Analgesic and anti-inflammatory activities of *Albizia gummifera* (J. F. Gmel) C.A. Sm. bark hydro-alcoholic extract and its fractions in rodents

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

By: Mesfin Tamirat (B.pharm)

Addis Ababa University

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College of Health Sciences

School of Pharmacy

Department of Clinical Pharmacy and Pharmacology

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School of Graduate Studies

This is to certify that the thesis prepared by Mesfin Tamirat Aba, entitled "Analgesic and anti-inflammatory activities of Albizia gummifera (J. F. Gmel) C.A. Sm. bark hydro-alcoholic extract and its fractions 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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Chair of Department

Abstract

Analgesic and anti-inflammatory activities of Albizia gummifera (J. F. Gmel) C.A. Sm. bark hydro-alcoholic extract and its fractions in rodents.

Mesfin Tamirat

Background: The majority of illnesses present as pain and inflammation. Despite advancement in pain medicines, there is a need for safe, effective analgesic drugs. In folkloric medicine, *A.gummifera* has been used for pain and inflammation, but not scientifically evaluated.

Objectives: This study aimed to investigate the analgesic and anti-inflammatory activities of *A.gummifera* bark crude extract and its fractions in rodents.

Methods: The analgesic activity of the *A.gummifera* was evaluated using an acetic-acid-induced writhing test and hot plate test by using acetyl salicylic acid 150 mg/kg and morphine 10 mg/kg as standard, respectively. Carrageenan-induced paw edema and cotton pellet granuloma methods were used to investigate the anti-inflammatory effect with Indomethacin 10 mg/kg and dexamethasone 0.5 mg/kg as the reference drugs, respectively. Three doses are selected based on the acute toxicity test result: 100 mg/kg, 200 mg/kg, and 400 mg/kg.

Results: The *A.gummifera* bark crude extract and its fractions did not show any sign of toxicity at 2000 mg/kg. The crude extract and its fractions showed statistically significant analgesic and anti-inflammatory activities as compared to the control group ($p<0.05$). The crude extract and methanol fractions reduced writhes by 66.68% and elongate latency period by 61.47% on 60 and 90 minute, respectively. The maximum edema inhibition was 62.15% in the aqueous fraction after 2 hours of administration. Aqueous fraction produces maximum exudate and granuloma inhibition (62.07% and 65.66%, respectively).

Conclusion: This study found that, *A.gummifera* bark crude extract and its fractions possessed promising analgesic and anti-inflammatory property.

Keywords: Pain, Analgesic, anti-inflammatory, *Albizia gummifera*.

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List of abbreviations & acronyms

AF	Aqueous fraction
AG	*Albizia gummifera*
C.A.	Cultivation of Albizia
CNS	Central nervous system
CD	Crohn's disease
CD 28	Cluster of differentiation 28
CE	Crude extract
COX	Cyclooxygenase
COPD	Chronic obstructive pulmonary disease
ERK	Extracellular signal regulated kinase
GRs	Glucocorticoid receptors
J.F.	Julibrissin Filippo
JNK	Jun N-terminal kinase
LD	Lethal dose
MAPK	Mitogen-Activated Protein Kinase Pathway
MF	Methanol fraction
NF-Kb	Nuclear Factor Kappa B
NAG	N- acetyl glucosaminidase
NMDA	N-methyl D-aspartic acid
OECD	Organization for economic cooperation and development
SEM	Standard error of mean
TNF	Tumor necrosis factor

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1. INTRODUCTION

1.1 Overview of pain and inflammation

Pain is a dreadful sensation related to a particular portion of the body.[1] The term also includes behavioral changes, neuroendocrine activation, and the stress response in addition to a disagreeable emotion or sensation associated with real or potential tissue injury.[2]

Irritation of pain receptors, which can be found in joints, skin, and many other organs, can produce pain. It is caused by mechanisms that either hurt or threaten to injure tissues.[3]Damage to the neurological system, peripheral nerves, brain, and spinal cord may also be the source of pain. Pain can exist without causing physical damage, and the patient refers to it as psychogenic pain.[4]

Based on etiology there are three types of pain.[5] The first is nociceptive pain, which is a protective sensation related to the detection of unpleasant stimuli that cause tissue damage. The second type of pain is inflammatory pain, which is linked with tissue injury and immune cell infiltration and can encourage repair by inducing pain hypersensitivity until healing happens. Pathological pain is a sickness state resulting from nerve injury (neuropathic) or abnormal dysfunctional function of the system of nerves.[6]

Pain is made up of three separate components. The first is protective pain, which is a physiological early-warning system for identifying and mitigating hazardous situations or unpleasant stimuli. The second sort of pain is a defense and adaptation mechanism. This pain is beneficial for the injured part of the body to recover by increasing sensory sensitivity following the inevitable tissue injury and establishing an environment that minimizes physical touch and activity. Finally, pathological pain is not protective, but rather destructive, as a result of a malfunctioning neuronal system.[7]

Based on its severity, pain can also be considered acute pain or chronic pain.[8] Acute pain is described as skeletal muscle spasm and sympathetic nervous system activation induced by a specific sickness or condition and is self-limiting. Chronic pain, on the other hand, is defined as pain that lasts longer than the normal recovery time after a disease or accident. Chronic pain has no biological role or clear endpoint, and it might be triggered by psychological reasons.[8]

Inflammation is a local response (reaction) of living vascularized tissues to endogenous and exogenous stimuli. It is characterized by pain, redness, swelling, and organ dysfunction, and is the natural result of the host's protective reactions to tissue harm induced by a variety of stimuli such as physical trauma, chemicals, and infectious agents.[9]

Inflammation can be caused by pathogens, harmful chemicals, or damaged cells. These factors can cause acute or chronic inflammation in the liver, heart, kidneys, lungs, pancreas, brain, intestines, reproductive system and etc. resulting in tissue damage or disease.[10]

The stages of inflammation are determined by the time it takes to carry out the process and many immunological variables, and there are two types of inflammation: acute and chronic processes. Acute inflammation is a brief operation that can last from a few minutes to a few days. Its primary traits are the leaking of fluid or plasma proteins and the translocation of leukocytes into an extravascular region.[11]

Inflammation is a necessary immune system reaction. Nonetheless, prolonged inflammation can cause several secondary effects on biological systems, including a greater likelihood of chronic illness and problems.[12]

1.2 Pathophysiology of pain and inflammation

The pain stimuli changed to electrical signal by nociceptor found in nerve endings of organs. The electrical signal (action potential) transmitted to the spinal cord through first order neuron. The first order neuron has two types of fibers which are called A delta fibers and C fibers. The A delta fibers are the myelinated type of fiber which transmit the sharp and fast type of signal while the C fibers are un myelinated and transmit the null and slow type of signal. The first order neuron relies into spinal cord through dorsal root ganglion and synapses with second order neuron.[14]

The second order neuron project into thalamus through dorsal horn of spinal cord and the signal transmitted to thalamus by ascending spino-thalamic fiber. Second order neuron synapse with third order neuron in the thalamus. Through third order neuron the signal transmitted to different part of cortex for integration and interpretation. Finally, the pain is modulated by the signal comes from brain through noradrenergic and serotonergic inhibitory fiber by release of endogenous opioid such as endorphins and enkalphins.[15]

The combined results of frequent or strong peripheral pain signals release excessive amounts of central nervous system neurotransmitters, including glutamate, which stimulates NMDA

and other receptors, resulting in increased neuronal sensitization in the spinal cord's dorsal horn.[16] Dorsal horn neuron sensitization can linger for hours, and it is believed to be responsible for pain that occurs outside of the site of tissue injury. Central sensitization differs from peripheral sensitization in that it allows for pain feelings caused by low-intensity stimuli.[17]

Inflammation is a set of changes that take place in a living tissue when it is injured, assuming that the harm is not severe enough to affect the tissue's form and vitality immediately, or as the reaction to injury to the live microcirculation and surrounding structures.[13]

The harm to the tissue and inflammation cause the stimulation and discharge of intracellular components from injured cells, inflammatory cells, and the main nerve fiber itself. Ions (H+, K+), prostaglandins (PGE2), bradykinin, neurotropic growth factors, cyclooxygenase, and cytokines (ILl, IL6, TNFa) release and propagate locally, sensitizing pain fibers to future painful and non-painful stimuli.[16] Degranulation of mast cells raises the local concentrations of 5-hydroxytryptamine and histamine. These compounds combine to form a "sensitizing soup," which lowers nociceptors' thresholds and activates "silent" nociceptors, intensifying the pain response. Plasma escape and local vasodilation amplify the inflammatory reaction and transmit hypersensitivity to neighboring tissues (secondary hyperalgesia).[15]

Acute inflammatory responses consist of three major steps: increased blood flow to the inflamed location, relaxation, and improved vascular permeability, which are associated with plasma escaping from microcirculation and the migration of phagocytic leukocytes to adjacent tissue.[18] The initial changes exhibited in the inflammation are alterations in the flow of fluid in the vessels and changes in the diameter of tiny blood vessels. Blood flow to this location is improved by newly formed capillaries and larger arterioles. Endothelial alterations enhance the microvasculature's vascular permeability, resulting in fluid escape into an extravascular location, which advances the next stage in the process.[11]

Fluid volume reduction in the artery lumen improves the viscosity of the blood, lowering the flow rate. Finally, as blood circulation changes, leukocytes interact and stick to the endothelium, rolling prior to adhering. They then migrate into the interstitial tissue via the artery wall. In the interstitial tissue, the important function of inflammation is obtained, which is to deliver plasma mediators and leukocytes to the affected area.[19]

During the inflammatory response, sensors, mediators, and target tissues differ depending on the type of infection.[20] These inflammatory products subsequently respond to target tissues such as local blood vessels, causing vasodilation, neutrophil extravasation, and plasma leakage into the infected region. The pathogens are then found and eliminated by tissue-resident macrophages, neutrophils, and mast cells. This process is aided by plasma components and begins with antibodies and their components. The classic complement pathway begins with the production of antibodies via C-reactive protein, natural antibodies, or serum amyloid protein.[21]

Chronic tissue inflammation occurs when inflammatory reactions occur when there is no actual trigger. It typically happens as a consequence of unresolved infections either by endogenous defense mechanisms or via another host protective barrier mechanism.[12] They can also occur as a result of physical or chemical factors that cannot be disassembled, along with genetic vulnerability. Foreign bodies, ongoing chemical exposures, recurring acute inflammatory responses, or specific microorganisms are all significant contributors to chronic inflammation.[22]

Chronic inflammation's molecular and cellular processes differ depending on the nature of the inflamed organs and cells.[23] Chronic liver disease, for example, is primarily associated with chronic hepatitis B and hepatitis C, drug or toxin-induced metabolic illness, alcohol consumption, and liver injury.[24]

1.3 Statement of the problem

Because of the subjective nature of the symptoms and the lack of consensus for precise diagnoses and diseases, defining the epidemiology of pain is challenging; therefore, discussing evidence for the true occurrence of most pain problems is problematic.

