Pharmacological Effect of Kaurenoic Acid in MSU-induced Animal Model of Gout

M. Phil Thesis

by

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2024

Pharmacological Effect of Kaurenoic Acid in MSU-induced Animal Model of Gout

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Registration No. 02332211022

to

Department of Pharmacy,

In Partial Fulfillment of Requirements of the Degree of

Master of Philosophy

in

Pharmacy (Pharmacology)

Department of Pharmacy
Faculty of Biological Sciences
Quaid-i-Azam University
Islamabad, Pakistan
2024

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Dedicated To

ALLAH Almighty who taught me with pen and taught things that I knew not. My humble gratitude to my family and who supported me in every walk of life and my best friend who believed in me.

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Acknowledgements

In the name of **ALLAH ALMIGHTY**, the most merciful, the most beneficent and with countless salutation upon our beloved **HOLY PROPHET HAZRAT MUHAMMAD** (PBUH). The dissertation you are looking at is the abstract of many desperate attempts, during which, I was guided, motivated, and supported by many people whom I feel to acknowledge. Deepest honor for the **Holy Prophet (PBUH), Khatam-ul-Nabiyeen**, who brought the ultimate guidance with him.

First of all, I am grateful to Almighty Allah, who gave me the strength and ability to accomplish this work successfully. Secondly, with utmost gratification. I would like to express my profound and intense sense of indebtedness to my ever-affectionate worthy supervisor, **Dr. Salman Khan,** Faculty of Pharmacy, Quaid-i-Azam University. His proficient counseling, valuable suggestions, boundless forbearance, indefatigable help with anything, anywhere, anytime, consummate advice, thought provoking instructions and giving me enough independence to decide the things throughout the study, helped me to grow in both my competence and in confidence as a researcher. I am unfathomable indebted to **Prof. Dr. Ihsan-ul-Haq,** Chairman, Faculty of Pharmacy, Quaid-i-Azam University for his encouragement, full support and cooperation during research work. I am very thankful to all my worthy teachers of Faculty of Pharmacy, Quaid-i-Azam University.

Maryam Jamil

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List of Abbreviations

Abbreviations	Description
ALT	Alanine Aminotransferase
ALU	Allopurinol
AST	Aspartate Transaminase
COX-2	Cyclooxygenase 2
CRP	C-Reactive Protein
DAP	3,3' Diaminobenzidine
EB	Evan Blue
ELISA	Enzyme-linked Immunosorbent Assay
EPO	Eosinophil Peroxidase
FTIR	Fourier-Transform Infrared Spectroscopy
i.art	Intra-articular
IHC	Immunohistochemistry
IκB	I-Kappa-B
IL-1β	Interleukin 1-Beta
iNOS	Inducible Nitric Oxide Synthase
i.p.	Intraperitoneal
KA	Kaurenoic Acid
LEKTI	Lympho-Epithelial Kazal Type Inhibitor
LFTs	Liver Function Tests
LPO	Lipid Peroxidation
MPO	Myeloperoxidase
MSU	Monosodium Urate
NF-κB	Nuclear Factor Kappa-light-chain-enhancer of activated B cells
NLRP3	Nucleotide-binding Domain, Leucin-rich-containing family, Pyrin Domain-containing 3
NO	Nitric Oxide
OD	Optical Density
PBS	Phosphate Buffer Saline
PGE2	Prostaglandins E2
PR3	Proteinase 3

RFTs	Renal Function Tests
ROS	Reactive Oxygen Species
SC	Subcutaneous
TLR4	Toll Like Receptor 4
TNF-α	Tumor Necrosis Factor Alpha
WBCs	White Blood Cells

Abstract

This study investigated the potential of kaurenoic acid (KA) in a gout model caused by monosodium urate (MSU) crystals. It was discovered that KA reduced gouty inflammation and reversed the structural, histological, and biochemical changes brought on by the MSU crystals. In this study, paw edema, joint thickness, and the frequency and duration of acute gout flare-ups were all considerably decreased by the administration of KA. Additionally, the treatment groups' behavioral parameters demonstrated considerable changes. A considerable reversal of inflammation and deterioration was seen in the KA-treated groups according to X-ray examination. The FTIR spectroscopy indicated the changes in the molecular makeup of tissues, modifications of biomolecules including proteins, lipids, and carbohydrates. Histopathological changes showed improvements in cellular infiltration in the paw, and inflammatory cell infiltration as well as chondrocyte irregularity on joint surface in the treatment groups While trichrome revealed suppressed collagen deposition and suppression of inflammation and tissue repair in paw and joint. In paw and joint tissues, the KA therapy up-regulated IκB-α expression while down-regulating TLR4, NF-κB, iNOS, and COX-2 expression. On the other hand, KA therapy greatly increased antioxidants and decreased oxidative stress indicators. According to Evans blue dye, the study shows that in the treatment groups' vascular permeability was intensely reduced in comparison to the diseased groups. Kaurenoic Acid appeared to have a high tendency for binding to protein targets, according to molecular docking research. KA was associated with drop in the cytokines such as TNF-α and IL-1β. In conclusion, KA significantly reduced inflammation in response to a gout attack induced by MSU.

CHAPTER 1

INTRODUCTION

1. INTRODUCTION

1.1. Background

In 2019, WHO defined "Gout" as an inflammatory disease in which the level of uric acid is increased in the body and crystals are formed especially around joints. First hyperuricemia caused arthritis-like symptoms and if it is not treated timely, it can lead to joint damage by tophi formation in the joints.

Gout results when there is metabolic failure which causes formation of crystals called MSU crystals. This leads to arthritis symptoms in joints. When purine metabolism is compromised, it leads to a higher level of uric acid in the body. This condition is accompanied by inflammation in the joints and ultimately there is tophi formation and arthritis (Kiyani *et al.*, 2019).

