterolateral columns. Most fibers terminate in the ventrobasal or posterior nuclei of the thalamus; few fibers terminate in the reticular areas. Signals are also sent to the somatosensory cortex. Glutamate is the neurotransmitter secreted in the spinal cord at A delta fibers (Santana, 2014).

The C-fibers which carry slow pain terminate in the substantia gelatinosa of dorsal horns in spinal cord. They also cross over to the opposite side and continue as anterolateral ascending tracts. The paleospinothalamic tract terminates in brain stem in one of the following areas:Reticular nuclei of medulla, pons and mesencephalon, Tectal area of mesencephalon deep.10-25% of the fibers pass to the thalamus, Periaqueductal gray region surrounding the aqueduct of Sylvius. The pain carried by slow chronic pathway is

poorly localised. Substance P is the neurotransmitter concerned with slow pain (Vikram, 2015).

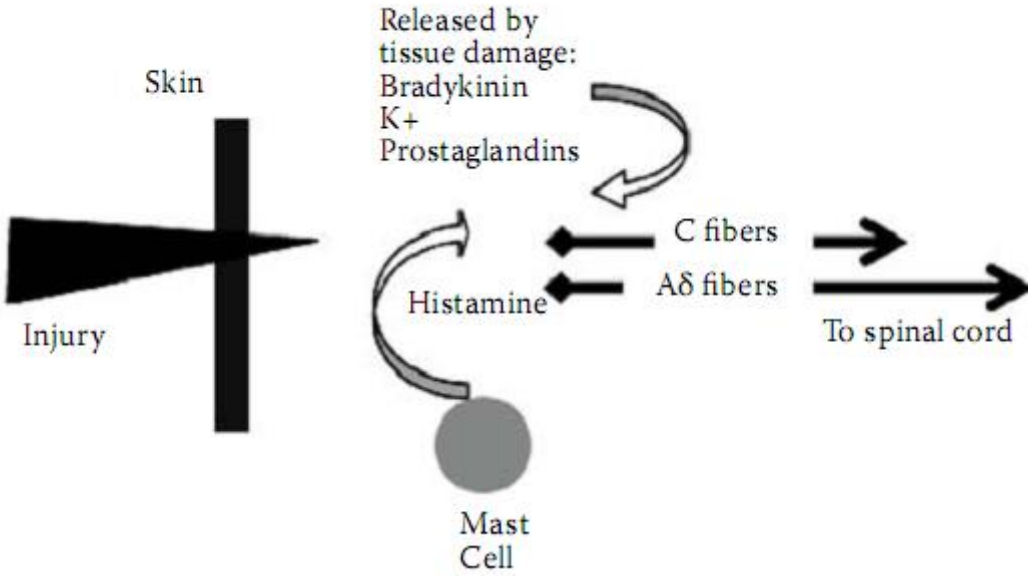


Figure 1:- Some Chemical released by tissue damage that stimulates nociceptors. In addition release of substance-P, along with histamine, produce vasodilation and swelling (Patel and Kopf, 2010).

1.4. Pain Epidemiology

The 2013 Global Burden of Disease Study estimated for asymptomatic permanent caries and tension-type headache of 2.4 billion and 1.6 billion, respectively. The distribution of the number of sequelae in populations varied widely across regions (Naghavi *et al*., 2015).

The burden of primary headache disorders in Addis Ababa in terms of missing working, school or social activities was 68.0%.This was 78.3% for migraine and 66.7% for tension type headache. Majority 92.0% of primary headache cases were not using health services and 66.0% did not use any drug or medications during the acute attacks and none were using preventive therapy (Mengistu and Alemayehu, 2013).

Prevalence of acute neuropathic pain in the developed world was estimates to be 1-3% of the population. Headache is one of the most frequent neurological disorders interfering

with everyday life. Approximately one-half of the adult population worldwide is affected by acute headache disorder (Hainer and Matheson, 2013). The one-year prevalence is 10–18 % in migraine and 31–90 % in tension-type headache. For Austria the one-year prevalence of migraine was 10 %. In a European survey migraine was found in 36 % (Zebenholzer *et al*., 2015). Migraine appears less prevalent, but still common, elsewhere in Asia (around 8%) and in Africa (3-7%), (Mengistu and Alemayehu, 2013).

Chronic pain is a multifactorial condition with both physical and psychological symptoms, it affects an estimated 20% of people worldwide and accounting for 15% to 20% of physician visits (Treede *et al.,* 2015; Park and Moon, 2010). The World Health Organization has estimated that 22% of the world's primary care patients have chronic debilitating pain (Gosset and Dietz, 2015). Neuropathic, Low back pain (LBP) and Cancer pain are mostly occur chronic pain.

Persistent post-surgical neuropathic pain (NP) is mostly an unrecognized clinical problem (Jain *et al.*, 2014). Approximately 20% of the adult European populations have chronic pain (Van Hecke *et al*., 2013).The American Chronic Pain Association estimates that more than 15 million people in the U .S. and Europe have some degree of neuropathic pain. More than two out of every 100 persons are estimated to have peripheral neuropathy; the incidence rises to eight in every 100 people for people aged 55 or older (Azhary *et al*., 2010).

A cross-sectional study to determine the prevalence of neuropathic pain (NP) in 80 recently treated leprosy patients in Ethiopia were showed that pain of any type was experienced by 60% of the patients. Pure nociceptive pain was experienced by 43%, pure NP by 11%, and mixed pain by 6%.The prevalence of NP is high in recently treated Ethiopian leprosy patients (Haroun *et al.*, 2012).

More than 80% of the population will experience an episode of LBP at some time during their lives. For most, the clinical course is benign; with 95% of those afflicted recovering within a few months of onset (Freburger *et al*., 2009). The 2010 Global Burden of Disease Study estimated that low back pain is among the top 10 diseases in the world. The lifetime

prevalence of non-specific (common) low back pain is estimated at 60-70% in industrialized countries (one-year prevalence 15 to 45%, adult incidence 5% per year). The prevalence rate for children and adolescents is lower than that seen in adults but is rising. Prevalence increases and peaks between the ages of 35 and 55 (Lozano *et al*., 2013).

Prevalence of chronic pain in the general population of Hong Kong was 34.9% reported pain lasting more than 3 months, having an average of 1.5 pain sites; 35.2% experienced multiple pain sites, most commonly of the legs, back, and head with leg and back being rated as the most significant pain areas among those with multiple pain problems (Wong and Fielding, 2011). In United States, adults weighted point-prevalence of chronic pain was 30.7%. Prevalence was higher for females (34.3%) than males (26.7%) and increased with age. The weighted prevalence of primary chronic lower back pain was 8.1% and primary osteoarthritis pain was 3.9% (Johannes *et al*., 2010).

The mean low back pain (LBP) point prevalence among Africa adolescents was 12% and among adults was 32%. The average one year prevalence of LBP among adolescents was 33% and among adults was 50%. The average lifetime prevalence of LBP among the adolescents was 36% and among adults was 62% (Louw *et a*l., 2007). The prevalence of BP (back pain) in developed countries has been estimated to be between 12% and 34%. The prevalence of BP in rural sub-Saharan Africa was 16.7%, which is within this range (El-Sayed *et al*., 2010).

A cross-sectional study on the prevalence and risk factors for LBP among nurses in a typical Nigerian (Murtala Muhammed Specialist Hospital [MMSH]) and Ethiopian (Jimma University Specialized Hospital [JUSH]) Specialized Hospitals was showed that the 12 month prevalence of low back pain (LBP) was 360 (70.87%). LBP was more prevalent among female nurses (67.5%) than the male nurses (32.5%) out of five hundred and eight respondents (178 [35%] males and 330 [65%] females) participated in the study (Sikiru and Shmaila, 2009).

According to study (International Association for the Study of Pain, 2013) globally pain related to the cancer vary widely, the range of reported prevalence of pain is highest for

the following tumors: head and neck (67–91%), prostate (56–94%), uterine (30–90%), genitourinary (58–90%), breast (40–89%) and pancreatic (72–85%).

1.5. Pharmacological Management of Pain

Drugs used in the management of pain and inflammation includes: the acetaminophen, the nonsteroidal anti-inflammatory drugs and the narcotic analgesics (SIGN; 2013).

The non-narcotic analgesics are a group of drugs used to relieve pain without the possibility of causing physical dependency, which can occur with the use of the narcotic analgesics. The non-narcotic analgesics can be divided into the salicylates, nonsalicylates (acetaminophen), the nonsteroidal anti-inflammatory drugs (NSAIDs) and other (Ford and Roach, 2010).

1. Acetaminophen: - represent *p*-aminophenol or pyrazolone derivatives with clinically useful analgesic and antipyretic efficacy. Its mechanism of action is not completely understood but thought to be mediated via inhibition of prostanoid formation by variants of COX enzymes (Bieger *et al*., 2011). Acetaminophen has analgesic and anti pyretic effects similar to NSAIDs, but it lacks a specific anti-inflammatory effect. It is a reasonable first-line option because of its more favorable safety profile and low cost. However, acetaminophen is associated with a symptomatic elevation of aminotransferase levels at dosages of 4 g /day even in healthy adults; a clinical significance of these findings is uncertain (Park and Moon, 2010). Acetaminophen showed slightly inferior pain relief to NSAIDs in patients with osteoporosis and chronic low back pain (SIGN; 2013). For acetaminophen is that it is quite useful as a mild-moderate analgesic agent, especially in patients with NSAID contraindications or in those with fever (Thomas, 2013).

2. **Nonsteroidal anti –inflammatory drugs (NSAIDs):-** All NSAIDs and COX-2 agents appear to be equally effective in the treatment of pain disorders that have three desirable pharmacological effects: anti-inflammatory, analgesic and antipyretic effects (Park and Moon, 2010). NSAIDs are characterized chemically by an acidic moiety linked to an aromatic residue; and by virtue of inhibiting cyclooxygenases (Bieger *et al*., 2011). NSAIDs are a necessary choice in pain management because of the integrated role of the

COX pathway in the generation of inflammation and in the biochemical recognition of pain (Ong *et al*., 2007). Cyclooxygenases (COX) localized to the endoplasmic reticulum is responsible for the formation from arachidonic acid of a group of local hormones comprising the prostaglandins, prostacyclin, and thromboxanes. These enzymes possess an elongated pore into which the substrate arachidonic acid is inserted and converted to an active product. NSAIDs penetrate into this pore and thus prevent access for arachidonic acid, leading to blockade of the enzyme (Bieger *et al*., 2011).

