Investigation of *In vivo* Analgesic and Anti-inflammatory Activities of 80% Methanol Extract of *Grewia feruginea* Bark, Identifying its Essential Oil Chemical Composition and *In Silico* Analysis of the Major Constituent

A Thesis submitted to the Department of Pharmaceutical Chemistry and Pharmacognosy of Addis Ababa University in Partial Fulfillment of the Requirements for the Degree of Master of Science in Medicinal Chemistry

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Abstract

Investigation of *In vivo* Analgesic and Anti-inflammatory Activities of 80% Methanol Extract of *Grewia feruginea* Bark, Identifying its Essential Oil Chemical Composition and *In Silico* Analysis of the Major Constituent

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Medicinal plants are most commonly used for the relief of pain and management of inflammation. *Grewia ferruginea* is traditionally used to treat cough, intestinal parasites, dandruff, malaria, scabies, diabetes mellitus, hemorrhoids, retained placenta, kidney infection, tooth ache, stomach ache, fire burn, wound and inflammatory disease conditions. However, there is no scientific validation that proves the traditional claims of *G. ferruginea* for the treatment of pain and inflammation. In this study animal models were used to investigate the analgesics and anti-inflammatory activities of 80% methanol crude extract of the bark of *G. ferruginea*. Either sex of swiss albino mice was randomly grouped into five groups of 6 mice per group. Distilled water (10ml/kg) was administered orally for the negative control group. Acetyl salicylic acid (150mg/kg), indomethacin (10mg/kg) and morphine (20 mg/kg) was used as a standard drug for positive control group. The remaining groups were treated with 80 % methanol bark extract of *G. ferruginea* at doses of 100, 200 and 400 mg/kg. Mices were then ordered to a battery of tests including acetic acid induced writhing and hot plate test for analgesics activity and carrageenan induced paw edema for acute anti-inflammatory tests. The crude extracts of *G. ferruginea* showed a dose-dependent significant reduction of pain in acetic acid induced analgesia models

($p<0.001$) with 400 mg/kg dose producing the highest reduction. The crude extract at all doses also showed a significant ($p<0.001$) analgesic effect in the hot plate method compared to the negative control, with maximum latency response observed at 120 minutes 32.32%, 40.32%, and 67.5% for 100mg/kg, 200mg/kg and 400 mg/kg extracts respectively. The crude extract significantly reduced carrageenan induced inflammation in a dose dependent manner, in which 400mg/kg crude extract shows the highest reduction of inflammation. The highest anti-inflammatory effect by the 100, 200 and 400 mg/kg of extracts were observed at 6hr post-induction with a value of 46.3%, 69.13 %, and 75.88% respectively, in carrageenan-induced paw edema model. Generally 80% methanol extract of *G. ferruginea* barks have potential anti-inflammatory and analgesics activities against stimuli in the rat and mice models which uphold the traditional use of the experimental plant. In this study a total of 20 components of essential oils were also identified using GC-MS methods, and trans β-ionone which is a major constituents of essential oil showed a good molecular docking results with PLA_2 enzymes.

Key words; Analgesic, Anti-inflammatory, *G. ferruginea*, *In silico*, Trans β-ionone

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List of abbreviations and Acronyms

AA- Arachidonic Acid

ADMET – Absorption Distribution Metabolism Excretion Toxicity

ATP- Adenosine Tri Phosphate

COX- Cyclooxygenase

CS – Corticosteroids

EO- Essential Oil

EPHI- Ethiopian Public Health Institute

GABA- Gama Amino Butyric Acid

GC –MS – Gas Chromatography Mass Spectrometry

IASP- International Association for Study of Pain

IL- Interlukin

IP- Intra Peritoneal

MPA- Maximum possible Analgesia

NF-κB -nuclear factor-kappa B

NGFs- Neutrophic Growth Factors

NSAIDs – Non Steroidal Anti-inflammatory Diseases

OECD- Organization of Economic Corporation and Development

PGs – Prostaglandins

PGE- Prostaglandin E

PLA_2- Phospho Lipase A_2

PO- Per Oral

QplogHERG- IC50 value for blockage of HERG K + channels

QPlogPo/w - Octanol/water partition coefficient

TNFα- Tumor Necrosis Factor alpha

WBC – White Blood Cell

WHO –World Health Organization

5-HT -5 Hydroxy Tryptamine

1. Introduction

1.1. Overview of pain

According to the International Association for Study of Pain (IASP) pain is an unpleasant sensory and emotional experience associated with actual or potential tissue damage or described in terms of such damage (Rajagopal, 2006). It is an unpleasant signal that something hurts. Pain is a complex experience that differs greatly from person to person, even between those with similar injuries or illnesses. Pain is a subjective result of nociception. Nociception is the encoding and processing of noxious stimuli in the nervous system that can be measured with electrophysiological techniques. Most of the nociceptors are polymodal, responding to noxious mechanical stimuli (squeezing, painful pressure or cutting of the tissue), noxious thermal stimuli (heat or cold), and chemical stimuli (Scholz & Woolf, 2002).

1.1.1. Pain pathway

The way the brain perceived a damaging stimulus in the body as painful is a complex process which is not still fully understood. This complex process which results from the nervous system is not being a hard wired system, although it exhibits plasticity that enables it to modify its function under different situations. Pain serves the purpose to protect the body and prevent tissue damage while it is healing. Sometimes, pain can become maladaptive and persist as chronic pain. These types of pain serve no protective function and are considered as pathological pain rather than physiological pain; it is then considered as a disease, no longer a symptom of another disease. Nociceptors detect painful stimuli; they are free nerve endings located in tissues and

organs. Nociceptors have a high thresholds and only respond to noxious stimuli (Fong & Schug, 2014).

Tissue injury may results from the release of inflammatory mediators, such as 5-Hydroxytryptamine (5-HT), bradykinin, histamine, ATP (Adenosine Tri Phosphate), nitric oxide, K+ and H+ from damaged cells. The arachidonic acid (AA) pathway plays a key role in many inflammatory diseases; Esterified AA on the cell membrane is hydrolyzed to its free form by phospholipase A2 (PLA2), further metabolized by lipoxygenases (LOXs) and cyclooxygenases (COXs) enzymes to a spectrum of bioactive mediators that includes lipoxins (LXs), prostanoids and leukotrienes (LTs). Most of this mediators are used as a novel preventive and therapeutic targets for different inflammatory diseases (Wang *et al*., 2021). Mediators like cytokine and growth factors are also released by immune cell activation. These inflammatory mediators provide an 'inflammatory soup' which produces a painful area of primary hyperalgesia and again spread into the tissues surrounding the initial area of injury to produce an area of secondary hyperalgesia (Dickenson, 2002).

1.1.2. Classification of Pain

Pain is often a debilitating symptom of different diseases and is considered a disease itself when it persists beyond recovery from an injury or illness. Pain can be very mild, almost unnoticeable, or explosive. Pain may be experienced as pricking, tingling, stinging, burning, shooting, aching, or electric sensations. Based on its duration pain can be majorly classified as acute or chronic (Merskey, 2008).

i. Acute pain

Acute pain is a painful condition with a rapid onset or of a short course to tissue damage resulting from injury, disease, overuse, inflammation or environmental stressors. This types of pain is self-limiting, however, can become chronic (Allegri *et al*., 2012).

ii. Chronic pain

Chronic pain is the pain that persists longer than the usual course of an acute injury or disease or the pain that recurs for months to years. It is disease in its own right and specific health care problem. The pathologic psycho behavioral manifestations of chronic pain is totally differ from acute pain (Feizerfan & Sheh, 2015). According to a 2014 study on the global burden of chronic pain, at least 10% of the world's population is affected by a chronic pain condition and every year, an additional 1 in 10 people develops chronic pain (Jackson *et al*., 2014).

1.1.3. Management of pain

Many substances are produced by the human body that influence the way in which pain messages are sent from the periphery to the brain and also the mechanism that the brain responds to these messages. Chemical messengers such as noradrenalin, GABA (Gama Amino Butyric Acid), 5-HT and endogenous peptides are some of this substances (Koneru *et al*., 2009). Drugs are external substances that can be used to supplement the body's inbuilt pain killing systems. The levels of the body's own painkiller system mimic or influenced by medications. The use of medication can have advantages of more profound pain relief effects, but this is often offset by different side effects. For instance the body's own endorphin levels suppressed by the use of opioids (Attal, 2001).

The application of massage, therapeutic touch, guided imagery, meditation, progressive relaxation, dreaming, rhythmic respiration, hypnosis, musical therapy, acupressure and cold-hot treatments are some of the non-pharmacological techniques of pain management. These non-invasive techniques control the gates to be transmitted to the brain and affect pain transmission or the release of endorphins and other natural opioids of the body (Demir, 2012; Helms & Barone, 2008)**.**

Acetaminophen, Nonsteroidal anti-inflammatory drugs (NSAIDs) and the narcotic analgesics are some of the commonly used drugs in the management of pain and inflammation (Colvin *et al*., 2014). NSAIDs are a group of drugs used to relief pain without causing physical dependency. Salicylates, nonsalicylates (acetaminophen) and NSAIDs are grouped under non-narcotic analgesics (Ford and Roach, 2021).

Pyrazolone or p-aminophenol derivatives acetaminophen has clinically useful antipyretic and analgesic efficacy but lacks a specific anti-inflammatory effect. It acts by inhibition of prostanoid formation by variety of COX enzymes (Bieger *et al*., 2011). Acetaminophen is considered as a first-line option due to its low cost and favorable safety profile. But at dosages of 4 g /day it is associated with a symptomatic elevation of aminotransferase levels even in healthy adults (Park & Moon, 2010). Patients treated with acetaminophen for chronic low back pain and osteoporosis showed slightly inferior pain relief than those treated by NSAIDs (Colvin *et al*., 2014). Acetaminophen is useful as a mild to moderate analgesic agent, especially for those with fever or NSAID contraindicated patients (Thomas, 2013).

NSAIDs which act by inhibiting COX enzymes are characterized structurally by an acidic moiety linked to an aromatic residue (Bieger *et al*., 2011). Both COX-1 and COX-2 inhibitor

NSAIDs appear to be effective in the treatment of pain disorders that have three desirable pharmacological effects which are analgesic, antipyretic and anti-inflammatory effects (Park & Moon, 2010).

Moderate to severe pain can be treated by narcotics analgesics which are controlled substances obtained from raw opium include codeine, morphine, camphorated tincture of opium and hydrochlorides of opium alkaloids (Ford and Roach, 2021). Analgesia produced by opioids through receptor-mediated blockade of pain transmission and neurotransmitter release (Thomas, 2013).

1.2. Overview of inflammation

Inflammation is an important response provided by the immune systems that ensures the survival during tissue injury and infection. Inflammatory responses are important to maintain normal tissue homeostasis. The mechanism of inflammation is a complicated process which is initiated by the recognition of specific molecular patterns associated with either infection or tissue injury. Inflammation is a degenerative process which is intense enough to cause local accumulation of low molecular weight catabolic products, which in turn elevates tissue osmotic pressure that attracts extra fluid, with or without heat release sufficient for significant elevation of tissue temperature (Ahmed, 2011).

Inflammation is a basic way in which the body reacts to irritation, infection or other injury, the key feature being pain, swelling, redness and warmth. (Stankov, 2012). Inflammation is defined by the presence of five macroscopic pathological phenomena, four of them proposed by Roman encyclopedist Aulus Cornelius Celsus in the first century AD. These are calor (elevated tissue temperature), tumor (swelling of the tissue), dolor (intensive sensation of a noxious stimulus),

rubor (blood color like redness of vascularized tissue), and impaired function of the affected organ. This cardinal signs have been considered as secondary to one primary pathophysiological events as a direct result of tissue damage (Xiao, 2017).

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

1.2.1. Phase of inflammation

Mechanism of inflammation represents a chain of organized, dynamic responses including both cellular and vascular events with specific humoral secretions. These pathways involve changing physical location of white blood cells (WBC) (monocytes, basophils, eosinophils, and neutrophils), plasma, and fluids at inflamed site (Abdulkhaleq *et al*., 2018). Immune defense cells release signaling molecules and secreted mediators such as serotonin, histamine, leukotrienes, PGs, oxygen and nitrogen derived free radicals principally in the mechanism which can contribute in the event of inflammation (Anwikar & Bhitre, 2010).