When studying the pathogenesis of gout, serum profile shows an increase in the level of uric acid which causes a condition in which crystals accumulate around joints. On microscopic examination, they are visible and called MSU crystals. The normal level of uric acid in a healthy individual is not more than 6.8 mg/dl. If the level is recorded more than 6 mg/dl and 7 mg/dl in females and males respectively, this means that there are increased chances of deposition of urate crystals and inflammatory arthritis can kick in any time. To prevent this attack appropriate measures should be taken timely (Patil *et al.*, 2021).

For proper understanding and treating the disease, it is pivotal to understand the mechanism and level to which disease is likely to be present in the body. Family and patient history can suggest the stage. Moreover, there is a level of disease in which there is an increase in MSU level in the body even formation of crystals, but arthritis is not present. Then comes the stage where the arthritic attack kicks in with symptoms of inflammation. Sometimes, it happens that when gout is cured completely it bounces back and recurrence occurs. This condition is called Intercritical gout (Richette *et al.*, 2019). It is more common in male population as compared to females affecting 4 and 1.4 out of 1000 of them respectively (Riaz *et al.*, 2022).

1.2. Prevalence of Gout

If we calculate the ratio with which males and females are affected, it comes out to be 10.1 to 3.1. Mostly, the attacks of gout are prevented or treated but sometimes it causes irreversible damage to the joints affecting quality of life. The population of gout-affected people is 1-3% and severe cases are reported only in 0.1-0.3% cases. The

occurrence of gout has also been studied age wise and under 80 years of age 11-13% and over 80 years of age 0.4% patients reported the symptoms. Geographically, studies have been conducted and showed that cases have been reported in US, Han Chinese, New Zealand, Maori and Asia. Recent studies have shown that the occurrence of disease is 0.9-2.5% in Europe, 3.9% in the US and over 6% in Oceanic Pacific (Patil *et al.*, 2021).

1.3. Risk Factors of Gout

Many factors are thought to affect the occurrence of gout, Environmental and Genetic factors being the leading ones. If there is constant stress in an environment and an unhealthy diet, it leads to the development of disease. Absence or mutations of genes such as urate transporter gene SLC2A9, ABCG2 and SLC22A12 can be leading cause of gout. Genetic mutations usually run in families where patients lack the ability to regulate the level of uric acid in serum. To maintain the uric acid level, balance should be maintained in its absorption and excretion from the body which is regulated by the above-mentioned genes as shown in figure 1.1. (Choi *et al.*, 2010).

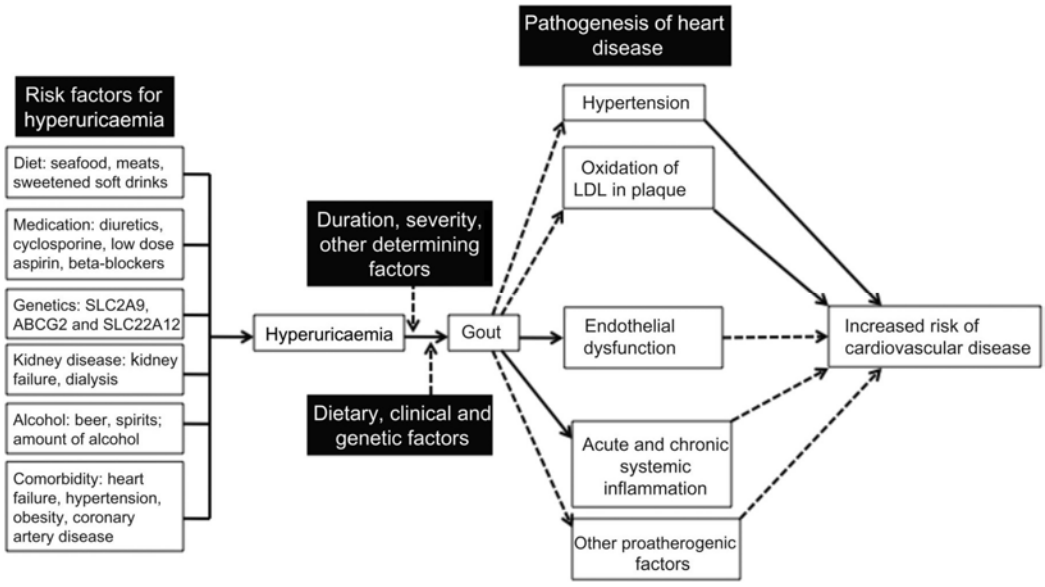


Figure 1.1. Risk factors associated with gout (Singh, 2015).

Uric acid can be highly controlled by diet. All the items which on metabolism release uric acid should be avoided in the people who are at increased risk of gout. These foods include meat, alcohol, seafood, and other such items. They release purine and cause accumulation in the high-risk population. Not only this, but foods having fructose in

them and drinks having high sugar content in them are also a risk factor for the development of gout (Zhang *et al.*, 2012a).

In addition to genetic and nutritional factors, underlying diseases pose a serious threat to the development of gout and onset of gout attacks. Diabetes is considered a serious threat that could raise the level of serum uric acid in the body. When oxidative phosphorylation is compromised, there is an increase in the level of adenosine. This further causes accumulation of uric acid because not only its synthesis is increased but its excretion is also impaired. When insulin is given, reabsorption of uric acid is increased. All these mechanisms make diabetes a major cause of gout. Other disorders that are linked to gout are kidney disorders, obesity, and hypertension. Hypertension is caused when there is reduction in blood flow to glomerulus and as a result the filtration is compromised, and an increase is observed in the blood pressure. Similarly uric acid interferes with proper filtration process and leads to the development of the disease. Patients on chemotherapy and immunotherapy after a transplant are also at high risk. People on other therapies such as diuretics can also cause uric acid levels to rise. Similarly, patients on ibuprofen are also considered on high alert (Roddy and Doherty, 2010).