3. The narcotic analgesics: - are controlled substances used to treat moderate to severe pain. The narcotics obtained from raw opium include morphine, codeine, hydrochlorides of opium alkaloids, and camphorated tincture of opium (Ford and Roach, 2010). Opioids provide analgesia through receptor-mediated blockade of neurotransmitter release and pain transmission (Thomas, 2013). Recently tapentadol and transmucosal immediate-release formulations of fentanyl are used to moderate to severe chronic pain management.

i. ***Tapentadol*** is a new centrally acting analgesic that relies on a dual mechanism of action. These are mu opioid receptor agonism and norepinephrine (noradrenaline) reuptake inhibition. It is therefore not a classical opioid, but represents a unique class of analgesic drug (Morlion, 2013).It is now registered for use in the treatment of moderate to severe chronic pain that proves unresponsive to conventional non-narcotic medications in many countries. Tapentadol has a much lower affinity (50 times less) to the mu receptor than morphine, but its analgesic effect is only around three times less than morphine (Afilalo *et al.*, 2010 ; Afilalo and Morlion, 2013).

ii. **Transmucosal immediate-release formulations of fentanyl (TIRF):-** Fentanyl is a commonly used synthetic phenylpiperidine derivative and μ -opioid receptor agonist that is highly lipophilic, thereby enabling rapid diffusion across the blood–brain barrier and diffusion into central nervous system structures. It is 100-fold more potent than morphine, (Smith, 2013). It was initially developed for parenteral administration, with the oral route being of limited use due to high first pass metabolism. However, its highly lipophilicity and high potency lend to other routes of administration suitable for both acute and chronic pain management. While transdermal fentanyl formulations for the management of cancer and chronic pain have been marketed for a considerable

time, a variety of immediate release formulations has become available recently (Schug and Goddard, 2014).

4. Cannabinoids: - It comprises a large group of chemical compounds that act upon the cannabinoid receptor. Cannabinoids represent a relatively new pharmacological option as part of a multimodel treatment plan (Lynch and Campbell, 2011). The pharmacology of natural and synthetic cannabinoid ligands is derived from their interaction with two cannabinoid receptor subtypes, CB_1 and CB_2. It is becoming apparent that the CB_2 receptor plays an important role in the mediation of pain processing. There is an emerging body of evidence to suggest that expression of the CB_2 receptor is unregulated as a consequence of tissue or nerve injury, supporting a potential role for CB_2-selective ligands in the treatment of inflammatory, postoperative and neuropathic pain (Yao *et al.*, 2008). Significant drug discovery efforts have been directed towards developing and characterizing CB_2-selective agonists both *in vitro* and *in vivo*. These efforts have sought to evaluate and validate the CB_2 receptor as an analgesic target. HU308 (4-[4-(1,1-diemethylheptyl)-2,6-dimethoxyphenyl]-6,6-dimethyl-bicyclo[3.1.1]hept-2-ene-2-ethanol) was the first CB_2-selective agonist exhibiting low affinity for CB_1 to be synthesized (Guindon and Hohmann, 2008).

5. Adjuvant analgesics and local anesthetic drugs: - Medications originally used to treat conditions other than pain but may also be used to help relieve specific pain problems; examples include some antidepressants and anticonvulsants. And medications with no direct pain-relieving properties may also be prescribed as part of a pain management plan. These include medications to treat insomnia, anxiety, depression, and muscle spasms (ACPA, 2016). The following are commonly used medicines.

i. **Co-analgesics Alpha-2-delta modulators (gabapentin and pregabalin):-** Gabapentin and pregabalin are anticonvulsant drugs that act by binding to the alpha-2-delta subunit of voltage gated calcium channels within the central nervous system. Thereby they are down- regulating calcium ion influx into neurons, subsequently reducing the release of a variety of excitatory neurotransmitters (in particular the excitatory amino acid glutamate) (CADTH,

2014). The initial indication for both compounds was neuropathic pain of various origins; there is now overwhelming evidence that gabapentin and pregabalin are effective in the treatment of neuropathic pain from a variety of causes (Schug and Goddard, 2014).

ii. **Serotonin-norepinephrine reuptake inhibitors (SNRIs):-** Antidepressants have long been used in the management of chronic pain, with tricyclic antidepressants (TCAs), in particular amitriptyline, commonly being utilized in the treatment of neuropathic pain (Schug and Goddard, 2014).

iii. **Ketamine**:-It was originally introduced into clinical practice as a dissociative anaesthetic. However, over recent years there has been an increasing interest in its use in the setting of pain management, as well in acute as in chronic pain states (Niesters *et al*., 2014). The mechanism of action of ketamine is primarily antagonism at the NMDA receptor, a calcium channel, for which glutamate is the natural ligand. This channel has also been linked to the phenomenon of central sensitization, a process associated with the development and maintenance of chronic pain (Borsook, 2009).

iv. **Topical treatments: -** For localized neuropathic pain, there is increasing interest in topical preparations such as lidocaine and capsaicin patches, in particular in view of their minimal systemic adverse effects (Schug and Goddard, 2014).

Recent advances in the pharmacological management of pain are not so much the result of new ‘miracle’ drugs, but new preparations and new ways to use old drugs in a variety of settings, often as components of a multimodal approach to pain relief.

1.6. Definition and Classification of Inflammation

The word inflammation comes from the Latin *inflammare* which means “to set on fire” (Scott *et al*., 2004). Inflammation is one of the non-specific physiological defensive responses that begin after cellular injury, which may be caused by microbes, physical agents (burns, radiation), chemicals (toxins, caustics), necrotic tissue and/or immunological reactions (Villarreal *et al*., 2001).

Inflammation is the protective phenomena and a response that occurs if an injury takes place due to some internal and external factors (Calder, 2006; Salzano, 2013). The main bases of inflammation functions are limiting damage and promoting repair of tissues. Although inflammation is beneficial in providing defense against infection invaders, it may become unchecked in case of pathogenesis of chronic inflammatory disease (Sachan and Singh, 2013). In general, it is one of the unique mechanism that help body to protect itself against burn, infection, allergens, toxic chemicals, or other noxious stimuli (Jadhav and Prabhavalkar, 2015). The main mechanism of inflammation is that the cell related with inflammation on cell membrane to cause the release of lysosomal enzyme, arachidonic acid and various eicosanoids are produced (Sachan and Singh, 2013). The five cardinal signs of inflammation are redness, heat, swelling, pain and loss of function. The redness phase is caused by an increase of the blood flow and vascular permeability. Histamine, prostaglandin and nitric oxide are chemical mediators of inflammation, inducing vasodilation and increased permeability (Salzano, 2013). Inflammation promotes the production of inducible nitric oxide synthase (iNOS) leading to local vasodilatation promoting metabolite delivery and export, and key components of the complement cascade important for antimicrobial activity (Parker and Clermont, 2010).The tissue swelling is caused by recruitment of inflammatory cells at the site of infection and accumulation of the exudate (fluid with high proteins content and antibacterial properties). The release of cytokines (IL-1 and TNF) increases the levels of leukocyte adhesion molecules on endothelial cells. Increased permeability of the blood vessels allows the passage of cells from the vessel into site of inflammation (Salzano, 2013). The essential components of the inflammatory reaction are the vascular and cellular responses. The cellular component involves the movement of white blood cells from blood vessels into the inflamed tissue. The white blood cells, or leukocytes, take on an important role in inflammation (Mohammed *et al*., 2015).

Inflammation can be classified, according to the time course and the tissue damage. Based on time of course it can be categorize into two; acute and chronic inflammation. Acute inflammation is an aggravating component of infections (Solano, 2013). Acute inflammation is immediate and early response to tissue injury and characterized by

vasodilation, vascular leakage, edema and leukocyte migration (Khan and Khan, 2010 and Sattar, 2011).

Inflammation is mediated by a number of chemical factors secreted by cells participating in the inflammatory process either directly and/or responding to the inflammatory stimulus (Olszowski, 2012).In general, inflammatory responses/mechanism occur in three distinct temporal phases, each apparently mediated by different mechanisms: (1) an acute phase, characterized by transient local vasodilation and increased capillary permeability; (2) a delayed, subacute phase characterized by infiltration of leukocytes and phagocytic cells; and (3) a chronic proliferative phase, in which tissue degeneration and fibrosis occur(Knollman *et al.*, 2011).

Chemical mediators release from leukocytes at the site of inflammation. These may include lipid mediators (e.g., prostaglandins (PGs), leukotrienes (LTs)), peptide mediators (e.g., cytokines), reactive oxygen species (e.g., superoxide), amino acid derivatives (e.g., histamine), and enzymes (e.g., matrix proteases) depending upon the cell type involved, the nature of the inflammatory stimulus, the anatomical site involved, and the stage during the inflammatory response (Calder, 2010).

Chronic inflammation is prolonged or persistent tissue injury and it characterized by lymphocyte, macrophage, plasma cell (mononuclear cell) infiltration; tissue destruction by inflammatory cells and attempts at repair with fibrosis and angiogenesis (Khan and Khan, 2010 and Sattar, 2011) and also chronic inflammation is characterized by the increased expression of multiple inflammatory genes that are regulated by pro-inflammatory transcription factors, such as nuclear factor-kappa B and activator protein-1,that bind to and activate co-activator molecules, which then acetylate core histones to switch on gene transcription (Barnes, 2006).

An ineffective or uncontrolled inflammatory response contributes to the cellular dysfunction, tissues damage that occurs in many chronic inflammatory diseases (e.g. rheumatoid arthritis, atherosclerosis, chronic hepatitis, pulmonary fibrosis) (Szliszka *et al.*, 2011).

1.7. Pharmacological Management of Inflammation

Nonsteroidal anti-inflammatory drugs (NSAIDs) are among the most widely used medications in the world because of their demonstrated efficacy in reducing pain and inflammation. NSAIDs as a class comprise both traditional nonselective NSAIDs (tNSAIDs) that nonspecifically inhibit both COX-1 and COX-2, and selective COX-2 inhibitors (Ong *et al*, 2007). All these drugs are well known for side effects such as intestinal tract ulcers and erosions of the stomach linings (Jadhav and Prabhavalkar 2015).

NSAIDs function by inhibiting prostaglandin-synthetase or cyclooxigenase (COX). COX exists in two isoforms, COX 1 and COX 2. COX 1 has homeostatic functions which includes the maintenance of gastric mucosa. COX 2 is implicated in inflammation and fever. NSAIDs can be non-selective inhibitors of COX, that is, they inhibit COX 1 and COX 2 and semiselective inhibitors of COX 2 (two or three times more selective in blocking COX 2 than COX 1) and highly selective inhibitors of COX 2 (seven times more selective in blocking the activity of COX 2) (Gómez-Moreno *et al.*, 2009). Most NSAIDs inhibit COX activity in a competitive fashion, where as Acetyl salicylic acid is an irreversible inhibitor of the enzyme (Wallace, 2013).

Acetyl salicylic acid is unique among non-selective NSAIDs in that it irreversibly acetyls COX 1 in platelets, which justifies its prescription as a cardioprotector. Regarding selective NSAIDs of COX 2, some have been withdrawn (like rofecoxib) because of the risk of severe thromboembolic phenomenon (Gómez-Moreno *et al.*, 2009).

Corticosteroids are the most effective anti-inflammatory therapy for many chronic inflammatory diseases, such as asthma. It works by decreasing inflammation and reducing the activity of the immune system. They are used to treat a variety of inflammatory diseases and conditions. However, they are relatively ineffective in other diseases such as chronic obstructive pulmonary disease (Barnes, 2006 and Barnes and Adcock, 2009).

1.8. Traditional Medicine in the Management of Pain and Inflammation

Traditional medicine has a long history. It is the sum total of the knowledge, skill, and practices based on the theories, beliefs, and experiences indigenous to different cultures, whether explicable or not, used in the maintenance of health as well as in the prevention, diagnosis, improvement or treatment of physical and mental illness(WHO report,2013). Whereas the USA National Center for Complementary and Alternative Medicine defines complementary and alternative medicine (CAM) as a group of diverse medical and health care systems, practices, and products that are not presently considered to be part of conventional medicine.CAM remedies include those practices that are thought to be outside of the dominant of conventional medical and psychological approach (Moss et al., 2011).