Acute and chronic phase of inflammatory response mediated by a different mechanism. Immune responses which involved in acute inflammation can be divided into cellular and vascular. The responses which occur in microvasculature normally appear in few minutes following microbial infection or tissue injury in the presence of other inflammatory stimuli. This process is rapid and eventually will lead to vasodilation and makes the vessels become more permeable. Then finally

inflammatory mediators can enter and produces interstitial edema (Lentsch & Ward, 2000; White, 1999).

Release of WBC from the circulatory system is essential during inflammatory responses (Farhangi *et al*., 2013). Chemotactic agents such as microbial endotoxins holding interleukins and amino terminal N-formyl methionyl groups along with the secretions of basophils such as leukotriene B, platelets activating factor and histamine can stimulate intense leukocytes infiltration within few minutes (Bitencourt *et al*., 2013). Neutrophils are the first inflammatory cells from the leukocytes that are recruited at the acute inflammation site (Curcic *et al*., 2015).

1.2.2. Mediators of inflammation

Tissue damage and inflammation result in activation and release of intracellular components from damaged cells, these includes histamine, serotonin, inflammatory cells (lymphocytes, neutrophils, macrophages), and the primary nerve fiber itself. The local release and spread of ions (H+, K+), prostaglandinE2 (PGE2) , bradykinin, COX, neurotrophic growth factors (NGFs) and cytokines, ILl (Interlukin 1), TNFa (Tumor Necrosis Factor a)) sensitizes pain fibers to subsequent painful and no painful stimuli (Scholz & Woolf, 2002).

PGs play an important role in the generation of the inflammatory response. PGs which are synthesized by many cell types are a group of long-chain fatty acids derived from AA. Their biosynthesis is majorly increased in inflamed tissue, and they contribute to the expression of the cardinal signs of acute inflammation. The role of PGs in the resolution of inflammation is more controversial even though the pro inflammatory properties of PGs during the acute inflammatory response are well established (Ricciotti & Fitzgerald, 2011)

In acute inflammation histamine is the best-known chemical mediator. Histamine causes vascular dilatation and the immediate transient phase of increased vascular permeability. It is stored in basophils, eosinophil, mast cells and platelets. Histamine which released from these sites is stimulated by lysosomal proteins and complement factors, which may increase vascular permeability, and neutral proteases, which finally activate the complement system (Lawrence *et al.,* 2002).

1.2.3. Management of inflammation

Steroidal drugs like prednisolone, dexamethasone and betamethasone (Barnes, 2006) or nonsteroidal drugs such as ibuprofen, indomethacin, aspirin, diclofenac, naproxen, nimesulide and celecoxib (Day & Graham, 2014) are used to treat both chronic inflammatory diseases such as osteoarthritis and rheumatoid arthritis; and acute inflammatory conditions (Suleyman *et al*., 2007). COX-1 and COX-2 isoforms are targets of NSAIDs. Both COXs are competitively inhibited by NSAIDs. The clinical efficacy of structurally distinct NSAIDs, all of which share this capacity for prostanoid inhibition, points to the importance of these mediators in the promotion of fever, pain and inflammation (Yuan *et al*., 2009). Corticosteroids (CS) are also the most effective and widely used drugs in many immune and inflammatory diseases. Endogenous corticosteroid production affected by CS therapy and has a suppressive effect on Hypothalamic-Pituitary Adrenal (HPA) axis. The actions of all CS are mediated by interaction of CS receptor with hormone, which regulates gene transcription (Barnes, 2006).

A number of side effects are associated with Prolonged use of these drugs for example, steroidal drug causes glaucoma, euphoria, adrenal atrophy, osteoporosis, cataracts, and suppression of response to infection or injury (Stanbury & Graham, 2015) ; and non-steroidal drug causes

bronchospasm and peptic ulcer due to blockade of both the physiological and inflammatory prostaglandins and concurrent production of leukotrienes (Suleyman *et al*., 2007).

1.3. Herbal analgesics and anti-inflammatory agents

Herbal medicines work in a mechanism that depends on an orchestral approach unlike that of the modern allopathic drugs. A variety of different molecules that is present in medicinal plants act synergistically on targeted elements of the complex cellular pathway. The search for new analgesic and anti-inflammatory drugs from traditional medicinal plant for pain relieve and treatment of inflammation is definitely logical and fruitful research strategy (Engels *et al*., 1991). By considering the high cost and adverse effects of synthetic conventionally available non-steroidal or steroidal drugs, the search for new analgesics and anti-inflammatory agents from herbal sources is getting popular with the objective to obtain better efficacy, greater safety and economical way to treat pain and inflammation (Gupta & Bhatia, 2008). Potent therapeutic agents developed from medicinal plants. New generation drugs with higher therapeutic value and low toxicity profile can synthesis from the templates of purified natural compounds that are isolated from plants (Kumar *et al*., 2013). St. johnswort, aloe, chickweed, ginger, licorice, meadowsweet, turmeric, and willow are some of the important medicinal plants which are found effective as anti-inflammatory (Chrubasik *et al*., 2007). Herbs like passionflower, aconite, hops, belladonna, henbane, lobelia, marijuana, tea, oats and wild lettuce are also have analgesic activity (Kaneria *et al*., 2007).

Over 6500 species of higher plants are found in Ethiopia which makes the country one of the most diverse floristic regions worldwide (Edwards and Asfaw, 1992). In Ethiopia traditional medicine practice most plants are commonly used as an anti-inflammatory and/or analgesic

agent. From the most commonly reported plants *A. sativum* (Giday, 2001), *Z. officinale, T. abyssinica and N. sativum* (Yirga, 2010), *M. stenopetala* (Teklehaymanot & Giday, 2010), *R. chalepensis* (Bekele & Reddy, 2015; Mesfin *et al*., 2009), *A. abyssinica* (Mesfin *et al*., 2013), *L. sativum* (Bekele & Reddy, 2015) and *Z. scabra* (Akele, 2012) were used as an anti-inflammatory and/or analgesic agents.

1.4. *Grewia ferruginea*

The genus *Grewia* from Tiliaceae family comprises approximately 150 species. *G. ferruginea* Hochst ex A Rich is Straggling shrub to tree up to 6 m high found in gallery forest near lakes, along rivers and in open Acacia-Combretum woodland, on dark brown soil; 1350-2700 m (Ullah *et al*., 2012). It occurs in most Ethiopian regions. In Ethiopia *G. ferruginea* has diferent vernacular names lenqoata or alenqoza in amharic, dokenu or ogomdi in oromifa, tsimkuya in tigregna (Flora of Ethiopia and Eretria). In Ethiopia different parts of *G. ferruginea* including its bark, leaves and roots were traditionally used for the treatment of different disease conditions. It was used for the treatment of taeniasis, dandruf, malaria, evil eye, scabicide, diabetes mellitus, hemorrhoids, toothache, intestinal parasites, retained placenta, tick and lice control, kidney infection, cough, stomach ache, fire burn, wound and inflammation (Fulas, 2001).

A variety of species from the genus *Grewia* have been reported to be used as medicinal agents to treat different diseases conditions and a number of compounds were isolated from various species of the genus *Grewia.* The extracts and preparations from the various plants, which are expectantly safe, exhibited various biological effects, e.g. anti-pyretic, anti-oxidant, antibacterial, hepatoprotective, anti-inflammatory, anti-emetic, anti-malarial and analgesic activities (Ullah *et al*., 2012).

Leaves and root extracts of *G. flava* possess significant antibacterial activity particularly against *S. aureus* and therefore could be used to combat bacterial diseases (Lamola *et al*., 2017). Wound, fracture and inflammatory disease conditions can be treated by *G. crenata* (Ukwuani *et al*., 2014). The anti-pyretic and analgesics activities of the aqueous extract of G. *tiliaefolia vahl* leaves were comparable to that of paracetamol (Sakat & Juvekar, 2009).

The dried fruits of *G. bicolor* are used as sweets; the roots, in poultice form, are applied to treat pustulent skin lesions and a decoction is given on the indication of delayed afterbirth to expel the placenta in humans and sometimes in cattle. There are a number of recent reports in the literature on the anti-inflammatory activities of triterpines, sterols and harman alkaloids (Jaspers *et al*., 1986).

G. ferruginea show activities against the bacterial strains *S. pygenes*, *P. aeruginosa and S. aureus* with 14-18.3 diameters of inhibitional zone at 25 mg/ml concentrations and also have significant inhibitory effect on *T. mentagrophytes* fungal strains (Sileshi *et al*., 2007). Significant *in vivo* laxative activity was observed from the leaf extracts of *G. ferruginea* by increasing gastrointestinal motility, fecal water content, stool weight, and intestinal fluid accumulation in mice (Tessema *et al*., 2020). Additionally the mucilage from *G. ferruginea* possesses pseudoplastic rheological behavior, good powder flow property, and swelling power. These characteristics of the mucilage reveals its potential for use as an excipient in formulating modified release dosage forms as well as semisolid and liquid dosage forms like suspensions (Haile *et al*., 2020).

Steroids, glycosides, flavones, lignans, phenolics, alkaloids, triterpenoids, lactones, anthocyanins and organic acids have been isolated from various species of this genus. Coumarinolignan were

isolated from *G. bilamellata* (Githinji *et al*., 2020). Hexanedioic acid, glutaric acid, 3,5 dihydroxy phenyl acrylic acid, (2,5 dihydroxy phenyl) 3',6',8'-trihydroxyl-4H chromen-4'-one were isolated from *G. optiva* and used in relieving the pathological symptoms and oxidative stress associated with excessive hydrolysis of acetyl and butyryl choline (Bari *et al*., 2019).

Vitexin, nitidanin, grewin, harman, contanoic acid, and gulonic acid have been isolated from different *Grewia* species (Ahamed *et al*., 2010; Waliullah *et al*., 2011). Heneicosanoic acid, β-sitosterol, propyl palmitate and catechin have been isolated from *G. Biloba* (Liu *et al*., 2008). Ahamed isolated γ-lactone, lactone and derythro- 2-hexenoic acid from *G. tiliafolia* in 2009 (Ahamed *et al*., 2010). Abirami reported the isolation of cyclopentadeca- 4, 12-dienone from *G. hirsute* (Natarajan *et al*., 2015). Mital also reported the isolation of octadecatrienoic acid, tridecanoic acid, octanoic acid and eicosanoic acid from *G. tenax* (Ullah *et al*., 2012). Glycosides like vitexin, lupeol, sitosterol, β-D-glucoside and isovitexin were isolated from *G. damin* (Jayasinghe *et al*., 2004)**.** Triterpenes like ursolic acid, betulin, betulinic acid, oleanolic acid , and the sterol; daucosterol were isolated from the stem bark of *G. optiva* (Uddin *et al*., 2011).

Phenols, flavonoids, steroids, saponins and tannins were reported by previous phytochemical screening test on methanol extract of the leaves of *G. ferruginea* (Haile *et al.*, 2020). Even though *G. ferruginea* is claimed to be used for its analgesics and anti-inflammatory activity by Ethiopian traditional healers, till now no studies have so far been carried out and there is no any report regarding the isolation of compound from the plant. This study therefore attempts to prove scientifically the traditional claim of *G. ferruginea* as analgesics and anti-inflammatory in Ethiopia.