Globally, it is believed that one in every five people suffers from pain, and another 1 in every 10 people is identified with the chronic type of pain every year.[25] The worldwide impact of chronic pain is rising. The most common symptomatic chronic condition is recurrent tension-type headaches, which, were determined to affect 1.9 billion individuals.[26]

While everyone suffers from pain, irrespective of age, gender, race, income, ethnicity, or region, pain is not dispersed evenly around the world. Acute, chronic, or intermittent pain, or a combination of the three, can be experienced by those who are in pain. Cancer,

osteoarthritis and rheumatoid arthritis, operations and traumas, and spinal disorders are the four most common causes of pain, making the etiology of pain complicated.[27]

Inflammatory bowel disease is a global health and economic burden that significantly lowers sufferers' quality of life. It is believed that over 3,000,000 individuals in the USA and Europe have IBD, and its prevalence in North America, Oceania, and several European countries exceeds 0.3%. In 2017, around the world, there were approximately 6.8 million cases of IBD.[28]

1.4 Inflammatory diseases

Inflammatory diseases are groups of clinical disorders that are characterized by abnormal inflammatory responses.[16]

Airways and allergic inflammation: Asthma, rhinoconjunctivitis, and COPD are all examples of airway and allergic inflammation. These three conditions are normally treated as different entities, but it's crucial to remember that allergic rhino conjunctivitis and asthma have a close relationship and that asthma and COPD may be symptoms of a broader illness. Allergic asthma is characterized by near-complete reversibility of bronchial obstruction and low inflammatory change while, COPD with no airflow restriction reversibility and noticeable inflammation and death of lung tissue.[29]

Osteoarthritis: It is a condition marked by progressive articular cartilage deterioration, new osteophyte formation, and synovial membrane inflammation, with the backbone of joint disease being cross-talk between articular cartilage and subchondral bone. Peripheral and central pain sensitivity are linked to osteoarthritic joints.[30]

Rheumatoid arthritis: It is a chronic inflammatory illness that mostly affects the hands and feet, causing cartilage and bone loss. It is commonly associated with small-intestinal discomfort and edema. Neuronal activity, which has a profile similar to that of other neuro-inflammatory states, is associated with initiating the joint actions of neurons and immune/vascular cells in the CNS, according to new research.[30]

Inflammatory bowel diseases (IBD): The two most common types of inflammatory bowel illnesses are Crohn's disease (CD) and ulcerative colitis (UC). Both CD and UC are chronic and acute debilitating inflammatory disorders with unclear causes.[31] IBD, according to current thinking, is a complex disorder involving both hereditary and environmental factors, with the result determined by an abnormal immunological response to naturally occurring

commensal microbiota in those with a compromised epithelial barrier. IBD is not curable; however, immunosuppressive and anti-inflammatory medications, and surgery are common maintenance treatments.[29]

Atherosclerosis: The main cause of cardiovascular disease is artery hardening. Most of the time, it's linked to the development of atheroma. Dysfunction of the endothelium is characterized by increased expression of adhesion molecules, increased attachment of leucocytes, and reduced responses to endothelium-dependent vasodilators, and is regarded as a precursor to atherosclerosis. Inflammatory mediators have a vital role in atherosclerosis from the initial recruitment of leukocytes through the atherosclerotic plaque rupture.[10]

1.5 Current treatment of pain and inflammation

1.5.1 Analgesics and anti-inflammatory drugs

Anti-inflammatory drugs work by interfering with the pathophysiology of inflammation to reduce tissue injury and increase patient quality of life. There are two types of anti-inflammatory medications: glucocorticoids and non-steroidal anti-inflammatory drugs. These are different in terms of how they work.[32]

Non-steroidal anti-inflammatory drugs (NSAIDs): NSAIDs suppress the enzyme cyclooxygenase and they are utilized in the treatment of moderate to mild pain and regulate body temperature.[33] NSAIDs have different effects, including analgesic, anti-inflammatory, antiplatelet, and antipyretic. NSAIDs are generally beneficial for postoperative pain control. The suppression of prostaglandin synthesis appears to be the primary analgesic impact of NSAIDs. Acetylsalicylic acid, Diclofenac, Fentiazac, Ketorolac, Bufexamac, Ibuprofen, Ketoprofen, Pyprophene, Indoprofen, Naproxen, Oxaprozin, and Tiaprofen are examples of non-steroidal drugs.[34]

Glucocorticoids: Glucocorticoids are an important class of drugs belonging to corticosteroids and over many years, their therapeutic application in the management of allergy, inflammation, and pain has spread. Glucocorticoids act by decreasing prostaglandins and proteins implicated in inflammatory processes, such as corticosteroids, that can be used to treat asthma, and the autoimmune inflammatory response, among other things.[35]

Opioids: The clinical application of opioids demonstrates a distinction between their clinical and laboratory pharmacology. When opioids are given to someone in pain, they have a different effect than when they are given to someone who is not in pain. Morphine,

Diamorphine, Pethidine, Meperidine, Methadone, Hydromorphone, Oxycodone, Fentanyl, and Buprenorphine are some of the most common opioids.[36]

1.6 Rationales of the study

Pain, fever, and inflammation are frequent markers and symptoms of the majority of diseases that afflict people. They cause injury, poor quality of life, depressive disease, and death, as well as a huge economic loss of income.

The treatment of pain, inflammatory disorders, and fever is a costly affair that necessitates the use of a variety of medications. Anti-inflammatory medications now in use have several serious adverse effects. Aspirin, for example, produces gastric ulcers, while paracetamol causes hepatotoxicity. Medicinal herbs are inexpensive and widely available, and they serve a vital role in primary health care in developing nations. As a result, strong anti-inflammatory medications with fewer adverse effects derived from plant extracts are required. The plant *Albizia gummifera* was selected for the current study because it has a traditional claim of using different parts of the plant for both analgesic and anti-inflammatory activity in different areas of Africa, and also because this plant is familiar for use in Ethiopian society. Therefore, conducting pharmacological studies is important to confirm the traditional claim.

2. LITERATURE REVIEW

2.1 Overview of *A.gummifera*

A.gummifera (J. F. Gmel) C.A. Sm. is a plant in the family Fabaceae. The genus was given its name after Filippo del Albizzi, a Florentine nobleman who introduced *Albizia julibrissin* into cultivation in 1749.[37] The species *A.gummifera* has been distributed in Angola, the Democratic Republic of Congo, Cameroon, Ethiopia, Madagascar, Kenya, Nigeria, Uganda, Tanzania, and Zambia. There are two varieties that have been identified. These include the gummifera variety, which has leaflets with auriculate margins near the base, and the ealaensis variety, which does not have leaflets with auriculate margins near the base. The current study was on a variety of gummifera. This plant is called peacock flower in English. [37] In Ethiopia, the local name of this plant is Mukarba in the Afaan Oromo and Sessa in the Amharic languages. [38]

Traditionally, it is used for the relief of tooth pain and rheumatism.[38] For traditional use, the leaves are crushed, dissolved in water, wrapped in cotton, and applied to the damaged teeth for relief of pain. The bark is chewed to relieve rheumatism. The bark decoction in the water is also used in the treatment of abdominal pain and inflammatory disease.[37] The plant is a huge deciduous tree with branches climbing to a flat top that grows to a height of 4.5-30 meters. The crown is flat, and the bark is smooth and grey, as shown in Fig.1, which was captured during plant collection.

Figure 1: The structural view of *A.gummifera* leaf and bark.

2.2 Phytochemical characterization of *A.gummifera* bark extracts

The bioactive substances of the *A.gummifera* aqueous and hydro-ethanol extracts were determined by the study conducted in Cameroon by Bayaga Hervé Narcisse.[38] From the qualitative tests, the metabolites found in the aqueous and hydro-ethanol extracts of

A.gummifera include alkaloids, flavonoids, cardiac glycosides, polyphenols, saponins, and tannins. From the quantitative test; the total tannins (mg Eq tannic acid/g) were 149.77 ± 3.72 in aqueous extracts and 126.65 ± 6.11 in hydro-ethanol extracts; the total flavonoids (mg Eq quercetin /g) were 60.98 ± 1.31 in aqueous extracts and 32.95 ± 4.55 in hydro-ethanol extracts; the polyphenols (mg Eq gallic acid/g) were 3.82 ± 0.11in aqueous extracts and 30.72 ± 0.75 in hydro-ethanol; the total flavonols (mg Eq gallic acid/g) were 373.06 ± 4.81 in aqueous extracts and 186.94 ± 4.81 in hydro-ethanol extracts.