CAM, which is noninvasive and generally considered to be relatively low toxicity, is used as an adjunct therapy with standard pain management techniques. The earliest systematic review showed that approaches such as acupuncture, massage therapy, mind-body interventions, and music therapy could effectively reduce pain and enhance quality of life (Bao *et al*., 2014). Although acupuncture was originally a feature of traditional Chinese medicine, it is now used worldwide. According to reports supplied by 129 countries, 80% of them now recognize the use of acupuncture (WHO, 2015)

The World Health Organization (WHO) reported that 80% of the emerging world's population relies on traditional medicine for therapy (Namuddu *et al*., 2011 and Kiefer *et al*., 2014). There has been a continuing demand for, and popular use of, traditional and complementary medicine worldwide. In some developing countries, native healers remain the sole or main health providers for millions of people living in rural areas. For instance, the ratio of traditional health practitioners to population in Africa is 1:500, whereas the ratio of medical doctors to population is 1:40 000. Over 100 million Europeans are currently users of traditional and complementary medicine (WHO, 2013).

During the past decades, the developed world has also witnessed an ascending trend in the utilization of CAM, particularly herbal remedies. Herbal medicine, also called

botanical medicine or phytomedicine, refers to using a plant's seeds, berries, roots, leaves, bark, or flower for medicinal purposes (Sarah, 2006). Approximately 20% of the US population regularly uses herbal medicine (Kiefer *et al*., 2014). Reports from Western Europe suggest that 20% (Netherlands) to 49% (France) of the population has used CAM at least once (Van Andel and Carvalheiro, 2013).While 90% of the population in Ethiopia use herbal remedies for their primary healthcare, surveys carried out in developed countries like Germany and Canada tend to show that at least 70% of their population have tried CAM at least once. It is likely that the profound knowledge of herbal remedies in traditional cultures, developed through trial and error over many centuries, along with the most important cures was carefully passed on verbally from one generation to another (Mahomoodally, 2013 and Moss *et al*., 2011).

In the ethnomedical system of Ethiopia, quite a large number of plants are used to treat ailments associated with pain like headache, stomachache, common wound such as *Ocimum lamiifolium, Deciliter laxata, Croton Macrostachys, Vernonia amygdalina, Carica papaya, Eucalyptus globules, Allium Sativam, Echinops maccrochaetus, Schinus molle,* and *Withania somnifera* were the commonly used plant species (Gabriel and Guji, 2014).

1.9. Leonotis ocymifolia

The Lamiaceae family also called Labiatae, has about 300 genera and more than 7500 species with cosmopolitan distribution (de Oliveira, et *al, .2015*). Leonotis is a genus frequently found in Africa and Leonotis (Lion's ear) consists of about 30 species of plants in the family Lamiaceae. Forexample, *Leonotis neptifolia* is native to tropical Africa and Southern India (Yeshitila, 2006). The name leonotis is derived from the Greek word "leon" (a lion) and "ous", "otis" (an ear), according to their flowers, which resemble lion's ears. The plant is a many-stemmed shrub, which usually attains a height of about 2.5 m (Yeshitila, 2006 and Vagionas *et al.*, 2007). The leaves are short, petiolate, obiong-lanceolate or lanceolate, about 5-10 cm long, slightly oblique at the throat, and have the short teeth on the rim of the leaf. The orange red flowers being grouped in dense clusters along the stems characterize the genus. The fruit consists of four little nutlets seated in the base of calyx tube (Figure: 2). Naturally it dies after flowering and new growth starts once more in the spring. Propagation is by means of cuttings, division of the rootstock or by seed (de Oliveira, *et al, .2015*).

Leonotis ocymifolia is indigenous to Eastern and Southern Africa, subshrub or shrub 1.5–2.5 m high, not very aromatic, and is occasionally cultured for its medicinal uses, which include acting as an ascaricide, an anticancer drug and as a treatment for ulcers and wounds. Labdane diterpenoids and other diterpenes have been isolated from the aerial parts of *Leonotis ocymifolia* (Vagionas *et al.,* 2007).

Leonotis leonurus, locally commonly known as "wilde dagga" (=wild cannabis), is traditionally used as a decoction, both topically and orally, in the treatment of a wide variety of conditions such as haemorrhoids, eczema, skin rashes, boils, itching, muscular cramps, headache, epilepsy, chest infections, constipation, spider and snake bites. The dried leaves and flowers are also smoked to relieve epilepsy. The leaves and flowers are reported to produce a mild euphoric effect when smoked and have been said to have a similar, although less potent, psychoactive effect to cannabis (Nsuala *et al.,* 2015).

They also have many medicinal uses. Cause effects such as mild auditory and/or visual hallucinations and increased euphoria. *Leonotis ocymifolia* is used more or less

interchangeably with *L. leonurus* in some areas, but specific uses include the treatment of diabetes, hypertension, anemia, eczema and other skin irritations (Yeshitila, 2006).

In Ethiopia *Leonotis ocymifolia (Burm. F)* commonly known as Ras-kimir or Yeferes Zeng. It is used traditional medicine for the treatment of headache and ulcer of the neck (d'Avigdor, *et al.*,2014), swelling (Yineger *et al.*,2007), blackleg (Lulekal, 2014) hookworm; gout, leishmaniasis, and it has anti-fertility properties (Tafesse *et al.*,2005), antemetic (Lulekal *et al.*,2013), and general malaise and in animals to expel out the parasite (Kefalew *et al.*,2015). People living around Bale area in southern Ethiopia use the leaves of this plant to expel intestinal parasites (Yineger *et al.,* 2007). The essential oils of the leaf and flower of *L. leonurus* and *L. Ocymifolia* growing in the Eastern Cape of South Africa exhibited a broad spectrum anti-bacterial activity against gram-positive (Bacillus subtilus, Bacillus cereus, Micrococcus kristinal, Staphytococcus aureus, etc) and gram-positive bacteria(Yeshitila, 2006).

Phytochemical screening showed the presence of phenols, flavonoids, alkaloids, saponin, glycoside and tannins (Sharma *et al.*,2013). Analysis of Ethiopian collections of leaves of *L.Ocymifolia* revealed the presence of five different labdane type diterpene lactones (Yeshitila, 2006).

Therefore, this study was primarily under taken to confirm the acclaimed analgesic effect and anti-inflammatory activity of the leaves extract of *Leonotis ocymifolia* based on their ethno medicinal use in Ethiopia.



Figure 2: Picture of *Leonotis ocymifolia*

1.10. Rationale of the Study

Almost 80% of world populations rely on traditional medicines for primary health care, most of which engross the use of plant extracts. Therefore, patients as well as physicians are increasingly interested to explore new options for the disease management using natural products with good effectiveness and fewer side effects (Lubna *et al.*, 2014). Despite the availability of sufficient drugs for treating pain and inflammation, majority of existing drugs suffer from adverse effects and causes physical dependency. In view of this, there is needs for the intensification of research into medicinal plants claim to be effective in the management of pain and inflammation.

A range of medicinal plants with analgesic and anti-inflammatory properties have been widely used by traditional healers (Kumar *et al*., 2015). But, therapeutic potentials of some of these medicinal plants have not been scientifically evaluated. Among these plants, the leaves extract of *Leonotis ocymifolia* has acclaimed folklore use as an analgesic and anti-inflammatory agent; however, there is no scientific evidence to substantiate such traditional claim. Hence, this study evaluates analgesic and anti-inflammatory activity of this plant with a view to validate its acclaimed use by the traditional practitioners. Besides, the finding of this study might provide a clue about the possible mechanisms of analgesic and anti-inflammatory action and may serve as a lead compound for the development of new analgesic and anti-inflammatory drugs. Furthermore, it might serve as baseline information for scientific community to further investigate the plant *Leonotis ocymifolia* by initiating advanced studies on molecular mechanisms with identification of the specific agents responsible for the analgesic and anti-inflammatory effect of the extract .

2. OBJECTIVE

2.1. General Objective

To evaluate the analgesic and anti-inflammatory activity of the 80% methanol leaf extract of *Leonotis ocymifolia (Burm.f.)* in vivo.

2.2. Specific Objectives

- To assess acute toxicity of the 80% methanol leaf extract of *Leonotis ocymifolia*,
- To evaluate the analgesic activities of the 80% methanol leaf extract of *Leonotis ocymifolia* using acetic acid induced writhing method,
- To evaluate the analgesic activities of the 80% methanol leaf extract of *Leonotis ocymifolia* using hot plate method,
- To evaluate the anti-inflammatory activities of the 80% methanol leaf extract of *Leonotis ocymifolia* using carrageenan induced paw edema,
- To evaluate the anti-inflammatory activities of the 80% methanol leaf extract of *Leonotis ocymifolia* using cotton pellet induced granuloma,
- To determine the phytochemical constituents of the 80% methanol leaf extract of *Leonotis ocymifolia*.

3. MATERIALS AND METHODS

3.1.Drugs and Chemicals

Carrageenan (Sigma Aldrich, Germany) was obtained from Ethiopian Public Health Institute (EPHI). Acetyl salicylic acid and morphine active ingredients were obtained from Ethiopian Pharmaceuticals Manufacturing (EPHARM) as a powder form. Dexamethasone (Medico Labs, Lot E6A00, Syria), Methanol (Carlo Erba, Italy), distilled water (EFMHACA), tween 80 and ammonia (Lobe chemi, India), glacial acetic acid, chloroform and sulfuric acid (Fisher Scientific, UK), acetic anhydride (Park Scientific, UK), picric acid (Sigma Aldrich, Germany) and ferric chloride solution (Finkem laboratory reagent, India).

3.2. Materials and Instruments

Rotary evaporator (Heidolph, Germany), lyophilizer (OPERON, OPR-FDU-5012, Korea), digital plethysmometer (Ugo Basile-Cat no 7140, Italy) electronic balance (KERN-ALJ 220-4, Germany), mini orbital shaker (SSM1-STUART), Tissue Drying Oven (Medite - Medizin technik, Germany), separatory funnel, flasks, Cotton pellets, syringes with needles, feeding tube, blunt forceps, scissors, suturing set.

3.3 Plant Material collection and authentication

The fresh leaves of *Leonotis ocymifolia* were collected from East showa zone in Ada'a District, Denkaka Kebele, Oromia region, Ethiopia, in January 2017. Identification and authentication of the plant specimens was done at the National Herbarium, Department of Plant Biology and Biodiversity Management, Addis Ababa University. A voucher specimen was deposited with voucher number AS 001/2017 for future reference. The leaves were washed gently by rinsing with distilled water without squeezing to remove debris and dust particles then dried at room temperature under shade and then size reduced into coarse powder with mortar and pestle.

3.4 Experimental Animals

Healthy Swiss albino mice, weighing 25–35g and aged 6–8 weeks were used for the experiment. Both male and female mice were used for the main study and male albino wistar rats weighing 180-220 g were used for the chronic anti-inflammatory study. Only female mice were used for the acute toxicity study. Animals were obtained from the

animal house of toxicology department of EFMHACA and from the animal house of School of Pharmacy, Addis Ababa University. Animals were kept in plastic cages at room temperature and on a 12 h light–dark cycle with access to pellet food and water *ad libitum*. They were acclimatized to the laboratory condition for one week before the commencement of the experiment. The care and handling of animals was performed following international guidelines (Worlein *et al*., 2011; National Research council, 2010).