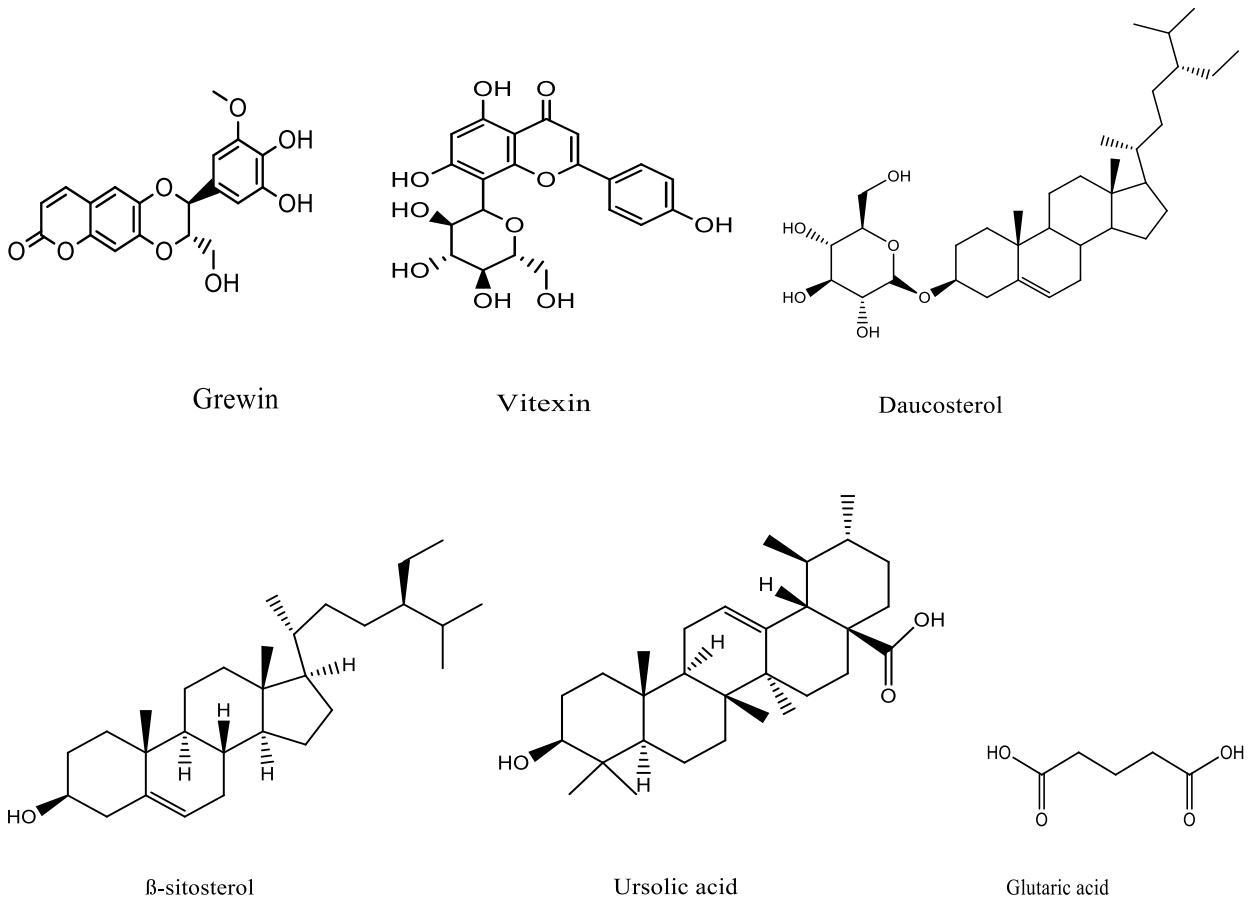

Figure 1: Compounds isolated from the genus *Grewia*

Figure 2: Photograph of *G. ferruginea*

1.5. Statement of the problem

Currently, a number of drug classes are available to manage inflammation and pain. However, the clinical uses of anti-inflammatory and analgesic agents are limited by their affordability, accessibility, adverse drug reactions and effectiveness (Yimer, 2019). Aspirin and morphine have been the most widely used analgesics drugs in recent decades. In most instances, these analgesic drugs, particularly opioids and NSAIDs, can only relieve 50% of the pain in about 30% of patients (Barrison and Wolfe, 1999).

A number of adverse effects such as hepatic injury, gastric ulcer, change in renal function, nephrotoxicity, bronchospasm, elevation of blood pressure, platelet inhibition, increased incidence of myocardial infarction and stroke which may result in increased bleeding are secondary to the use of NSAIDs (Ong *et al*., 2007; Stanos, 2013).

Corticosteroids are also associated with a variety of side-effects such as glucose intolerance/diabetes mellitus, immune suppression, hypertension, obesity, osteoporosis, glaucoma, and growth retardation in children (Rhen & Cidlowski, 2005; Spies *et al*., 2011). On the other hand opioid analgesics cause tolerance and physical dependence (Shih, *et al*., 2007; Yasmen *et al.,* 2018).

1.6. Significance of the study

Traditional medicines are used to treat different types of human disease. 80% of the total population of Ethiopia used these herbs for the treatment of different disease conditions (Bekele, 2007). Because of the great potential that herbal medicines hold in combating various diseases conditions, WHO (World Health Organizations) encourages the use of this plants to prove efficacy and safety in the healthcare programs (WHO, 2013).

A range of medicinal plants are believed to be an important source of new chemical substances with potential anti-inflammatory and analgesics therapeutic effects which have been widely used by traditional healers (Farnsworth, 1988). But, therapeutic potentials of some of these medicinal plants have not been scientifically evaluated. Among these plants *G. ferruginea* has traditional claim to use as an anti-inflammatory and analgesics agent (Megersa *et al*., 2019; Zenebe *et al*., 2012)

This traditional claim is not validated by scientific evidence. So, the present studies investigate the anti-inflammatory and analgesics activity of *G. ferruginea* with a view to validate its use by the traditional healers and communities. This experiment might also provide a hint about the possible mechanisms of anti-inflammatory and analgesics action and may serve as baseline information for further identification and investigation of compound for the development of new anti-inflammatory and analgesics drugs with low toxicity profile and high activity.

2. Objective

2.1. General objective

To investigate *in vivo* analgesic and anti-inflammatory activities of 80% methanol extract of *G. feruginea* bark, to identify its essential oil chemical composition and *in silico* analysis of the major constituent

2.2. Specific objectives

- To assess acute toxicity of the 80% methanol bark extract of *G. ferruginea*
- To investigate the analgesic activities of the 80% methanol bark extract of *G. ferruginea* by using acetic acid induced writhing test
- To investigate the analgesic activities of the 80% methanol bark extract of *G. ferruginea* using hot plate test
- To investigate the anti-inflammatory activities of the 80% methanol bark extract of *G. ferruginea* using carrageenan induced paw edema test
- To identify the essential oil chemical constituents of the dried bark of *G. ferruginea*
- To investigate *in silico* analysis of the major constituents of essential oil

3. Materials and Methods

3.1. Chemicals, drugs and reagents

Indometacin, morphine and acetyl salicylic acid in powder form, and distilled water were obtained from Ethiopian Pharmaceuticals Manufacturing (EPHARM). Hexane (Laboratory Fine Chemicals Pvt Ltd India), normal saline (Fresenius Kabi, India), glacial acetic acid, and methanol (Carlo Erba group reagents, Italy) were purchased from pharmaceutical vendors. Carrageenan (Sigma Aldrich, Germany) was obtained from Ethiopian Public Health Institute (EPHI)

3.2. Materials and instruments

Hot plate, measuring cylinder, flasks, clavanger, rotary evaporator (Buchi Rota Vapor R-200, Switzerland), gas Chromatograph 7890A System (Agilent Technologies, Greece), mass spectrometer (5975C VL MSD, Agilent Technologies, Greece), electronic balance (KERN-ALJ 220-4, Germany), drying Oven (Medite - Medizin technik, Germany), separatory funnel, syringes with needles, feeding tube, examination gloves, whatman filter paper no.1 and stopwatch were used to conduct the study.

3.3. Collection of plant materials

The fresh bark of *G. ferruginea* were collected from shebel, East Gojjam zone, Amhara regional state, Ethiopia around 290 km north of Addis Ababa in may 2021. Authentication and identification of the plant specimens was done at Addis Ababa University, college of natural sciences, national herbarium by a taxonomist, a voucher specimen was deposited with voucher number YY 002/2022 for additional reference. Firstly dust and debris particles are removed from

the bark by rinsing it with distilled water without squeezing and then dried at room temperature under shade conditions and finally mortar and pestle were used to reduce the size in to coarse powder.

3.4. Experimental animals

Analgesics activity study was performed by using healthy male and female swiss albino mice with 20–30g weights and 6–8 weeks age. Male albino wistar rats which weigh 180-220 g were used for the study of acute anti-inflammatory activity. Acute toxicity study was also assessed by using female mice. Rats were purchased from the animal house of EPHI and also mice were obtained from the animal house of School of Pharmacy, Addis Ababa University. Rats and mices were kept in plastic cages on a 12 hr light–dark cycle at room temperature and with access to pellet food and water *ad libitum*. They were weighed and fasted overnight before each laboratory experiment. Before the beginning of the experiment mice were acclimatized to the laboratory condition for one week. Handling and care of animals was performed according to international guidelines (OECD, 2008).

3.5. Preparation of plant Extracts

The bark of *G. ferruginea* was cleaned, air dried under shade and milled into coarse powder using a mortar and pestle. 600 gram of *G. ferruginea* bark was used and divided into two halves for the extraction process. The first half 300 gram of bark powder was macerated in 2 liter 80% methanol solvent. The remaining half 300 gram sample was also macerated by the same volume of solvent which are used before. The maceration was aided by occasional shaking for 72 hr at room temperature. Then, the extract was filtered first using muslin cloth and then using whatman filter paper no 1.

The marc was re-macerated twice using the same volume of solvent to exhaustively extract the plant material with the whole extraction taking a total of 9 days. After collection of the filtrate rotatory evaporator were used to concentrate at 40 ºC under reduced pressure. Then the concentrate was dried in an oven at reduced temperature. After water removal, a dark brown powder residue were obtained and kept in tightly stoppered bottles and stored at 2 ^{0}C (deep freezer) until use. Another 300 grams of *G. ferruginea* bark was macerated with 80% methanol solvent for the same period of time and with the same ration.

The following formula was used to calculate the yield of the extract

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

3.6. Hydro distillation

The clean dried barks of *G. ferruginea* were selected for oil extraction for the determination of essential oil components. Extraction of essential oil was performed by hydro distillation using Clevenger-type apparatus. The distillate was mixed with n-hexane in a separatory funnel in order to remove residual water from the oil. Then, the organic phase was separated and filtered using whatman No. 1 filter paper. Then, the organic solvent was evaporated in a rotary evaporator under reduced pressure at a temperature not exceeding 30 °C. The essential oil was weighed and stored in a refrigerator at -2°C in a tightly closed amber colored glass bottle for further investigation.

3.7. Acute toxicity tests

The limit test recommendations of Organization of Economic Corporation and Development (OECD) guideline 425 was used to perform acute toxicity test (OECD, 2008). 6-8 weeks age 12

hr fasted female albino mice were used to assess the toxicity study. Sighting study was firstly performed to determine the starting dose. 2000 mg/kg of the crude extract was given orally to a single female mouse as a single dose by using oral gavage. No death was recorded within 24 hr. The same dose of extract (2000mg/kg) was administered for additional four mices and the animals were observed sequentially for 4 hr with 30 min interval and then for a total of 14 consecutive days with a 24 hr interval for the general symptoms and signs of toxicities like convulsions, weight loss, behavioral pattern, salivation, diarrhea, tremor, lethargy, paralysis, food and water intake, changes in skin, eyes, mucous membranes and mortality. Three dose levels were selected based on the acute toxicity test results, which are a middle dose, which was one-tenth of the maximum dose obtained during acute toxicity study test; a high dose which was twice of the middle dose and a low dose which was half of the middle dose

3.8. Animal grouping and dosing

Mice of both sexes were randomly assigned into five groups. Each group contains six mice to investigate the analgesic activity test. Rats were used for anti-inflammatory activity. The first group which is negative control group received distilled water 10 ml/kg orally. The second group positive control group received standard drugs 150 mg/kg acetyl salicylic acid for acetic acid induced writhing test, 20 mg/kg indometacin for carrageenan induced paw edema model and 20 mg/kg morphine for hot plate method. The remaining three groups received different doses (100 mg/kg, 200 mg/kg and 400 mg/kg) of the extract orally by using an oral gavage. Acute toxicity test was used to determine the dose of crude extract.