1.4. Pathophysiology of Gout

Gout is an inflammatory condition that can impair quality of life and currently it affects a large proportion of the population highlighting the need to raise awareness for preventing this disease. To develop effective treatment and potential therapies, pathophysiology of the disease must be studied. Gout is clinically manifested in four different stages.

“Pre-gout stage “is the first to occur where there is an increase in the level of uric acid in serum. This increase is generally an indicator of the sever gout attacks soon. If the level of uric acid is controlled, these attacks can be prevented leading to improved quality of life. Secondly, “Asymptomatic gout” occurs where there is an early formation of crystals in joints in addition to the rise in serum levels of uric acid. There is absence of these crystals in the first, but here these formations show that the disease is progressing. Although there is no pain and distortion, appropriate measures should be taken immediately. Then there is the “Gouty Flares phase” which is characterized by the severe pain in joints as result of formation of MSU crystals in and around the joints. There are episodes of acute pain and difficulty carrying out normal functioning. Then

comes the final and most severe phase of gout characterized by the formation of tophi which is visible in radiological examination. Tophi formation causes severe pain and discomfort and ultimately deformity of the affected joint. The quality of life is seriously compromised as it is the chronic phase.

Hyperuricemia is a condition in which the level of uric acid has been increased from the normal range that is 408 mol/L or 6.8 mg/dl. Once the set value is exceeded, there is an increase in the level and ultimately formation of uric acid crystal around joints. This imbalance in the body occurs either dure to increase from the normal production in the liver or the decrease in the excretion and abnormalities in reabsorption process in kidney. The major cause of high uric acid level in serum is dysfunctioning of excretion processes. Uric acid is excreted mainly from the kidneys leading to 1/3 of its excretion while the rest is excreted from the gut. Abnormalities in the kidney are the leading cause of development of gout. In the kidney, proximal convoluted tubule is responsible for maintaining the excretion and reabsorption of uric acid through the involvement of various channels and transporters. If these transporters are impaired, this leads to an increase in uric acid and gout appearance. These include reabsorptive urate-anion exchangers, URAT1/SLC22A12, OAT4/SLC22A11, and OAT10/SLC22A3 and secretory anion exchange transporters like OAT1, OAT2, and OAT3 as shown in figure 1.2.

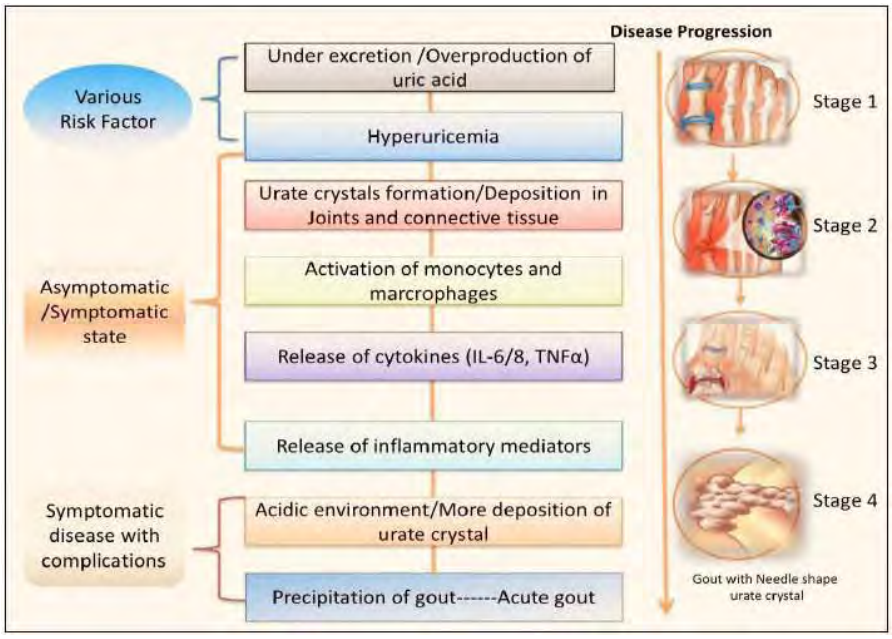


Figure 1.2. Pathogenesis and different steps of progression of gout (Basnet *et al.*, 2021).

When the level of MSU is raised around joints, this causes initiation of the inflammatory process. To initiate inflammation, there is activation of NLRP3 inflammasome and interaction of MSU with macrophages at the site of inflammation. NLRP3 causes Caspase 1 to convert pro-interleukin 1 to IL-1. Free long-chain fatty acids also cause the release of pro-inflammatory cytokines. This process requires acetylation of tubulin protein by mitochondria. Eventually, proinflammatory cytokines, chemokines and other substances contributing to inflammation such as ROS, PGE2 and enzymes from lysosomes are produced. There is activation of mast cells and neutrophils at the site of inflammation. These are the immune cells that upon activation will cause severe inflammatory cytokines and further produce immune response. Neutrophils form NETs that will resolve the by releasing lipid mediators as well as anti-inflammatory cytokines.

When uric acid is raised for a long period of time, it leads to the formation of tophi, the chronic phase of gout. In tophi formation, innate and adaptive immunity are involved. There are inflammatory mediators such as IL-1β and TNF-α but the anti-inflammatory mediators such as TGF-β1 are also present. Both these when present at the site of MSU deposition led to tophi formation. Not only this formation of NETs also contributes to the synthesis of tophi. This tophi synthesis in and around bones and joints leads to chronic fourth stage of gout (Hutton *et al.*, 2018).