2.3 Medicinal uses of *A.gummifera*

Leishmaniasis: The *A.gummifera* seed ethanol extract, n-butanol, and aqueous fractions have anti-promastigote activity with a low toxicity profile, according to an *in vitro* investigation.[40]

Anthelmintic activity: In *in vitro* egg hatch and larval growth experiments, extracts of *A.gummifera* displayed different potencies in nematodes of sheep.[41]

Antitrypanosomal activity: The screened ethanol extract of *A.gummifera* leaf at a dosage of 25 mg/ml was found to have good trypanocidal activity *in vitro*. The bioactive component in the extract is responsible for this observation.[42]

Antimicrobial activity: Because of the synergistic action seen, the blend of *A.gummifera* and *Spathodea campanulata* in 25/75 proportions proved to be the optimal association or combination. The combo of *A.gummifera* and *Spathodea campanulata* in proportions of 75/25 is the unique recipe employed by traditional practitioners of healing, which may or may not be effective as a result of the opposing effects.[43]

The low minimum inhibitory activity and minimum bactericidal activity values obtained against all *Salmonella serovars* tested indicated that *A.gummifera* methanol crude extract showed significant antibacterial activity. The extract was not mutagenic. It did, however, show signs of cytotoxicity.[44] Antibacterial activity of solvent fractions in their crude and semi-purified states of *A.gummifera* and *C. macrostachyus* against clinical isolates and a reference strain of *N. gonorrhoeae* has been discovered.[45]

2.4 Animal models for screening analgesic activity

2.4.1. Acetic acid-induced writhing model

A writhing test is a chemical approach for inducing peripheral discomfort in mice by administering irritant chemicals like phenyl quinone or acetic acid. The analgesic effect of the

test compound is estimated by a decrease in the frequency of writhing. Shivaji et al originally reported that in mice, abdominal writhings manifest as back arching, hind limb extension, and abdominal muscle contraction.[46]

The writhing resulted by parenteral administration of acetic acid is the result of severe endogenous long-term discomfort. Due to their irritating nature, these principles are also prone to producing lesions. Writhing is an obvious reply to acute pain generated by irritating principles via the nociceptors, which exhibit peritoneal retraction and stretching of the back legs. Signals sent to the CNS as a result of irritation cause a discharge of mediators like prostaglandins, which lead to enhanced nociceptors sensitivity.[47]

2.4.2. Hot plate model

A manual hot plate test can be applied to individual mice or with the use of automated devices that may test as many as sixteen mice at the same time. In this test, an animal is placed on a heated surface that is kept between 52 and 55 degrees Celsius. To prevent the animal from falling, a cylinder made of translucent plastic is wrapped around it. As the heat of the surface becomes painful, the animal will lift and lick a paw after a brief duration, often several seconds. The animal is then removed from the equipment right away.[48]

The time it takes to lick the paw, or latency is the dependent variable in this test, and it is manually recorded using a timer. Some mice will leap or vocalize, and these behaviors might be employed instead of licking their paws. To reduce the potential for tissue injury from extended exposure to the heated surface, a cut-off period, usually 15 seconds, must be established. The automated version of this test measures the time it takes for an animal to jump and records it automatically.[49]

When an analgesic, such as morphine, is given, the time it takes for an animal to respond to being placed on a warm surface is lengthened. For the first time when using the hot plate test, or evaluating a new strain of an animal, attention must be given to determining the optimum quantity of stimulation (i.e., surface temperature) that will induce the intended reaction (i.e., paw licking or jumping). To ensure that the anticipated hot plate temperature will result in the appropriate sensitivity, one should also examine the effects of a reference analgesic such as morphine.[50]

2.5 Anti-inflammatory activity screening models

2.5.1 Carrageenan-induced paw edema model

Carrageenan has long been utilized as a toxic agent for generating inflammation in lab animals and screening compounds with anti-inflammatory properties. When this chemical is put into an experimental animal, it causes a significant inflammatory response.[51] A carrageenan solution containing 1-3 percent carrageenan is typically utilized and administered to experimental animals at a dose of 50-150 microliters.[52]

Carrageenan causes edema in rat models in two phases, depending on the experimental animal's age and weight.[53] The release of mediators like serotonin and histamine causes central nociceptor neurons to become sensitized in the first phase (0-2 hours). In the second phase, the protease, lysosome, prostaglandins, and bradykinin are released in large amounts. Clinically utilized anti-inflammatory medications are susceptible to the second phase (2.5-5 hours) of edema. Prostaglandins are important mediators of the inflammatory response during the second phase, and they can trigger nociceptors, causing pain.

2.5.2 Cotton pellet-induced granuloma model

In this approach, cotton pellets are implanted subcutaneously in the dorsal area of a laboratory animal to produce granulomas. The proliferative phase of inflammation is assessed using this model. Inflammation causes macrophages, neutrophils, and fibroblasts to proliferate, resulting in the formation of granulomas.[54]

The first events involve the accumulation of fluid and protein-aqueous substances, as well as the infiltration of neutrophils. By day 7, the granuloma will develop a capsule with fibrous vessels that contain infiltrating mononuclear cells and fibroblasts high in N-acetyl glucosaminidase (NAG). Dry weight measurements are used to assess granuloma formation, and the action of NAG and the overall content of nucleic acid are used to evaluate cellular content.[55]

3. OBJECTIVES

3.1 General Objective

- To investigate the analgesic and anti-inflammatory activity of *A.gummifera* bark hydro-alcoholic extract and its fractions in rodents.

3.2 Specific Objectives

- To determine the acute oral toxicity of the hydro-alcoholic bark extract of *A.gummifera* in mice.
- To evaluate the analgesic activity of *A.gummifera* bark extract and its fractions using an acetic acid-induced writhing test in mice.
- To evaluate the analgesic activity of *A.gummifera* bark extract and its fractions using a hot plate test in mice.
- To investigate the anti-inflammatory activity of *A.gummifera* bark extract and its fractions using the carrageenan-induced paw edema model in rats.
- To investigate the anti-inflammatory activity of *A.gummifera* bark extract and its fractions in a cotton pellet-induced granuloma model in rats.

4. MATERIALS AND METHODS

4.1 Drugs and chemicals

Drugs and chemicals that were utilized include: acetylsalicylic acid powder (Oxford Lab Fine Chem LLP, Enland), indomethacin powder (Sawraj pharmaceutical, India), morphine sulfate (Hameln pharma ltd,UK), dexamethasone (Drugfarm laboratories, India), normal saline (Vidharbh pharma LLP, India), carrageenan powder (Sigma Aldrich, Germany), methanol (Haihang industry, China), distilled water, glacial acetic acid 1% (Jagdamba Chemicals, India), hexane (Overlack AG, Germany), chloroform (Krada CPS Industry, Spain), and ethyl acetate (Shandong Minglang chemical Co.Ltd).

4.2 Materials and instruments

Digital plethysmometer, rotary evaporator (Heidolph, Germany), hot plate, electronic balance, mini orbital shaker, nylon gauze, plastic containers, tissue drying oven, cotton pellets, chromic catgut (0/4 metric-1/2 circle), and Whatman filter paper no. 1.

4.3 Collection and preparation of *Albizia gummifera*

The fresh bark of *A.gummifera* was collected from Worra Jarso District, North Shoa Zone of Oromia Regional State in Ethiopia, which is located 180 km from Addis Ababa. The plant collection was conducted during March-April 2022, a season when the community herbalists thought the medicinal plant's therapeutic activity would be at its peak. A sample of the plant bark was presented to a taxonomist for botanical verification and authentication. The plant samples were deposited for future reference with the code MT001 at Addis Ababa University's National Herbarium, College of Natural and Computational Sciences.

4.4 Extraction

A.gummifera bark was dried at room temperature under the shade. The bark of *A.gummifera* was crushed using a mortar and pestle. The crushed plant material (600g) was macerated with 80% methanol (6 liters) three rounds within three consecutive days at room temperature and it was shaken every 6 hrs. The extract was filtered with nylon and gauze before being passed through Whatman No. 1 filter paper. Following the extraction, methanol was removed under vacuum using rotavapor at 40°C. After that, the extract was dried in a hot oven at 40°C. Until used in bio-screening investigations, the concentrate was kept in sealed containers at 4°C temprature.

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

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

% Yield= (45 g)/ (600 g) X 100 = 7.5%

4.5 Fractionation

The extract (20g) was mixed with water (250 ml), and partitioning was done using a separating funnel and washed with several organic solvents (hexane, chloroform, ethyl acetate, methanol, and water) in ascending polarity order. Unfortunately, there was no extracted mass remaining after removing the solvents hexane, chloroform, and ethyl acetate. But from the hydro-methanol solution the dry crude extract was recovered by removing the solvents with a rotary evaporator and hot oven. The recovered crude extract was dissolved in methanol and washed many times until a clear solvent of methanol was obtained. Then the remaining mass of crude extract was completely dissolved in water. Finally, the water and methanol fractions were obtained. The methanol fractions of the plant extract were dried by evaporating methanol using a rotary evaporator and both fractions were dried in a hot oven set at 40°C. Until further investigation, all extracts were kept in a 4°C refrigerator.

4.6 Experimental animals

The experimental animals were obtained from the animal house of Addis Ababa University's School of Pharmacy, College of Health Sciences. Analgesic activity tests were performed on both sexes of Swiss albino mice, aged 6–8 weeks and weighing 25–35 g. In the acute and chronic anti-inflammatory models, equal ratios of male and female rats weighing 180–220g were used. The experimental rodents were kept in a clear plastic cage at room temperature for 12 hours in the dark, followed by 12 hours in light cycles. The rodents were fed a rodent pellet diet and had unlimited access to water. Before the experiments, the rodents were acclimatized to the laboratory environment one week prior to experment.

The study was conducted in accordance with the National Research Council's Guide for the Care and Use of Laboratory Animals and the Organization for Economic Cooperation and Development's (OECD) requirements.[56] Ethical approval with a reference number of ERB/SOP/467/14/2022 (Annex: 1) was acquired from the School of Pharmacy's Research Review Committee.

4.7 Acute toxicity test

The OECD guideline 425/2022 was followed for acute toxicity testing (OECD, Test No. 425: Acute Oral Toxicity: Up-and-Down Procedure, OECD Publishing, 2022).[57] The toxicity study used 6-8 weeks female albino mice that had been fasted. Only one female mouse was given a single dose of 2000 mg/kg crude extract via oral gavage. There was no fatality within the first 24 hours. After that, four more mice were enrolled in the extract therapy at a similar dose.

The animals were seen for general toxicity signs and symptoms for 4 hours with 30 minutes gap and thereafter for 14 days with a 24-hour period for general toxicology signs and symptoms. The signs and symptoms, including changes in the eyes mucous membranes, skin, behavioral patterns, somatomotor activity, salivation, diarrhea, weight loss, tremor, convulsions, paralysis, lethargy, and death, were monitored. But these signs and symptoms did not occur. Three treatment doses were chosen based on the results of 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).[54]

4.8. Pilot study

Six rats and six mice were grouped into three groups with an equal ratio of male and female participants as part of a pilot study to test the pharmacological activities of *A.gummifera*. Rats were utilized in the carrageenan-induced paw edema, while mice were used in the acetic acid-induced writhing test. Two animals per group received the crude extract at dose of 100, 200, and 400 mg/kg for each of the three dose levels. According to the findings, *A.gummifera* has analgesic and anti-inflammatory properties. The largest percentage of analgesic effect for the acetic acid model was 41.7% for 100 mg/kg, 45.6% for 200 mg/kg, and 53.2% for 400 mg/kg. Additionally, the crude extract of *A.gummifera* had an anti-inflammatory effect at all dosages used. The highest levels of paw edema inhibition measured at different dose levels were 47.2% for 100 mg/kg, 51.3% for 200 mg/kg, and 56.7% for 400 mg/kg.

