Evaluation of analgesic and anti-inflammatory activities of 80% methanol extract of *Vernonia filigera* leaves in rodents.

A Thesis Submitted to the Department of Pharmacology and Clinical Pharmacy, School of Pharmacy, College of Health Sciences, Addis Ababa University in Partial Fulfillment of the Requirements for the Master of Science Degree in Pharmacology

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School of Graduate Studies

This is to certify that the thesis prepared by Abenezer Asfaw Laemengo, entitled "Evaluation of analgesic and anti-inflammatory activities of 80% methanol extract of *Vernonia filigera* leaves in rodents" 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 concerning originality and quality.

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Abstract

Evaluation of analgesic and anti-inflammatory activities of 80% methanol extract of *Vernonia filigera* leaves in rodents

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Addis Ababa University 2024

Background: Traditional medicine practitioners in Ethiopia mostly use medicinal herbs to treat pain and inflammation**..** In Ethiopian traditional medicine practice, *Vernonia filigera* is used to alleviate headache and inflammation. However, its analgesic and anti-inflammatory activities are not scientifically investigated yet.

Objective: The present study aims to evaluate the analgesic and anti-inflammatory activities of the 80% methanol extract of *Vernonia filigera* leaves using rodent models.

Methods: Eddy's hot plate and acetic acid -induced writhing test were used to assess the extract's central and peripheral analgesic efficacy. Carrageenan -induced paw edema and cotton pellet granuloma were used to test the extract's anti-inflammatory efficacy. Morphine 10 mg/kg, acetyl salicylic acid 150 mg/kg, indomethacin 10 mg/kg and dexamethasone 0.5 mg/kg were standard drugs for hotplate test, acetic acid -induced writhing test, carrageenan -induced paw edema and cotton pellet models, respectively. Three doses (100, 200 and 400 mg/kg) of the extract were chosen for each group based on the results of the acute toxicity test.

Results: In all the test groups, the crude extract and its fractions demonstrated statistically significant analgesic and anti-inflammatory activities compared to the control groups ($p<0.05$). The crude extract of *Vernonia filigeria* leaves at a dose of 2000 mg/kg did not show any signs of toxicity in the acute oral toxicity test. In the acetic acid induced model, the crude extract showed significant analgesic activity at all doses tested compared with the negative control. Its percentage inhibition of writing was 41.81, 53.62 and 64.53% at 100, 200 and 400 mg/kg, respectively. In

hot plate model, the crude extract at (400 mg/kg) showed analgesic effect (64.84%) which is highest of all treatment groups. In carrageenan induced paw edema, from the treatment groups, the highest edema inhibition (59.05%) was recorded 5 hours after administration of high dose crude extract (CE) (400 mg/kg) of the crude extract. The maximum percentage inhibition of exudate and granuloma formation was obtained by CE 400mg/kg (56.03% and 62.26%), followed by methanol fraction (MF) 400mg/kg (51.54% and 56.88%) and aqueous fraction (AF) 400mg/kg (50.15% and 51.47%), respectively.

Phytochemical screening of the extract showed the presence of tannins, alkaloids, flavonoids, and saponins in the plant extract.

Conclusion: The results of this study support the plant's traditional use by showing that the extract has significant analgesic and anti-inflammatory properties.

Keywords: Analgesic, Anti-inflammatory, *Vernonia filigeria*, 80% methanol extract, solvent fractions

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List of Abbreviations& Acronyms

AF	Aqueous fraction
ANOVA	Analysis of variance
ASA	Acetyl salicylic acid
CNS	Central nervous system
CE	Crude extract
COX	Cyclooxygenase
GRs	Glucocorticoid receptors
CGRP	Calcitonin gene related peptide
CNS	Central nervous system
COX	Cyclooxygenase
DRG	Dorsal root ganglion
EFMHACA	Ethiopian food, medicine and healthcare administration and control authority
5-HT	5-Hydroxytryptamine
IASP	International association for study of pain
IL	Interleukin
LD	Lethal dose
MF	Methanol fraction
NSAIDs	Non-steroidal anti-inflammatory drugs
OECD	Organization for economic cooperation and development
PAG	Periaqueductal grey matter
PG	Prostaglandin
SEM	Standard error of the mean
SPSS	Statistical package for social science
TGF –α	Transforming growth factor -α
TNF	Tumor necrosis factor
VF	Vernonia filigera

1. INTRODUCTION

1.1. Overview of Pain and Inflammation

Pain is a Greek word that denotes punishment. According to Plato, it originates from within the body, implying that pain is more of an emotional experience (Kumar & Elavarasi, 2016). It is a frequently occurring medical symptom that requires medical attention (Finnerup *et al.*, 2018). The major difficulties that are accompanied by defining pain are: (1) pain has always had a subjective nature; objectivity is also needed to standardize it; and (2) the compulsory linking of pain with "tissue damage," which, although denied, again focuses attention on a problem of or in the body as distinct from a problem with the body. (3) implied equivocation about its veracity as a form of distress when there is no obvious nociception. In 1979, the International Association for the Study of Pain (IASP) defined pain as "an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage (Cohen *et al*., 2018). Pain affects the whole world population, regardless of their sex, race, religion, economic status, and location. The main causes of pain are arthritis, cancer, operations and trauma, and spinal or back problems (Public & Priority, 2011). It has a beneficial role for the body to understand what is going on inside the body and to take the correct medical measures (Raffaeli & Arnaudo, 2017).

Inflammation is the body's reaction to harm to cells and tissues. as a result of a variety of circumstances, such as infections, chemicals, and thermal and mechanical injuries (Kaushik *et al*., 2012). The ancients defined inflammation by five cardinal indications based on visual observation: redness (rubor), swelling (tumor), heat (calor; solely applicable to the body's extremities), pain (dolor), and loss of function (functio laesa) (Punchard *et al*., 2004).

1.2. Classification of Pain and Inflammation

Pain is difficult to classify, but it can be categorized based on its duration, anatomical location, disease condition, type of patient, and intensity (Thienhaus, 2002). There are various classifications for pain, but the most important in terms of therapeutic application is the distinction between nociceptive and neuropathic pain. Nociceptive pain is defined as pain that is induced predominantly by nociceptor stimulation. If pain is induced by something other than a stimulus given to the nociceptor by generating impulses along the route close to the nociceptor (this could be a nociceptor in the nerve, the spinal cord, or the brain), it's known as neuropathic pain (Anaesth

& Rajagopal, 2016). Nociceptive pain is caused by noxious stimulation of the tissue's sensory endings, while neuropathic pain is caused by injury or disease to the neurons of the central or peripheral nervous system

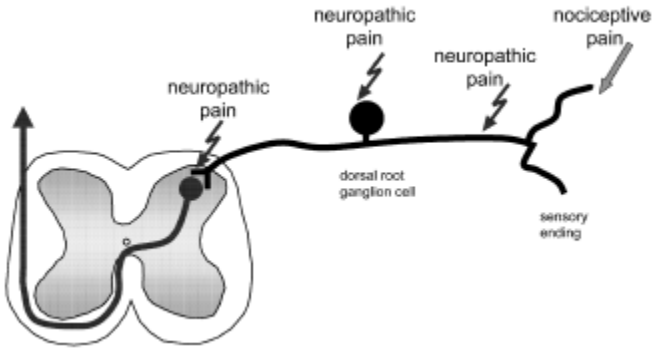

Figure 1 : Sketch of a nociceptive afferent with its synapse in the dorsal horn of the spinal cord (Schaible & Richter, 2004).

Noxious stimulation of the thermociceptor at its sensory end causes nociceptive pain. Pathological stimulation of the axon, the dorsal root ganglion (DRG), or neurons in the central nervous system causes neuropathic pain.

There are two types of pain based on duration: acute and chronic. Acute pain has a specific cause and takes a short time to alleviate (Eke & Briggs, 2019). Chronic pain occurs due to some chronic diseases that cause persistent pain and last more than 30 days (Anaesth & Rajagopal, 2016). The causal link between nociception and pain in many chronic pain states is shaky, and the pain does not reflect tissue damage. Rather, psychological and social factors appear to influence the outcome. In many situations of low back discomfort, for example, chronic pain can also be caused by a long-term illness. Might be the outcome of long-term nociceptive stimulation. processes. It's possible that it'll be accompanied by neuroendocrine symptoms. Dysregulation, weariness, dysphoria, and physical impairment are all symptoms of dysregulation as well as mental performance (Schaible & Richter, 2004).

Acute and chronic inflammation are two types of inflammation. Acute inflammation is defined by the exudation of fluid and plasma proteins (oedema) as well as the emigration of leukocytes, primarily neutrophils. Chronic inflammation is defined as inflammation that lasts for a long time (weeks or months) and is characterized by active inflammation, tissue destruction, and attempts at

repair all occurring at the same time. Chronic inflammation includes some of the most common and disabling human diseases, such as rheumatoid arthritis, atherosclerosis, tuberculosis, and chronic lung diseases (Achar *et al*., 2010).

1.3. Pathophysiology of Pain and Inflammation

Pain comprises different entities for its cause; these are cognitive, sensory, affective quality, and motivational (Kumar & Elavarasi, 2016). It is caused by mechanisms that either injure or have the potential to damage tissues. Such harmful impulses are referred to as "noxious" and are sensed by sensory receptors known as "nociceptors." There are several phases in the neural processing of noxious signals that might result in the sensation of pain. These are Transduction is the process by which unpleasant sensations are transformed into electrical signals in the nociceptors. Transmission is the second stage of the processing of noxious signals. Information from the periphery is relayed to the spinal cord, next to the thalamus, and lastly to the cortex. Modulation is the third and most significant part of noxious stimulus processing. This mechanism depicts changes in the nervous system in reaction to unpleasant stimuli, allowing noxious messages to pass through to be selectively received at the dorsal horn of the spinal cord, blocking signal transmission to higher centers. Modified. Descending modulatory systems: Activation of the descending system by endorphins occurs through specific receptors called ‘‘opioid receptors’’. These systems are activated in and around the periaqueductal gray (PAG) region of the midbrain. Such neurons then project to sites in the medullary reticular formation and the locus ceruleus the major source of serotonin and norepinephrine cells in the brain, respectively. through uncertain circuitry (probably through the disinhibit ion, that is, inhibition).

of a tonically active inhibitory interneuron) (Vanderah, 2007) C-fibers and Ad-fibers are two types of nociceptors. Nociceptors are sensory receptors that respond only to noxious stimuli. These nociceptors are nerve endings that originate in the dorsal root ganglia and terminate in the superficial layer at the dorsal horn of the spinal cord's They are relaying information such as glutamate, substance P, and calcitonin gene-related peptide (CGRP), and other neurotransmitters are released to send messages (Jeftinija *et al*., 1991;Lawson et al., 1997;Lawson *et al*., 2002).These "pain" neurotransmitters activate second-order neurons by binding to their receptors. The second-order neuron crosses the spinal cord to the contralateral side and travels up the spinothalamic tract

until it reaches the thalamus." The third-order neuron is then triggered and travels from the thalamus to the somatosensory cortex, allowing pain experience. Second-order neurons in the spinal cord cause direct activation of lower motor neurons in the ventral horn of the spinal cord, causing a reflex withdrawal from the area of noxious stimulus. Similarly, there are interneurons at the spinal cord level that moderate incoming pain input (Vanderah, 2007). Nonmyelinated fibers with a conductivity of 0.5 to 2 m/sec are known as C-fibers. Mechanical, thermal, and chemical stimuli all cause noxious information to be transmitted through nociceptive C fibers. As a result, they are called C-polymodal nociceptors. A-delta fibers are myelinated fibers with a conductivity of 2 to 20 m/sec. High-threshold mechanoreceptors refer to fibers that respond to high-intensity mechanical stimulation. Some, but not all, A-delta fibers also respond to thermal stimuli; they are referred to as mechanothermal receptors (Kopf & Patel, 2010).