3.5 Preparation of Plant Extracts

The leaves of *Leonotis ocymifolia* was air dried under shade and pulverized using a mortar and pestle to get a coarse powder used for the extraction. 250grams of *Leonotis ocymifolia* leaf was used and divided into two halves for the extraction process. The first half (120 g of the leaf) was macerated in 600ml of 80% methanol and the same volume of the solvent was used for macerating the other remaining half. The maceration was undertaken with occasional shaking using mini orbital shaker being tuned to 120 rpm for 72 hrs at room temperature. Then, the extract was filtered first using muslin cloth and then using Whatman filter paper (No 1).Filtration and collection of the extract was done three times with the whole extraction taking 9 days. After the extraction, methanol was evaporated under vacuum using rotavapor at 40^0C. The resulting solution was placed in a deep freezer operating at negative $20C^0$ till it forms solid ice and then the remaining solvent (water) was removed using lyophilizer . After water removal, a light green black powder residue weighing 27.46g was obtained, giving rise to a percentage yield of 11.44%. The powder residue was then stored at 4^0C (deep freezer) until use. The extract was dissolved in 2% Tween 80 in DW for the subsequent tests. Another 250grams of *Leonotis ocymifolia* leaf was macerated with distilled water with the same ration and for the same period of time.The filtrates from the three batches of the aqueous extract were also combined, placed in a deep freezer at -20C to form an ice and lyophilized. The water extract provides a yield of 17.26gm of black powder (6.9% w/w). The powders were kept in tightly stoppered bottles and stored in a in a deep freezer at $-20C^0$ till commencement of the pilot study.

The yield of extract was calculated using the following formula:

$$\% \text{ Yield} = \frac{\text{Weight of extracted material}}{\text{Weight of original plan material used}} \times 100 \ldots\ldots \text{ (Ezeja } et\ al.\text{, 2013)}$$

% Yield= 27.46g /250gX100= 11.44%

3.6 Acute Toxicity Study

Acute toxicity test was performed according to the Organization of Economic Corporation and Development (OECD) guideline 425(2008). Fasted female albino mice of 6-8 weeks were used for the toxicity study. First, a sighting study was performed to determine the starting dose, in which, a single female mouse was given 2000 mg/kg of the extract as a single dose by oral gavage. Since no death was observed within 24 h, additional four mice were used for the extract and administered the same dose of extract. The animals were observed continuously for 4 h with 30 min interval and then for 14 consecutive days with an interval of 24 h for the general signs and symptoms of toxicities such as changes in skin and fur, eyes and mucous membranes, somatomotor activity and behavioral pattern, salivation and diarrhea, weight loss, tremor and convulsions, lethargy and paralysis, food and water intake and mortality. After acute toxicity test, three dose levels were chosen. A middle dose, which was one-tenth of the maximum dose obtained during acute toxicity study; a low dose, which was half of the middle dose, and a high dose which was twice of the middle dose.

3.7 Pilot study

Pilot study was done using acetic acid induced model and Carrageenan-induced paw edema acute model of inflammation on 80% methanol leave extract (80ME) and aqueous leave extracts(AE) of *Leonotis ocymifolia*. The two extracts were administered at doses of 100, 200 and 400mg/kg and two animals per group were used in all the dose levels. The result indicated that both have analgesic and anti-inflammatory activity; even though the

80ME had shown a better analgesic effect at all employed doses (maximum % analgesic effect for acetic acid model were = 33.6% for 100mg/kg, 43.3% for 200mg/kg, and 60.5% for 400mg/kg) than the AE (% analgesic effect for acetic acid model were = 30.8% for 100mg/kg, 42.5% for 200mg/kg, and 58.3% for 400mg/kg). And also 80ME had shown a better anti-inflammatory effect (%A) at all employed doses (maximum %A at the 6^{th} h = 45.75% for 100mg/kg, 69.15% for 200mg/kg, and 74.47% for 400mg/kg) than the AE (maximum %A at the 6^{th} h = 43.52% for 100mg/kg, 50% for 200mg/kg, and 60.19% for 400mg/kg). Therefore, 80ME was chosen for this study.

3.8 Grouping and Dosing of animals

Mice of both sexes (3 mice from each sex) were randomly assigned into five groups of six mice in each group to perform the analgesic and anti-inflammatory activity test and for chronic inflammatory model only male rats were used. The first group was assigned as negative control and received the vehicle (2% Tween 80) was administered orally at a volume of 10 ml/kg. The second group was assigned as positive control and administered with standard drugs (150 mg/kg and 100 mg/kg Acetyl salicylic acid for acetic acid induced writhing and for carrageenan induced paw oedema model (acute anti inflammatory models) respectively, 20 mg/kg morphine for hot plate method and Dexamethasone (0.5 mg/kg p.o. in chronic anti inflammatory model) was administered to this group. The other three groups were given different doses (100 mg/kg, 200 mg/kg and 400 mg/kg) of the extract orally. Dose selection was made based on acute toxicity test and pilot experiments. All administrations were carried orally using an oral gavage.

3.9 Evaluation of Analgesic Activity of the Extract

3.9.1Acetic acid induced writhing method

The method of Arul *et al*., (2005) was used with slight modification. Mice of either sex were divided into five groups of each consisting of six animals. Three groups were given different dose of the plant extract (determined based on acute toxicity), while one group was given a vehicle (negative control) and the other group was given a standard drug Acetyl salicylic acid, 150mg/kg (positive control) one hour before acetic acid

administration. Sixty minutes later, Analgesic activity of *Leonotis ocymifolia* was assessed by counting the number of writhes induced by 0.6% acetic acid (10 ml/kg, i.p.) (Lohdip and Aguiyi, 2013). Five minutes after the acetic acid injection i.p., the animal was placed in a glass jar individually and the contractions of abdominal muscles together with stretching of the hind limbs were cumulatively counted over a period of 30 minutes. Percentage protection against writhing was taken as an index of analgesia (Kumar *et al.*, 2010 and Le Bars et al, 2001) and calculated using the following formula

$$\% \text{ Analgesic Activity} = \frac{\text{Mean writhing count (Control group – Treated group)}}{\text{Mean writhing count of control group}} \times 100$$

3.9.2.Hot plate method

This test consists of introducing a mouse into an open-ended cylindrical space with a floor consisting of a metallic plate that is heated by a thermode (Woolfe and MacDonald, 1944; Eddy and Leimbach, 1953; O'Callaghan and Holzman, 1975). A plate heated to a constant temperature produces two behavioral components that can be measured in terms of their reaction times, namely paw licking and jumping. Both are considered to be supraspinally integrated responses (Le Bars *et al*., 2001). Mice of either sex were divided into five groups of each consisting of six animals. All animals were fasted overnight. Three groups were given different doses of the plant extract (determined based on acute toxicity, p.o.), while one group was given a vehicle (negative control, p.o.) and the other group was given standard drug morphine (positive control, 20mg/kg oral). The animals were placed on a hot plate maintained at a temperature of 55 ± 1ºC (Yadav *et al*., 2011*).* Before the treatment, the reaction time of each animal was recorded. The latency to lick the paw or jump from the hot plate was noted as the reaction time. The reaction times were noted at 30, 60, 90 and 120 min. The cut off time was considered as 15 s (Neto *et al*., 2005).

Percentage increase in reaction time or pain threshold inhibition, was derived, using the formula (Dash *et al*., 2015)

$$\textbf{Elongation (\%)} = \frac{\text{Latency (test)} - \text{Latency (control)}}{\text{Latency (test)}} \times 100$$

3.10 Evaluation of anti-inflammatory activity of the extract

3.10.1. Carrageenan induced mice paw oedema

The anti-inflammatory activity was determined in mice according to the method of Winter *et al* (1962). Mice were fasted for 12 h with free access to water until the experiment starts. Acute inflammation was produced by injection of carrageenan (1% w/v carrageenan in normal saline, 50 µl) in the right hind paw of the mice. Carrageenan was injected one hour after oral administration of the extract (Rahman *et al.*, 2011). The inflammation was quantitated in terms of ml i.e., displacement of water by oedema using a digital plethysmometer at time 0, 1, 2, 3, 4,5 and 6h after carrageenan injection (Okokon *et al*., 2012). The percent inhibition of oedema was calculated in comparison to the control animals and was calculated using the following formula (Balamurugan *et al.*, 2012):-

$$\textbf{\%Percentage inhibition} = \frac{\text{Mean paw volume control - Mean paw volume treatment}}{\text{Mean paw volume in control group}} \times 100$$

3.10.2.Cotton pellet induced granuloma method

The method previously described by (Afsar *et al*. 2013) was used to assess the transudative and proliferative (granulomatous) components of chronic inflammation. Male albino wistar rats (180-220 g) were fasted for 12 h with free access to water until commencement of the experiment. The control, standard and test groups of rats received 2% tween 80, dexamethasone (0.5 mg/kg p.o.) and crude extracts.

Sterile cotton pellet weighing 10 ± 1 mg were prepared by rolling o f a cotton piece of 10mg and sterilized by autoclaving for 30 min at 120 ^{0}C under 15 lbs pressure. Twenty minutes after treatment with the standard drug and extracts, the rats were anesthetized

with diethyl ether and subcutaneous tunnel was made aseptically using blunted forceps in both sides of previously shaved groin region of each rats. Two sterilized cotton pellets weighing 10±1 mg each were then implanted bilaterally in the subcutaneous tunnel and sutured with chromic catgut (0/4metric-1/2 Circle). Treatment with the standard drug (dexamethasone) and extracts continued for seven consecutive days (p.o., once a day). On the 8th day, the rats were sacrificed with ether anesthesia, thereafter; the pellets surrounded by granuloma tissue were dissected out carefully and freed from extraneous tissue. The wet weight of the cotton was taken immediately after removal and then dried at 60^0C for 24hrs and the net dry weight, that is, after subtracting the weight of the cotton pellets, was determined.

The exudate amount (mg), granulation tissue formation (mg), and percent inhibition of exudate and granuloma tissue formation were calculated according to the formula given by Aziz *et al*., (2014):

$$\text{Exudates inhibition (\%)} = \left(1 - \frac{\text{Exudates in treated group}}{\text{Exudates in controls}}\right) \times 100$$

$$\text{Granuloma inhibition (\%)} = \left(1 - \frac{\text{Granuloma in treated group}}{\text{Granuloma in controls}}\right) \times 100$$

Where: Measure of exudates formation = immediate wet weight of pellet - Constant dry weight of pellet

Measure of granuloma tissue formation = Constant dry weight - Initial weight of cotton pellet.

3.11. Preliminary phytochemical screening

The qualitative phytochemical screening of aqueous leaf extracts *Leonotis ocymifolia* were tested for the presence of active principles such as glycosides, saponins, alkaloids, flavonoids, phenols and tannins. Following standard procedures were used.

Test for Steroids and Triterpenoids: (Liebermann Burchard test) – 0.25g extract was mixed with 2ml of acetic anhydride, boiled and cooled. 2ml Concentrated sulphuric acid was then added from the sides of the test tube and observed for the formation of a brown

ring at the junction of two layers. Green coloration of the upper layer and the formation of deep red color in the lower layer would indicate a positive test for steroids and triterpenoids respectively (Wani *et al*., 2011 and Bhandary *et al*., 2012).