1.4.1. Acute gout pathophysiology

When the level of uric acid is raised in the body, it starts getting deposited around joints. As a result, the phagocytic cells are activated in the synovial joint region and produce chemokines and other inflammatory mediators and lead to inflammation. Uric acid forms cross-linking with the proteins and lipid bilayer of the membranes of phagocytic cells which is another mechanism through which uric acid produces inflammation. Many proteins are involved such as G proteins, phospholipases A2, C, and D, tyrosine kinase, and other kinases which include mitogen-activated kinases (ERK1/ERK2, p38) and c-Jun N-terminal kinases. IL-8 is released, and neutrophils are activated. Then monocytes and pole cells kick in the pathogenic phase of gouty arthritis. In the pre-gout and inter-critical stages, the macrophages are involved, and uric acid crystals are entrapped within the macrophages without exacerbating the condition.

When urate crystals are taken up in the synovial joint region, monocytes produce IL-1, IL-6, IL-8 and TNF-α and mast cells, IL-1 and histamine. As a result of this dilatation

of vessels occur and their permeability is increased. Although the immune responses involved worsen the condition, they are the cells that will ultimately fight the condition and provide relief in the end of the inflammatory process.

Upon activation, mast cells, monocytes and endothelial cells causes migration of neutrophils to the site of synovial joints and cause inflammation by releasing chemotactic factors like leukotrienes, IL-8 and other interleukins and platelet activating factor causing symptoms of acute disease. The acute symptoms generally disappear within a few days. Understanding the mechanism of acute gout will lead to opening of many potential targets to mitigate the symptoms and ultimately resolve the pathological condition. Colchicine is the drug which interferes with IL-8 production and endothelial cells and neutrophils activation.

When the crystals of uric acid are removed, macrophages play their role to stop the acute attack by either no chemokines release or by removing cellular debris from the site of inflammation. When macrophages release TGF-β and the level of cytokines is reduced by break down of proinflammatory cytokines, it leads to the suppression of inflammation. Vasodilation and vascular permeability are involved in inflammatory processes when gout attack occurs, but they are the ones causing apoptosis of the inflammatory materials in the synovium at the end of the acute attacks as shown in figure 1.3.

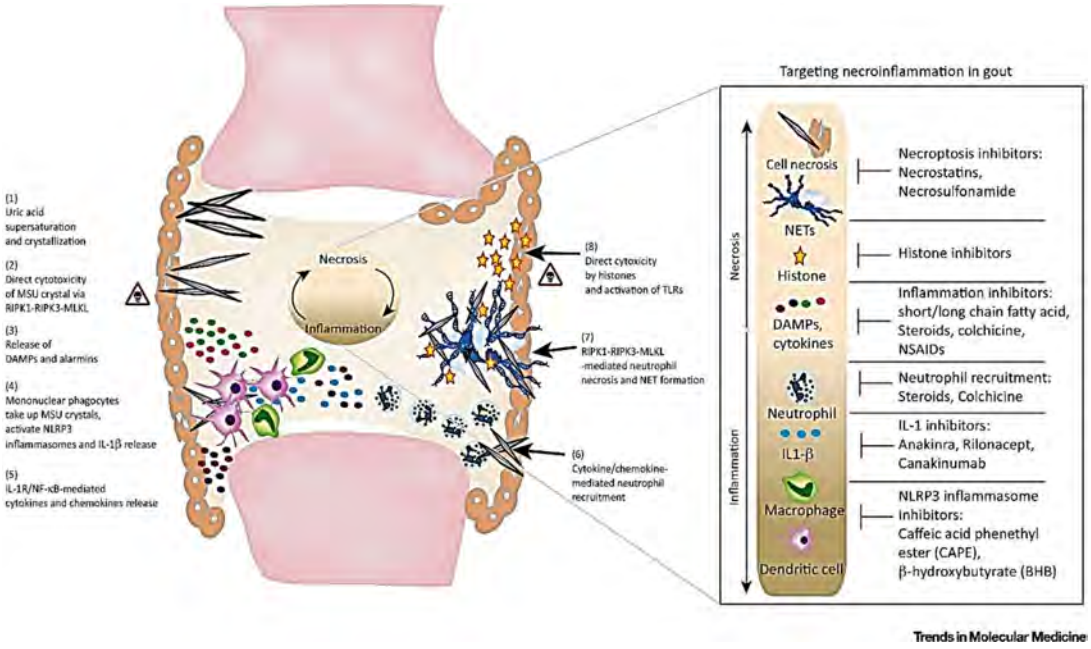


Figure 1.3. Pathophysiology of acute gout (Desai *et al.*, 2017).

1.4.2. Pathogenesis of chronic gout

Gout is mainly presents in the chronic stage. When the level of uric acid is raised and sudden release of inflammatory mediators occurs, it is acute at that point. If this level of uric acid is persistently high in the body it leads to formation of tophi, chronic inflammation of synovium and ultimately deposits in the bones. There are many underlying mechanisms for development of chronic disease such as release of cytokines, NO production and enzymes proteases involving metals in their catalytic mechanism all leading to a functional and structural abnormality of joints and ligaments.

The erosion of bones and joints and maturation of osteoclasts precursors to mature osteoclasts is caused by IL-1 and RANK and RANK-RANKL pathway. Osteoblast's function is compromised, and they start releasing proinflammatory cytokines and overhanging edges of cells in synovium play pivotal role in chronic inflammation. Intercritical stage is marked by the absence of severe gout attack but the proinflammatory cytokines are still present in small quantities causing irritation and poor quality of joints and bony structures. Drugs that lower the level of uric acid are the drugs of choice along with that of anti-inflammatory drugs such as colchicine for the proper management of chronic disorder. In recurring attacks patients, improper management is usually the root cause in the patient.