Mechanism of pain sensation

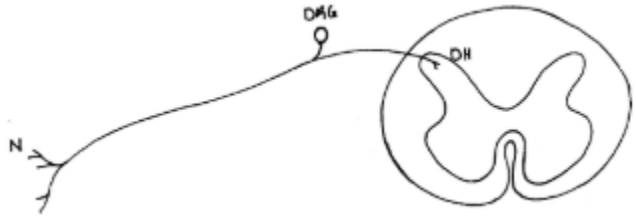

Figure 2: depicts an oversimplified pain pathway from the periphery up to the dorsal horn of spinal cord (Anaesth & Rajagopal, 2016).

The peripheral bare nerve ending subserving pain is called the nociceptor. An electrical impulse is generated when the nociceptor is stimulated mechanically, thermally, electrically, or chemically. and transmitted up the peripheral nerve via A-delta and C fibers to the dorsal horn of the spinal cord. In the dorsal horn cells, the impulses get modified before onward transmission to the thalamus and the cerebral cortex, where the pain is appreciated as stimuli normally, but are activated in the inflamed tissues. Once they are recruited, the same degree of peripheral stimulus generates a greater number of electrical impulses, thereby resulting in the worsening of pain (Fig. 2) (Anaesth & Rajagopal, 2016).

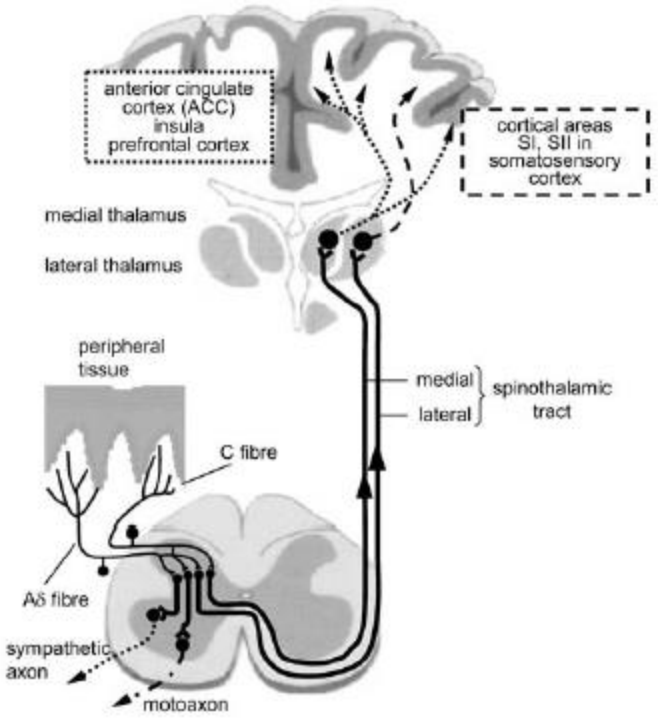

Figure 3: Scheme of the nociceptive system, showing afferent nerve fibers and their synapses in the dorsal horn of the spinal cord, and nociceptive free nerve endings in the peripheral tissue (Schaible & Richter, 2004**).**

From there the medial and lateral spinothalamic tracts ascend to the medial and lateral thalamus, and interneurons project into motor and sympathetic reflex pathways
(Anaesth & Rajagopal, 2016).

The causes, and physiological and pathological outcomes, of inflammation are described in Figure 4 below.

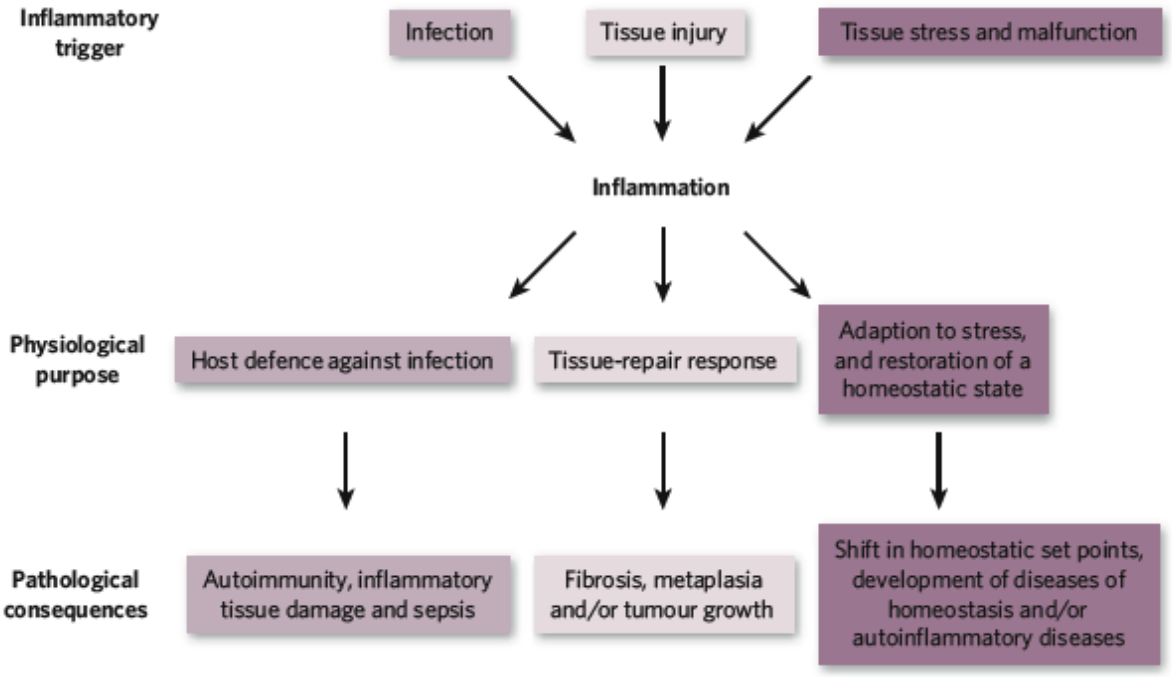

Figure 4: Causes, and physiological and pathological outcomes, of inflammation (Medzhitov, 2008).

The inflammatory response, triggered by infection, involves the production of pro-inflammatory mediators like NO and PGE2, as well as cytokines like TNFα (Sheeba & Asha, 2009). Lipopolysaccharide (LPS), an endotoxin in gram-negative bacteria's outer membrane, controls these mediators, activating innate immunity and causing different physiological purposes and pathological consequences (Liu *et al*., 2007)

Inflammation is histologically characterized by the dilatation of the vascular components, that is, arterioles, capillaries, and venules, associated increase in permeability and blood flow exudation of fluids this activation and release of immunologically active initiators causes activation of hormonal response mechanisms by the leukocyte migration to the inflammatory focus (Stanley & Weaver, 1998).

1.4. Epidemiology of Pain and Inflammation

In communities all throughout the world, pain is regarded as a major clinical, social, and economic issue. Because of, the subjective character of the symptoms and a lack of consensus on diseases with specific diagnoses and definitions of the subject, describing the epidemiology of pain is difficult (Henschke et al., 2015). Low back pain, headaches, and abdominal pain are the most prevalent reported ailments in children and adolescents (Calvo-Muñoz *et al*., 2013) (Swain *et al*., 2014). Globally, it is estimated that one out of every five adults suffers from pain, with another one out of every ten adults being diagnosed with chronic pain each year. Cancer, osteo- and rheumatoid arthritis, operations and traumas, and spinal issues are the four most common causes of pain, making the genesis of pain a difficult, multidisciplinary endeavor (Public & Priority, 2011). Persistent pain was widespread among primary care patients in a variety of settings around the globe. The prevalence of persistent pain for all centers combined was 21.5%, with prevalence rates ranging from 5% to 33% (Gureje *et al.*, 1998). According to a study at the Gondar hospital on adult HIV patients complaining about pain, that gave a 51.2% (46.4%–55.9%) prevalence of pain. The parts of the body commonly involved were the head 47 (17.9%), followed by the abdomen 41 (15.6%), and the back 35 (13.3%) (Azagew *et al*., 2017). According to a study on patients with Parkinson's disease in Addiss Ababa, 84% had symptoms of pain. The prevalence of pain in Parkinson's disease patients in Ethiopia is among the highest in the world and is under recognized and undertreated (Hirsi *et al*., 2019). According to research on the prevalence of low back pain, Low back pain is the most common musculoskeletal ailment, affecting people from all socioeconomic groups around the world. Low back pain (LBP) affects roughly 60%–80% of people at some point in their lives. The 12-month prevalence of LBP varies from 3.21% in office workers to 82.93% in nurses. The mean annual prevalence of LBP was 50.69% ± 17.75. (Jegnie & Afework, 2021).

Crohn's disease and ulcerative colitis are examples of inflammatory bowel diseases (IBD), which are conditions characterized by persistent inflammation of the gastrointestinal tract and remissions and relapses(Ponder & Long, 2013). A major global health and economic burden, inflammatory bowel disease (IBD) dramatically reduces the quality of life for those who have it. IBD is thought to affect about 3,000,000 people in the USA and Europe, and it is more common than 0.3% in

North America, Oceania, and numerous European nations. There were over 6.8 million IBD cases worldwide in 2017(Alatab et al., 2020).