4.9 Methods for the determination of analgesic activity

4.9.1 Acetic acid-induced writhing test

The analgesic effect of *A.gummifera* bark extract was tested in experimental animals as described by Collier *et a1.*,by inducing pain with acetic acid.[58]

The experimental animals were grouped into eleven groups, each with six mice. The experimental mice were fasted for 12 hours before pain induction and the administration of doses but were permitted access to water. The pain was generated by injecting a 0.6 percent acetic acid solution into the left side of the abdomen intraperitoneally at a dose of 10 ml/kg body weight. Abdominal muscular contraction in the belly and twisting of the body trunk of the animal were seen as signs of pain shortly after injection with acetic acid.

The different groups were treated as summarized in Table 1:

Table 1: Treatment protocol in different groups to test the analgesic activity of *A.gummifera* by acetic-acid-induced model

S.No	Group	Treatment protocol
1	I (Negative control)	DW 10 ml/kg
2	II (Positive control)	ASA 150 mg/kg (p.o)
3	III	CE 100 mg/kg
4	IV	CE 200 mg/kg
5	V	CE 400 mg/kg
6	VI	MF 100 mg/kg
7	VII	MF 200 mg/kg
8	VIII	MF 400 mg/kg
9	IX	AF 100 mg/kg
10	X	AF 200 mg/kg
11	XI	AF 400 mg/kg

DW= Distilled water, ASA=Acetyl salicylic acid, CE= Crude extract, MF=Methanol Fracction, AF= Aqeous Fraction

Each mouse in each group was injected with 0.6% acetic acid intraperitoneally into the stomach's left side at a dose of 10 ml/kg to elicit an unpleasant sensation 30 minutes after receiving different treatments. For 30 minutes, the number of abdominal constrictions

(writhes) in each mouse was tallied, starting 5 minutes after the intraperitoneal injection of acetic acid.

$$\%\ Analgesic\ activity = \frac{Mean\ writhing\ count(Control\ -\ Treated\ group)}{Mean\ writhing\ count\ of\ Control\ group} X100$$

4.9.2 Hot plate test

Analgesic activity was determined by the Hot Plate Test in experimental animals using the approach outlined by Care *et a1*.[59]

This test involves placing a mouse in an open space with a metallic plate as a floor that is kept at a constant temperature. This results in two behavioral elements, paw licking and jumping, which are evaluated by reaction times. These are thought to be responses that are supraspinally integrated.[57]

Mice of both sexes were placed into eleven groups, each with six mice. All of the animals were fasting for the entire night. Group I was given distilled water (10 ml/kg), and Group II was given the conventional drug morphine (reference group, 10 mg/kg p.o). Group III up to Group V were given varying doses of the crude extract (100 mg/kg, 200 mg/kg, and 400 mg/kg respectively). Group VI up to Group VIII were given varying doses of the methanol fraction (100 mg/kg, 200 mg/kg, and 400 mg/kg, respectively) and Group IX up to Group XI were given varying doses of the water fraction (100 mg/kg, 200 mg/kg, and 400 mg/kg, respectively). The animals were put on a heated plate that was kept at 55 degrees Celsius.

Each animal's response time was recorded prior to therapy. The response time was measured as the time it took to jump or lick the paw. At 30, 60, 90, and 120 minutes, the time taken to react was recorded. The time limit was set at 15 seconds.

$$\%\ Elongation = \frac{Latency\ of\ Test\ group\ -\ Latency\ of\ Control\ group}{Latency\ of\ Test\ group} X100$$

4.10 Methods for determination of anti-inflammatory activity

4.10.1 Carrageenan-induced paw edema

The acute anti-inflammatory activity was measured in rats using the Mccarson & Fehrenbacher method [60]. Rats were fasted for 12 hours and allowed to drink water freely until the experiment began. The rats were grouped into eleven groups, and distilled water was given to the negative control group, indomethacin 10 mg/kg was given to the positive control

group, and different doses of the crude extract, methanol fraction, and water fraction were given to the other groups separately. Carrageenan in 0.05 ml of a 1% solution was administered by injecting into the right hind paw of the mouse to cause acute inflammation. Carrageenan was injected 30 minutes after the extract was administered orally. Using a digital plethysmometer, the inflammation or displacement of water by edema in milliliters was measured at 0, 1, 2, 3, 4, 5, and 6 hours after the injection of carrageenan. In comparison to the control animals, the edema inhibition percentage was computed using the following formula:[54]

$$\%\ Inhibition = \frac{Mean\ paw\ volume\ of\ control\ group\ -\ Mean\ paw\ volume\ of\ test\ group}{mean\ paw\ volume\ of\ control\ group} X100$$

4.10.2 Cotton pellet-induced granuloma method

An approach described by Bailey *et al. (1982)* was utilized to evaluate chronic inflammation granuloma component.[55] For 12 hours, male albino Wistar rats (180-220 g) were fasted and then provided with unlimited water access until the investigation began. Distilled water was given to the negative control group, and dexamethasone (0.5 mg/kg oral) was given to the positive control group. Different doses of the extract and its fractions were given to the rest of the groups of rats separately. A sterile cotton pellet weighing 10 mg was made by rolling cotton and sterilized in an autoclave at 120°C and 15 pounds of pressure for 30 minutes.

Ketamine was used to sedate the rats 30 minutes after receiving the reference drug and the extracts, and a tunnel beneath the skin was created using sharp forceps in an aseptic manner on both sides among each rat's previously shaven region. The subcutaneous tunnel is then inserted bilaterally with two sterile cotton pellets, each weighing 10 mg, sutured by chromic catgut. The reference medicine (dexamethasone) and extracts and their fractions were administered for seven consecutive days (p.o/day).

The rats were euthanized on the eighth day and the pellets enclosed with granuloma tissue were meticulously removed from the superfluous tissue and cut out. The wet weight of the cotton was collected just after it was removed, weighed, and then dried at 60°C for 24 hours to get the net dry weight, which was calculated after removing the weight of the cotton pellets. According to the formula below, the amounts of exudate (mg), development of tissue granulation (mg), and percentage of exudate inhibition and tissue granuloma formation were computed as follow:

$$\%\ Exudates\ inhibition = 1 - \frac{Exudates\ in\ treated\ group}{Exudates\ in\ control\ group} X100$$

$$\%\ Granuloma\ inhibition = 1 - \frac{Granuloma\ in\ treated\ group}{Granuloma\ in\ control\ group} X100$$

Where: - Exudates = the pellets immediate wet weight − the cotton constant dry weight

- Granuloma = the cottons constant dry weight − the initial weight of the cotton pellet

4.11 Statistical analysis

The mean and standard error of the mean (SEM) were used to express the findings. Multiple mean difference and response comparisons of various groups with controls and extracts of various doses were carried out using SPSS and one-way analysis of variance (ANOVA), subsequent to the Tukey post hoc test for multiple comparisons of the mean differences of different treatments with the control group and extracts of different doses. At the 95 percent confidence interval, a "p" value of less than 0.05 was deemed significant. Tables and graphs were used to present the analyzed data.

5. RESULTS

5.1 Acute toxicity test

The acute oral toxicity test of *A.gummifera* bark crude extract at a dose of 2000 mg/kg did not show serious change in behavior, harmful consequences, or death within 24 hours or over the next 14 days. As stated in OECD Guideline 425 (2008), the limit test at 2000 mg/kg, it is plausible to deduce that the oral lethal dose in 50% (LD_{50}) of the crude extract in mice is more than 2000 mg/kg. Therefore, the results indicate that the extract of *Albizia gummifera* has a low toxicity level.

5.2 Analgesic activity of *A.gummifera*

5.2.1 The effects of *A.gummifera* extract and its fractions on inhibition of writhing in the acetic acid-induced writhing test model

The acetic acid-induced writhing test showed that the crude extract, the methanol fraction and the aqueous fraction of *A.gummifera* produced significant analgesic activity ($p<0.001$) as compared to the negative control group as shown in table 2.

Table 2: The number of writhing induced by acetic acid and the effect of *A.gummifera* bark the crude extract, methanol fraction, and water fraction

Groups	Number of writhing (Mean± SEM)
DW 10 lm/kg (a)	38.00±1.41
ASA 150 mg/kg (b)	11.71±0.7 a^{2} c^{2} d^{1} f^{1} i^{2} j^{1}
CE 100mg/kg (c)	24.50±1.338 a^{2} b^{2} e^{2} g^{1} h^{2} k^{1}
CE 200mg/kg (d)	20.33±1.28 a^{2} b^{1} e^{1} h^{1}
CE 400mg/kg (e)	12.66±0.66 a^{2} c^{2} d^{1} f^{1} i^{2} j^{1}
MF 100mg/kg (f)	21.00±1.41 a^{2} b^{1} e^{1} h
MF 200mg/kg (g)	16.35±1.77 a^{2} c^{1} i
MF 400mg/kg (h)	12.66±0.80 a^{2} c^{2} d^{1} f^{1} i^{2} j^{1}
AF 100mg/kg (i)	25.00±2.08 a^{2} b^{2} e^{2} g^{1} h^{2} k^{1}
AF 200mg/kg (j)	20.00±2.64 a^{2} b^{1} e^{1} h^{1}
AF 400mg/kg (k)	16.00±0.57 a^{2} c^{1} i^{1}

DW= Distilled water, CE= Crude extract, MF=Methanol fraction, AF=Aqueous fraction

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} $p<0.001$

5.2.2 Analgesic activity of *A.gummifera* extract and its fractions in acetic acid-induced writhing test model

The percentage of analgesic activity of *A.gummifera* crude extract and its fractions in the acetic acid-induced writhing model are presented in Table 2 below. Higher analgesic activity was observed in the standard group (ASA 150 mg/kg, 69.18%). Next to that, the high dose of the crude extract and the methanol fraction of the plant *A.gummifera* bark extract produce higher analgesic activity (66.68%) as compared to the other groups. The analgesic activity within the group was increased with the increasing dose, from a lower dose to a higher dose. The crude extract and methanol fraction groups showed the same analgesic activity.