Test for Cardiac Glycosides: (Keller Killiani Test) – 0.5g extract was dissolved in 2ml of glacial acetic acid containing one drop of and Ferric chloride solution and mixed.1ml Concentrated sulphuric acid was added, and observed for the formation of two layers. Lower reddish brown layer and upper acetic acid layer which turns bluish green would indicate a positive test for glycosides (Maobe *et al*., 2013 and Bhandary *et al*., 2012).

Test for Saponins: (Foam Test) – 0.25g LO was mixed with 5 ml of distill water and shaken and observed for the formation of froth, which is stable for 15 minutes for a positive result (Singh and Bag *et al*., 2013).

Alkaloids: (Hager's Test) – About 25 mg of the LO was treated with 3ml of Hager's reagent (saturated picric acid solution).Formation of yellow precipitate would show a positive result for the presence of alkaloids (Bhandary *et al*., 2012).

Test for Flavonoids: 5 ml of dilute ammonia solution were added to a 0.2g LO of methanolic extract followed by addition of 1ml concentrated H_2S0_4. A yellow coloration observed in solution indicated the presence of flavonoids. The yellow coloration disappeared on standing (Wadood *et.al.,* 2013).

Tannins: About 0.25 g of 80ME and each fraction was boiled in 10 ml of distill water in a test tube and then filtered with filter paper (Whatman No. 1). A few drops of 0.1% ferric chloride were added to the filtrate. A brownish green or a blue-black precipitate indicated the presence of tannins. (Sumathy,V. *et al.,* 2011 and Singh and Bag *et al*., 2013).

Test for Phenols: (Ferric chloride test): The 0.25g LO of extract was treated with 5 % ferric chloride and observed for formation of deep blue or black color.

Test for Anthraquinone: 5 ml of chloroform was added to 0.25g of extract. The resulting mixture was shaken for 5 minutes after which it was filtered. The filtrate was then shaken with equal volume of 10 % ammonia solution. The presence of a bright pink colour in the aqueous layer indicated the presence of anthraquinones (Uddin. *et al*., 2011).

3.12. Statistical Analysis

The data were analyzed using SPSS version 20.0 for Windows. The experimental results are expressed as mean ± standard error of the mean (S.E.M) and statistical analysis was carried out by employing one way analysis of variance (ANOVA) followed by Tukey Post Hoc Test for multiple comparisons to compare results among groups, where p values < 0.05 were considered statistically significant. The analyzed data were then presented using tables. Linear regression was also used where appropriate.

4. RESULTS

4.1 Acute Toxicity Study

The acute oral toxicity test of 80% methanolic extract of leaves of *Leonotis ocymifolia* at a dose of 2000 mg/kg did not show gross behavioral changes, toxic effects or mortality within 24 h and in the next 14 days. According to the "Limit Test at 2000 mg/kg" of OECD guideline 425 (2008), it can be concluded that the oral LD_{50} of crude 80ME is greater than 2000 mg/kg in mice. Hence, the result suggests that the extract has a low toxicity profiles.

4.2 Analgesic Activity

. In acetic acid induced writhing and hot plate method the crude extract showed a significant analgesic activity. In acetic acid induced writhing method, the extract at all doses significantly ($p<0.001$) reduced the number of writhes in mice as compared to negative control (Figure 3). The higher (400 mg/kg) dose of the extract showed a significant ($p<0.001$) difference compared to the lower and middle (100 mg/kg and 200 mg/kg) doses of the extract. Acetyl salicylic acid, on the other hand, showed a significant ($p<0.001$) difference from the 100 mg/kg and 200mg/kg dose of the extracts. However, no significant difference was observed with that of 400mg/kg doses of the extract.

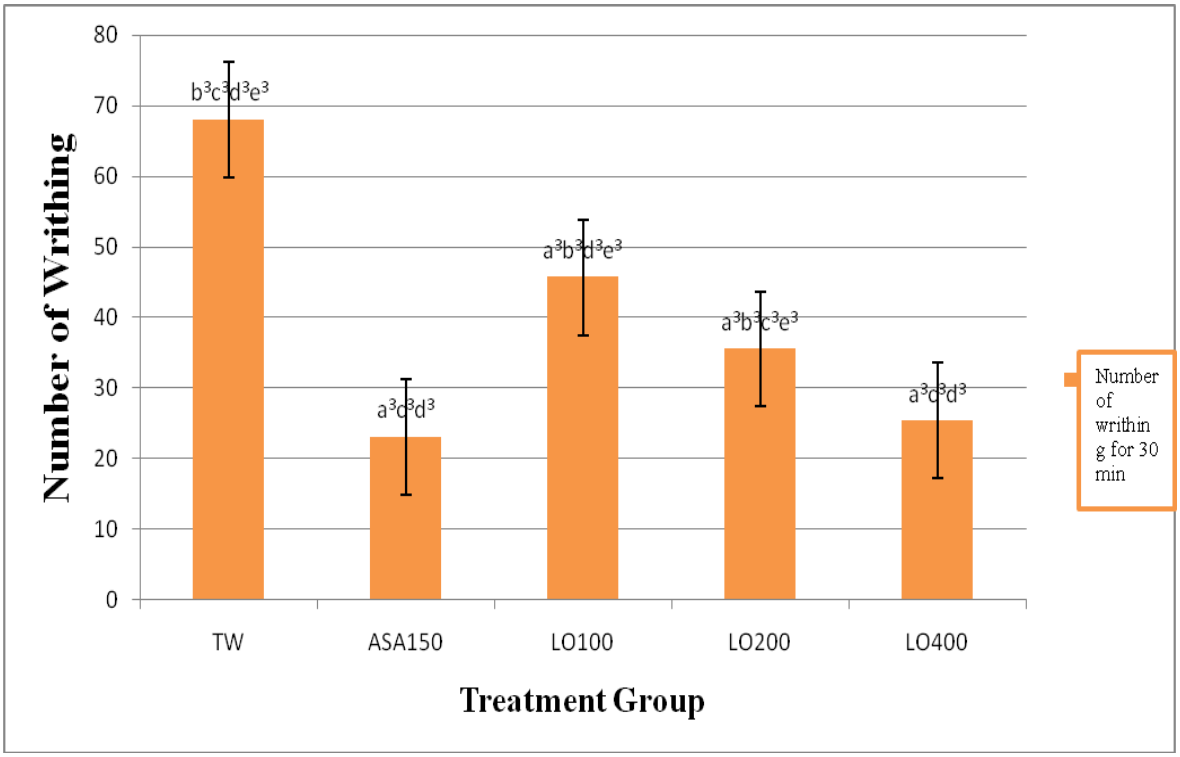


Figure 3: Effect of 80% Methanol leaf extract of *Leonotis ocymifolia* on acetic acid induced writhing model in mice. Data is mean ±SEM, n=6 TW(10 ml/kg)=Tween 80; ASA= Acetyl salicylic acid (150 mg/kg); LO100= *Leonotis ocymifolia* Extract (100 mg/kg); LO200 =(200 mg/kg); LO400, =(400 mg/kg); a, compared to control; b, compared to ASA, c compared to100mg/kg, d compared to 200mg/kg, e compared to 400mg/kg, $^{3}p<0.001$.

Maximum analgesic effect (%A) by the 100, 200 and 400 mg/kg of LO was observed at 30min post- induction, with respective values of 32.8%, 47.9%, and 62.8%, and the effect at this minute was found to increase in dose dependent manner ($R^2 = 0.762$). Intergroup comparison among doses of the LO also showed a statistically significant difference in 200 versus 100mg/kg ($p < 0.001$), 400 versus 100mg/kg ($p < 0.001$) and 200 versus 400mg/kg ($p < 0.001$). However, no significant difference was observed between ASA 150 versus 400mg/kg dose groups (figure 4).

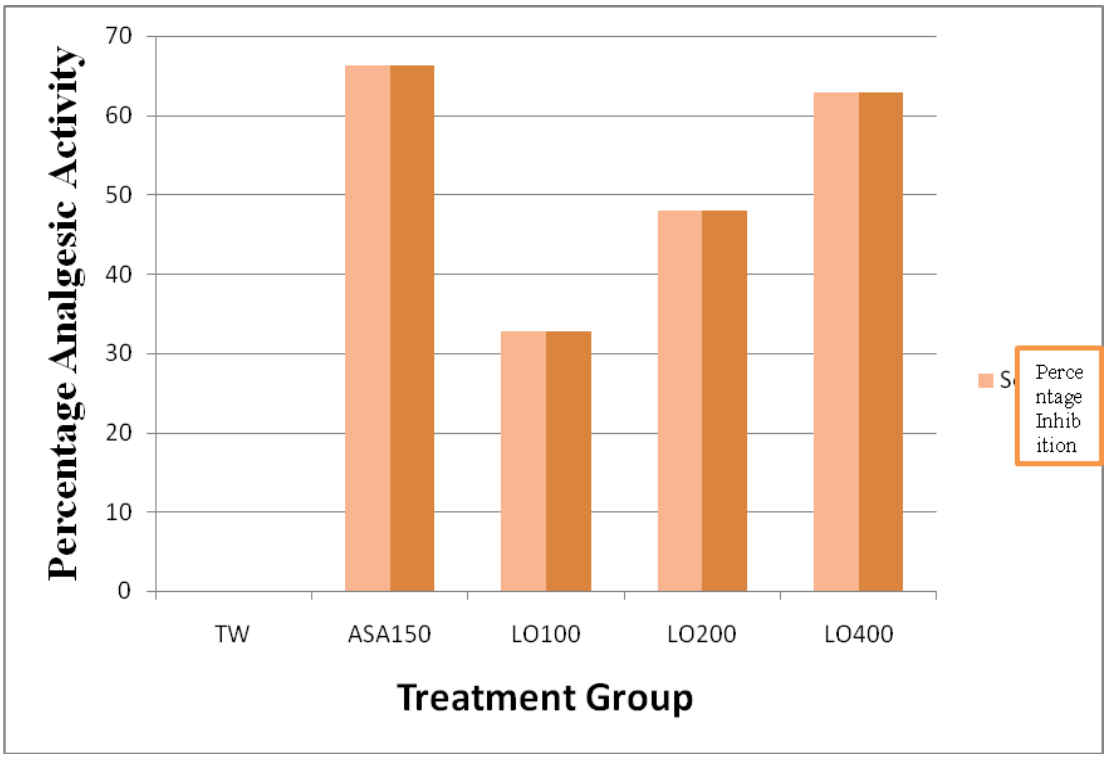


Figure 4: Percentage analgesic activity of 80% Methanol leaf extract of *Leonotis ocymifolia* on acetic acid induced writhing model in mice. TW (10 ml/kg); ASA, Acetyl salicylic acid (150 mg/kg); LO100, Extract (100 mg/kg); LO200, Extract (200 mg/kg); LO400, Extract (400 mg/kg);

In the hot plate method, the extract at all doses produced a significant ($p<0.001$) analgesic effect compared to negative control, with a maximum effect observed at 120 min for 400mg/kg and morphine which produced a maximum analgesic effect at 120 min (72.84%) (Figure 5). Morphine, on the other hand, showed a significant ($p<0.001$) difference from 100 and 200 mg/kg but it showed comparable effect with 400 mg/kg of LO ($p<0.05$) at 120 min.