1.5. Management of Pain and Inflammation

Pain must be treated because it causes suffering and muscle spasms, untreated pain will keep getting worse, causes neuroanatomical re-organization, Anatomical and genetic changes (Anaesth & Rajagopal, 2016). Various pain management protocols have been developed, including pharmacologic and non-pharmacologic approaches. And they are now commonplace, particularly in the developed world (Eke & Briggs, 2019). Non-pharmacological therapies are divided into two categories: 1. Physical (sensory) interventions. Physical (sensory) interventions are typically patient-specific and block nociceptive input and pain perception. Massage, positioning, heat and cold treatment, transcutaneous electrical nerve stimulation (TENS), acupuncture, and progressive muscle relaxation are some methods for reducing pain intensity and improving patient quality of life. 2 Psychological interventions: Continuous pain can lead to the development of maladaptive states and behaviors, which can wreak havoc on daily functions, increase distress, and heighten pain perception. Cognitive behavioral therapy, mindfulness-based stress reduction, acceptance and commitment therapy (ACT), guided imagery, and biofeedback are the most often utilized psychological interventions. 3. Others, music therapy is also used for in pain management (Hassan *et al*., 2012)

Strong opioids, mild opioids, and non-opioids are the three types of analgesics. Antidepressants and anticonvulsants, as well as N-methyl-D-aspartate (NMDA) receptor antagonist and cannabinoid drugs, are occasionally utilized as adjuvants for the treatment of persistent neuropathic pain and/or pain caused by neuralgia (Abdool *et al*., 2020).

Non-opioid analgesics, such as paracetamol, aspirin, and NSAIDs, is mediated centrally and may involve direct and indirect suppression of central cyclooxygenases (COX), activation of the endocannabinoid system, and spinal serotonergic pathways (Graham & Davies, 2013). COX inhibition and the subsequent lowering of prostaglandins in tissue are how these drugs work (Enamandram et al., 2018) (Yamamoto, 1996). Opioids' analgesic effects are mediated via direct inhibition of pain-transmitting neurons and opioid-receptor-mediated activation of pain-inhibitory

neurons in the central nervous system (Bannister *et al*., 2017). Both acute and chronic pain can be effectively treated with opioids (A. Kumar *et al*., 2017). Neuropathic pain can be effectively treated with tramadol, an opioid receptor agonist and serotonin (HT)-noradrenalin reuptake inhibitor, especially that of the peripheral nervous system (O'Connor & Dworkin, 2009) (D'Mello & Dickenson, 2008).Tapentadol is a novel painkiller that combines an opioid receptor agonist with a noradrenalin reuptake inhibitor that lacks the pain-facilitating activity of elevated serotonin, secondary to serotonin reuptake inhibition (Bee et al., 2011). (Martini *et al*., 2015). Antidepressants boost endogenous serotonin levels (Costigan *et al*., 2009), while anticonvulsants are effective in the treatment of lancinating pain (Bennett & Woods, 2014). NMDA receptor antagonists are neurotransmitters that are released in the spinal cord, affecting central sensitization (Collins *et al*., 2010).

Non-steroidal anti-inflammatory drugs (NSAIDs), like aspirin, are frequently used to treat inflammatory illnesses.Corticosteroids or even disease-modifying anti-rheumatic medications are sometimes used in an attempt to reduce other symptoms of the disease (Punchard *et al*., 2004). NSAIDs prevent the enzyme cyclooxygenase, which is also found in the gastric mucosa. Due to the discovery that the cyclo-oxygenase (COX2) in inflammatory lesions differed from the COX1 in the stomach, specific COX2 inhibitors, like celecoxib, were developed. These medications relieve a lot of arthritic symptoms, but they hardly ever lead to stomach ulcers (Hawkey *et al*., 2001).

1.8. Traditional Medicine in the Management of Pain and Inflammation

The medicinal use of plants as analgesic drugs in folk medicine is an ancient tradition, far older than the current sciences of medicine in developing countries (Ullah *et al*., 2015). According to estimations, up to 70,000 plant species are used ethno medicinally (Haq *et al*., 2012). About 80% of the global population utilizes medicinal plants of family (Lamiaceae) as analgesic or anti-nociceptive agents (Uritu *et al*., 2018). Plant materials containing acetyl salicylic acid (ASA) have been traditionally used since 400 BC for the treatment of pain and fever (Rao & Knaus, 2008). Aspirin, an anti-inflammatory drug, is synthesized from salicin, a naturally occurring substance. It is generally considered to have been first isolated from the bark of the willow tree, *Salix alba*

*(*Dias *et al*., 2012). Aspirin was really named after the glycoside of methyl salicylate known as spiraein, which was discovered from Spiraea ulmaria L. (now known as Filipendula ulmaria (L.) Maxim.) (Van Wyk & Gorelik, 2017*).*

1.9. Overview of *Vernonia filigera*

William Vernon, an English botanist who collected and identified this genus in Maryland in the late 1600s and died in 1711, is the name of the genus Vernonia. (Quattrocchi, 1999). Vernonia (Asteraceae) is the largest genus in the tribe Vernoniae, with close to 1000 species.). Numerous species in the genus Vernonia are used in food, medicine, and industry. Vernonia colorata and Vernonia amygdalina, for instance, are eaten as leafy vegetables. (Burkill, 1985). Vernonia filigera is native to Ethiopia and widely distributed. It is shrub or rarely herb, 1-2 5(-4) m high with a thick rootstock, stem branches, stout, erect, densely leafy, terete, striate, minutely ashen-tomentose. Leaves alternate, petiolate, membranous, ovate-oblong -elliptical or lanceolate, grey green above with boary mid vein, pale grey-whitish with dense sessile resinous gland(Tadesse, n.d.). In Ethiopia, the local name of *Vernonia filigera* plant is Rejicho in the Sidammu Afu and Daba Keded in the Amharic languages. Traditionally, it is used for the relief of leg pain and inflammation (mich) by sprinkling powder on burning charcoal and smoke is inhaled nasally. *Vernonia filigera* (VF) Oliv & Hiern is also mostly utilized in ethno-veterinary medicine for the treatment of trypanosomiasis (Tefera & Kim, 2019,Teklehaymanot, 2009). As the most researched chemicals in the genus, the vernolides are a class of sesquiterpene lactones that exhibit intriguing bioactivity in tests for cytotoxicity, antibacterial, anti-inflammatory, anti-leishmanial, and antiplasmodial effects (Toyang & Verpoorte, 2013).In phytochemical experiments, the pharmacologically important sesquiterpene lactones vernolepin (1) shown a competitive antagonistic effect against histamine (2) a biphasic enhancement/inhibition of coaxial stimulation of guinea pig ileum; (3) an antiaggregating and disaggregating effect against rabbit platelet aggregation caused by arachidonic acid without inhibition of cyclc-oxygenase or lipoxygenase.(Weigensberg *et al*., 1984).and A set of East African Vernonia species are known to produce unique secondary metabolites, and from their above-ground parts, vernodalin has been isolated (Abegaz *et al*., 1994).

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Figure 5: Picture of *Vernonia filigera*

1.10 Rationale of the Study

Pain has a significant impact on people's daily lives. The negative impacts of pain include difficulty doing daily tasks, frequent prescription medication use, health-care costs, disability, and the burden of unemployment (Turk, 2002). The use of NSAIDs is linked with a range of side effects, including gastrointestinal irritation and ulcers, changes in renal function, blood pressure effects, hepatic damage, and platelet inhibition, which can lead to increased bleeding (Keeley, 2016).On the other hand, side effects from corticosteroids can vary depending on the dosage and length of treatment. Increased gluconeogenesis and improved catabolic action on muscle, skin, lymphoid, adipose, and connective tissue are the effects of their influence on the metabolism of

carbohydrates, lipids, and proteins. Steroid use can lead to many complications such as physical weakness, mental responses, hypertension, osteopenia, and the emergence of latent diabetes mellitus(Paradkar, 2019). Furthermore, steroidal anti-inflammatory drugs can cause immune suppression by disrupting cytokine networks involved in lymphocyte function (Barrison & Wolfe, 1999). There has been growing issues in the treatment properties of traditional medicines due to increasing problems associated with allopathic medicines, including the emergency of bacterial resistance, poor effectiveness of some drugs, and harmful side effects that are associated with many synthetic drugs (Khumalo *et al*., 2022).

Currently, modern anti-inflammatory drugs are associated with some severe side effects. Therefore, the development of potent anti-inflammatory drugs with fewer side effects is necessary. *Vernonia filigera* has long been used to treat pain and inflammation, but no scientific studies have been conducted to back up this traditional claim. The purpose of this study will be to investigate anti-inflammatory and analgesic effects of *Vernonia filigera*.

2. OBJECTIVES

2.1. General Objective

To evaluate analgesic and anti-inflammatory activities of 80% methanol extract of *Vernonia filigera* leaves in rodents.

2.2. Specific Objectives

- ✓ To determine acute oral toxicity of the 80% methanol leaf extract of *Vernonia filigera* leaves,
- ✓ To evaluate analgesic activity of the leaf crude extract and fractions of *Vernonia filigera* using an acetic acid-induced writhing test in mice,
- ✓ To evaluate the analgesic activities of the 80% methanol leaf crude extract and fractions of *Vernonia filigera* using the hot plate method,
- ✓ To investigate the anti-inflammatory activities of the 80% methanol leaf crude extract and fractions of *Vernonia filigera* using carrageenan-induced paw edema,
- ✓ To investigate the anti-inflammatory activities of the 80% methanol leaf crude extract and fractions of *Vernonia filigera* using cotton pellet-induced granuloma,
- ✓ To investigate secondary metabolites of the leaf extract of *Vernonia filigera* through preliminary phytochemical analysis

3. MATERIALS AND METHODS

3.1. Chemicals and Instruments

Materials and instrument that were used during the study are; Rotary evaporator (Heidolph, Germany), 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), cotton pellets, flasks, separatory funnel , syringes with needles, suturing set, blunt forceps, scissors, feeding tube , nylon and gauze Whatman filter paper no. 1., hot plate, chromic catgut (0/4 metric-1/2 circle), glove, aluminum foil.