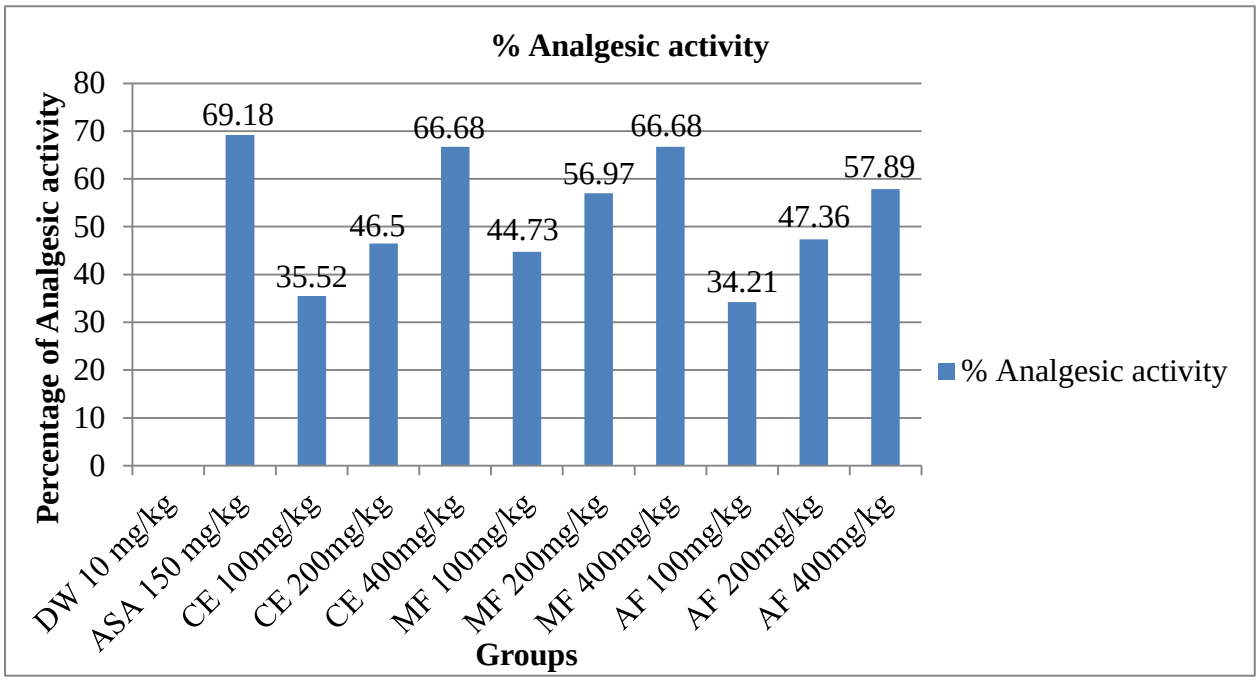

Figure 2: Analgesic activity of *A.gummifera* bark crude extract and its fraction by acetic acid induced writhing method.

DW= Distilled water, CE= Crude extract, MF=Methanol fraction, AF=Aqueous fraction, ASA= Acetyl Salicylic Acid

5.2.3 The effects of *A.gummifera* extracts and its fractions on elongation of the latency period in the hotplate model

In the hot plate test, in all groups, *Albizia gummifera* bark 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 3.

Table 3: Hot plate latencies of *A.gummifera* bark crude extract and its fractions

Groups	Latency time in sec (Mean ± SEM)				
	0 min	30 min	60 min	90 min	120 min
DW 10 ml/kg (a)	3.66±0.33	4.33±0.33	4.33±0.42	4.00 ±0.36	3.66±0.33
Morphine 10 mg/kg (b)	4.66±0.21	11.33±1.08 a^2 c^2 d^1 e^1 f^2 g^1 h1 i^2 j^2 k^2	12.33±0.88 a^2 c^2 d^2 e^1 f^2 g^2 h^2 i^2 j^2k^2	9.66±0.66 a^2 c^2 d^1 i^1 j^2 k^2	8.83±1.10 a^2c^1 i^2j^1 k^1
CE 100mg/kg (c)	4.83±0.30	6.33±0.42 b^2 d^1e^1 f g^1h^1	6.50±0.22 a^1 b^2 d^1 e^2 g^1	6±0.36 a^1 b^2 e^2 f^1 g^1 h^1	6±0.36 a^1 b^1 e^2
CE 200mg/kg (d)	4.50±0.22	8.66±0.49 a^2 b^1 c^1	8.66±0.42 a^2 b^1 c^1 i^1	7.16±0.16 a^2 b^1 e^1 h^1	7.00 ±0.25 a^2 c^2 e^1 i^2 j^1 k^1
CE 400mg/kg (e)	4.66±0.21	8.66±0.21 a^2 b^1 c^1	9.5±0.22 a^2 b^1 c^2 i^2 j^1 k^1	9.33±0.21 a^1 c^2 d^1 i^1 j^1 k^1	9.33±0.21 a^2 c^2 d^1 i^2 j^1 k^1
MF 100mg/kg (f)	4.17±0.30	7.50±0.42 a^1 b^2	7.50±0.22 a^2 c^1	8.66±0.42 a^2 c^1 j^1	7.33±0.33 a^2
MF 200mg/kg (g)	4.66±0.21	9.00±0.36 a^2 b^1 i^1	8.66±0.42 a^2 b^2 c^1 i^1	8.00±0.36 a^2 b^1	7.33±0.33 a^2
MF 400mg/kg (h)	4.16±0.30	8.66±0.21 a^2 b^1 c^1	8.50±0.61 a^2 c^2 d^1	9.50±0.56 a^2 c^2 d^1 i^1 j^1 k^1	8.00±0.36 a^2 i^1
AF 100mg/kg (i)	4.66±0.33	6.66±0.21 a^1 b^2 g^1	6.5±0.22 a^1 b^2 d^1 e^2 g^1	7.00±0.36 a^2 b^1 e^1 h^1	5.66±0.33 a^1b^2 e^2 h^1
AF 200mg/kg (j)	4.66±0.21	7.33±0.42 a^1 b^2 e^1	7.16±0.16 a^1 b^2 e^1	6.66±0.33 a^1 b^2 e^1 f^1 h^1	6.5±0.34 a^1 b^1 e^1
AF 400mg/kg (k)	4.83±0.16	7.33±0.33 a^1 b^2	6.83±0.30 a^1 b^2 e^1	6.83±0.30 a^2 b^2 e^1 h^1	6.50±0.34 a^1 b^1 e^1

DW= Distilled water, CE = Crude extract, MF=Methanol fraction, AF=Aqueous fraction

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} $p<0.001$

5.2.4 Analgesic activity of *A.gummifera* extract and its fractions in hot plate model

The percentage of elongation of the latency period of *A.gummifera* crude extract and its fractions in the hotplate model are presented in Fig. 4 below. The maximum percentage of elongation of the latency period (70.31%) 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 (61.47) was gained with a higher dose (400 mg/kg) of the crude extract group on 60 minutes and with a higher dose (400 mg/kg) of the methanol fraction group on 90 minutes. The percentage of elongation of the latency period increased with the increasing dose within the same group. The higher percentage of elongation of latency time was observed during 60 and 90 minutes as compared to the other time.

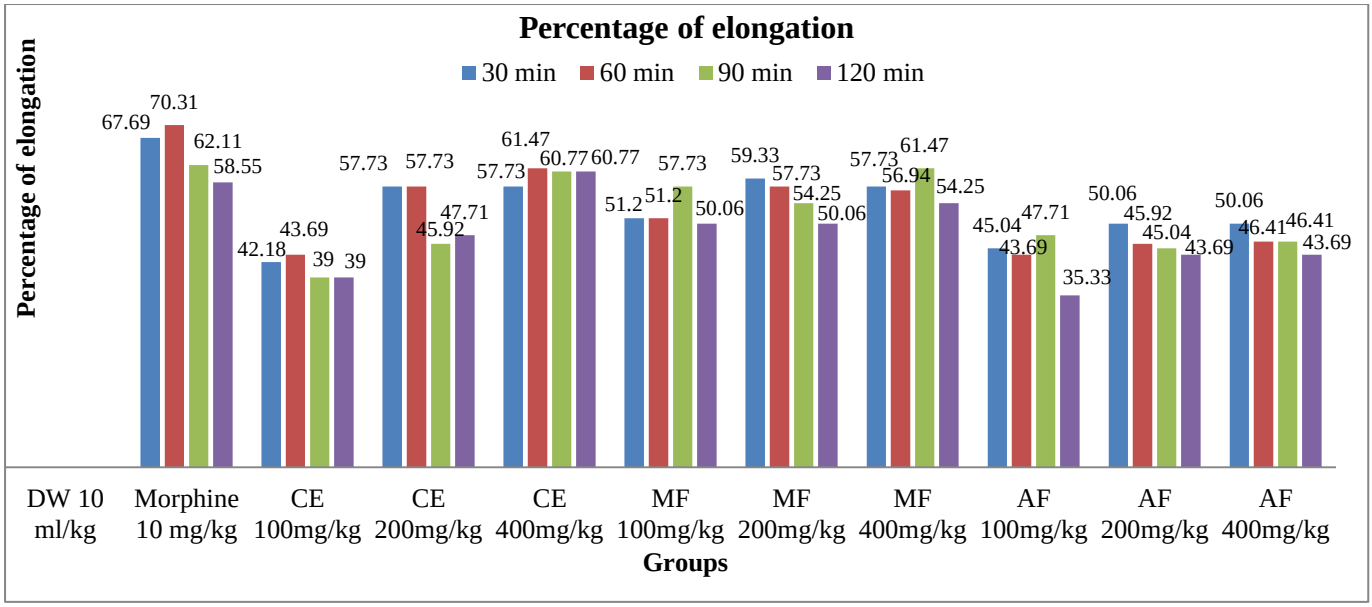

Figure 3: Percentage elongation of time latency on a hot plate by the crude extract of *A.gummifera* bark and solvent fractions of the crude extracts

DW= Distilled water, CE = Crude extract, MF=Methanol fraction, AF=Aqueous fraction

5.3 Anti-inflammatory activities of *A.gummifera*

5.3.1 The effects of *A.gummifera* extracts and its fractions on edema inhibition in the carrageenan-induced paw edema model

In the Carrageenan-induced paw edema method, 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 *A.gummifera* bark crude extract and its fractions on carrageenan-induced Paw edema in rats is shown below (Table 4). All the treatment groups produced statistically significant ($p<0.001$) effects as compared to the negative control group. In

addition, the comparison of different groups also shows a statistically significant effect ($p<0.05$).