3.9. Evaluation of Analgesic Activity of the Extract

3.9.1. Acetic acid induced writhing test

The analgesic activity of the crude extract was evaluated by slight modification of Arul *et al* method. Acetyl salicylic acid (150mg/kg) as a standard was given for positive control group. The mice were received intraperitoneal (i.p) injection of 0.6% v/v (10 ml/kg) acetic acid solution after one hour treatment of the extract. After five minutes administration of acetic acid, the number of stretches or writhes (characterized by the abdominal musculature contraction followed by extension of hind limbs) was counted for 20 minutes (Cavalcante-Silva *et al.*, 2012). A reduction in the number of writhes as compared to the control group was considered as evidence for the presence of analgesia, expressed as percent inhibition of writhing, which was calculated using the following formula:

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

3.9.2. Hot plate test

All mice were fasted overnight before the experiment was begun. The standard drug morphine (20 mg/kg po) was administered to the positive control group to investigate the analgesic activity. Pain was induced by placing the mice on a hot plate maintaining at 55 ±1 ∘C within the restrainer. The reactions time (in seconds) or latency period were determined when the mice react to the thermal pain by jumping or licking their paws. Then the reaction times were recorded at 0, 30, 60, 90 and 120 minutes after the administration of the crude extracts. In order to avoid injury to the tissues of the paws the cut off time were 15 sec. The maximum possible analgesia (MPA) should be calculated by the following formula (Fan *et al*., 2014):

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

3.10. Evaluation of Anti-inflammatory activity of the extract

3.10.1. Carrageenan induced paw edema test

The method of Winter *et al* was used to determine the anti-inflammatory activity of the crude extract in rat model. Administration of 100µl carrageenan (1% w/v carrageenan in normal saline) in the hind paw of the rat produced acute inflammation (Deng *et al.*, 2011). After one hour oral administration of the standard drug, vehicle and crude extract carrageenan was injected into the right hind paw of the rat. The displacement of water by edema using a digital plethysmometer was recorded as inflammation in terms of ml at 0, 1, 2, 3, 4, 5 and 6 hrs after carrageenan injection (Sharma *et al.*, 2010; Liao *et al.*, 2012). Percentage inhibition of edema was calculated in comparison to the control group using the following formula (Olukunle *et al.*, 2011; Saini & Singhal, 2012).

$$\%\ \text{Inhibition} = \frac{\text{Mean paw volume (control} - \text{ treatment)}}{\text{Mean paw volume in contro group}} \times 100$$

3.11. Gas chromatography/Mass spectrometry (GC/MS) analysis

GC-MS analysis was carried out on 7890A GC System (Agilent Technologies, Greece) fitted with a non-polar column; HP-5 MS, 0.25 µm film thicknesses and capillary column size of 30 m x 0.25 mm I.D. The essential oil (EO) was diluted with n-hexane in 10:1 ratio prior to analysis. The diluted EO (1µl) was injected at 240 °C by using helium as carrier gas at flow rate of 0.9 ml/min. Then the gas chromatograph oven temperature was programed to 60 °C for 1 min, and then 3 °C/min until the temperature reached 240 °C. The interface temperature was adjusted

at280 °C. When the GC separated component of the EO reached the mass spectrometer (5975C VL MSD, Agilent Technologies, Greece) it was ionized by 70 eV electrons with source temperature of 230 °C. Then ionized samples were separated and accelerated according to mass to charge ratio (m/z) by quadrupol with a temperature of 150 °C. Finally, the ionized species were detected by MS detector with scanning capacity of 40 - 400 amu. The compounds were identified by comparing the obtained mass spectra with the mass spectra from the database sources.

3.12. *In silico* study

3.12.1. Molecular docking study

Molecular docking studies were conducted in order to explore potential biological targets for the ligand compound. After initial docking assessments on potential targets, trans β-ionone was specifically docked onto the Phospholipase A_2 (PLA_2) enzymes, chosen for its significance in inflammatory mediators and as a potential target for analgesics and anti-inflammatory activity investigation (Jabeen *et al*., 2005).

3.12.1.1. Protein preparation

The Crystal structure of PLA_2 in complex with niflumic acid (PDB ID: 1TD7) was retrieved from the Protein Data Bank (PDB). Subsequently, protein preparation was performed using the Schrödinger Suite 2023 version 1 program (Schrödinger Suite Release 2023-2, 2022). This process included the addition of hydrogen's, addressing issues such as missing side chains, assigning correct bond orders, and adjusting ionization and tautomeric states utilizing the Epik tool. All water molecules beyond a 5 Å radius were removed. Optimization and subsequent

minimization of all atoms were carried out using OPLS3 force fields, with the convergence of heavy atoms to RMSD set at 0.3 Å.

3.12.1.2. Ligand preparation

The structure of trans β-ionone was drawn using ChemDraw Ultra (2019) in MDL SD file format. The Ligprep module of Maestro V13.5, Schrödinger Suite 2023-1 was used to perform ligand preparation (Schrödinger Suite Release 2023-2, 2022). Different ionization states and stereoisomers for ligand structure were generated.

3.12.1.3. Receptor grid Generation

In the docking experiment, the active sites within the PLA_2 structure were anticipated using the Receptor Grid Generation tool in Maestro (Schrödinger Suite Release 2023-2, 2022). A grid box, defined by x, y, and z coordinates, was created at the binding site of the co-crystal ligand for the purpose of docking, featuring a 6.0 Å radius. To mitigate the nonpolar aspects of the receptor, the van der Waals radii of the receptor atoms were adjusted, employing a partial atomic charge scaling factor of 0.8 and a partial cut-off of 0.15.

3.12.1.4. Ligand Docking

Employing the glide software in extra precision (XP) mode, the co-crystallized ligand was docked into the active site of the enzyme or protein to predict binding affinity and molecular interactions. Then after we conducted docking of the trans β-ionone compound into the active binding site of the prepared protein. Docking scores, measured in kcal/mol, were utilized for the analysis of the binding pose. All procedures and analysis were carried out using the Schrödinger suite of programs.

3.12.2. Pharmacokinetics Prediction study

Absorption, Distribution, Metabolism, Excretion and Toxicity (ADMET) analysis was employed to evaluate the drug-like characteristics of trans β-ionone, utilizing Lipinski's rule of five (Lipinski, 2004). The QikProp module within Schrödinger Maestro (v13.5) was utilized in this study to assess various ADMET properties of trans β-ionone (QikProp, 2015). These properties include molecular weight, hydrogen bond donor count, hydrogen bond acceptor count, lipophilicity and others (Lipinski, 2004). Qp log HERG was also considered to assess the toxicity profile.

3.13. Statistical analysis

SPSS version 20.0 was used to analyze the data. Statistical analysis was carried out by employing one way analysis of variance (ANOVA) followed by tukey post hoc test for multiple comparisons to compare results among groups. The experimental results are expressed as mean ± standard error of the mean (S.E.M), where p values < 0.05 were considered statistically significant. The analyzed data were then presented using tables.

4. Results and Discussions

4.1. Yields of crude extract and essential oil

The barks of *G. ferruginea* were subjected to two distinct extraction methods: maceration and hydro distillation. As a result, maceration involving dried and powdered *G. ferruginea* barks in 80% methanol led to a dark brown-colored extract with a percentage yield of 10.66% (w/w). On the other hand, hydro distillation of the dried bark yielded a pale yellow-colored essential oil with a distinct smell, and the yield was 0.33% (w/w).

4.2. Acute toxicity study

The acute oral toxicity test on the 80% methanol extract of *G. ferruginea* bark at 2000 mg/kg, following the Limit Test as per OECD guideline 425, revealed no adverse effects or mortality like sedation, sleep, hyperactivity, twitching, rigidity, jumping, irritability, abnormal secretion or death within the first 24 hours and the subsequent 14 days. Thus, it can be concluded that the oral LD_{50} of the crude extract exceeds 2000 mg/kg in mice, indicating a low or negligible toxicity profile. Therefore, the current study for the bark of *G. ferruginea* can be considered as safe, according to the 2008 OECD (OECD, 2008).

4.3. Analgesics activity

Two animal models were used to evaluate the analgesics activity of the crude extract. The first one was Acetic acid-induced writhing response which was a chemical method used to observe central and peripheral analgesic effects. The second method was hot plate test, thermal stimulus to investigate its central analgesic activity (Galani & Patel, 2010).

4.3.1. Acetic acid induced writhing test

The peripheral analgesics activity of different compounds was evaluated by writhing test or abdominal contraction test in rodent models. Acetic acid-induced writhing model produces pain sensation by triggering inflammatory response and such pain stimulus leads to release of arachidonic acid from tissue. Additionally the pain induction effects of acetic acid are manifested due to liberation of mediators such as cytokines, histamine, eicosanoids, serotonin, bradykinin, and prostaglandins particularly PGE2 and PGF2 as well as lipoxygenase products. These mediators promote increase vascular permeability, stimulate the nervous terminal of nociceptive fibers and reduce the threshold of nociception. This response is manifested by abdominal contractions characterized by movements of the hind paws particularly (Le Bars *et a*l., 2001; Kumar *et al*., 2009). The method was selected because of, its ability to mimic human clinical pain conditions and sensitivity to mild analgesics (Sharma *et al*., 2012).

In this study, the peripheral analgesics activity was evaluated by measuring number of writhes reduction in an acetic acid-induced writhing test. Results from the experiment are summarized in Table 1. It is interesting to note that the mice group treated with the 80% methanol extract of *G. ferruginea* significantly reduced the mean number of writhing ($p<0.001$) across all doses compared to the negative control group.

The crude extract exhibited percentage inhibitions of 38.94%, 51.3%, and 62.18% at doses of 100 mg/kg, 200 mg/kg, and 400 mg/kg, respectively (Table 1). Specifically, the higher dose (400 mg/kg) demonstrated a significant difference compared to both the lower extract dose (100 mg/kg) ($p<0.001$) and the middle dose (200 mg/kg) ($p<0.05$) of the extract. The analgesic effect of the crude extract showed a dose-dependent relationship, with the highest percentage inhibition

observed at 400 mg/kg, reaching 62.18%. However, the extracts demonstrated lower effectiveness compared to acetyl salicylic acid (positive control). There was a significance difference ($p<0.001$) between acetyl salicylic acid and the 100 mg/kg and 200 mg/kg extract doses, and a notable difference ($p<0.05$) compared to the 400 mg/kg extract dose.

Acetic acid induced writhing response inhibition was observed by both the standard drug acetyl salicylic acid and the crude extracts of *G. ferruginea*. The anti-nociceptive effect of *G. ferruginea* seen in this study may be mediated through suppression of prostaglandin pathway and or peripheral mechanism of pain inhibition (Ferdous *et al*., 2008).

Table 1: Effects of 80% methanol crude extract of the bark of *G. ferruginea* in acetic acid induced writhing test

Group	Mean no of writhing ± SEM	% Inhibition
DW	44.50 ± 1.63 $b^3c^3d^3e^3$	-
ASA 150	11.50 ± 0.76 $a^3c^3d^3e^1$	74.16
GF 100	27.17 ± 1.14 $a^3b^3d^1e^3$	38.94
GF 200	21.67 ± 1.31 $a^3b^3c^1e^1$	51.30
GF 400	16.83 ± 0.48 $a^3b^1c^3d^1$	62.18

Values are expressed as mean ± S.E.M (n=6); DW = Distilled water(10 ml/kg); ASA= Acetyl salicylic acid (150 mg/kg); GF100= *G. ferruginea* extract (100 mg/kg); GF200 =(200 mg/kg); GF400, =(400 mg/kg); a, compared to control; b, compared to ASA, c compared to100mg/kg, d compared to 200mg/kg, e compared to 400mg/kg,$^1p < 0.05$, $^2p<0.01$, $^3p<0.001$.

4.3.2. Hot plate test

The hot plate method was employed to evaluate the central analgesics effects of the crude extract. Pain is centrally modulated via a number of complex processes including opiate, dopaminergic, noradrenergic and serotonergic systems. It was selected for this study because of

several advantages including sensitivity to strong analgesics, accuracy of results, less time consuming and limited tissue damage because of a cutoff point i.e. 15 seconds that is usually applied to decrease the amount of time the mouse spends on the hot plate (Sharma *et al*., 2012). In this model, a plate heated at a constant temperature produces two supra-spinally integrated behavioral responses that can be measured in terms of their reaction times, namely jumping and paw licking. (Milind & Monu, 2013).