The main chemical and drugs that were used during the study are; Morphine (Martindale pharma, UK) Indomethacin and Acetylsalicylic acid (Reckitt Benckiser Pharmaceutical ,South Africa) ,Methanol from (Carlo Erba, Italy), Carrageenan (Sigma Aldrich, Germany), diethyl ether, Dexamethasone (Medico Labs, Lot E6A00, Syria), Distilled water (Ethiopian pharmaceutical Manufacturing Factory, Ethiopa) ,Glacial acetic acid from (Fisher Scientific, UK), 1%, Hexane, Chloroform, 1 %, Ethyl acetate (Finkem Laboratory Reagent, India**)**.

3.2 Plant Material

3.2.1. Collection and Authentication

The leaves of *Vernonia filigera* were collected from Leku town located in the Northern Sidama zone of Sidama region, 298 km south of Addis Ababa. Identification & authentication of the plant specimens were performed by a taxonomist and voucher specimen (AA001) were deposited for the future reference at the National Herbarium, College of Natural and Computational Sciences, Addis Ababa University, Addis Ababa, Ethiopia.

3.2.2. Preparation of the Extract

The leaves of *Vernonia filigera* were air dried under the shade at room temperature and pulverized using a mortar and pestle into a coarse powder. The 400 g of plant powder was macerated for 72 hours (three times) at 120 rpm with frequent shaking using a mini orbital shaker to extract 80% methanol. The resultant liquid was then filtered with nylon and gauze before being passed through (Whatman No. 1 filter paper). After the extraction, methanol was evaporated under vacuum using Rota vapor at 40°C. The remaining solvent (water) was removed using hot oven at 40°C. The

powdered extract was then packed into a glass vial, properly labeled, and stored at 4°C in the refrigerator until used in bio-screen investigation.

The percentage yield of the plant extract was computed as follows-

$$\% \text{ Yield} = \frac{\text{Weight of extracted material}}{\text{Weight of original plant material used}} \times 100$$

$$\% \text{ Yield} = \frac{(93\ g)}{(400\ g)} \times 100 = 23.25\%$$

3.2.3. Fractionation

The extract (20g) was dissolved in 250 ml of water, and using a separating funnel, partitioning was carried out and washed many times until a clear solvent was obtained in the mixture with a number of organic solvents in ascending polarity order, including hexane, chloroform, ethyl acetate, methanol, and water. The methanol fractions of the plant extract were dried by evaporating methanol using a rotary evaporator and other fractions were dried in a hot oven set at 40°C. Until further investigation, all extracts were kept in a 4°C refrigerator. There was extracted mass remaining after removing the solvents hexane, methanol, and water. Unfortunately, very little amount was obtained from ethyl acetate and chloroform.

3.3 Experimental Animals

Healthy both sexes Swiss albino mice (8–12 weeks, weighing 25–35 grams) and, male and female rats weighing 180–220g were obtained from the animal house of the School of Pharmacy, Addis Ababa University, and/or or purchased from the animal unit of the Ethiopian Public Health Institute were used in this study. The animals were maintained under standard laboratory conditions (room temperature with a 12-hour light-dark cycle) and provided with standard animal feed and water *ad libitum*. Prior to the experiment animals were acclimatized to the laboratory conditions for 7 days. Every animal used in this study were handled in accordance with the internationally accepted standard guidelines for the use of animals in research (OECD 2008) (Committee *et al*., 2010; Carbone, 2012). Moreover, ethical approval was acquired from the School of Pharmacy's Ethics Committee with a reference number of ERB/SOP/464/14/2022 (Annex: 1).

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3.4 Acute Toxicity Study

For acute toxicity testing, the Organization for Economic Co-operation and Development's (OECD) guideline 425/2022 was adhered to (OECD, Test No. 425: Acute Oral Toxicity: Up-and-Down Procedure, OECD Publishing, 2022). (Sewell et al., 2023). For the toxicity study fasted female albino mice aged 6–8 weeks were used. First, a sighting study was performed to determine the starting dose, in which a single female mouse received 2000 mg/kg of the extract as a single dose by oral gavage. In a 24-hour period, no deaths were noted; four more mice were employed and given the same dose of extract. The animals were observed continuously for 4 hours with a 30-minute interval and then for 14 consecutive days with an interval of 24 hours. The general toxicological signs and symptoms included alterations in the eyes and mucous membranes, skin and fur; changes in somatomotor activity and behavior; diarrhea and salivation, weight loss, tremor and convulsions, paralysis and lethargy, changes in food and water intake; and mortality, were observed.

3.5 Animal Grouping and Dosing

Swiss albino mice weighing 25–35 g and rats weighing 180–220 g of either sex were randomly divided into fourteen groups of six mice/rats per group. Group I received vehicles and was designated as the negative. The group II was served as positive control and was treated with standard drugs; morphine (10 mg/kg) for hot plate test, indomethacin (10 mg/kg) for carrageenan induced paw edema test, aspirin (150 mg/kg) for acetic acid induced writhing test and dexamethasone (0.5 mg/kg p.o.) for cotton pellet induced granuloma. The crude extract and fractions were given orally to the other test groups (III- XIV) at varying doses (100, 200, and 400 mg/kg). Pilot studies and an acute toxicity test were used to select the dose. All administrations were performed orally using an oral gavage.

3.6. Pilot Study

Three groups were formed, each containing six rats (utilized in the carrageenan-induced paw edema) and six mice (used in the acetic acid-induced writhing test) with an equal ratio of male and

female participants, as part of a pilot study to test the pharmacological activities of *Vernonia filigera*. Two animals per group received the crude extract at doses of 100, 200, and 400 mg/kg for each of the three dose levels. The findings of the study support the idea that *Vernonia filigera* has analgesic and anti-inflammatory properties. The maximum percentage of analgesic effect for the acetic acid model was 42.3% for 100 mg/kg, 48.36% for 200 mg/kg, and 58.24% for 400 mg/kg. Additionally, the crude extract of *Vernonia filigera* had an anti-inflammatory effect at all dosages used. The highest levels of paw edema inhibition measured at different dose levels were 49.2% for 100 mg/kg, 53.49% for 200 mg/kg, and 56.67% for 400 mg/kg.

3.7 Evaluation of Analgesic Activity

3.7.1 Acetic acid induced writhing method

According to (Hayfaa *et al*., 2013), the analgesic activity of *V. filigeria* leaves extract was evaluated in laboratory animals by causing pain with acetic acid. Fourteen groups of experimental animals were formed, each with six mice and set to receive: (1) vehicle DW 10 ml/kg delivered orally; (2) ASA 150 mg/kg delivered by oral route; (3) *V. filigeria* leaves crude extract, hexane, methanol, and aqueous fractions each with 100, 200, and 400mg /kg doses. Animals were placed separately in a cage and allowed to acclimate for at least 10 minutes. Mice were given distilled water, acetylsalicylic acid, leaves crude extract, hexane, methanol, and aqueous fractions each with 100, 200, and 400mg doses 1 hour prior to induction of visceral pain. An intraperitoneal injection of a glacial acetic acid solution in normal saline at a 0.6% volume per volume (10 mL/kg) induced visceral pain. After being put back into their cages, the animals were watched for writhing, which was identified by the abdomen and at least one hind limb being stretched simultaneously. The number of writhing responses was counted for 15 minutes, starting directly after the acid injection. The presence of analgesia was demonstrated by a decrease in the number of writhes in comparison to the control group. This was stated as a percent inhibition of writhing, which was computed using the following formula.:

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

3.7.2 Hot plate method

The mice were placed on a hot plate maintained at 55 °C within the restrainer (Fan *et al*., 2014). Reaction times were recorded at different times to the mice's fore- and hind paw licking or jumping following oral administration of the crude extract, hexane, methanol, and aqueous fractions each with 100, 200, and 400mg, morphine (10 mg/kg, s.c.), or vehicle (DW 10ml/kg). Each mouse's latency time, or reaction time (measured in seconds), on the hot plate was recorded at intervals of 30, 60, 90, and 120 minutes following administration of the corresponding agent. (Dash *et al*, , 2015). A 15-second cut-off period was followed to prevent paw harm (Yimer *et al*., 2020). The percentage increase in reaction time or pain threshold inhibition was calculated using the formula

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

3.8 Evaluation of anti-inflammatory activity

3.8.1 Carrageenan-induced rat paw edema

By using the Yimer *et al*., 2020 approach, the acute anti-inflammatory activity was assessed. Rats were starved for the entire night and had free access to water were used in this procedure to induce acute inflammation in their paws. Carrageenan (1% w/v carrageenan in normal saline, 0.05ml) was injected into the mice's right hind paw to induce acute inflammation. Before the induction of inflammation, the paw will be marked with ink at the level of the lateral malleolus. Carrageenan was injected one hour after oral administration of the crude extract, hexane, methanol, and aqueous fractions each with 100, 200, and 400mg, the vehicle, and the standard drug indomethacin (10 mg/kg) with the respective groups of mice. Inflammation was measured in terms of ml, i.e., displacement of water by edema, using a digital plethysmometer at times 0, 1, 2, 3, 4, 5 and 6 after carrageenan injection. The percentage inhibition of edema was determined in comparison to the control mice using the formula.

$$\text{\% Edema inhibition} = \frac{\text{PEC} - \text{PET}}{\text{PEC}} \times 100$$

Where; PEC paw edema in control group PET paw edema in test group

3.8.2 Cotton pellets-induced granuloma

Albino Wistar rats (180-220 g) were fasted for 12 h allowed free access to water until commencement of the experiment. Rats were given the crude extract, hexane, methanol, and aqueous fractions each with 100, 200, and 400mg, negative control, and standard dexamethasone (0.5 mg/kg p.o.). After a cotton piece weighing 10 mg was rolled, it was autoclaved for 30 minutes at 120 °C and 15 pounds of pressure to create sterile cotton pellets with a weight of 10 ± 1 mg. The rats were given diethyl ether anesthesia twenty minutes after receiving the standard drug and extracts. A subcutaneous tunnel was then aseptically created in each rat's both sides of the previously shaved groin area using blunted forceps. Two sterilized cotton pellets weighing 10±1 mg each were then implanted bilaterally in the subcutaneous tunnel and sutured with chromic catgut (0/4 metric-1/2 circle). Treatment with the standard drug (dexamethasone), crude extracts and fractions was continued for seven consecutive days (p.o., once a day). The animals were sacrificed with ether anesthesia on the 8th day, and cotton pellets were removed surgically and made free from extraneous tissues. Following removal, the cotton was weighed wet and dried at 60°C for 24 hours, and the net dry weight, that is, after subtracting the weight of the cotton pellets, was calculated (Afsar *et al*., 2013). 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

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

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

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.