In the severe attack of gout, uric acid level is lowered in the biochemical reports. For the proper management of disease, first NSAIDs or colchicine are given followed by the administration of Allopurinol or Febuxostat for lowering the level of uric acid. Sometimes, a sudden drop in the level of uric acid triggers an acute attack. This might happen during surgery or other such conditions. So, careful monitoring through pathological reports should be monitored.

Within the normal range, uric acid is an essential requirement of the body as it lowers oxidative stress and is also important for normal functioning of the endothelium. But once the level of uric acid exceeds the normal range, it becomes toxic causing inflammation in the body. Hence, for maintenance of homeostatic condition and vascular endothelium, uric acid level should be closely monitored. Studies have shown that when hyperuricemia occurs and xanthine oxidase inhibitors are used, the risk of cardiovascular damage is reduced as the vascular function is normalized, but only if there is underlying hyperuricemia as shown in figure 1.4. (Ragab *et al.*, 2017).

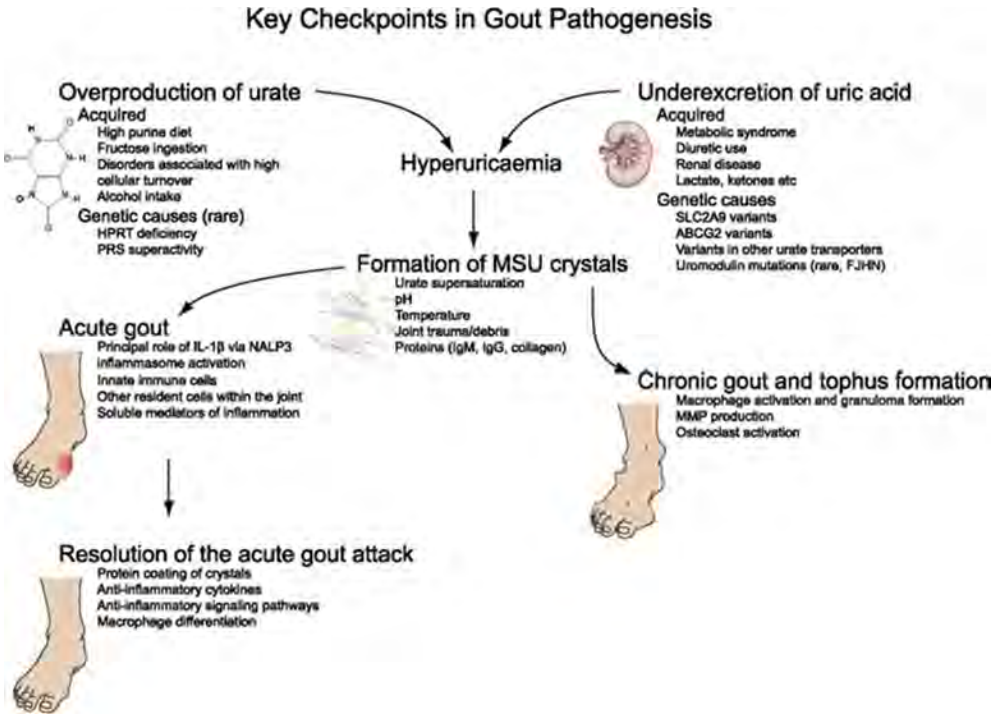


Figure. 1.4. Key checkpoints in gout pathogenesis (Merriman and Dalbeth, 2011).

1.5. Immune Mechanism of Gout

When MSU crystals are accumulated in the potential targets of inflammation, they activate the monocytes and macrophages. On the membrane TLRs are present that indirectly get activated and produce inflammatory cytokines. In the direct mechanism, dendritic cells are activated nonspecifically, it leads to inflammation via multiple signaling pathways. When the MSU crystals come in contact with the cholesterol in the membrane, it leads to activation of certain protein kinases involved in the inflammatory mechanisms. Upon presence in the targeted area, MSU crystals cause the excessive activation of phagocytic cells and cross-linking with the membranes is produced due to involvement of cytoskeleton and ultimately the activation of IL-1β. Drugs such as Colchicine and Cytochalasin D inhibit this very process making them effective for the treatment of gout. The Colchicine mechanism involves not only the inhibition of IL-1β but also by directly sensing the crystals hence no need of blocking pathways. Once the activation of phagocytes occurs, ROS, loss of potassium and lysosomal cathepsin production and other stress signals are released which activate the inflammasomes. It releases IL-1β and IL-18 upon sensing abnormality and comes in action to produce innate and adaptive immunity. The most common being NLRP3 in gout which is also being produced in response of asbestos, alum, and silica.

NLRP3 is poorly understood while certain studies have shown that its production is inversely related to antioxidants. So, the mechanism of action of NLRP3 has something to do with the redox signal transduction, ROS, and loss of potassium.

The main cytokine involved in gouty attacks is IL-1β. It can be produced by either the action of Caspase-1 protease or the neutrophilic production of PR3 and finally IL-1β release and inflammatory response.

So, NLRP3 activates and releases cytokines specially IL-1β the main culprit of gouty attacks and as a result inflammatory condition progresses and converts to chronic from acute (Busso and So, 2010).

Acute inflammatory response is produced when the gout starts in its acute phase. The joint cells lining observe an acute increase in the growth with lots and lots of penetration of neutrophils, phagocytes, and lymphocytes. Many risk factors are associated with this abrupt increase in the uric acid level such as any surgery, accident, diseases and drug and alcohol abuse. As result of any of the factors, new crystals form or tiny particles are released from already present crystals. Some risk factors also control crystal production such as interstitial fluid, temperature, and pH at the site of potential target of inflammation and level of uric acid. Mast cells produce histamine, other enzymes, TNF-α and other inflammatory products. And its degranulation and activation produce inflammation.