The analgesic potential of the bark of *G. ferruginea* crude extract was assessed in mice using the hot plate test. All doses of the extracts in this study suppressed pain sensation and showed a dose-dependent increase in latency time, indicating significant analgesic activity ($p < 0.001$) at the 120 min observation point compared to the negative control.

The 80% methanol extract of *G. ferruginea* demonstrated maximum analgesic effects at 120 min with values of 32.32%, 40.32%, and 67.50% for doses of 100, 200, and 400 mg/kg, respectively. The analgesic effect was observed to increase in a manner dependent on the dosage (Table 3). At the 400 mg/kg dose, the extract exhibited a significantly different analgesic effect compared to the lower and middle doses ($p < 0.001$) at 30, 60, 90, and 120 min of observation. However, there was no significant difference between the 100 mg/kg and 200 mg/kg doses throughout the observation period from 30 to 120 min.

Morphine, the standard drug, demonstrated a significant difference in analgesic activity compared to the negative control, lower dose, and middle dose of the extracts ($p < 0.001$) at 30, 60, 90, and 120 min of observation. It also exhibited a significant difference from the higher extract dose of 400 mg/kg ($p < 0.001$) at 30 and 60 min. However, there was no significant

difference between the standard and the 400 mg/kg dose of extracts at 90 and 120 min of the observation period.

Generally, hot plate exhibits neurogenic pain while acetic acid causes inflammatory pain by inducing capillary permeability (Bittar *et al*., 2000). Both types of pain were inhibited by the crude extract of *G. ferruginea.* The anti-nociceptive effect of the extract in these two models reveals dual action through peripheral and central mechanism.

Table 2: Effect of 80%methanol crude extract of the bark of *G. ferruginea* in hot plate test

Group	Mean latency time in seconds ± SEM				
	0 min	30 min	60 min	90 min	120 min
DW 10 ml/kg	3.017±0.0843	3.267±0.1022	3.550±0.0619	3.633±0.1174	3.700±0.0931
MO 20 mg/kg	3.450±0.1327	7.733±0.2824 $a^3c^3d^3e^1$	9.550±0.2432 $a^3c^3d^3e^1$	10.517±0.3701 $a^3c^3d^3$	12.183±0.2937 $a^3c^3d^3$
GF 100	3.050±0.1621	4.200±0.1633 b^1e^1	5.017±0.1108 $a^3b^3e^3$	5.233±0.2092 $a^2b^3e^3$	5.467±0.1783 $a^3b^3e^3$
GF 200	3.117±0.1138	4.317±0.2286 $a^1b^3e^3$	5.317±0.2040 $a^3b^3e^3$	6.017±0.4086 $a^3b^3e^3$	6.200±0.3225 $a^3b^3e^3$
GF 400	3.350±0.1138	6.633±0.3116 $a^3b^1c^3d^3$	8.683±0.2548 $a^3b^1c^3d^3$	9.833±0.2124 $a^3c^3d^3$	11.383±0.2937 $a^3c^3d^3$

Data represent mean± S.E.M (n = 6) ; $^1p<0.05$, $^2p<0.01$, $^3p<0.001$; a-relative to control; b-relative to standard; c- relative to 100 mg/kg; d- relative to 200 mg/kg; e-relative to 400 mg/kg; DW – Distilled water 10ml/kg; MO - morphine 20 mg/kg; GF 100 – G. *ferruginea* 100 mg/kg; GF 200 – *G. ferruginea* 200 mg/kg; GF 400 – *G. ferruginea* 400mg/kg.

Table 3: Percentage protection of 80% Methanol extracts of *G. ferruginea* on latency time of hot plate method in mice

Group	% Protection			
	30 min	60 min	90 min	120 min
MO	57.75	62.83	65.46	69.63
GF 100	22.21	29.24	30.58	32.32
GF 200	24.32	33.23	39.62	40.32
GF 400	50.75	59.12	63.05	67.50

MO - Morphine (20 mg/kg); GF 100 - Extract (100 mg/kg); GF 200 - Extract (200 mg/kg) and GF 400 - Extract (400 mg/kg).

4.4. Anti-inflammatory activity

Carrageenan-induced paw edema model was used to evaluate the acute anti-inflammatory activity of the crude extract. It is is a prototype of exudative phase of acute inflammation (Divakar *et al*., 2010). Carrageenan-induced rat hind paw edema has been widely used for the discovery and evaluation of anti-inflammatory drugs, since the relative potency estimates obtained from most drugs tend to reflect clinical experience (Winter *et al*., 1962). These method has been usually used to investigate the anti-edematous effect of natural products (Panthong *et al*., 2007). Additionally, carrageenan as a phlogistic agent is non-antigenic and is devoid of apparent systemic effects and exhibits a high degree of reproducibility (Kale *et al*., 2007). The injection of carrageenan into the sub-plantar tissue of the right hind paw of rats in the control group led to the development of edema, peaking at 0.95 ml at 2 hrs post carrageenan injection.

Inflammation induced by carrageenan develops immediately following injections. There are two phases that come after the release of inflammatory mediators secondary to the injection of carrageenan. The first phase, which occurs 0 up to 2.5 hr after the injection of carrageenan, has been attributed to the action of mediators like bradykinin, histamine and 5-HT on vascular

permeability. 5-HT and histamine are released during the first 1.5 hr while bradykinin is released between 1.5 and 2.5 hr after the injection of carrageenan on the right hind paw of the rat (Tamaddonfard *et al*., 2013).

Pro-inflammatory PGs and nducible COX-2 products with infiltration of PMN leucocytes (neutrophils) are released in the second phase which is 2.5 up to 6 hrs post carrageenan injection in the hind paw, (Dawson *et al*., 1991). The peak inflammation is usually seen approximately

After 2 up to 3 hr post carrageenan injection the peak inflammation was observed and is characterized by the release of PGs (Kumar *et al*., 2009). The second phase considered to be the most interesting phase in terms of inflammatory processes. The effect of NSAIDs which mainly inhibit the COX pathway has been evaluated by carrageenan-induced paw edema model. Most clinically effective anti-inflammatory drugs suppress carrageenan-induced hind paw edema in the second phase which correlates with therapeutic doses of clinically effective anti-edematous drugs (Mathew *et al*., 2014; Panthong *et al*., 2007). This phase is sensitive to most clinically effective anti-inflammatory drugs, which have been frequently used to assess the anti-edematous effect of natural products (Fernandes *et al*., 2010; Marrassini *et al*., 2010).

In this study indomethacin was used as reference drug. In the carrageenan-induced paw edema, the 80% methanol extract from the bark of *G. ferruginea*, at all tested doses, significantly reduced paw edema starting from 1 hr and lasting for 6 hrs after induction ($p < 0.001$) compared to the control group. Indomethacin (25 mg/kg), the standard drug, exhibited significant anti-inflammatory activity compared to the lower and middle doses of the extracts ($p < 0.001$). It also showed a statistically significant effect compared to 400 mg/kg ($p < 0.05$ at 1 and 4 hrs; $p < 0.01$ at 5 and 6 hrs).

The dose-dependent effect of the crude bark of *G. ferruginea* was evident from the 1st to the 6th hr, reaching its peak effect (55.66% inhibition) at the dose of 400 mg/kg at the 6th hr. The maximum anti-inflammatory effect for doses of 200 and 400 mg/kg of *G. ferruginea* was observed at 6 hours after induction, with values of 42.7% and 55.6%, respectively. In contrast, the maximum effect for the 100 mg dose (23.1%) was recorded at 5 hours, and the effect at this hour increased in a dose-dependent manner (Table 5).

Lower dose (100 mg/kg) of crude extract did not show sufficient anti-inflammatory activity at 1, 2, 3, 4 and 5 hr post carrageenan injection but percentage inhibition of inflammation was relatively highest at 6 hr (22.9%). This is because of the presence of little concentration of active ingredients in 100 mg/kg of crude extracts. Intergroup comparisons among different doses of *G. ferruginea* revealed that there were statistically significant effects observed between 100 and 200 mg/kg ($p < 0.001$), 100 and 400 mg/kg ($p < 0.001$) at all-time intervals. Additionally, there was a significant effect between 200 mg/kg and 400 mg/kg ($p < 0.001$ at 1, 2, and 3 hrs; $p < 0.01$ at 4, 5, and 6 hrs). There is no a significant differences between the standard drug indomethacin (10 mg/kg) and 400 mg/kg crude extract at 2 and 3 hrs interval.

This experiment may also give a clue about the duration of action and onset of the crude extract. Even though it is a challenge to forecast the time of onset for anti-inflammatory activity, the crude extracts of the bark of *G. ferruginea* for anti-inflammatory and analgesics activity showed rapid onset, and the duration seems to be fairly long. The observed edema inhibition was prominent in the later phase of inflammation, which was similar to the effect of NSAIDs indicating that the anti-inflammatory activity is possibly mediated through inhibition of pro-inflammatory metabolites like PGs and COX enzyme (Burke *et al*., 2006).

Generally the increased paw volume during the early and late phases of carrageenan-induced inflammation was effectively inhibited by 80% methanol crude extract of *G. ferruginea*. This may be due to active components of the crude extract suppress both phases of inflammation by inhibiting the activity and/or release of the inflammatory mediators involved in carrageenan induced paw edema such as COX, 5-HT, histamine PG, bradykinin, leukotriens and eicosanoids (Ma *et al*., 2013; Saini & Singha, 2012).

Table 4: Effects of 80% methanol extract of *G. ferruginea* on carrageenan induced paw edema model in rat

Group	Mean paw volume (ml) (Mean ± SEM)						
	0 h	1 h	2 h	3 h	4 h	5 h	6 h
DW	0.8933±0.0076 $b^3c^3d^3e^3$	0.9533±0.0120 $b^3c^3d^3e^3$	0.9433±0.0072 $b^3c^3d^3e^3$	0.9317±0.0130 $b^3c^3d^3e^3$	0.9283±0.0079 $b^3c^3d^3e^3$	0.9233±0.0084 $b^3c^3d^3e^3$	0.9133±0.0084 $b^3c^3d^3e^3$
Indo 10	0.4117±0.0105 $a^3c^3d^3e^3$	0.4517±0.0060 $a^3c^3d^3e^1$	0.4117±0.0075 $a^3c^3d^3$	0.3767±0.0080 $a^3c^3d^3$	0.3450±0.0115 $a^3c^3d^3e^1$	0.3400±0.0110 $a^3c^3d^3e^2$	0.2983±0.0095 $a^3c^3d^3e^2$
GF 100	0.7883±0.0166 $a^3b^3d^3e^3$	0.8250±0.0195 $a^3b^3d^3e^3$	0.7600±0.0234 $a^3b^3d^3e^3$	0.7417±0.0257 $a^3b^3d^3e^3$	0.7333±0.0239 $a^3b^3d^3e^3$	0.7100±0.0224 $a^3b^3d^3e^3$	0.7033±0.0208 $a^3b^3d^3e^3$
GF 200	0.6650±0.0205 $a^3b^3c^3e^3$	0.7083±0.0230 $a^3b^3c^3e^3$	0.6383±0.0247 $a^3b^3c^3e^3$	0.5733±0.0247 $a^3b^3c^3e^3$	0.5483±0.0292 $a^3b^3c^3e^2$	0.5350±0.0317 $a^3b^3c^3e^2$	0.5233±0.0297 $a^3b^3c^3e^2$
GF 400	0.5150±0.0165 $a^3b^3c^3d^3$	0.5283±0.0154 $a^3b^1c^3d^3$	0.4800±0.0121 $a^3c^3d^3$	0.4492±0.0078 $a^3c^3d^3$	0.4333±0.0067 $a^3b^1c^3d^2$	0.4250±0.0096 $a^3b^2c^3d^2$	0.4050±0.0120 $a^3b^2c^3d^2$

Data is expressed as mean ± S.E.M. (n =6), a- compared to control; b- compared to indometacin, c-compared to100mg/kg, d -compared to 200mg/kg, e- compared to 400mg/kg, $^1p<0.05$, ^2p 0.01 $^3p<0.001$.