Table 4: Carrageenan-induced Paw edema in rats and the effect of *A.gummifera* bark crude extract and its fractions

Groups	**Paw edema in ml (Mean ± SEM)**						
	0 hrs.	1 hrs.	2 hrs.	3 hrs.	4 hrs.	5 hrs.	6 hrs.
DW 10 ml/kg (a) Control	1.09±0.05	2.56±0.05	3.25±0.14	3.54±0.11	3.70±0.08	3.87±0.03	4.01±0.03
Indomethacin 10 mg/kg (b)	1.14±0.04	1.25±0.04 a^2 g^1	1.33±0.01 a^2 c^2 f^2 g^1	1.45±0.01 a^2 c^2 f^2 g^1h^1 i^1	1.58±0.02 $a^2c^2d^2f^2$ $g^2h^1i^1j^1$	1.77±0.03 $a^2c^2d^2$ $f^2g^2h^1i^1$	1.98±0.01 $a^2c^2d^2e^2$ $f^2g^2h^1$
CE 100mg/kg (c)	1.12±0.03	1.37±0.01 a^2 $e^1g^1k^1$	1.84±0.07 $a^2b^2e^1j^1k^2$	2.02±0.09 $a^2b^2e^2j^1k^1$	2.15±0.07 $a^2b^2e^2j^1k^2$	2.31±0.07 $a^2b^2e^1h^1$ $i^1j^1k^2$	2.5±0.05 $a^2b^2e^2$ $f^1g^1h^2$
CE 200mg/kg (d)	1.12±0.03	1.30±0.03 a^2 g^1 k^1	1.61±0.07 a^2 k^1	1.82±0.05 a^2b^1	2±0.03 $a^2b^1k^1$	2.21±0.05 a^2b^2 k^1	2.32±0.04 $a^2b^2i^1j^1k^1$
CE 400mg/kg (e)	1.12±0.04	1.15±0.01 $a^2c^2g^2i^1$	1.43±0.03 $a^2c^1f^1$	1.56±0.04 $a^2c^2f^1g^1$	1.75±0.05 $a^2c^2f^1$	2±0.03 $a^2c^1f^1$	2.21±0.02 $a^2b^1c^2$
MF 100mg/kg (f)	1.11±0.03	1.39±0.04 $a^2e^1k^1$	1.81±0.06 $a^2b^2e^1j^1k^2$	1.96±0.08 $a^2b^2e^1k^2$	2.09±0.08 $a^2b^2e^1k^2$	2.26±0.03 $a^2b^2e^1j^1k^2$	2.31±0.05 a^2b^2c1 $i^1j^1k^1$
MF 200mg/kg (g)	1.08±0.02	1.56±0.05 $a^2b^2c^1d^1$ $e^2h^2j^1k^2$	1.70±0.03 a^2b^1 k^2	1.87±0.04 $a^2b^1e^1k^1$	2.00±0.04 $a^2b^1k^1$	2.21±0.03 $a^2b^2k^1$	2.31±0.02 $a^2b^2c^1i^1j^1k^1$
MF 400mg/kg (h)	1.11±0.04	1.30±0.04 $a^2g^1k^1$	1.61±0.03 a^2 k^2	1.78±0.02 a^2b^1	1.88±0.02 a^2b^1	2.05±0.03 $a^2b^1c^1$	2.21±0.04 $a^2b^1c^1$
AF 100mg/kg (i)	1.13±0.03	1.4±0.01 $a^2e^1k^1$	1.62±0.01 a^2 k^2	1.77±0.03 a^2b^1	1.94±0.02 a^2b^2	2.04±0.06 $a^2b^1c^1$	2.11±0.02 $a^2c^2f^1g^1$
AF 200mg/kg (j)	1.12±0.02	1.33±0.02 $a^2g^1k^1$	1.50±0.02 a^2c^1 f^1	1.67±0.02 a^2c^1	1.86±0.05 $a^2b^1c^1$	2.01±0.05 $a^2c^1f^1$	2.10±0.04 $a^2c^1d^1f^1g^1$
AF 400mg/kg (k)	1.1±0.02	1.12±0.02 $a^2c^1d^1f^2$ $g^2h^1i^2j^1$	1.23±0.01 $a^2c^2d^1$ $f^2g^2h^2$	1.53±0.05 $a^2c^2f^2g^1$	1.67±0.07 $a^2c^1d^1f^2g^1$	1.86±0.07 $a^2c^2d^2f^2g^1$	2.08±0.03 $a^2c^2d^1f^1g^1$

DW= Distilled water, CE= Crude extract, MF=Methanol fraction, AF=Aqueous fraction

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} $p<0.001$

5.3.2 Anti-inflammatory activities of *A.gummifera* extract and its fraction in carrageenan-induced paw edema model

The maximum percentage of inhibition of inflammation was observed in 400 mg/kg of the aqueous fraction (62.15%) after 2 hours of drug administration. Also, the inflammation and swelling of the rat's hind paw were significantly inhibited with the higher dose of the crude extract (56.00%) after 2 hours of drug administration, as shown in Fig. 5 below. The maximum percentage of edema inhibition was produced after 2 and 3 hours, as compared to the other times.

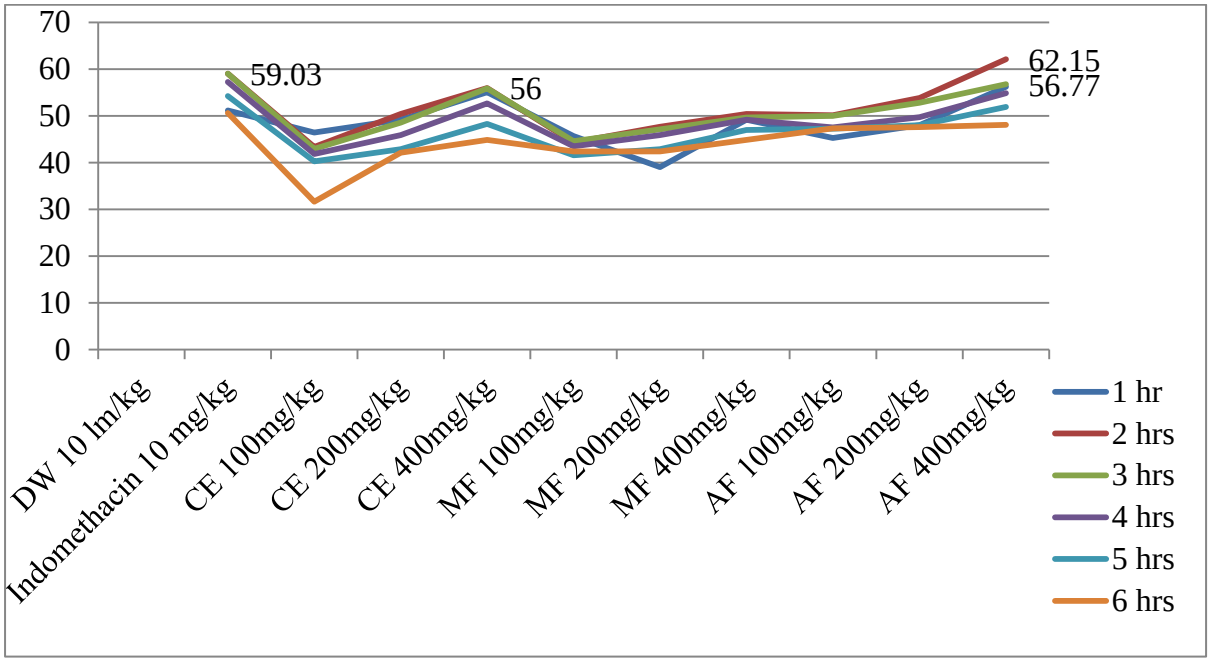

Figure 4: Percentage inhibition of inflammation in carrageenan-induced paw edema in rats by *A.gummifera* bark methanol extract and its solvent fractions

5.3.3 The effects of *A.gummifera* extracts and its fractions on exudate and granuloma inhibition in the cotton pellet-induced granuloma model

In the cotton pellet-induced granuloma method, the insertion of a sterile cotton pellet into the subcutaneous tunnel formed in the groin region of the rat induces inflammatory exudation and granuloma formation. The effect of *A.gummifera* bark crude extract and its fractions on cotton pellet-induced granuloma formation in rats is shown in Table 5. 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.

Table 5: Weights of exudate and granuloma in mg (Mean ± SEM) induced by the cotton pellet method in rats and the effect of *A.gummifera* bark crude extract and its fraction

Groups	Weight of exudates in mg (Mean ±S.E.M)	Weight of granuloma in mg (Mean ±S.E.M)
DW 10ml/kg (Control) (a)	57.96±0.88	30.58±1.93
Dexa 0.5 mg /kg (b)	20.5±0.76 $a^2c^2d^2e^1f^2g^2h^1i^2j^1$	10.25±0.60 $a^2c^1f^2g^1i^1$
CE 100mg/kg (c)	39.5±2.06 $a^2b^2e^1h^1j^1k^2$	16.38±0.89 $a^2b^1e^1k^1$
CE 200mg/kg (d)	36.7±0.77 $a^2b^2h^1k^1$	13.6±0.38 a^2f^1
CE 400mg/kg (e)	30.61±1.21 $a^2b^1c^1i^1k^1$	10.5±0.5 $a^2c^2f^2g^1i^1$
MF 100mg/kg (f)	37.33±2.02 $a^2b^2h^1k^1$	18.05±0.69 $a^2b^2d^1e^2h^1j^1k^2$
MF 200mg/kg (g)	34.7±0.77 $a^2b^2k^2$	15.93±1.26 $a^2b^1e^1k^1$
MF 400mg/kg (h)	28.45±1.47 $a^2b^1c^2d^1f^1i^1$	13.25±0.92 a^2f^1
AF 100mg/kg (i)	38.33±2.07 $a^2b^2e^1h^1j^1k^2$	15.83±0.60 $a^2b^1e^1k^1$
AF 200mg/kg (j)	30.36±1.90 $a^2b^1c^1i^1k^1$	13.18±0.64 a^2 f^1
AF 400mg/kg (k)	21.98±1.88 $a^2c^2d^2e^2f^2g^2i^2j^1$	10.5±0.42 $a^2c^1f^2g^1i^1$

DW= Distilled water, CE = Crude extract, MF=Methanol fraction, AF=Aqueous fraction

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} $p<0.001$

5.3.4 Anti-inflammatory activities of *A.gummifera* extract and its fraction on Cotton pellet-induced granuloma method

The percentage of exudate and granuloma inhibition in the cotton pellet-induced granuloma model is shown below in Table 6. The maximum percentage of inhibition of exudate (64.63%) and granuloma (66.48%) was observed in the standard group. Also, a high percentage of exudate inhibition was produced with the higher-dose aqueous fraction (62.07%), and similar granuloma inhibition was produced with the crude extract and aqueous fraction (65.66%). The inhibition of exudation and granulomas was found to increase in a dose-dependent manner.