3.9 Preliminary phytochemical screening

Secondary metabolites perform a variety of defensive roles in the human body, including strengthening the immune system, shielding the body from free radical damage, eliminating harmful bacteria, and much more They also help to maintain physical fitness. Alkaloids, tannins,

flavonoids, and phenolic compounds are the most bioactive secondary metabolites(Anulika NP. *et al*., 2016). The literature states that: Alkaloids are analgesics, like morphine; Flavonoids have antioxidant, anti-carcinogenic, anti-allergic, anti-inflammatory, and anti-viral properties; Phenols have biological properties that include antioxidant, anti-inflammatory, and anti-carcinogenic effects; plants containing tannins are used as astringents to prevent diarrhea; and glycosides have biological properties that include hemolytic, antimicrobial, and antioxidant(Justin *et al*., 2014)

The 80% methanol extracts of *Vernonia filigera* leaf extracts were subjected to a qualitative phytochemical screening process to determine the presence of active components such as glycosides, saponins, alkaloids, flavonoids, phenols, and tannins. Standard operating procedures were followed (Pooja & Gm, 2016).

Test for Alkaloids:(Hager's Test): A few drops of Hager's reagent (saturated picric acid solution) were added to 2 mg of the extract that was taken in a test tube. The presence of alkaloids was established by the formation of a yellow precipitate(Pooja & Gm, 2016).

Test for Anthraquinones: Borntrager's Test About 50 mg of powdered extract was heated with a 10% ferric chloride solution and 1 ml of concentrated HCl. The extract was cooled and filtered, and the filtrate was shaken with diethyl ether. Strong ammonia was used to extract the ether extract further, and the presence of anthraquinone was indicated by the aqueous layer's pink or deep red colors (Tyagi, 2017).

Test for Cardiac Glycosides: Keller-Killiani Test 2ml of extract was treated with 1ml of glacial acetic acid, 1 drop of 5% FeCl3 and Conc. H2SO4 and the observed for the formation of reddish brown color appears at junction of the two liquid layers and upper appears bluish green indicates the presence of Cardiac glycosides(Sasikumar *et al.*, 2015**).**

Test for Flavonoids: 2 ml of each extract was added with few drops of 20% sodium hydroxide, formation of intense yellow color is observed. To this few drop of 70% dilute hydrochloric acid were added and yellow color was disappeared. Formation and disappearance of yellow color indicate the presence of flavonoids in the sample extract(Mital & Jha, 2021)

Test for Phenols: Ferric chloride test: Extracts were treated with 3-4 drops of ferric chloride solution. Formation of bluish black color indicates the presence of phenols(Tyagi, 2017)

Test for Saponins: Foam test: A drop of solution containing sodium bicarbonate was put into a test tube that held approximately 5 ml of extract. After giving the test tube a good shake, it was left for 3 minutes. The appearance of foam resembling honeycomb suggested the presence of saponins(Sasikumar *et al*., 2015)..

Test for Steroids and Triterpenoids: Liebermann-Burchard's test: After dissolving 2 mg of dry extract in acetic anhydride, heating the mixture to boiling, cooling it down, and then adding 1 ml of pure sulfuric acid along the test tube's edges, Steroids were identified by the formation of a green color, while triterpenoids were indicated by the formation of a violet-colored ring(Pooja & Gm, 2016)..

Test for Tannins: Ferric chloride test: A few drops of a 5% w/v FeCl3 solution were added to 1-2 ml of the extract. Galactotannins were represented by a green color, whereas pseudotannins were shown by a brown color (Pooja & Gm, 2016).

3.10. Data analysis

The Statistical Package for Social Science (SPSS) Windows version 26 was used to analyze the data. The results of the study were expressed as mean ± standard error of the mean (SEM). To analyze differences among the groups, a one-way analysis of variance (ANOVA) was employed. Multiple comparison tests with Tukey's were used to perform subgroup analysis. A one-way analysis of variance (ANOVA) was used to analyze differences among groups. Subgroup analysis was done using Tukey's multiple comparison tests. P-values of less than 0.05 will be considered statistically significant. Graphs and tables were used to present the analyzed data.

4. RESULTS

4.1 Acute toxicity test

At a dose of 2000 mg/kg *Vernonia filigera* leaf crude extract did not result in any significant behavioral changes, harmful consequence, or death over the first 24 hours or the next 14 days. It is reasonable to assume that the oral lethal dose in 50% (LD50) of the crude extract in mice is greater than 2000 mg/kg, as indicated by OECD Guideline 425 (2008), which sets the limit test at 2000 mg/kg. Consequently, the findings suggest that *Vernonia filigera* leaf extract has low toxicity.

Three treatment doses were chosen based on the acute toxicity test: a middle dose that was one-tenth of the maximum dose obtained during the acute toxicity study (200 mg/kg); a low dose was half of the maximum dose obtained during the acute toxicity study (100 mg/kg); and a high dose was double of the highest dose acquired from the acute toxicity study (400 mg/kg) (Latha *et al*., 2010).

4.2 Analgesic activity of *Vernonia filigera*

4.2.1 Analgesic effects against acetic acid-induced writhing test

The crude extract, n-hexane fraction, the methanol fraction (MF), and the aqueous fraction (AF) of *Vernonia filigera* produced significant analgesic activity ($p<0.001$) as compared to the negative control group in the acetic acid-induced writhing test, as shown in Table 1. According to the percentage of analgesic activity of Vernonia filigera crude extract and its fractions in the acetic acid-induced writhing model presented in figure 6 below, maximum analgesic activity was observed in the standard group (ASA 150 mg/kg, 69.55%). Beside the standard group, the high dose of the crude extract (400 mg/kg) of the plant Vernonia filigera leaf extract produced higher analgesic activity (64.53%) as compared to the other groups. The analgesic activity within the group increased with the increasing dose, from a lower dose to a higher dose. The hexane fraction, methanol fraction, and aqueous fraction groups show comparative analgesic activity

Table 1: The effects of Vernonia filigera leaf crude extract, n-hexane, methanol and aqueous fractions against acetic acid induced writing.

Treatment groups	Number of writhing (Mean± SEM)	% Analgesic Activity
ASA 150 mg/kg(b)	11.16±2.22 a2c1	69.55
DW 10 ml/kg (a)	36.66±1.54	
CE 100 mg/kg(c)	21.33±1.97 a2 (be)1	41.81
CE 200 mg/kg(d)	17±2.44 a2	53.62
CE 400 mg/kg(e)	13±0.73 a2 c1	64.53
HF 100 mg/kg(f)	30±1.03 (b c de)2	18.16
HF 200 mg/kg(g)	27.83±1.35 a1 (b d e)2	24.08
HF 400 mg/kg(h)	23.83±2.22 (ab e)2	34.99
MF 100mg/kg(i)	29±1.39 a1 b2 c1(de)2	20.89
MF 200 mg/kg(j)	25±1.06 (a2 b)2 d1 e2	31.8
MF400 mg/kg(k)	22.66±1.2 (a b e)2	38.18
AF100 mg/kg(l)	31.16±1.13b2 c1(d e)2	15
AF 200mg/kg(m)	29±0.96 a1 b2 c1(d e)2	20.89
AF 400 mg/kg(n)	25.66±0.88 (ab)2 d1 e2	30

DW= Distilled water(a), CE (100, 200, 400) = Crude extract (c, d, e), HF (100, 200, 400) =Hexane fraction (f, g, h), MF (100, 200, 400) =Methanol fraction (i, j, k), AF (100, 200, 400) =Aqueous fraction (l, m, n). Data is stated as Mean ± SEM of observations and all groups compared to the negative control group and each other. [1]$p<0.05$, [2] $p<0.001$

4.2.3 The effects of *Vernonia filigera* extracts and its fractions on elongation of the latency period in the hotplate model

All groups in the hot plate test, *Vernonia filigera* leaf extract, and fractions of the extract showed significant analgesic activity ($p<0.001$) after 30 min as compared to the negative control group, as shown below in Table 2. The percentage of elongation of the latency period of *Vernonia filigera* crude extract and its fractions in the hotplate model are presented in Fig. 7 below. The higher percentage of elongation during the latency period (72.06%) was produced with the standard group (morphine 10 mg/kg) 60 minutes after administration. Next to it, a comparable percentage of elongation of the latency period (64.84%) was gained with a higher dose (400 mg/kg) of the crude extract group over 90 minutes. The percentage of elongation during the latency period increased with the increasing dose within the same group. The lower percentage of elongation of latency time was observed during 30 minutes as compared to the other time in grou

Table 2: Hot plate latencies of *Vernonia filigera* leaf crude extract and its fractions