Table 5: Percentage protection of 80% methanol extract of *G. ferruginea* on carrageenan induced paw edema model in rat

Group	Anti-inflammatory activity (%A)						
	0 h	1 h	2h	3 h	4 h	5 h	6 h
DW	-	-	-	-	-	-	-
Indo 10	53.91	52.62	56.36	59.57	62.84	65.34	67.34
GF 100	11.75	13.46	19.43	20.34	21.00	23.10	22.99
GF 200	25.56	25.70	32.33	38.47	40.94	42.06	42.70
GF 400	42.34	44.58	49.11	51.78	53.32	53.97	55.66

Data represent mean ± SEM (n=6); Indo 10 – Indomethacin 10mg/kg; GF100 - Extract (100 mg/kg); GF 200 - Extract (200 mg/kg) and GF 400 - Extract (400 mg/kg).

4.5. Chemical composition of the essential oil

GC-MS is widely utilized for identifying individual components in essential oils. In this study, we conducted GC-MS analysis on the essential oil extracted from the bark of *G. ferruginea*. The identification of individual compounds was achieved through the use of retention index (RI) and mass spectral (MS) data. The analysis revealed a total of 20 compounds, constituting 84.06% of the total oil. Sesquiterpinoids are the major components of the identified essential oils, which accounts 20.044% of the total identified essential oil followed by hydrocarbons (11.718%), fatty acids (7.004%), diterpinoids (4.739%), monoterpines (4.414%) and alkylated phenols (3.363%). The relative percentages of the identified constituents are detailed in Table 6.

Although no single compound emerged as the dominant component in the essential oil, trans-β-ionone (7.505%), 5E, 9E-farnesylacetone (4.805%), phytol (4.739%), methylhexadecanoate (4.113%), di-tert-butylphenol (3.363%), 6, 10, 14-trimethyl-2-pentadecanone (3.310%), methyl-9-octadecenoate (2.891%), trans α-ionone (2.729%), and neryl acetone (2.725%) were identified as main components in the essential oil of *G. ferruginea*. The essential oil also contained minor

compounds, including trans nerolidol (1.695%), trans eugenol (1.151%), β-damascenone (0.246%), cis/trans-linalol oxide (0.175%) and α-pinene (0.117%).

Trans-β-ionone was reported from the different aerial parts of *Smirnovia turkestana* from the Fabaceae family by GC-MS analysis (Akhbari *et al*., 2018). It was also extracted from the Tiliaceae family *Triumfetta rhomboidea* (Mevy *et al*., 2006). Hexadecane, octadecane, 1-octadecene and phytol were synthesized from *G. bulot* (Pham *et al*., 2023). Phytol and 6, 10, 14-trimethyl-2-pentadecanone were also reported from *Grewia lasiocarpa* (Akwu *et al*., 2019).

α -pinene which is found in the oils of many plants have an inhibitory effects of inflammatory responses induced by lipopolysaccharide (LPS) using mouse peritoneal macrophages. It also significantly Inhibit inflammatory mediators like IL-6, TNF- α, nitric oxide (NO), COX2, mitogen-activated protein kinases (MAPKs) and the nuclear factor-kappa B (NF-κB) (Karthikeyan *et al*., 2018; Kim *et al*., 2015). α- pinene and linalool exhibit significant anti-inflammatory and analgesic effects through inhibiting nociceptive stimulus-induced inflammatory infiltrates and COX-2 overexpression (Li *et al*., 2016). Eugenol is an Inhibitor of pro-inflammatory mediators, including IL-1β and IL-6, TNF-α, PGE2, NF-κB, and leukotriene C4 and 5-lipoxygenase (5-LOX). Its anti-inflammatory activity is associated with preventing neutrophil/macrophage chemotaxis and inhibiting the synthesis of inflammatory neurotransmitters, such as PGs and leukotrienes (Andrade & Mendes, 2020). Trans nerolidol, β-damascenone and di-tert-butylphenol were also reported for their analgesics and anti-inflammatory activities (Chan *et al*., 2016; Moore & Swingle, 1982; Pan *et al*., 2019).

The *Grewia* species contains biologically significant amounts of primary metabolites such as minerals, fiber, carbohydrates, protein, amino acids and ash, but low contents of fats and fatty

acids. These make the species a good choice for weight conscious people and healthy life. Additionally, a number of compounds are isolated from the various parts of the genus *Grewia* such chlorogenic acid, caffeic acid, gallic acid, quercetin, myricetin, vitexin, catechins and morin; can be used for the development of nutraceuticals in order to address life threatening ailments (Qamar *et al*., 2021). They are also a source of a number of essential oils.

This study presents, for the first time, the separation and identification of the essential oil components from the bark of *G. ferruginea* using GC-MS. The chemical structures and classifications of the identified constituents are compiled in Annex 1 as a supporting document. Additionally, gas chromatograms of the oil are included in Annex 2.

Table 6: Essential oil components of the bark of *G. ferruginea*

No.	Components	RT	RI(HP5-MS)	Area%
1	α-pinene	5.195	931	0.117
2	not identified (mixture)	10.756	1106	0.294
3	cis/trans-Linalool oxide	13.419	1172	0.175
4	trans-Eugenol	21.129	1357	1.151
5	β-Damascenone	22.197	1382	0.246
6	Alkane	22.599	1392	1.050
7	Trans- α-lonone	23.959	1425	2.729
8	Neryl acetone	25.041	1452	2.725
9	Trans- β -lonone	26.300	1483	7.505
10	Di-tert.-butylphenol	27.443	1512	3.363
11	trans-Nerolidol	29.389	1563	1.695
12	1-Hexadecene	30.517	1592	2.506
13	Hexadecane	30.806	1600	0.689
14	1-Octadecene	37.731	1793	2.598
15	Octadecan	37.975	1800	0.710
16	6,10,14-Trimethyl-2-pentadecanone	39.466	1844	3.310
17	5E,9E-Farnesylacetone	41.855	1917	4.805
18	Methylhexadecanoat	42.165	1926	4.113
19	Alkene	44.312	1994	4.165
20	Methyl-9-octadecenoate	47.535	2099	2.891
21	phytol	47.897	2111	4.739

In addition to the essential oils which are discussed above, preliminary phytochemical screening was previously performed for the bark of *G. ferruginea*; and the presence of terpenoids, flavonoids, saponins and tannins was reported (Tura *et al*., 2017). The anti-inflammatory and the analgesics activities of the crude extract may be also by the presence of these secondary metabolites. These phytochemicals are well known for their ability to inhibit inflammatory conditions as well as pain perception due to their inhibitory effects on enzymes involved in the production of the inflammatory chemical mediators (Kaushik *et al*., 2012).

The analgesics and anti-inflammatory effect of the crude extract may possibly be associated with the activities of secondary metabolites whose protection against inflammation is reported somewhere else; terpenoids (Cadirci *et al*., 2012; Li *et al*., 2009), flavonoids (Chen *et al*., 2018; Liu *et al*., 2014; Moscatelli *et al*., 2006; Narayan & Kumar, 2013), saponins (Hernandez-Ortega *et al*., 2012; Kothavade *et al*., 2015), and tannins (Hernandez-Ortega *et al*., 2012; Ma *et al*., 2013). Flavonoids and terpenoids can significantly inhibit a number of inflammatory mediators and prevent the synthesis of prostaglandins (Awad *et al*., 2004; Das *et al*., 2012; Sowemimo *et al*., 2013).

Generally, the presence of a combination of different phytoconstituents in the experimental plant contributes for the anti-inflammatory action of the crude extract in carrageenan induced paw edema test

4.6. *In silico* analysis

4.6.1. Molecular docking analysis

Molecular docking is important for accelerating drug discovery by saving time and money, and also offering valuable insights into how ligands interact with target proteins. It avoids unnecessary costs associated with biological assays of compounds with a high likelihood of presenting future pharmacokinetic problems, as these parameters have an impact on pharmacokinetic properties. The selected target was PLA2, which is a known inflammatory mediators involved in the production of eicosanoids. As it is a crucial enzyme in the regulation of inflammation its inhibition leads to the treatment of inflammatory disease conditions. The native structures of PLA2 and NSAIDs such as niflumic acid (2-[3-(trifluoromethyl) anilino] nicotinic acid) remain unaltered upon binding to each other, indicating a high complementarity between the substrate-binding site of PLA2 and the structure of niflumic acid (Jabeen *et al*., 2005).

Figure 2 depicts both 2D and 3D representations of trans β-ionone docked in the active site of PLA_2. The molecular docking study reveals that trans β-ionone show good docking scores which is 5.787kcal/mol. The investigation identifies hydrogen bond and hydrophobic interactions between trans β-ionone and specific residues in the PLA2 binding site. As shown in Figure 2, trans β-ionone forms a hydrogen bond with the amino acid residue TYR 64 in the PLA2 active site, This molecular docking study implies that trans β-ionone may have the potential to inhibit PLA2. The anti-inflammatory mechanism of action of trans β-ionone was predicted by docking of trans β-lonone in the active site of PLA_2 (PDB ID: 1TD7) using Schrödinger 2023-1 Suite version-1.

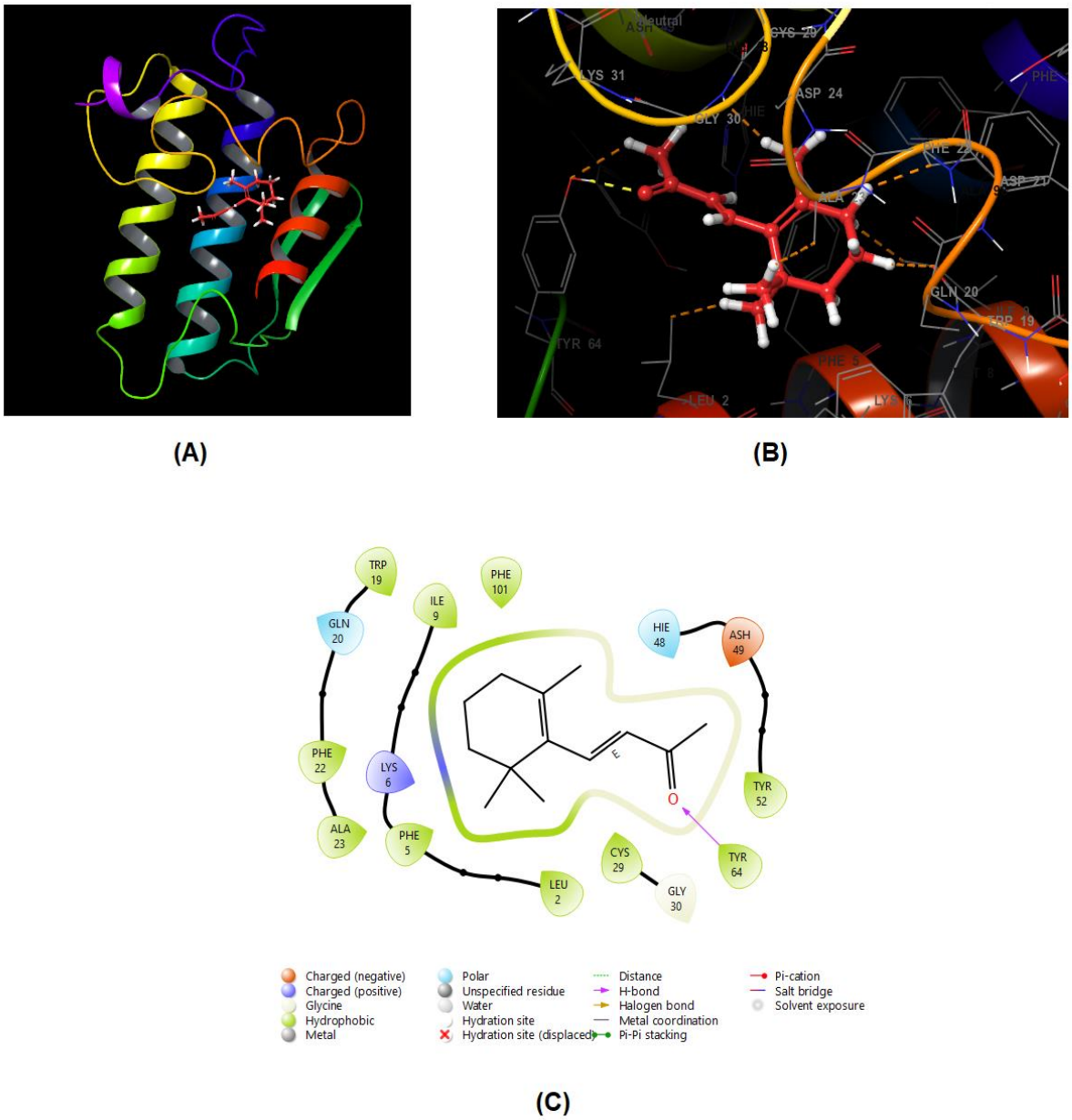

Figure 3: Docking of trans β-ionone with PLA2

(A): 3D representation of trans β-ionone docked within the active site of PLA_2;

(B): The 3D zoomed view of the PLA_2 interaction;

(C): 2 D model of trans β-ionone showing interactions with residues at the PLA_2 enzyme.