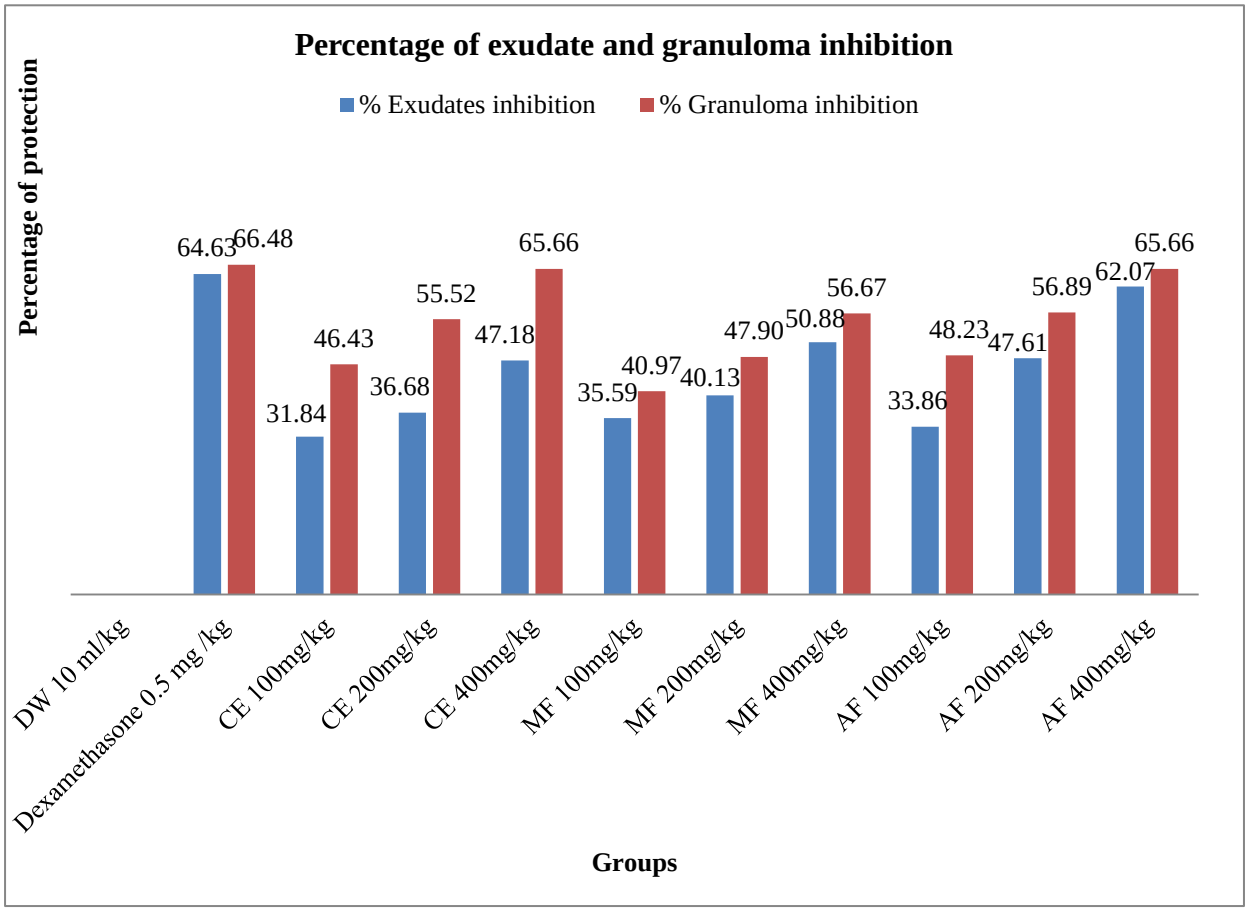

Figure 5: Percentage of exudate and granuloma inhibition by *A.gummifera* crude extract and its fractions

DW= Distilled water, CE= Crude extract, MF=Methanol fraction, AF=Aqueous fraction

6. DISCUSSION

This study was aimed at investigating the *in-vivo* analgesic and anti-inflammatory activities of *A.gummifera* bark crude extract and fractions. *A.gummifera* is used in traditional medicine for the management of pain and inflammation.[38] But the analgesic and anti-inflammatory activities of this plant were not approved scientifically by using models of pain and inflammation. Therefore, in the current study, the analgesic and anti-inflammatory activity of *A.gummifera* bark extract was evaluated to confirm its traditional claim. Our findings showed that the crude extract of the bark of *A.gummifera* and its methanol and aqueous fractions had analgesic and anti-inflammatory activities.

The acute toxicity study conducted in this study revealed that the plant *A.gummifera* bark methanol extract did not cause any sign of toxicity or mortality at a high dose of 2000 mg/kg in mice. This indicates that *A.gummifera* might have a good safety profile for use. Therefore, the plant *A.gummifera* is a promising plant for future use after a detailed safety profile study with sub-acute and chronic toxicity tests.

There is evidence that several pharmacological drugs may be beneficial in alleviating pain in various models.[61] So in this study, different models were used to detect the peripheral and central analgesic activity of *A.gummifera* bark extract.

The writhing response provoked by acetic acid is a chemical procedure for eliciting pain from peripheral sources in mice with acetic acid injection.[46] Hence, the acetic acid model was used to detect the analgesic activity of *A.gummifera* bark extract. It was proven that, in the acetic acid model, cytokines such as interleukin-1(IL), tumor necrosis factor (TNF), and chemokines work together to generate the writhing response.[62] Writhing in mice caused by acetic acid is mediated by spinal Mitogen-Activated Protein (MAP) kinases (ERK, JNK, and p38), PI3K, and microglia.[62] Similarly, in this study, the injection of 0.6 percent acetic acid intraperitoneally at a dose of 10 ml/kg body weight induced a pain sensation and a writhing response.

In this study, the writhing test triggered by acetic acid demonstrated that the crude extract of *A.gummifera* bark and its methanol fraction produced significant analgesic activity compared to the control group ($p<0.001$). The crude extract and the methanol fraction of the plant *A.gummifera* bark extract produced higher analgesic activity (66.68%) in comparison to the negative control group.

Non-steroidal anti-inflammatory drugs (NSAIDs) have been shown to alleviate pain and reduce edema by inhibiting prostaglandin synthesis and preventing the activity of the enzymes Cyclooxygenases (COX-1 and COX-2).[63] Aspirin has anti-inflammatory, analgesic, and antipyretic properties .[64] In this study, acetyl salicylic acid was used as a standard, and it showed significant analgesic activity ($p<0.001$) when compared to the control group. *A.gummifera* possesses analgesic activity comparable to the standard group. This may indicate that the extract might exert its action in a similar way to the standard drug.

The hot-plate test simulates acute thermal pain.[59] This model was chosen due to its sensitivity to analgesic action and the safety of the time restriction for applying heat stimulation to mice.[65] The mice responded to thermal stimulation on the hotplate by jumping and licking their paws.

Morphine, an opiate receptor agonist, can reduce pain-evoked responses in several supraspinal locations, including the thalamus and primary and secondary somatosensory cortex.[66] Morphine mediates its effects by activating the μ - opioid receptor.[67] Therefore, morphine was used in this study as a standard, and it produced higher analgesic activity after 60 minutes of administration (70.31%). The extract of *A.gummifera* shows comparable analgesic activity with morphine; this indicates that the plant has active constituents that may act in a similar manner on opioid receptors.

The hot plate test proved that all the crude extract and its solvent fractions showed significant analgesic activity ($p<0.001$) in comparison to the negative control group. Analgesic activity within the group increased with the increasing dose, from 100 mg/kg to 400 mg/kg. The activities increased with the increasing times, from 30 minutes up to 90 minutes, and then slightly decreased after 90 minutes. The effect of the extract increases in a dose-dependent way.

When we compare the percentage of elongation between the crude extract, methanol fraction and aqueous fractions; the crude extract and methanol fraction showed a higher percentage of elongation of the latency period (61.47%) at 60 minutes and 90 minutes, respectively. This may indicate that the active constituent of the plant extract that produces the analgesic activity may be found in the methanol fraction in higher amounts than in the water fraction.

Various studies show that an inflammatory reaction at the cellular and tissue levels includes a sequence of events such as dilatation of valves and arterioles, increased permeability of blood

vessels and blood flow, leukocyte diffusion into tissues, and edema development.[21] Accordingly, acute anti-inflammatory activity was investigated in the carrageenan-induced paw edema model.

The inflammation in the carrageenan-induced model was caused by Carrageenan is widely used to induce a condition of prolonged pain marked by changes in behavior such as allodynia, hyperalgesia, and other pain behaviors. While sensitized behaviors and associated central gene and physiological modifications within the dorsal horn of the spinal cord have been studied.[68]

The initial steps of the inflammatory response include the accumulation of fluid and protein-aqueous substances, as well as the influx of neutrophils.[55] The collection of this fluid results in increasing edema from the time of carrageenan injection into the right hind paw. Other mediators are released as a result of the release of arachidonic acid metabolites, adhesion chemokines, molecules, cytokines, and platelet-activating factors, and chemotaxis is initiated.[68] Consequently, in this study, the injection of a 1% solution of carrageenan (0.05 ml) into the right hind paw of a rat caused acute inflammation and induced progressive edema.