Maximum analgesic effect (%A) by the 100, 200 and 400 mg/kg of LO was observed at 120 minute, with respective values of 59.34%, 69.86 %, and 71.99%, and the effect at this minute was found to increase in dose dependent manner ($R^2 = 0.918$). Intergroup

comparison among doses of the LO also showed a statistically significant different effect in 200 versus 100mg/kg ($p < 0.001$ from 30 min – 120 min), 400 versus 100mg/kg ($p < 0.001$ from 30 min – 120 min) and 20mg/kg morphine and 400 mg/kg ($p < 0.001$ from 30 min – 90 min and $p < 0.05$ at 120 min) dose groups.

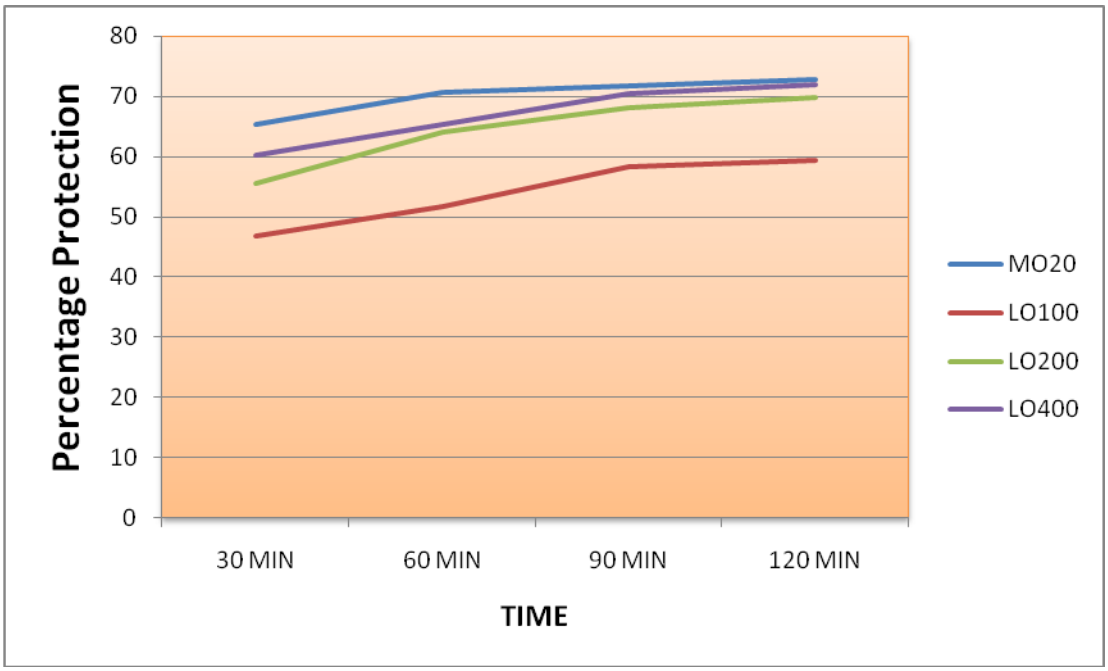


Figure 5: Percentage protection of 80% Methanol extracts of *Leonotis ocymifolia* on latency time of hot plate method in mice. MO, Morphine (20 mg/kg); LO100, Extract (100 mg/kg); LO200, Extract (200 mg/kg) and LO400, Extract (400 mg/kg).

Table 1: Effects of 80% methanol extract of *Leonotis ocymifolia* on hot plate latency time in Mice.

Treatment Group	Hot plate latencies in sec.(Mean±SEM)				
	0min	**30min**	**60min**	**90 min**	**120min**
TW	2.0650± 0.0173	2.3617 ± 0.0236	2.3367±0.0209	2.3367±0.0208	2.3300±0.0278
Morphine	2.1017±0.01014	6.7917 ± 0.0212 $a^3c^3d^3e^3$	7.9683±0.0098 $a^3c^3d^3e^3$	8.2650±0.0267 $a^3c^3d^3e^3$	8.5783±0.0140 $a^3c^3d^3e^1$
LO100	2.0983±0.0182	4.4283± 0.0169 $a^3b^3d^3e^3$	4.8250±0.0118 $a^3b^3d^3e^3$	5.6050±0.0076 $a^3b^3d^3e^3$	5.7300±0.0118 $a^3b^3d^3e^3$
LO200	2.1000±0.0192	5.3033±0.0199 $a^3b^3c^3e^3$	6.5100±0.0139 $a^3b^3c^3e^3$	7.3500±0.0159 $a^3b^3c^3e^3$	7.7333±0.0112 $a^3b^3c^3e^3$
LO400	2.0750±0.0152	5.9367±0.0159 $a^3b^3c^3d^3$	6.7350±0.0173 $a^3b^3c^3d^3$	7.9283±0.0087 $a^3b^3c^3d^3$	8.3233±0.1178 $a^3b^1c^3d^3$

Data is expressed as mean ± S.E.M. of six observations. a, compared to control; b, compared to Morphine 20mg/kg, c compared to100mg/kg, d compared to 200mg/kg, e compared to 400mg/kg, $^1p<0.05$ and $^3p<0.001$.

4.3 Anti-inflammatory Activity

Subplantar injection of 0.05 ml of 1% carrageenan to the mice hind paw produced a progressive increment of paw thickness that reached its maximum value after 3h of induction in 2% tween vehicle controls (Table 2). All tested doses of the 80% methanol extract (100, 200 and 400mg/kg of LO) showed a significant inhibition of paw edema that starts from 1 h ($p < 0.001$) and the effect lasted till 6 h post- induction ($p < 0.001$ from 1^{st} - 6^{th} h) as compared to the 2% tween vehicle control.

Maximum anti-inflammatory effect (%A) by the 100, 200 and 400 mg/kg of LO was observed at 6h post induction, with respective values of 46.3%, 69.13 %, and 75.88%, and the effect at this hour was found to increase in dose dependent manner ($R^2 = 0.916$). Intergroup comparison among doses of the LO also showed a statistically significant effect in both 200 versus 100mg/kg ($p < 0.001$ from 1^{st} - 6^{th} h), and 400 versus 100mg/kg ($p < 0.001$ from 1^{st} - 6^{th} h). However, no significant difference was observed between 200 versus 400mg/kg dose groups.

In the carrageenan induced paw oedema model, 200 and 400mg/kg of the LO showed higher anti inflammatory effect (%A) than that of 150mg/kg of Acetyl salicylic acid throughout the observation period, whereas 100mg/kg of LO had shown a comparable anti-inflammatory effect with 150mg/kg of Acetyl salicylic acid (Figure 6).

Table 2: Effects of 80% methanol extract of *Leonotis ocymifolia* on carrageenan induced paw oedema model in mice.

Treatment Group	Mean increase in paw volume (ml)(Mean±SEM)					
	Mean at 1hr	**2hr**	**3hr**	**4hr**	**5hr**	**6hr**
TW	0.5883±0.0087 $a^3b^3c^3d^3e^3$	0.5667±0.0088	0.5883±0.0189	0.5100±0.0275	0.4600±0.0195	0.4283±0.0154
ASA	0.3450±0.0062 a^3c^3	0.3233±0.0088 $a^3c^3d^2e^3$	0.3083±0.0105 $a^3c^3d^1e^3$	0.2733±0.0076 $a^3c^1d^3e^3$	0.2283±0.0060 $a^3d^2e^3$	0.1483±0.0079 a^3c^3
LO100	0.4467±0.0092 $a^3b^3d^3e^3$	0.4217±0.0095 $a^3b^3d^3e^3$	0.4083±0.0101 $a^3b^3d^3e^3$	0.3367±0.0102 $a^3b^1d^3e^3$	0.2717±0.0111 $a^3d^3e^3$	0.2300±0.0107 $a^3b^3d^3e^3$
LO200	0.3367±0.0067 a^3c^3	0.2683±0.0095 $a^3b^2c^3$	0.2500±0.0093 $a^3b^1c^3$	0.1700±0.0124 $a^3b^3c^3$	0.1483±0.0119 $a^3b^2c^3$	0.1322±0.0100 a^3c^3
LO400	0.3283±0.0108 a^3c^3	0.2300±0.0116 $a^3b^3c^3$	0.2167 ±0.0080 $a^3b^3c^3$	0.1333±0.0076 $a^3b^3c^3$	0.1183±0.0108 $a^3b^3c^3$	0.1033±0.0126 a^3c^3

Data is expressed as mean ± S.E.M. of six observations. a, compared to control; b, compared to ASA, c compared to100mg/kg, d compared to 200mg/kg, e compared to 400mg/kg, $^1p<0.05$ and $^3p<0.001$.

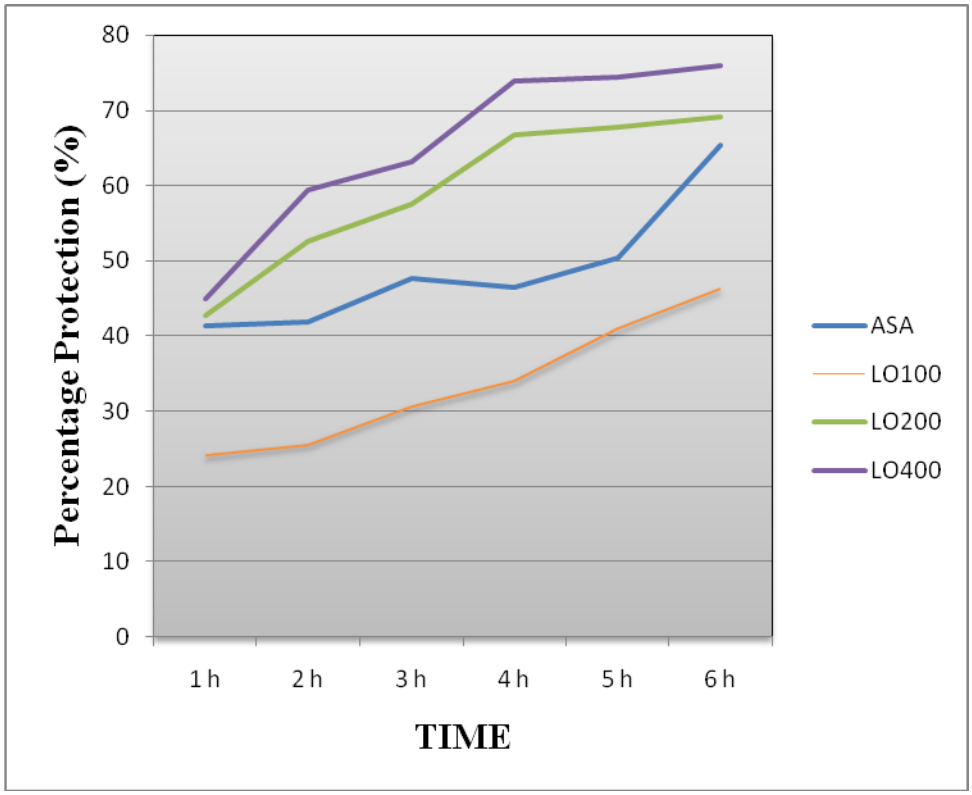


Figure 6: Percentage protection of 80% methanol extract of *Leonotis ocymifolia* on carrageenan induced paw oedema model in mice. Acetyl salicylic acid 100mg/kg, LO100, Extract (100 mg/kg); LO 200, Extract (200 mg/kg) and LO 400, Extract (400 mg/kg).