Once the endothelial cells are activated in response to inflammatory initiation and TNF-α and magnification by leukocytes, it causes dilation and increased vascular permeability causing proteins from plasma and WBCs at the targeted site. Upon encountering the WBCs, wither the crystals are taken up by the giant WBCs or they interact with receptors on surface. The role of neutrophils is clearly making the response even more prominent by releasing various proinflammatory cytokines such as NO, ROS, PGE2, LTB4, IL-1, enzymes and chemokines causing vessels to dilate, local redness, erythematous tissue formation and pain at the targeted site. Complementary pathways are also involved in the activation of inflammatory response causing chemokines production such as C3a and C5a for leukocytes.

Bradykinin is also considered a key mediator for endothelium activation and hence extravasation from vessels by dilating them and production of pain. This is produced when MSU crystals form vasoactive peptide bradykinin by activating kininogen in the plasma.

Many factors such as PGE2, bradykinin, substance P, a neuropeptide, and activation of nociceptors lead to generation of intense pain in gout attacks.

Already marketed drugs for the management of gouty arthritis are colchicine, NSAIDs countering the inflammation process. The pathogenesis of gout is studied extensively to discover new drugs after revealing of various targets (Dalbeth and Haskard, 2005) as shown in figure 1.5.

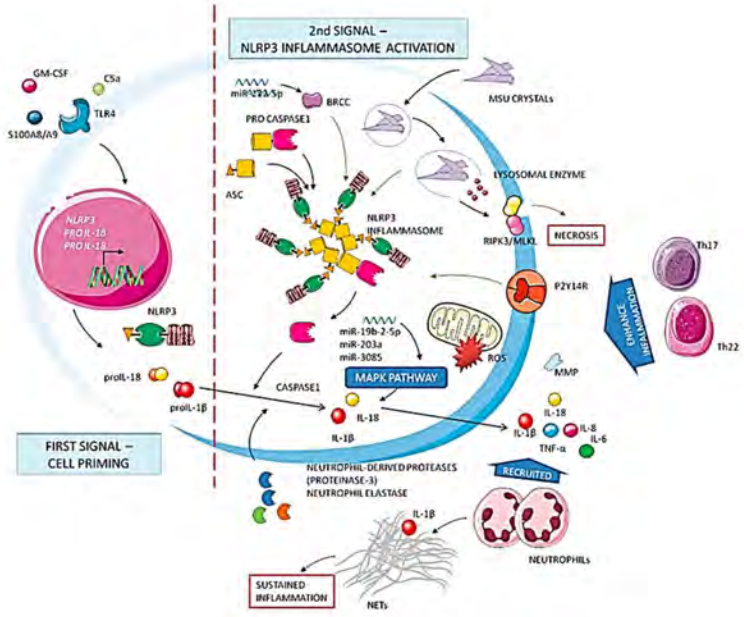


Figure. 1.5. Complex network of molecular mechanisms implicated in gout. Inflammation has been shown in two stages. Cell priming production of precursors of cytokines and inactive inflammasome molecules needs the subsequent activation step after Signal 2. IL-1β is critical to the upregulation of inflammatory process (Galozzi *et al.*, 2021).

1.6. Clinical Manifestations of Gout

In case of gout, many symptoms are observed by extremely severe pain in joints which leads to immobile joints is the main and most concerning one. The patients report difficulty in motion of the targeted joint. In some patients, the onset of symptoms is sudden, but others complain about mild to moderate pain, itching and irritation in the affected joints. Other than these main symptoms, inflammatory signs are observed such as calor, dolor and rubor, the typical indicators. There is throbbing pain or burning and discomfort when the attack sets in and lasts within 24 hours and recorded to be a seven on the pain scale.

The most affected joint is MTP, mostly the foot region is affected. In the majority of cases, tenderness and pain occur at night causing difficulty in falling or staying asleep,

condition usually lasting within 15 days. Many factors can aggravate the condition such as medical history, diet rich in purines, alcohol, and severe dehydration.

When the gout persists for a long time, it leads to the formation of tophi in the foot, elbows, hands etc. Mostly they are non-painful but sometimes when they are inflamed, there is risk of pain. Besides this the presence of tophi-like structures causes social stigma embarrassing the patients and causes difficulty in gripping objects, tenderness of joints and inability to wear normal footwear. Although it is painless in most cases, it is prune to infections and repeatedly patient gets sick and sometimes it starts releasing white substance from it making quality of life difficult.

When gout attack occurs, there are signs of clear inflammation such as infections of the joint membranes, rubor, dolor, calor and tenderness and difficulty in moving even bruises are possible showing vascular extravasation. To prove that it is an inflammatory process, temperature is raised. Once the attack is covered, the skin at the previously infected site shows peeling effect and vessels are visible throughout the process at the site of inflammation. The most affected surface where tophi are commonly observed are Achilles tendon, MTP joint peroneal tendon, helix of the ear, olecranon bursa, and foot pad and they are visible as granules and nodules under the surface of the skin (Grassi and De Angelis, 2011).