Mean Latency Time (sec) ± S.E.M (Percentage of Elongation)					
Groups	0 min	30 min	60 min	90 min	120 min
Morphine10mg/kg(b)	3.23±0.11	11.11±0.29 $^{(acde)\,2}$ (72)	10.63±0.94$^{(acd)2e1}$ (72.06)	9.98±0.66 $^{(acd)2e1}$(70.94)	8.18±0.46$^{(ac)2}$ (5.77)
DW 10 ml/kg (a)	3.11±0.03	3.07±0.04	2.97±0.07	2.9±0.15	2.8±0.15
CE100 mg/kg(c)	3.25±0.15	5.98±0.29$^{(a\,b\,e)2}$(48.66)	6.15±0.41$^{(a\,b)2\,e1}$(51.7)	6.21±0.38$^{(ab)2\,e1}$(53.3)	5.26±0.33$^{(ab)2d1e2}$(46.76)
CE 200 mg/kg(d)	3.55±0.07	7.11±0.33$^{(ab)2}$ (56.87)	7±0.33$^{(ab)2}$ (57.57)	7.31±0.41$^{(ab)2}$ (60.32)	6.9±0.3 ^{a2} (59.42)
CE 400 mg/kg(e)	3.55±0.04	8.13±0.3$^{(abc)2}$(62.23)	8.08±0.3 $^{a2(bc)1}$(63.24)	8.25±0.26^{a2c1} (64.84)	7.8±0.4$^{(ac)2}$ (64.1)
HF 100 mg/kg(f)	3.23±0.08	5.03±0.27$^{a1(bde)2}$(38.96)	5.36±0.26$^{a1\,(be)2}$(44.58)	5.36±0.25^{a1b2d1e2}(45.89)	4.93±0.32^{a1b2d1e2}(43.2)
HF 200 mg/kg(g)	3.25±0.09	5.38±0.28$^{(ab)2d1e2}$(42.93)	5.46±0.24 $^{a1(be)2}$(45.6)	5.6±0.18$^{(abe)2}$(48.21)	5.6±0.22$^{(ab)2\,e1}$(50)
HF 400 mg/kg(h)	3.38±0.13	6.05±0.32$^{(abe)2}$ (49.25)	6.28±0.35$^{(abe)2}$(52.7)	6.08±0.44$^{(ab)2\,e1}$(52.3)	6.18±0.45 $^{a2\,b1}$(54.69)
MF 100mg/kg(i)	3.23±0.11	5.13±0.34$^{(a\,be)2}$ (40.15)	5.36±0.33^{a1b2e1}(44.18)	5.38±0.36^{a1b2d1e2}(46.69)	5.26±0.41$^{(abe)2}$ (46.76)
MF 200 mg/kg(j)	3.23±0.13	6.21±0.31 $^{a2\,b1}$(50.55)	6.41±0.22$^{(ab)2}$ (53.66)	6.36±0.24$^{(ab)2}$ (54.4)	6.3±0.22 $^{a2\,b1}$(55.55)
MF400 mg/kg(k)	3.31±0.15	6.9±0.3$^{(ab)2}$(55.5)	7.08±0.41$^{(ab)2}$ (58.05)	6.78±0.5$^{(ab)2}$ (57.22)	6.41±0.47$^{(ab)2}$(56.31)
AF100 mg/kg(l)	3.4±0.12	5.21±0.27$^{(ab)2d1e2}$(41.07)	5.35±0.28^{a1be2}(44.48)	5.46±0.29$^{(ab)2d1e2}$(46.88)	5.16±0.34^{a1b2d1e2}(45.73)
AF 200mg/kg(m)	3.2±0.1	5.33±0.3$^{(ab)2d1e2}$ (42.4)	5.4±0.21^{a1b2e1}(45)	5.46±0.23$^{(ab)2d1e2}$(46.88)	5.55±0.36$^{(ab)2e1}$(49.54)
AF 400 mg/kg(n)	3.38±0.13	5.96±0.34$^{(abe)2}$(48.48)	5.91±0.39$^{(ab)2e1}$ (49.74)	5.95±0.33$^{(ab)2e1}$ (51.26)	6.11±0.27^{a2b1}(54.17)

DW= Distilled water(a), CE (100, 200, 400) = Crude extract (c, d, e), HF (100, 200, 400) =Hexane fraction (f, g, h), MF (100, 200, 400) =Methanol fraction (i, j, k), AF (100, 200, 400) =Aqueous fraction (l, m, n)

Data is stated as Mean ± SEM of six observations and all groups compared to the negative control group and each other. 1p<0.05, 2 p<0.001

4.3 Anti-inflammatory activities of *Vernonia filigera*

4.3.1 The effects of *Vernonia filigera* extracts and its fractions on edema inhibition in the carrageenan-induced paw edema model

The injection of carrageenan 0.05 ml of 1% solution into the right hind paw of the rat causes acute inflammation and induces progressive edema. The effect of *Vernonia filigera* leaf crude extract and its fractions on carrageenan-induced paw edema in rats is shown below (Table 3). All the treatment groups produced statistically significant effects as compared to the negative control group ($p<0.001$). The percentage inhibition of inflammation of Vernonia filigera crude extract and its fractions in the carrageenan-induced paw edema model are presented in Fig. 8 below. The maximum percentage of inhibition of inflammation was observed in Indomethacin 10 mg/kg after 4 hours of drug administration (62.5%).Additionally, following five hours of medication administration, the higher dose(400 mg/kg) of the crude extract considerably reduced the rat's hind paw's edema and inflammation (59.07%)., Almost all treatment groups have progressive protection as time goes on.

Table 3: Carrageenan-induced paw edema in rats and the effect of *Vernonia filigera* extract crude extract and its fractions

Paw edema in ml (Mean ± SEM) (Percent Inhibition %)							
Groups	0 hrs.	1 hrs.	2 hrs.	3 hrs.	4 hrs.	5 hrs.	6 hrs.
Indomethacin 10mg/kg(b)	1.22±0.00	1.27±0.00$^{(ac)21}$ (54.96**)**	1.3±0.01$^{(ac)2d1}$ (58.59**)**	1.33±0.01$^{(ac)21}$ (62**)**	1.38±0.01$^{(ac)21}$ (62.5**)**	1.47±0.02$^{(ac)2d1}$ (62.4**)**	1.56±0.01$^{(ac)2\ d1}$ (61.38**)**
DW10 ml/kg(a)	1.24±0.01	2.82±0.06	3.14±0.06	3.5±0.07	3.68±0.08	3.91±0.07	4.04±0.03
CE 100 mg/kg(c)	1.22±0.00	1.47±0.00^{a1b2e1} (47.87**)**	1.68±0.02$^{(abe)2}$ (46.49**)**	1.73±0.03$^{(ab)2(de)1}$ (50.57**)**	1.77±0.02$^{(ab)2(de)1}$ (51.9**)**	1.9±0.01$^{(ab)2\ d1e2}$ (51.4**)**	1.96±0.01$^{(ab)2e1}$ (51.48**)**
CE 200 mg/kg(d)	1.23±0.00	1.4±0.03 ^{a2b1} (50.35**)**	1.5±0.02 $^{a2\ b1\ c1}$ (52.22**)**	1.53±0.02 $^{a2\ b1\ c1}$ (56.28**)**	1.59±0.01 $^{a2\ b1\ c1}$ (56.79**)**	1.66±0.01 ^{a2b1} (57.54**)**	1.8±0.02 ^{a2b1} (55.44**)**
CE 400 mg/kg(e)	1.22±0.01	1.35±0.00^{a2c1} (52.12**)**	1.43±0.00^{a2c1} (54.45**)**	1.48±0.01 $^{a2\ c1}$ (57.71**)**	1.53±0.01 $^{a2\ c1}$ (58.42**)**	1.6±0.01$^{(ac)2}$ (59.07**)**	1.72±0.01 $^{a2\ c1}$ (57.42**)**
HF 100 mg/kg(f)	1.22±0.01	1.72±0.03$^{(abcde)2}$ (39**)**	1.79±0.03$^{(abde)2}$ (42.99**)**	1.87±0.03$^{(abde)2}$ (46.57**)**	2.01±0.02$^{(abde)2}$ (45.38**)**	2.14±0.03$^{(ab)2c1(de)2}$ (45.26**)**	2.25±0.04$^{(abcde)2}$ (44.3**)**
HF 200 mg/kg(g)	1.23±0.02	1.62±0.01$^{(abcde)2}$ (42.55**)**	1.76±0.06$^{(abde)2}$ (43.94**)**	1.87±0.05$^{(abde)2}$ (46.57**)**	1.97±0.04$^{(ab)2c1(de)2}$ (46.46**)**	2.11±0.04$^{(ab)2c1(de)2}$ (46.03**)**	2.21±0.03$^{(abde)2c1}$ (45.29**)**
HF 400 mg/kg(h)	1.22±0.01	1.62±0.03$^{(ab)2c1(de)2}$ (42.55**)**	1.71±0.04$^{(ab)2d1e2}$ (45.54**)**	1.8±0.03$^{(ab)2d1e2}$ (48.57**)**	1.92±0.01$^{(abde)2}$ (47.82**)**	2.07±0.02$^{(abd\ e)2}$ (47.05**)**	2.17±0.02$^{(ab)2c1(de)2}$ (46.28**)**
MF 100 mg/kg(i)	1.22±0.00	1.56±0.01$^{(ab)2\ d1e2}$ (44.68**)**	1.69±0.02$^{(ab)2d1e2}$ (46.17**)**	1.86±0.06$^{(abde)2}$ (46.85**)**	1.99±0.04$^{(ab)2c1(de)2}$ (45.92**)**	2.09±0.03$^{(ab)2c1(de)2}$ (46.54**)**	2.22±0.02$^{(ab)2c1(de)2}$ (45.04**)**
MF 200 mg/kg(j)	1.25±0.01	1.39±0.01 $^{a2\ b1}$ (50.70**)**	1.46±0.01 $^{a2\ b1c1}$ (53.50**)**	1.57±0.01 $^{a2\ b1}$ (55.14**)**	1.63±0.02 $^{a2\ b1}$ (55.7**)**	1.74±0.04 $^{a2\ b2}$ (55.49**)**	1.84±0.03 $^{a2\ b2}$ (54.45**)**
MF 400 mg/kg(k)	1.25±0.18	1.37±0.00^{a2}(51.41$^{)}$	1.44±0.01^{a2c2}(54.14**)**	1.49±0.00^{a2c1}(57.42**)**	1.55±0.01^{a2c1}(57.88**)**	1.65±0.02$^{a2\ c2}$(57.8)	1.73±0.02^{a2c1}**(**57.17**)**
AF 100 mg/kg(l)	1.26±0.02	1.54±0.02$^{(a\ b)2\ d1\ e2}$ (45.39**)**	1.72±0.04$^{(ab)2d1e2}$ (45.22**)**	1.86±0.06$^{(abde)2}$ (46.85**)**	1.96±0.06$^{(ab)2c1(de)2}$ (46.73**)**	2.06±0.04$^{(abde)2}$ (47.31**)**	2.24±0.04$^{(ab\ cde)2}$ (44.55**)**
AF 200mg/kg(m)	1.24±0.01	1.41±0.01 $^{a2\ b1}$(50$^{)}$	1.48±0.01^{a2c1} $^{(}$52.86)	1.56±0.02^{a2b1}(55.42**)**	1.64±0.03$^{(ab)2}$(55.43**)**	1.77±0.05$^{(ab)2e1}$(54.73)	1.91±0.07$^{(ab)2e1}$(52.72**)**
AF 400 mg/kg(n)	1.26±0.01	1.39±0.00 ^{a2}(51.41$^{)}$	1.45±0.01$^{(ac)2}$(54.14)	1.51±0.01^{a2c1}(56.85**)**	1.56±0.01^{a2b1c1}(57.6**)**	1.65±0.02$^{(ab)2}$(57.8**)**	1.82±0.04$^{(ab)2}$(54.95)

DW= Distilled water(a), CE (100, 200, 400) = Crude extract (c, d, e), HF (100, 200, 400) =Hexane fraction (f, g, h), MF (100, 200, 400) =Methanol fraction (i, j, k), AF (100, 200, 400) =Aqueous fraction (l, m, n)

Data is stated as Mean ± SEM of six observations and all groups compared to the negative control group and each other. [1]$p<0.05$, [2] $p<0.001$

4.3.3 The effects of *Vernonia filigera* extracts and its fractions on exudate and granuloma inhibition in the cotton pellet-induced granuloma model

The effect of *Vernonia filigera* leaf crude extract and its fractions on cotton pellet-induced granuloma formation in rats is shown in Table 4. The crude extract and its fractions in all the tested doses significantly reduced the inflammatory exudation and granuloma ($p<0.001$) as compared to the negative control group. Also, the comparison between different groups shows statistically significant effects ($p<0.05$) in reducing the weight of exudate as well as in inhibiting granuloma. The percentage of exudate and granuloma inhibition in the cotton pellet-induced granuloma model is shown below in figure 9. The maximum percentage of inhibition of exudate and granuloma was observed in the standard group (62.18% and 69.11%, respectively). Also, a high percentage of exudate inhibition was produced with the higher-dose (400 mg/kg) crude extract (56.03%), and similarly granuloma inhibition was observed with the crude (62.26%) ,followed by methanol fraction (400 mg/kg) exudate and granuloma inhibition (51.54% and 56.88%) and aqueous fraction (400 mg/kg) (50.15% and 51.47%) respectively The inhibition of exudation and granulomas was found to increase in a dose-dependent manner.