4.6.2. Pharmacokinetics prediction

The most commonly used ADMET analysis is important as it clarifies how a pharmaceutical compound is processed within an organism's body, thereby influencing the pharmacological action of the compound. We used Maestro V.13.5's Qikprop (ligand-based ADMET analysis) module for this investigation, which offers ranges for comparing a given molecule's properties with those of 95% of known drugs (Lipinski, 2004). Our compounds submit to an ADMET analysis and the compounds exhibit good drug-like characteristics, and Lipinski's rule of five violations was not seen, as outlined in Table 7.

Table 7: ADMET data of trans β-ionone calculated using Qik Prop Simulation

Descriptor	Predicted values	Recommended values (QikProp, 2015)
Molecular weight (g/mol)	192.3	130 - 725
Hydrogen bond donor	0	0 - 6
Hydrogen bond acceptor	2	2- 20
High lipophilicity (QPlogP_o/W)	-3.111	-2 – 6.5
QplogHERG	-3.353	< -5
QPlogBB	-0.031	-3.0 to 1.2
QPPCaco	3396.435	<25 poor, >500 great
Human oral absorbtion	3	1, 2, or 3 for low, medium, or high
QPlogS	-3.456	–6.5 – 0.5
#metab	2	1-8

5. Conclusion

According to the observed results from the experiment, we can conclude that the 80% methanol crude extract of *G. ferruginea* increase hot plate latency, suppress abdominal writhes and suppress carrageenan-induced inflammation. The result from the data indicate the 80% methanol crude extract of *G. ferruginea* possesses both analgesic and anti-inflammatory properties, which are mediated by both peripheral and central inhibitory mechanisms as well as via inhibition of PG synthesis. The investigation of anti-inflammatory and analgesics activity of the plant support the traditional use of the crude extract of *G. ferruginea* barks for relieving inflammation and pain in Ethiopian traditional medicine. Most of the constituents of essential oils were also previously reported for their analgesics and anti-inflammatory activity. The finding of analgesics and anti-inflammatory activities of the test was supported by *in* silico molecular docking analysis.

6. Recommendations

- Identification and isolation of pharmacologically active principles which are responsible for anti- inflammatory and analgesics activities of the plant should be further studied.
- Additional toxicological and pharmacological studies should be conducted in order to give a detailed information about this plant for clinical use
- Additional work should be done to understand the mechanism of the crude extract on anti-inflammatory and analgesic action.
- Further investigation of anti-inflammatory and analgesics activity of the plant on chronic inflammatory and pain models.

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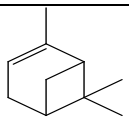
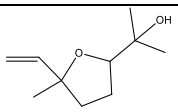
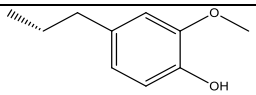
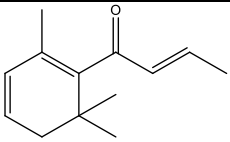
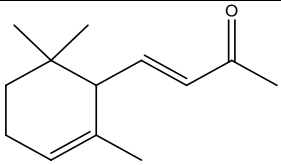
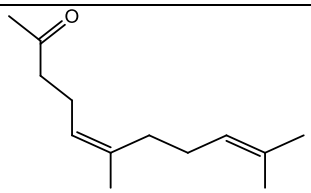
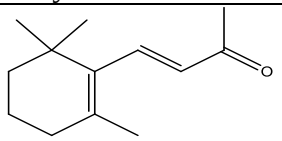
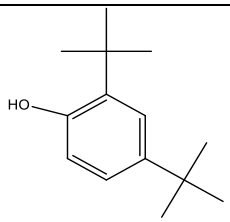
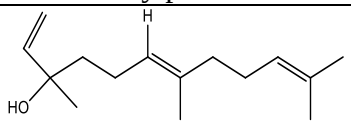
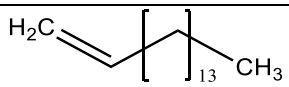
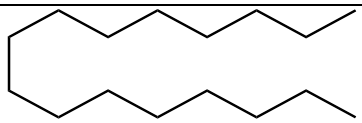
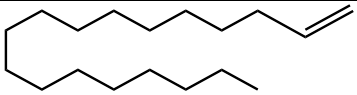
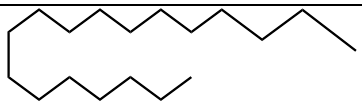
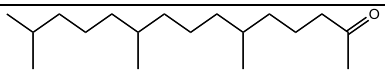
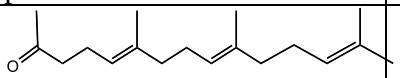
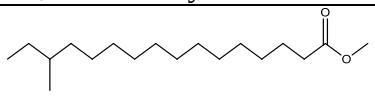
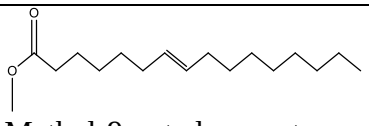
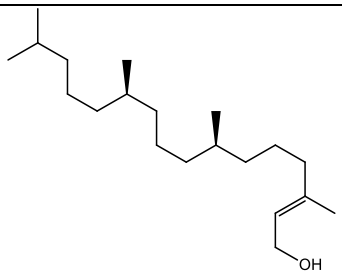
8. Appendices

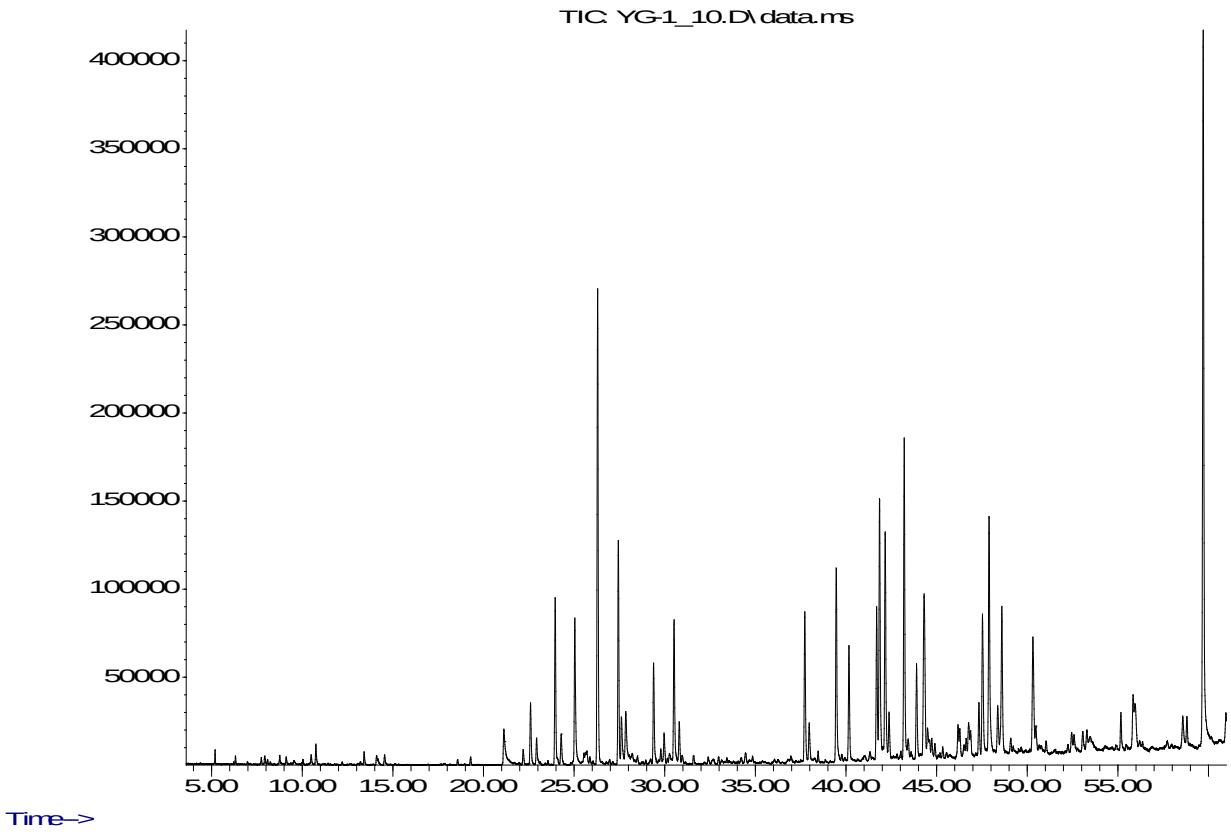
Annex 1: Name and chemical structure of constituents in essential oil from the bark of *G. ferruginea*

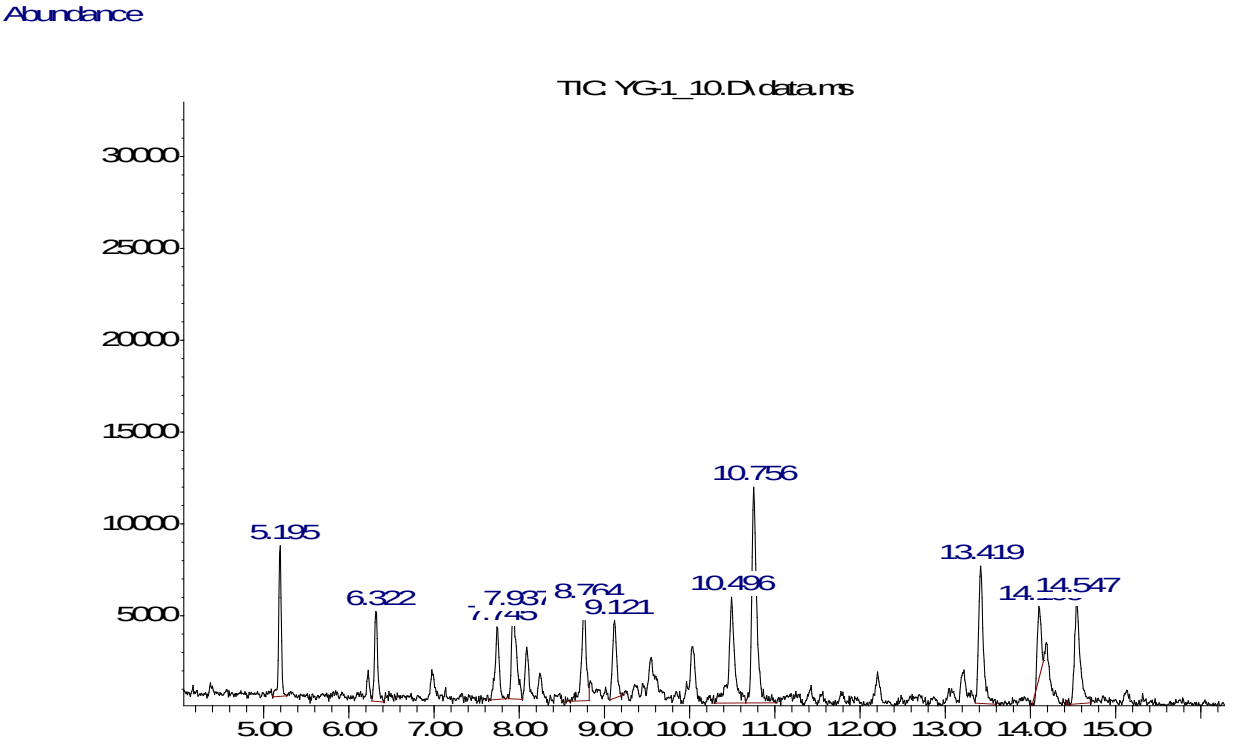
No	RI (HP5-MS)	Compound	Class of compounds	Area % of total
1	931	 α-Pinene	Monoterpine	0.117
2	1172	 cis/trans Linalool oxide	Acyclic Monoterpenoid	0.175
3	1357	 trans-Eugenol	Phenolic Monoterpenoid	1.151
4	1382	 β-Damascenone	Cyclic monoterpene ketone	0.246
5	1392	C_nH_{2n+2} Alkane	Hydrocarbons	1.050
6	1425	 Trans- α-lonone	Sesquiterpenoids	2.729
7	1452	 Neryl acetone	Monoterpene ketone	2.725
8	1483	 Trans- β- Ionone	Sesquiterpenoids	7.505