1.7. Clinical Assessment and Diagnosis Presentation

In gout, swelling and inflammation is observed in the lower limb areas. The cause is underlying high level of uric acid that has not been addressed and ultimately caused an attack. If it is treated on first attack, it will last only two weeks. But if hyperuricemia is not treated properly, it will lead to recurrent attacks with intercritical phases and ultimately other joints will be affected as well, even the upper extremities. In some cases, it causes tophi formation and chronic symptoms. Tenderness in joints causing difficult movement and structural abnormalities of joint are all the aggravated forms of joint. If the disease progresses to a highly advanced level, it affects the gut functioning called tophaceous sickness. Not only this other region can be affected such as viscera, spine, nose, and eyes. Gout affects the quality of life and muscle strength in patients with recurring gout attacks. As a result, patients' social life, job and appearance at other events is compromised even social stigma can happen if it is too much visible on face and hands. If there are other underlying diseases, quality of life is further compromised (Grassi and De Angelis, 2011).

1.7.1. Diagnostic investigation

There are different diagnostic procedures which can be used. One of the important methods for diagnosis is placing the synovial fluid obtained from the synovial joint or the tophi secretion under the microscope and observe for the signs of inflammation. The presence of cloudy, non-viscous yellow appearance with lots and lots of neutrophils and other white blood cells and needle shaped crystals with 1-2 μm in length all give the hint of development of gout.

For diagnosing an acute attack, it is important to consider the level of uric acid in the serum as well. This is because the synovial fluid will show visible crystals in patients with hyperuricemia even in the absence of attack and even if the disease has been cured from the attack. So, if the level of uric acid in serum is observed to be less than 360 mol/L, the patient is diagnosed as patient under attack even if the synovium is showing crystals in scans.

Not only this, but uric acid in serum is also not the sole determinant of gouty disease because as it has been mentioned earlier, during the acute attack, the level of serum uric acid is observed to be lower. So, false negative results can be obtained. So, continuous monitoring through serum level of uric acid as well as fluid observation is monitored closely. During acute attack, general symptoms of inflammation will be observed such as increase in the level of neutrophils and C- reactive proteins classifying gouty attack as an inflammatory response of the body.

Many laboratory tests are used to determine gout and other underlying diseases that commonly occur with gout. Serum obtained from blood gives an estimation of the rise of uric acid in patients. Along with that RFTs are performed to investigate the renal health and creatinine can also be determined through its level in serum. Not only this lipid and glucose profiles should be checked routinely to see for the signs of dyslipidemia and diabetes mellitus by screening of lipids, HbA1c and fasting glucose in blood.

Other tests which are used for diagnosis are the radiological tests as they are more preferred since there is no need of takin out fluids from the joints increasing patient compliance and the results of the tests shows that the bones are showing signs of erosion and clearly overhanging in the corners is present.

Ultrasound imaging is considered as an advanced tool to observe joints. When the ultrasonic waves are subjected to fall on affected joints, there are images of contour signs on hyaline cartilage. Similarly articular cartilage also shows the presence of

crystals. Even tophi are visible. If there is presence of heterogenous materials or blizzard appearance all are the signs of gout. More advanced techniques such as DECT dual energy is also used in recent times (Parthasarathy and Vivekanandan, 2018).

1.7.2. Clinical diagnosis

1.7.2.1. Asymptomatic hyperuricemia

When an acute attack of gout occurs, there is inflammation, pain, rubor, calour and dolor and swelling in the affected joint lasting within 24 hours. Generally, the joint movement is compromised, and tenderness is observed. All joints in the body are susceptible to the deposition of crystals or the onset of the disease but the most involved joint is MTP joint. Sometimes even hip and shoulder joint get involved. Usually, a single joint is affected but soft tissue is commonly involved causing bruising and infections of tendons. General symptoms of inflammation are common such as fever and restlessness. In women, it mostly occurs after menopause. Signs and symptoms should be observed closely to find out any comorbid disease.

The techniques used for the detection are described below. Analyzing the synovial fluid under microscope is the important technique confirming the presence of crystals in the joint. The features of the fluid and crystals are described earlier and are used in diagnosing gouty attacks.

Moreover, the analysis of serum uric acid is a pivotal marker in the diagnosis of the disease. Diagnosing step is very important because sometimes the uric acid level is raised but no symptoms are observed and sometimes, the values are normal and there is gouty attack.

1.7.2.2. Intercritical period

When the colchicine and NSAIDs perform their role and the flare is gone, there is this phase with minimal symptoms. Proper management is quite important because if it fails, there will be another attack and so on until proper interventions are made.

1.7.2.3. Chronic tophaceous gout

If there is treatment failure, the disease will continue and eventually the crystals start forming tophi in the joint and they are visible showing that the disorder has reached to a later stage in the patient. Even abdominal conditions appear and sometimes tophi can be observed facially and affect nose, eyes, and ears. Tophi are observed

microscopically. For cleat biopsy, joint aspiration is the important step so that MSU tophi can be distinguished from other tophi and nodules and better diagnosis is the key to cure the disease.

Pathological identification is used most for the diagnosis of diseases. But for the proper diagnosis, not only serum level but synovial fluid examinations are also important. This examination is still considered as the most important tool and the patient is not said to have gout until the reports of microscopic examination make it clear.

Another important thing is to rule out all other conditions in which tophi-like or nodule-like structures appear, also rheumatoid arthritis should be ruled out properly so that appropriate diagnosis could be made. Sometimes tophi appear on the onset of gout showing person to person variation in the course of the disease (Ragab *et al.*, 2017).

1.7.3. Laboratory diagnosis

The most reliable method of diagnosis is the observation under the light microscope after the removal of the fluid from the synovium. The fluid is also subjected to other testing other than the microscopic examination. Analyzing the number of leukocytes and determining for the presence of any cell cultures. Urine analysis is also an important test for evaluating uric acid in the body. Moreover, radiological analysis is another important test for detecting the presence of gout. Tests should be repeated for better diagnosis. As technology is improving, better diagnostic tools are being developed for better diagnosis treatment (Ragab *et al.*, 2017).