Indomethacin is a non-steroidal anti-inflammatory drug (NSAID) with very effective analgesic, antipyretic, and anti-inflammatory activity.[69] Hence, Indomethacin was used as a standard in the carrageenan-induced method and showed a significant percentage of inflammatory edema inhibition ($p<0.001$) in comparison to the negative control group. The high dose (400 mg/kg) of the aqueous fraction produces a statistically significant effect in comparison to the negative control group ($p<0.001$). The maximum percentage of edema inhibition (62.15%) was observed in the aqueous fraction after 2 hours of administration.

The investigation of isolated crude saponins from *Chlorophytum borivilianum* roots revealed that saponins have anti-inflammatory properties by suppressing inflammatory mediators such as histamine and prostaglandin and inhibiting fibroblast growth without influencing exudation. The inhibition of inflammatory mediators could be related to a change in gene expression.[70] Similarly, the plant *A.gummifera* contains saponin, which may contribute to its anti-inflammatory properties through suppression of inflammatory mediators.

Inflammation can be acute or chronic, and it happens in three stages. Inflammation begins with increased vascular permeability, followed by leukocyte infiltration, granuloma

development, and tissue healing.[68] In the current study, the cotton pellet-induced granuloma procedure was used, and the subcutaneous incorporation of a cotton pellet into a rat led to the formation of a granuloma at the location of the implant. This model has been used to determine the transduative and proliferative aspects of chronic inflammation. The transductive phase results in an increase in cotton pellet weight and the formation of granulomas as a result of an inflammatory response from fibroblasts, lymphocytes, and macrophages.[71] Therefore, in this model, the dry weight measure of granuloma was used to evaluate the chronic anti-inflammatory activity of *A.gummifera* extract and its fractions.

In the cotton pellet-induced granuloma model of the current study, dexamethasone was used as the standard, and it showed a statistically significant effect in comparison to the negative control group ($p<0.001$). Dexamethasone is effective in reducing inflammatory processes because it inhibits naive T-cell proliferation and differentiation via inhibiting CD28 co-stimulation required for effective T-cell priming and expansion.[72] Dexamethasone interacts with cytosolic glucocorticoid receptors (GRs), causing heat shock proteins to dissociate from the receptors, according to a molecular-level study. This separation causes conformational changes in the GRs, allowing them to enter the nucleus. GCs bind to GC response sites in the cell nucleus, altering transcription and inhibiting inflammatory granuloma formation.[73] The crude extract and the methanol and aqueous fractions of *A.gummifera* also showed significant anti-inflammatory activity as compared to the negative control group ($p<0.001$). A high percentage of exudate and granuloma inhibition was produced with the 400mg/kg of the aqueous fraction (62.07% and 61.87%, respectively). The inhibition of exudation and granulomas was observed to rise in a dose-dependent way. This indicates that it may act similarly to dexamethasone and produce a significant effect on the reduction of edema.

The plant *A.gummifera* contains several metabolites that may contribute to its analgesic and anti-inflammatory activities. Mainly, alkaloids, saponins, tannins, flavonoids, and phenols were the most common metabolites, which are expected to have analgesic and anti-inflammatory activity. Various studies show that saponin and flavonoids possess the potential to block pain sensation and inflammation by inhibiting the inflammatory mediators involved in pain and inflammation processes.[74] Tannins act against the inflammatory response via their free radical scavenging property.[75] In addition to this, tannins also have anti-inflammatory activity by suppressing arachidonic acid metabolism and reducing prostaglandin production.[76] The anti-inflammatory activity of flavonoids was linked to their mechanism of suppression of pro-inflammatory enzymes such as lipooxygenase, cyclooxygenase-2, and inducible NO

synthase.[77] Hence, the hyroalcoholic extract of the bark of *A.gummifera* might possess its analgesic and anti-inflammatory activity because it has metabolites that protect against pain and inflammation in high amounts.

Another plant in a similar genus, *Albizia,* was also reported to have significant analgesic and anti-inflammatory activity. The study conducted in Bangladesh on *Albizia lebbeck* reported that the plant's extract possesses high analgesic and anti-inflammatory activity.[78]

In a carrageenan-induced paw edema model, the extract of *A.lebbeck* showed 36.68% and significant edema inhibition at 400 mg/kg. In the acetic acid-induced writhing model, it showed a 39.9% and 52.4% reduction of writhing with 200 and 400 mg/kg, respectively. This suggests that due to the similarity of the main components of the plant constituents, they show similar activity, and the presence of these metabolites contributes to the analgesic and anti-inflammatory activity. As compared to *A.lebbeck*, *A.gummifera* possesses highly significant analgesic and anti-inflammatory activity.

Another species of the genus *Albizia*, *Albizia procera* 100 and 200 mg/kg, p.o., demonstrated considerable analgesic effect ($p<0.001$) in mice against formalin-induced pain sensation.[79] These findings suggest that *A.procera* contains bioactive chemicals with anti-inflammatory and analgesic properties. This indicates that several species of the genus Albizia possess considerable analgesic and anti-inflammatory activities.

7. CONCLUSION AND RECOMMENDATION

7.1 Conclusion

In conclusion, the current study demonstrated that *A.gummifera* bark extract and its solvent fractions possessed promising analgesic and anti-inflammatory activities. It extended the latency period, reduced the number of writhings, decreased inflammatory edema, and suppressed cotton pellet-induced exudate and granuloma formation. This finding shows that the traditional use of *A.gummifera* for pain and inflammation was sensible and scientifically supported.

7.2. Recommendations

The findings of this study on *A.gummifera* should serve as a foundation for further research into its analgesic and anti-inflammatory activities. As a result, additional research is required.

- To determine the mechanism by which *A.gummifera* exerts its analgesic and anti-inflammatory activity
- To determine the safety profile of *A.gummifera* through sub-acute and chronic toxicity testing in animal models

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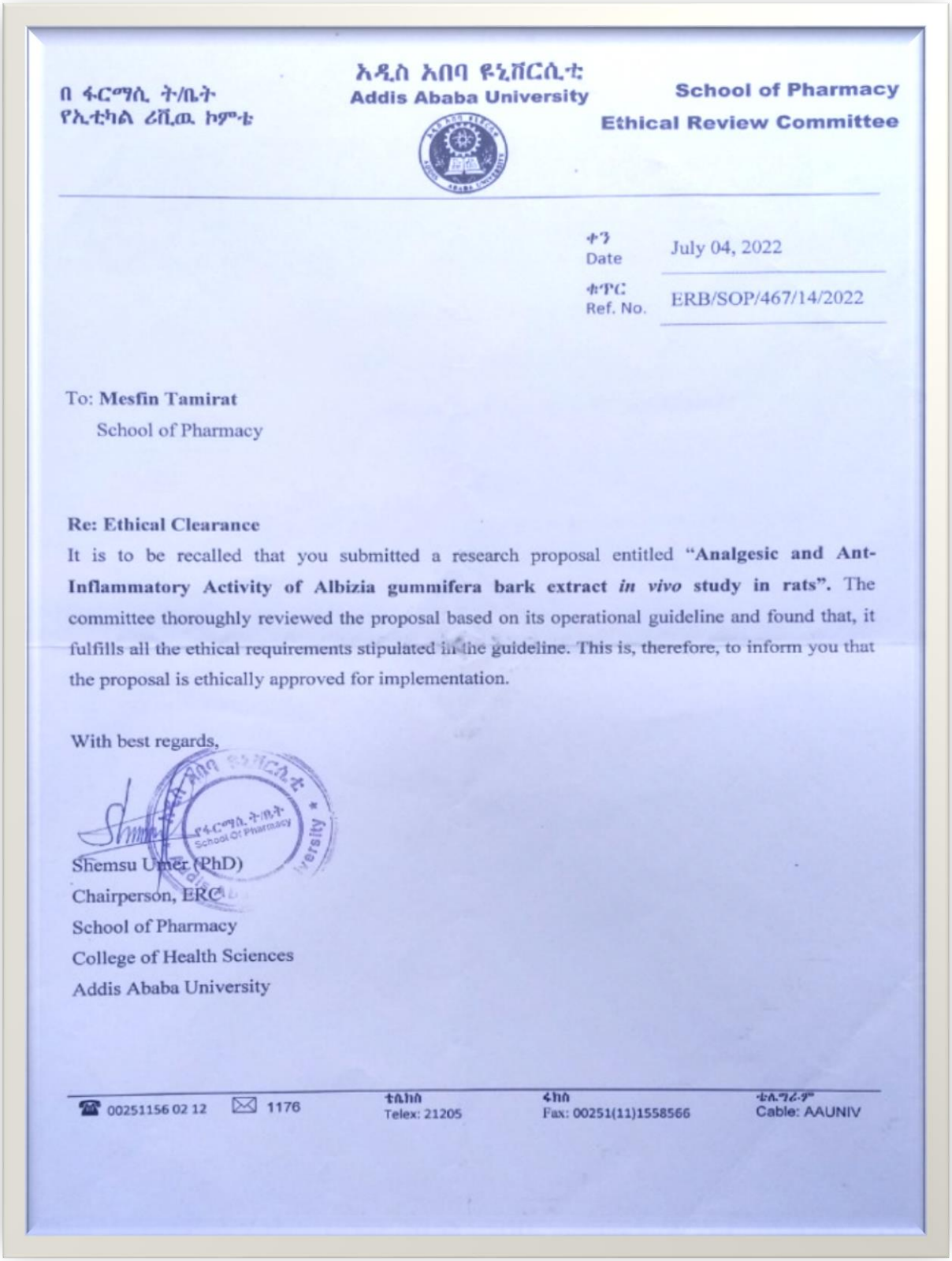
Annex 1: Ethical clearance



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Addis Ababa University

School of Pharmacy
Ethical Review Committee

ቀን Date: July 04, 2022

ቁጥር Ref. No.: ERB/SOP/467/14/2022

To: **Mesfin Tamirat**
School of Pharmacy

Re: Ethical Clearance

It is to be recalled that you submitted a research proposal entitled **"Analgesic and Ant-Inflammatory Activity of Albizia gummifera bark extract *in vivo* study in rats".** 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

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