9	1512	 Di-tert-butylphenol	Alkylated phenol	3.363
10	1563	 trans-Nerolidol	Sesquiterpene alcohol	1.695
11	1592	 1-Hexadecene	Hydrocarbons	2.506
12	1600	 Hexadecane	Hydrocarbons	0.689
13	1793	 1-Octadecene	Hydrocarbons	2.598
14	1800	 Octadecan	Hydrocarbons	0.710
15	1844	 6, 10, 14-Trimethyl-2-pentadecanone	Sesquiterpenoids	3.310
16	1917	 5E, 9E-Farnesylacetone	Sesquiterpene	4.805
17	1926	 Methylhexadecanoat	Fatty acyls	4.113
18	1994	C_nH_{2n} Alkene	Hydrocarbons	4.165
19	2099	 Methyl-9-octadecenoate	Fatty acid methyl ester	2.891

20	2111	 Phytol	Acyclic diterpenoids	4.739
Total surface area				84.066

Annex 2: Gas Chromatograms of the essential oil of *G. ferruginea*

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20000
15000
10000
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6.322
7.937
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9.121
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10.756
13.419
14.547
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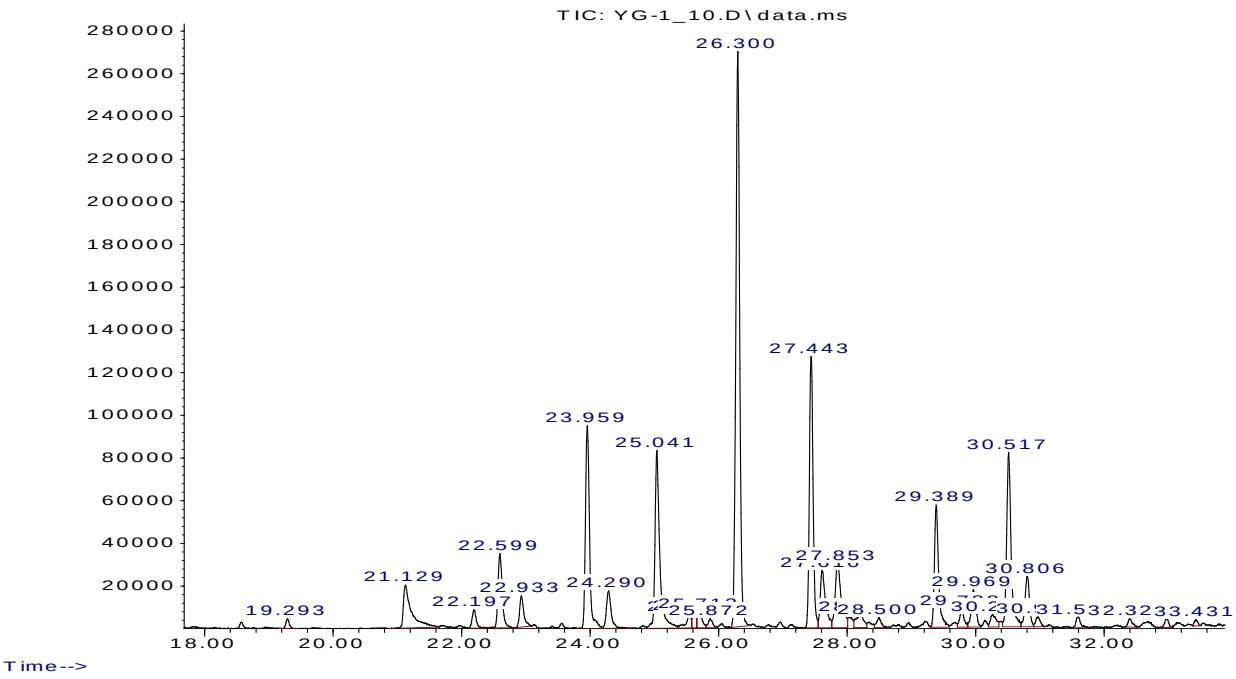
11.00
12.00
13.00
14.00
15.00
Time-->


Abundance
TIC: YG-1_10.D\data.ms
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240000
220000
200000
180000
160000
140000
120000
100000
80000
60000
40000
20000
26.300
27.443
23.959
25.041
30.517
29.389
22.599
21.129
22.933
24.290
22.197
19.293
25.872
27.853
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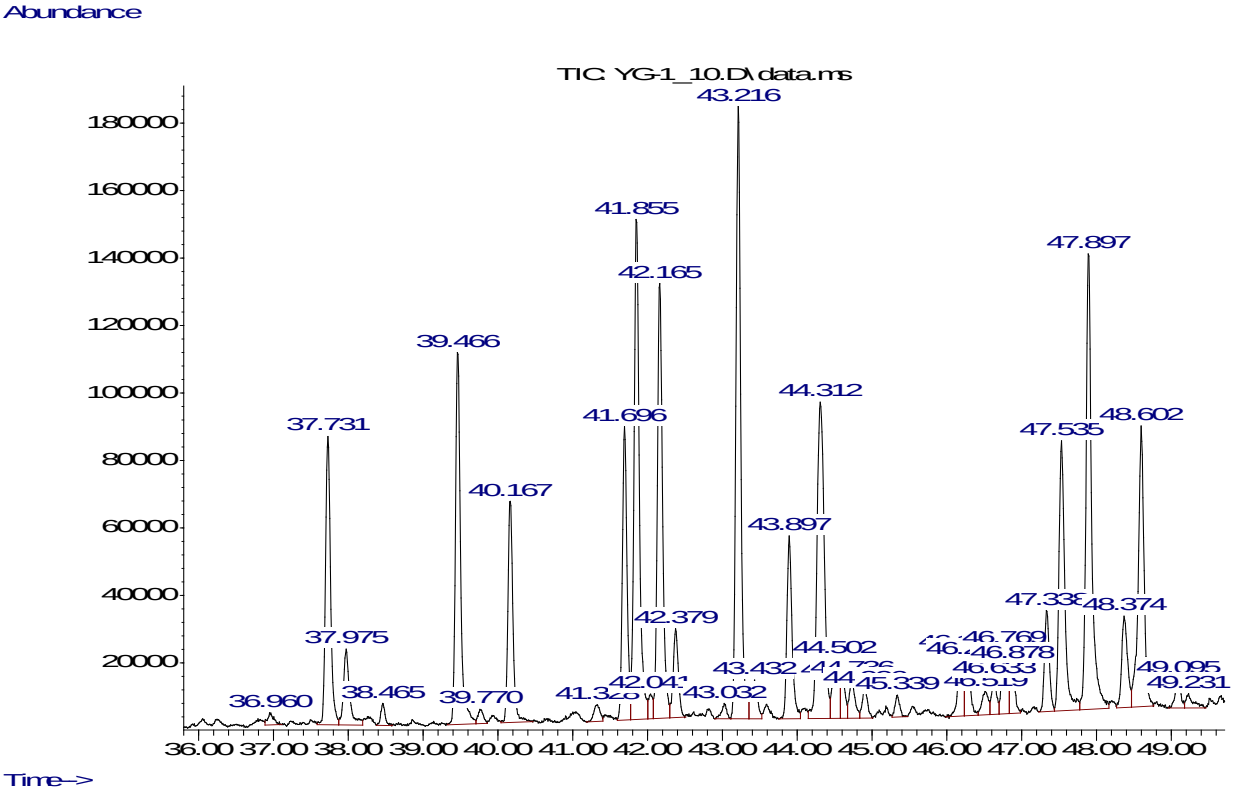
18.00
20.00
22.00
24.00
26.00
28.00
30.00
32.00
Time-->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42.165
39.466
44.312
41.696
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47.535
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43.897
42.379
47.338
48.374
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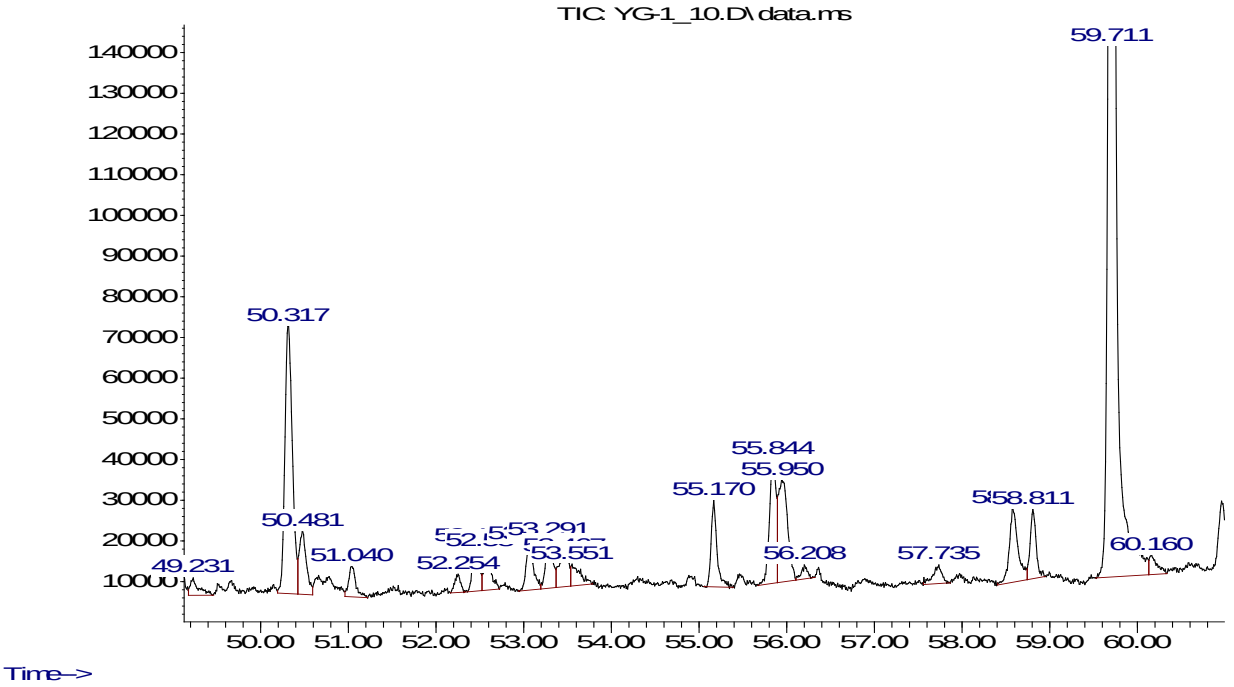
36.960
38.465
39.770
43.032
45.339
Time-->


Abundance
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55.170
58.811
50.481
60.160
49.231
51.040
52.254
53.551
56.208
57.735
Time-->