Table 1: Weights of exudate and granuloma in mg (Mean ± SEM) induced by the cotton pellet method in rats and the effect of *Vernonia filigera* leaf crude extract and its fraction

Treatment groups	Mean weight of exudate(mg) ± S.E.M	% Inhibition of Exudation	Mean weight of (mg) ± S.E.M	% Inhibition of Granulation
Dexamethasone 0.5 mg /kg(b)	22.5±0.42$^{(ac)2\ d1}$	62.18	10.5±0.42$^{(ac)2\ d1}$	69.11
DW 10 ml/kg (a)	59.5±1.33		34±0.96	
CE 100 mg/kg(c)	35.16±0.47$^{(ab)2\ d1e2}$	40.9	20.33±1.02 $^{(ab)2d1\ e2}$	40.2
CE 200 mg/kg(d)	29.16±1.64^{a2b1c1}	50.99	16±0.73 $^{a2\ b1\ c1}$	52.94
CE 400 mg/kg(e)	26.16±0.70 $^{(ac)2}$	56.03	12.83±0.6$^{(ac)2}$	62.26
HF 100 mg/kg(f)	45.16±1.49 $^{(a\ b\ c\ d\ e)2}$	24.1	29±0.81 a1 $^{(bcde)2}$	14.7
HF 200 mg/kg(g)	39.33±0.76 $^{(abde)2}$	33.89	25.83±0.6$^{(ab)2c1(de)2}$	24.02
HF 400 mg/kg(h)	37.16±0.87$^{(abde)2}$	37.54	23.83±0.83$^{(abde)2}$	29.91
MF 100 mg/kg(i)	37.16±0.83$^{(abde)2}$	37.54	20.83±0.87$^{(ab)2d1e2}$	38.73
MF 200 mg/kg(j)	34.16±0.65$^{(ab)2d1e2}$	42.58	18.16±0.6$^{(ab)2\ e1}$	46.58
MF400 mg/kg(k)	28.83±0.87 $^{a2\ b1\ c1}$	51.54	14.66±0.88 $^{a2b\ c1}$	56.88
AF100 mg/kg(l)	38.5±1.11$^{(abde)2}$	35.29	23.33±1.25 $^{(abde)2}$	31.38
AF 200mg/kg(m)	36.5±1.05$^{(abde)2}$	38.65	19.5±0.76$^{(abe)2}$	42.64
AF400 mg/kg(n)	29.66±0.84 $^{(ab)2\ c1}$	50.15	16.5±0.76 $^{(ab)2}$	51.47

DW= Distilled water(a), CE (100, 200, 400) = Crude extract (c, d, e), HF (100, 200, 400) =Hexane fraction (f, g, h), MF (100, 200, 400) =Methanol fraction (i, j, k), AF (100, 200, 400) =Aqueous fraction (l, m, n). Data is stated as Mean ± SEM of six observations and all groups compared to the negative control group and each other. [1]$p<0.05$, [2] $p<0.001$

4.4. Phytochemical screening

Anthraquinones, cardiac glycosides, steroids, and sterols were absent from the aqueous extract of *Vernonia filigera* leaves, but saponins, alkaloids, flavonoids, tannins, terpenoids, and phenols were present.

Table 2: Preliminary phytochemicals screening of 80% methanol extract of the leaves of *Vernonia filigera*

Secondary metabolite	Results
Alkaloids	+
Anthraquiones	-
Cardic Glycosides	-
Flavonoids	+
Phenols	+
Saponins	+
Steroids	-
Tannins	+
Terpenoids	+

+ = indicating presence **-** = indicating absence

5. DISCUSSSION

Traditionally, *Vernonia filigera* was used to treat pain and inflammation in Ethiopia (Tefera & Kim, 2019). Even though its analgesic and anti-inflammatory activity is not proven scientifically, people utilize it. The present study aims to investigate the traditional claim of *Vernonia filgera* leaves for their analgesic and anti-inflammatory activity by using experimental models.

To study the safety profile, a high dose of 2000 mg/kg *Vernonia filigera* leaf extract was administered to mice. However, it doesn't result in any toxic symptoms, such as sedation, hyperactivity, twitching, stiffness, irritability, jumping, drowsiness, or mortality. According to the 2008 Organization for Economic Cooperation and Development (OECD), the current investigation so demonstrated that the extract has no indication of acute toxicity. This indicates that the LD50 of the extract is greater than 200mg/kg.

The analgesic activity of *Vernonia filigera* leaf extract was evaluated by using models like acetic acid-induced writhing and the hot plate model. A peripheral analgesic activity test was performed by the acetic acid-induced writhing test (Saha *et al*., 2007). The acetic acid-induced writhing test is a chemical method that causes writhing or pain of peripheral origin due to the injection of acetic acid. The signals sent to the central nervous system in response to pain due to irritation caused by the release of mediators such as prostaglandins, which contributes to the increased sensitivity of nociceptors (Gawade, 2012). Acetic acid in the writhing model causes nociceptive activity because of the release of TNF-α, interleukin 1β, and interleukin 8 by resident peritoneal macrophages and mast cells (Ribeiro *et al*., 2000).

The crude extract of the test plant showed significant analgesic activity against acetic acid induced writing at different doses, (100, 200, and 400 mg/kg), when compared to the negative control. The percentage of inhibition of acetic induced writing of the tested doses was 41.81% 53.62% and 64.53%, respectively. The results were found to be significant ($P < 0.05$) in comparison to the control. This result showed that the extract exerted analgesic activity in a dose-dependent manner. The findings of the experiment suggested that the analgesic effect of *Vernonia filigera* may be due to peripheral pain mechanisms or suppression of the prostaglandin pathway. A decrease in the number of writhing tells us that the possible mechanism of action is the same as with aspirin.

The maximum inhibition was observed with the highest dose (400 mg/kg) of the 80% methanolic leaf extracts of (55.59%) analgesic activity in a study done in Ethiopia on another genius of *verinonia, V. auriculifera,* at the acetic acid-induced writhing test. This increased inhibition of writhing was shown in a dose-dependent manner. As compared to *V. auriculifera*, *Vernonia filigera* has better activity.

A hot plate test was conducted using acute thermal stimulus to measure the likely effect of the test compound (Bannon, 2001). This test is recommended for assessing centrally acting analgesic profile of drugs (Yam *et al*., 2020). When animal paws are exposed to thermal stimuli on a hot plate, an acute, non-inflammatory nociceptive response develops. (Dutra *et al*., 2008). Enkephalin, opiate drugs, like morphine, interact with opioid receptors and produce analgesia by the same mechanisms, i.e., hyperpolarization of interneurons and depressing the release of transmitters associated with the transmission of pain. Morphine also interacts with opioid receptors found in the supraspinal structures and activates the supraspinal system. It has already been understood that morphine interacts with μ receptors to produce analgesia (Lipp, 1991). The standard drug morphine produces its maximum analgesic activity at 90 minutes (72.06%), and from the crude and its fractions, the crude extract had the highest analgesic effect (64.84%), The crude extract and its fractions displayed significant analgesic activity ($p<0.05$) in comparison to the negative control group. Analgesic activity within the group increased with increasing dose and time, at 100 mg/kg, 200 mg/kg, and 400 mg/kg. The *Vernonia filigera* extract produced a dose-dependent prolongation of hot plate latency. Among the fractions, MF 400 mg/kg produced the highest percentage of inhibition (58.05%). The finding shows that the plant *Vernonia filigera* may have a similar mechanism of analgesia as morphine.

lowest dose of all fractions showed a significantly smaller increase in latency time than the highest dose ($p < 0.05$). In addition, at the 60th, 90th, and 120th minutes, the highest doses of the fractions significantly increased the latency time compared to the middle doses ($p < 0.05$)(Ashenafi et al., 2023).

Carrageenan (CAR)-induced paw edema is commonly used to identify the acute phase of inflammation. (Odabasoglu *et al*., 2011). Carrageenan-induced inflammation comprises of two phases (Wang *et al*., 2015): the first phase (first 2 h after carrageenan injection) is attributed to the

release of pro-inflammatory mediators like histamine and serotonin, and the second phase (3–5 h after carrageenan injection) is mainly mediated by kinins, prostaglandin, nitric oxide, cyclooxygenase cytokines, and neutrophil-derived free radicals (Niu *et al*., 2013). Apart from its widely recognized ability to inhibit cyclooxygenase, indomethacin also has other actions that include lowering cerebral blood flow (CBF), blocking nitric oxide (NO) pathways, and lessening oxidative stress(Villar- et al., 2021). Nonsteroidal anti-inflammatory drugs (NSAIDs) like indomethacin have strong analgesic, antipyretic, and anti-inflammatory properties(Lucas, 2016).