Cotton pellet induced granuloma

Subcutaneous implantation of two pellets of cotton each weighting 10 ± 1 mg in groin region of rats induced granulomatous inflammation with a maximum granuloma weight and exudates observed in 2% tween 80 received negative controls as shown in Table 3. The 80% methanol extract of *Leonotis ocymifolia* at all tested doses significantly inhibited the formation of inflammatory exudates ($p < 0.001$) and granuloma mass ($P < 0.001$) as compared to the negative control.

Intergroup comparisons among doses of the LO revealed a statistically significant effect in100 versus 200 mg/kg ($p < 0.05$ in exudates inhibition; $p < 0.001$ in granuloma inhibition), 100 versus 400mg/kg ($P < 0.001$ in both exudates and granuloma inhibition) and 200 versus 400mg/kg ($P < 0.05$ in both exudates and granuloma inhibition). Furthermore, the anti-inflammatory effect of the LO was found to increase in dose dependent manner ($R^2 = 0.558$).

Table 3: Effects of 80% methanolic extract of *Leonotis ocymifolia* on cotton pellet induced granuloma model in rats.

Treatment group	Mean weight of exudates (mg) ± S.E.M	Mean weight of granuloma (mg) ± S.E.M
TW	51.300±0.3688 $b^3c^3d^3e^3$	29.417±0.6514 $b^3c^3d^3e^3$
DEXA0.5	24.172±0.8510 $a^3c^3d^3$	6.017±0.3833 $a^3c^3d^3e^3$
LO100	36.467±0.9025 $a^3b^3d^1e^3$	22.350±0.2975 $a^3b^3d^3e^3$
LO200	31.967±0.7974 a^3b^3 c^1e^1	17.600±0.3670 a^3b^3 c^3e^1
LO400	27.750±1.4630 a^3 c^3d^1	14.517±1.1876 a^3b^3 c^3 d^1

Data expressed as mean ± S.E.M. of six observations. a, compared to control; b, compared to Dexamethasone 0.5mg/kg, c compared to100mg/kg, d compared to 200mg/kg, e compared to 400mg/kg, $^1p<0.05$ and $^3p<0.001$.

The percentage inhibition of the extract was 28.91%, 37.68% and 45.91% for exudates and 24.03%, 40.18% and 50.65% for granuloma at 100 mg/kg, 200 mg/kg and 400 mg/kg respectively.Whereas the percentage inhibition of dexamethasone was 52.89% for exudates and 79.54% for granuloma. All dose of the extract showed a significant effect when compared to negative control (figure 7).

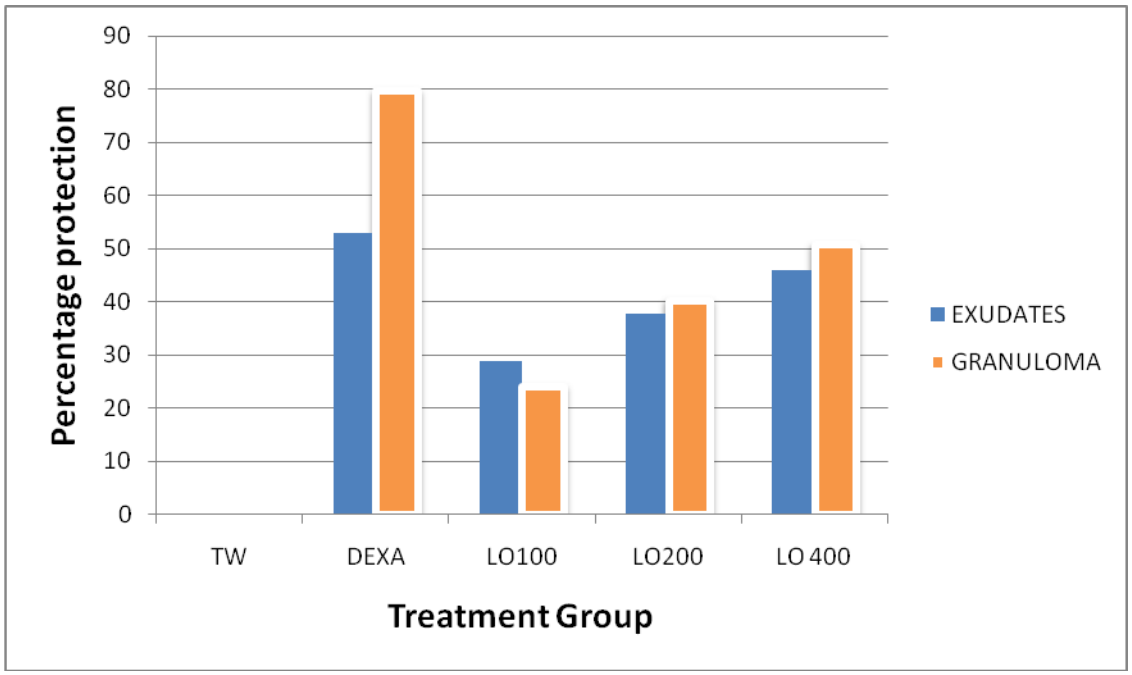


Figure 7: Percentage inhibition of 80% methanol extract of *Leonotis ocymifolia* on cotton pellet induced granuloma model in rats. Data is expressed as mean±SEM,n=6; TW, 2% Tween 80 (10 ml/kg); Dexamethasone (0.5 mg/kg); LO100, Extract (100 mg/kg); LO200, Extract (200 mg/kg); LO400, Extract (400 mg/kg)

4.4 Phytochemical Sreening

Preliminary chemical screening of aqueous extract of the leaves of *Leonotis ocymifolia* showed the presence of saponins, alkaloids, flavonoids, tannins, terpenoids, phenols and the absence of anthraquinones, cardiac glycosides, steroids and sterols (table 4).

Table 4: Preliminary phytochemical screening of 80% methanol extract of the leaves of *Leonotis ocymifolia*

Secondary metabolite	Result
Saponins	+
Alkaloids	+
Tannins	+
Cardiac Glycosides	-
Phenols	+
Terpenoids	+
Flavonoids	+
Anthraquiones	-
Steroids and sterols	-

+ = indicating presence
- = indicating absence

5. DISCUSSION

Leonotis ocymifolia is used in folk medicine for pain and inflammation in Ethiopia (d'Avigdor *et al*, 2014 and Yineger, H. *et al*, 2007.). However, no report about the anti-nociceptive and anti-inflammatory activity of this plant in experimental models of pain and inflammation is reported. In the present study, analgesic and anti-inflammatory activities of the leaves extract of *Leonotis ocymifolia* leaves were evaluated to verify its traditional claims.

The plant, *Leonotis ocymifolia*, is safely used for treatment of human and animal diseases. 80% methanol extract of LO at a high dose of 2000 mg/ kg for the acute toxicity did not cause sign of toxicity or mortality in mice like hyperactivity, twitching, rigidity, irritability, jumping, sleep, sedation, abnormal secretion or death. Therefore, the current study of plants leaf can be considered as safe, according to the 2008 Organization for Economic Cooperation and Development (OECD).

In this study, the peripheral and central analgesic activity of *Leonotis ocymifolia* was evaluated using acetic acid induced writhing method and hot plate method in mice. Hot plate method is sensitive to centrally acting analgesics whereas acetic acid induced writhing test is used for detects both peripheral and central analgesics (Galani and Patel, 2011).

Acetic acid induced writhing in mice attributed visceral pain finds much attention of screening analgesic drugs (Hasan *et al*., 2010). The crude extracts of the plants showed significant analgesic action compared to the negative control. In acetic acid induced writhing model the extract showed 32.8%, 47.9%, and 62.8% inhibition of writhing response at 100, 200 mg and 400 mg/kg, respectively. The results were found to be highly significant ($P < 0.001$) in comparison to the control. The above values suggested that the extract has analgesic activity in dose dependent manner ($R^2 = 0.762$). i.e. by increasing the dose, the analgesic activity also increases. The number of writhing's decreased by the extract in dose dependent manner compared to control group.

Pain sensation in acetic acid induced writhing method is elicited by triggering localized inflammatory response resulting in the release of free arachidonic acid from tissue phospholipid via cyclooxygenase (COX), and prostaglandin biosynthesis (Jayanthi and Jyoti,2012) and the pain induction effects of acetic acid are produced due to liberation of mediators such as histamine, serotonin, bradykinin, cytokines and eicosanoids. These factors promote increase o f vascular permeability as well as reduce the threshold of nociception and stimulate the nervous terminal of nociceptive fibers (Thamizhselvam *et al.*, 2012). In other words, the acetic acid induced writhing has been associated with increased level of PGE and PGF2a in peritoneal fluids as well as lipoxygenase products. The increase in prostaglandin levels within the peritoneal cavity then enhances inflammatory pain by increasing capillary permeability (Kumar *et al.*, 2009). It was found that both *Leonotis ocymifolia* and Acetyl salicylic acid caused an inhibition on the writhing response induced by acetic acid. The administration of Acetyl salicylic acid, a well known 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, where it was shown BK and PGs play an important role (Gisèle *et al.*, 2014). The analgesic effect of *Leonotis ocymifolia* seen in this experiment may be mediated through peripheral pain mechanism and or through suppression of prostaglandin pathway since it has been observed that any agent that decreases the number of writhing will demonstrate analgesia preferably by inhibition of prostaglandin synthesis, a peripheral mechanism of pain inhibition (Ezeja *et al.*, 2011).

In hot plate test, nociceptive reaction against thermal stimuli in rodents is a well-established model for detection of opiate analgesic as well as several types of analgesic drugs from spinal origin (Qamruzzamaa and Ansarib, 2012). The hot plate method has been found to be suitable for evaluation of centrally acting analgesics (Kumari *et al*., 2013). Pain is centrally modulated via a number of complex processes including opiate, dopaminergic descending noradrenergic and serotonergic systems. The analgesic effect produced by the tests and standards may be via central mechanisms involving these receptor systems or via peripheral mechanisms involved in the inhibition of

prostaglandins, leukotrienes, and other endogenous substances that are key players in pain (Mishra *et al*., 2011).

The hot plate test was selected because the test is sensitive to strong analgesics and limited tissue damage because of a cutoff point that is usually applied to limit the amount of time the mouse spends on the hot plate (Ibironke and Ajiboye, 2007). The paws of mice are very sensitive to heat at temperatures which are not damaging the skin. The responses are jumping, withdrawal of the paws and licking of the paws. The time until these responses occur is prolonged after administration of centrally acting analgesics (Kanazi *et al*., 2010).In this study, the extract at all doses produced a significant ($p<0.001$) analgesic effect from 30 min onwards compared to negative control, maximum analgesic effect (%A) by the 100, 200 and 400 mg/kg of LO was observed at 120 minute, with respective values of 59.34%, 69.86 %, and 71.99%, while morphine which produced a maximum analgesic effect at 120 min (72.84%).Therefore, the extract showed comparable effect with morphine. The LO extract produced a dose dependent prolongation of hot plate latency ($R^2 = 0.918$).

In general, acetic acid causes inflammatory pain by inducing capillary permeability, while hot plate exhibits neurogenic pain (Maheen *et al.*, 2015). *Leonotis ocymifolia* has shown inhibition of both types of pain. The analgesic effect of the plants in both models reveals that they have dual action through central and peripheral mechanism.