In the carrageenan-induced model, all the test groups showed statistically significant activities ($p<0.05$). The maximum percentage of protection from paw edema was recorded 4 hours after administration of indomethacin (62.5%), which is a statistically significant effect in comparison to the negative control group ($p<0.001$). From the treatment groups, the highest edema inhibition (59.05%) was recorded at 5 hours after administration of high-dose CE (400 mg/kg). This is also a statistically significant effect in comparison to the negative control group ($p<0.001$).From the fractions MF 400 mg/kg has (57.88%)) of edema inhibition at 4hrs and followed by AQ 400 mg/kg has (57.8%) of edema inhibition at 5 hrs., which is not statistically significant with crude extract especially as dose increase. All the treatment groups have dose dependent effect. In the carrageenan-induced paw edema model, 80% methanolic leaf extracts of *V. auriculifera* showed values of 38.13, 44.06, and 50% for 100, 200, and 400 mg/kg, respectively; this implied the maximum percent inhibition at 4 hours after treatment (Ashenafi *et al*., 2023). When we compare it with Vernonia auriculifera, Vernonia filigera had significant analgesic and antinfilamatory activity at CE 400mg (64.53%) and CE 400mg at 5 hrs. (59.07%), respectively

Vernonia glaberrima's n-butanol fraction has strong analgesic and anti-inflammatory properties and the largest percentage suppression of inflammation from the second to fourth hour was seen in the carrageenan-induced paw edema model (Nasir *et al*., 2019).

The chronic anti-inflammatory investigations employed the cotton pellet-inducing granuloma test (Hosseinzadeh *et al*., 2012). The inflammatory response to a cotton pellet inserted subcutaneously in the body occurs in three stages: (A) a transudative phase that lasts for the first three hours; and (B) an exudative phase that lasts for the next three to seventy-two hours after the pellet is implanted, (C) a proliferative phase, which is defined as the rise in the granuloma's dry weight that

happens three to six days following implantation (Vittalrao *et al*., 2011). Numerous mediators, including chemokines, eicosanoids, and cytokines, have been discovered to be involved in the formation of the granuloma. Granulomas are highly vascularized reddish masses of tissue that develop as a result of inflammation and healing processes that include the growth of tiny blood vessels, neutrophils, fibroblasts, and macrophages (Ghaisas *et al*., 2010). Exudative material and the amount of granulomatous tissue are correlated with the weight of the wet cotton pellets and the dry pellets, respectively. The weight of granuloma development may decrease as a result of the proliferative phase being suppressed (Anuja *et al*., 2014). A class of steroid hormones known as GCs have strong anti-inflammatory properties(Jeklova et al., 2008). Strong corticosteroid dexamethasone binding affinity for intracellular glucocorticoid receptors enhances transcription of anti-inflammatory cytokines like interleukin 1 receptor antagonist (IL-1Ra) and interleukin 10 (IL-10), and represses transcription of pro-inflammatory cytokines like interleukin 1β (IL-1β), interleukin 6 (IL-6), cyclooxygenase 2 (COX2), and tumor necrosis factor α (TNFα)(Huebner et al., 2014)

All the tested doses of the extract in the cotton pellet-induced granuloma model showed a statistically significant inhibition ($p<0.001$) of both exudates and granuloma formation. The standard drug dexamethasone had the highest inhibition of exudate and granuloma formation (62.18% and 69.11%, respectively). The maximum percentage of the test of inhibition of exudate and granuloma inhibition was obtained at CE 400 mg/kg, followed by MF 4000 mg/kg and AF 400 mg/kg (56.03, 51.54, and 50.15%, respectively) for exudate formation and 62.26, 56.88, and 51.47%, respectively, for granuloma formation, which has a statistically significant effect in comparison to the negative control group ($p<0.001$).

All tested doses significantly inhibited granuloma mass formation, only the middle and highest doses of the EAF significantly inhibited the formation of inflammatory exudate when compared to 2% tween 80 ($p < 0.05$). In terms of inhibition of granuloma mass formation, all doses of the three fractions exhibited statistically significant inhibition when compared to 2% tween 80 ($P < 0.05$)(Ashenafi et al., 2023).

The analgesic properties of medicinal plants have been attributed to alkaloids, tannins, flavonoids, and saponins (Asante *et al*., 2019). It has been demonstrated that several medicinal plants contain

flavonoids that have anti-nociceptive and/or anti-inflammatory properties (Eldahshan & Azab, 2012). Flavonoids prevent prostaglandin generation that is are implicated in a variety of immunologic responses. Another class of regulatory enzymes impacted by flavonoids is protein kinase. Flavonoids also reduced inflammatory processes by inhibiting these enzymes (Eldahshan & Abdel-Daim, 2015).

Because of their inhibitory effects on enzymes involved in the formation of the chemical mediator of inflammation, flavonoids and saponins are well known for their potential to reduce both pain perception and inflammation (Kaushik *et al.*, 2012) Flavonoids and tannins are also responsible for the free radical scavenging effects. Moreover, flavonoids exhibit activities like anti-allergic, anti-inflammatory, antimicrobial and anticancer activity. Numerous metabolites found in *Vernonia filigera* may be involved in the plant's analgesic and anti-inflammatory properties. The most prevalent metabolites were primarily alkaloids, saponins, tannins, flavonoids, and phenols; these compounds are anticipated to have analgesic and anti-inflammatory properties.

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6.CONCLUSION

The 80% methanol crude extract of *Vernonia filigera's* leaves and its solvent fractions showed statistically significant analgesic and anti-inflammatory properties. It prevented exudate and granuloma formation caused by cotton pellets, prolonged the latency period, decreased inflammatory edema, and decreased the frequency of writhing's. The findings of this study demonstrated the rationale and scientific backing for the traditional usage of *Vernonia filigera* as a remedy for pain and inflammation.

7. RECOMMENDATION

The findings of this study on *Vernonia filigera* ought to serve as a foundation for additional research into the plant's analgesic and anti-inflammatory properties. Therefore, more research is required to

- Determine and isolate the specific ingredient or ingredients that provide the anti-inflammatory and analgesic effects.
- Ascertain the exact mechanism of action by which *Vernonia filigera possesses* its anti-inflammatory and analgesic properties
- Determine the *Vernonia filigera* leaves' sub-acute and chronic toxicity

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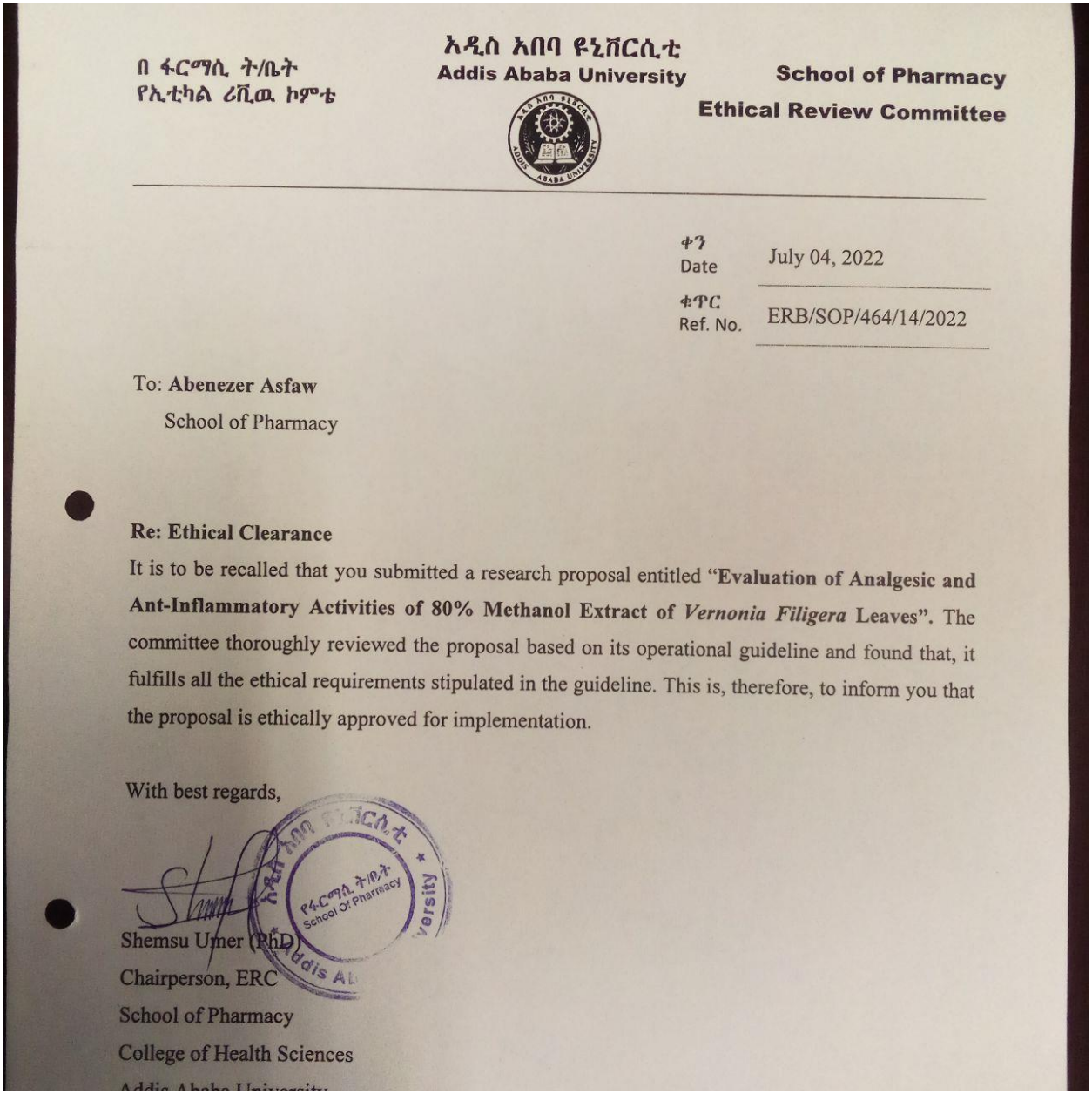
Annex 1: Ethical clearance



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Addis Ababa University

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School of Pharmacy
Ethical Review Committee

ቀን Date: July 04, 2022

ቁጥር Ref. No.: ERB/SOP/464/14/2022

To: **Abenezer Asfaw**
School of Pharmacy

Re: Ethical Clearance

It is to be recalled that you submitted a research proposal entitled "**Evaluation of Analgesic and Ant-Inflammatory Activities of 80% Methanol Extract of *Vernonia Filigera* Leaves".** The committee thoroughly reviewed the proposal based on its operational guideline and found that, it fulfills all the ethical requirements stipulated in the guideline. This is, therefore, to inform you that the proposal is ethically approved for implementation.

With best regards,

Shemsu Umer (PhD)
Chairperson, ERC
School of Pharmacy
College of Health Sciences
Addis Ababa University