In the study of anti-inflammatory activity, *Leonotis ocymifolia* was evaluated the extracts against carrageenan induced paw oedema for acute inflammation and cotton pellet induced granuloma for chronic inflammation. The inflammatory response is a polyphasic tissue reaction, which ranges from short lived increase in vascular permeability to a prolonged cellular infiltration and proliferation. So it is important to evaluate the performance of agents claimed for anti-inflammatory effect via a battery of tests valid for various phases of inflammation (Kumar *et al*., 2012).

The carrageenan induced paw edema model is a prototype of exudative phase of acute inflammation (Garg *et al*., 2010 and Elumalai *et al*., 2012). Carrageenan is the phlogistic agent of choice for testing anti-inflammatory drugs as it is not known to be antigenic and is devoid of apparent systemic effects (Solanki *et al*., 2015). Moreover, the experimental model exhibits a high degree of reproducibility. Carrageenan induced paw edema model has been commonly used as an experimental model for sub-acute inflammation and is believed to be biphasic. Hence, injection of carrageenan induces localized inflammation in two different phases through sequential release of several mediators. The initial phase, which occurs between 0 and 2.5 h after carrageenan injection, has been attributed to the action of mediators such as histamine, serotonin and bradykinin on vascular permeability. Histamine and serotonin are mainly released during the first 1.5 h while bradykinin is released from 1.5 to 2.5 h after carrageenan injection (Tamaddonfard *et al.*, 2013).

The second phase (2.5 - 6 h post-carrageenan injection) is a result of overproduction of COX-2 and its pro-inflammatory PG products, with infiltration of polymorphonuclear leucocytes (neutrophils) (Solanki *et al*., 2015). The peak inflammation is usually seen approximately 2 - 3 h post carrageenan injection and is attributed to PG release (Khobragade *et al*., 2013 and Jadhav and Prabhavalkar, 2015). All these mediators include prostaglandins, histamine, bradykinins, leucotrienes and serotonin, which cause pain and fever.Inhibitions of these mediators from reaching the injured site or from bringing out their pharmacological effects normally improves the inflammation and other symptoms (Jami *et al.*, 2014). Hence, the second phase appears to be the most interesting phase in terms of inflammatory processes. The carrageenan-induced paw edema is known to be sensitive to COX inhibitors but not to 5-LOX inhibitors, and hence has been used to evaluate the effect of NSAIDs which primarily inhibit the COX pathway, i.e. single-action NSAIDs such as Acetyl salicylic acid. It has been demonstrated that the suppression of carrageenin-induced hind paw edema after the 3rd h correlates reasonably with therapeutic doses of most clinically effective antiinflammatory agents (Mali *et al.*, 2013). Production of arachidonic metabolites via the COX-2 enzyme is the main factor responsible for the late phase of carrageenan induced inflammation (Hajhashemi *et al.*,

2007).It is well documented that centrally acting drugs such as narcotics inhibit both phases equally while peripherally acting drugs such as NSAIDs inhibit the late phase (Saleem *et al.*,2015).hence, in this study carrageenan-induced hind paw edema was used as an appropriate acute model of inflammation and Acetyl salicylic acid was opted as reference drug.

The present study of anti-inflammatory activity of 80% methanol extract of *Leonotis ocymifolia* against carrageenan induced paw edema shows that the extracts have significant effect on inflammation and markedly reduced the swelling. The percentage protection in the paw volume in the group of animals treated with *Leonotis ocymifolia* extract 100mg, 200mg and 400mg 46.3%, 69.13 %, and 75.88% respectively at 6 hours. 200 and 400mg/kg of the LO showed higher anti inflammatory effect (%A) than that of 150mg/kg of Acetyl salicylic acid throughout the observation period, whereas 100mg/kg of LO had shown a comparable anti-inflammatory effect with 150mg/kg of Acetyl salicylic acid its percentage paw volume reduction was 65.35%. 80% methanol extract of the leaves of *Leonotis ocymifolia* showed a significant inhibition of paw edema that starts from 1 h ($p < 0.001$) and the effect lasted till 6 h post induction ($p < 0.001$ from 1^{st} - 6^{th} h) as compared to the negative control. However, the greatest inhibition was observed at 6h. The effect at this hour was found to increase in dose dependent manner ($R^2 = 0.916$).

In the carrageenan induced paw oedema model, 200 and 400mg/kg of the LO showed higher anti inflammatory effect (%A) than that of 150mg/kg of Acetyl salicylic acid throughout the observation period, whereas 100mg/kg of LO had shown a comparable anti-inflammatory effect with 150mg/kg of Acetyl salicylic acid

Other plants in the Lamiaceae family such as Leonotis leonurus (Ojewole.,2005;Maphosa *et al*. 2012) and *Leonotis neptifolia* (Vasuki *et al*., 2015) were also reported to have analgesic and anti-inflammatory activities. The aqueous extract of Leonotis leonurus with 50, 100 and 200mg/kg acetic acid model showed that decreases the number of writhes significantly ($p<0.05$) and exhibited a high inhibition of 96.2, 100 and 100%, respectively and also showed significant anti-inflammatory effect at 200 mg/kg with percentage protection of 86.4% at 3h (Maphosa *et al*. 2012). Leonotis leonurus produced

dose-dependent and significant ($p < 0.05$-0.001) antinociceptive effects against thermally and chemically induced nociceptive pain stimuli in mice (Ojewole.,2005).

Cotton pellet induced granuloma model is one of the most commonly employed models in animal research to screen for chronic anti-inflammatory activity of drugs and novel natural products (Singh *et al*., 2014). This model has been employed to assess the transudative and proliferative components of chronic inflammation (Sengar *et al*., 20 15). The weight of the wet cotton pellets correlates with exudative material and the weight of dry pellets correlates with the amount of granulomatous tissue. The suppression of proliferative phase could result in decrease in the weight of granuloma formation (Anuja *et al*., 20 14).

In this model, the transudative phase causes an increase in the wet weight of the cotton pellet while hosting inflammatory response to the implanted cotton between days 3 - 6 causes granuloma formation due to proliferation of fibroblasts and infiltration of modified macrophages and lymphocytes. Hence, the increase in dry weight is considered as a measure of proliferative component of chronic inflammation (Bagad *et al*., 2013). This model was, therefore, used for further verification of the anti-inflammatory activity of extract of *Leonotis ocymifolia* on the transudative and proliferative components of chronic inflammation. Steroidal antiinflammatory drugs were found to demonstrate higher activity in this model (Andrade *et al*., 2007 and Farias *et al*.), and hence dexamethasone was opted as a reference drug. dexamethasone treatment was the most effective, because of the drug's mechanism of action, which inhibits the inflammatory process in the very beginning, inactivating the enzyme phospholipase A_2 (Padilha *et al*., 2009).

In the cotton pellet induced granuloma model, all tested doses of the extract showed a statistically significant inhibition of both exudates and granuloma formation. In this model, the significant ($P < 0.001$) inhibitory effect of the LO on formation of exudates (Table 3) substantiates the finding of the carrageenan induced acute model, i.e. both findings solidify the effectiveness of the LO in inhibiting the exudative and transudative component of inflammation. The inhibitory values for 100, 200, and 400 mg/kg of the

extract were 28.91%, 37.68% and 45.91% for exudates and 24.03%, 40.18% and 50.65% for granuloma formation.

Flavonoids and saponins are well known for their ability to inhibit pain perception as well as anti-inflammatory properties due to their inhibitory effects on enzymes involved in the production of the chemical mediator of inflammation (Mollika *et al.,* 2014).Tannins and flavonoids show anti- inflammatory property by inhibiting arachidonic acid metabolism pathway and synthesis of prostaglandins (Mali *et al.,* 2013). Plants which have analgesic and anti-inflammatory activity contain mainly alkaloids, flavonoids, saponin, tannins phenolic compound, glycosides, coumarins and triterpenoids chemical constituents (Sengupta *et al.,* 2012).

The present study focused mainly on the phytochemical screening of major classes of phytochemicals with known analgesics and anti-inflammatory activities such as; saponins, alkaloids, flavonoids, tannins. Anti-inflammatory molecular mechanisms of flavonoids have been suggested to include: inhibition of pro-inflammatory enzymes, such as cyclooxygenase-2, lipoxygenase and inducible NO synthase, inhibition of NF- k B and activating protein-1 (AP-1) and activation of phase II antioxidant detoxifying enzymes, mitogen-activated protein kinase (MAPK), protein kinase C and nuclear factor-erythroid 2-related factor 2 (Serafini *et al*., 2010). Evidence for the anti-inflammatory properties of saponins has been provided by several studies using different models of pain and inflammation and NO inhibition has been observed with saponin secondary metabolites (Moharram and El-Shenawy, 2007 and Hassan *et al*., 2012). Tannins could affect the inflammatory response via their radical scavenging activities. Inducible nitric oxide synthase (iNOS) is produced in response to a variety of inflammatory cytokines as well as lipopolysaccharide (LPS). Tannin is responsible for its anti-inflammatory properties seen as the inhibition of iNOS in macrophages that have been activated with LPS (Jeffers, 2006).

The preliminary phytochemical analysis of the leaves of *Leonotis ocymifolia* 80% methanol extract was performed as per the standard qualitative methods. A series of chemical tests were carried out for presence of saponins, alkaloids, flavonoids, tannins,

terpenoids, phenols and the absence of anthraquinones, cardiac glycosides, steroids and sterols. The result of the current study is therefore; in agreement with a study conducted by Yeshitila, 2006.

Hence, the presence of alkaloids, saponin, flavonoids and tannins in the extract could be attributed for observed significant analgesic and anti- inflammatory activities (Kumar *et al.,* 2015 and Mali *et al.,* 2013). The present study has shown that the extracts of *Leonotis ocymifolia* leaf posses analgesic and anti-inflammatory activity. The analgesic and anti-inflammatory activity of *Leonotis ocymifolia* may be due to the presence of phytoconstituents like flavanoids, tannins, saponins and alkaloids as reported in phytochemical investigation. From the present results, it can be concluded that *Leonotis ocymifolia* possesses aanaalgesic and anti-inflammatory effect. However, further investigations are needed to determine the active chemical constituent(s) responsible for the analgesic and anti-inflammatory activities.

6. CONCLUSION

From the results observed from the current investigation, it is concluded that the 80% methanol extract of *Leonotis ocymifolia* suppress abdominal writhes, increase hot plate latency, suppress carrageenan-induced inflammation and cotton pellet induced granuloma chronic inflammation model. The current findings demonstrated scientific rationale for the traditional use of this plant in painful and inflammatory conditions. But the exact mechanism by which *Leonotis ocymifolia* exerts its anti-inflammatory and analgesic activity is not determined yet and needs further investigation to elucidate the active constituent(s) and underlying mechanism(s).

7. RECOMMENDATION

The results presented in this study regarding *Leonotis ocymifolia* should be taken as a basis for further investigation of its analgesic and anti-inflammatory action. Hence, further studies are needed

- To identify and purify the active constituent(s) responsible for the analgesic and anti- inflammatory effect
- To determine the exact mechanism of action of the active constituent(s)
- To determine the long term safety profile of the plant
- further pharmacological and toxicological studies should also be conducted to give a detailed profile of the compound screened from this plant for clinical use

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