1.8. Management of Gout

For the proper management of gout, treatment of the acute attack of gout as well as minimizing the chances of recurrence are important. The level of uric acid in the body should be controlled and the already developed crystals should be dissolved for effective treatment. The uric acid should be controlled to such a level that concentration is reduced to 6mg/dL or 360mol/L or even lesser. This is done by using drugs that will lower uric acid. Adherence and compliance of the patient is monitored through laboratory testing and repeated checking on the patients.

For the patients coming with the acute attack of gout, managing, and relieving the symptoms of attack is the first line of treatment. The inflammation must be controlled

as soon as possible, and the pain has to be managed. For this purpose, NSAIDs, colchicine and corticosteroids such as Dexamethasone will be used.

Along with taking proper medications, some changes in the lifestyle should be made so that treatment works effectively. Diet should be healthy, and purines should be totally cut off and weight should also be managed properly. These changes have proved to be effective in managing acute attack as well as dissolution of MSU crystals. So, all the healthcare workers such as doctors, nurses and nutritionists will collaborate for effective management.

Underlying disorders such as hypertension, diabetes mellitus and metabolic disorders like dyslipidemia and all the factors that will lead to development of the disease should be managed. If all these factors are effectively controlled, this will lead to positive impact on the health of the patients and ultimately reduce burden from the healthcare.

Not only this, but the patients should be approached repeatedly, and tests should be performed to see if the medication is working. Joints should be examined for the signs of swelling and appearance of tophus and the level of disorder. Giving the charge to the patient after giving them complete insight of the disease is the most effective of all.

There are two approaches for curing gout. One is the short-term goal which management of pain and control gout attacks at first. Then comes the long-term goal which is stopping the recurrence of attack and lowering the level of uric acid in body. Further role of diet, weight loss and management of underlying diseases has been discussed earlier for positive outcomes (Cronstein and Terkeltaub, 2006).

1.9. Natural Products in Gout

To observe the roles of natural substances in gout, different studies were conducted. In 2012, a study was conducted to observe the effect of intake of cherry on the recurrent attacks of gout in patients already suffering from the disease. It was revealed that the recurrence of attacks was reduced by 35% showing its use can be beneficial. With urate lowering drugs such as Allopurinol, cherry administration was even more effective.

Another study was conducted in 2014 which revealed the useful effect of tart cherry juice in gouty arthritis as it will reduce the level of uric acid and inflammation

associated with gout. Another data from 2019 RCT highlights that cherry and cherry product significantly reduced uric acid in serum (Zhang *et al.*, 2012b).

The seed extracts of celery have been used since ages in ancient medicines due to its role in countering inflammation and having a diuretic effect resulting in secretion of uric in urine. In 2019, Evidence-based Complementary and Alternative Medicine study revealed the impact of celery on gout in rodents. Anti-inflammatory and urate lowering effect was observed in that study (Hardani *et al.*, 2015).

Extract obtained from Urtica dioica also known as Nettle leaf showed effectiveness in gout and countering of inflammation. Phytotherapy Research published the beneficial effect and potential decrease in inflammatory markers production through Urtica dioica in murine model of gout in 2016 (Roschek Jr *et al.*, 2009).

The anti-inflammatory effect of Boswellia serrata has been studied and its role in gout is considered. A study conducted in 2017 suggested that it should be added in the treatment chart along with the medications (Siddiqui, 2011).

Curcuma longa derived Turmeric contain curcumin as an active ingredient. They have a clear anti-inflammatory role and antioxidant potential have also been observed. In gout, it has been shown to be extremely helpful in managing the symptoms in pre-clinical and clinical trials (Funk *et al.*, 2006).

Zingiber officinale gives ginger with analgesic and anti-inflammatory potential. Cell based studies and preclinical studies have shown that ginger has promising results in inflammatory diseases such as arthritis and gout (Al-Nahain *et al.*, 2014).

Omega- fatty acids and other fish supplements have shown their effectiveness in inflammatory diseases and have analgesic effects as well as reduce inflammation potential (Cai *et al.*, 2019).

1.10. Treatment of Gout

For the proper management of gout, proper diagnosis is required and after that the level and stage of the disease, underlying diseases, and variations from person to person all play a significant role in the development of patient care plan and resolving symptoms as soon as possible.

Colchicine is considered as the drug of choice in the cure of gout attacks. It acts by reducing inflammation and preventing the WBCs from rushing to the affected area. The level of drug should be maintained in a therapeutic level in blood for which after initial dose of 1-2 mg, it is administered on hourly basis in amount of 0.5-0.6 mg., but it has a limitation as it cannot be given to heart patients, kidney failure patients or patients with abdominal diseases.

NSAIDs such as naproxen, ibuprofen, and indomethacin and COX2 inhibitors such as celecoxib are also effective in gout as they help in pain management by PGE2 inhibition. If NSAIDs cannot be used in cases where gastrointestinal bleeding is present, COX-2 inhibitors can still be used.

Corticosteroids are used for management of gout when there are underlying diseases and when other drugs are not suitable or prohibited. They reduce swelling and inflammation and even pain is cured when given through oral, IM and IA routes. Prednisolone in a dose of 30-40 mg is given orally initially for 1 week or 10 days.

IL-1 inhibitors such as canakinumab which is monoclonal antibody inhibiting IL-1β can be used if other drugs cannot be used due to any reason. It cures inflammation in gout cases and is approved by the European Medicines Agency.

As it has been already mentioned that goal of long-term therapy is lowering the level of uric acid in recurrent attacks and dissolution of tophi. In either cases, the level of uric acid is lowered to less than 6 mg/Dl and prevents further accumulation (Pillinger and Mandell, 2020).

Allopurinol inhibits xanthine oxidase and leads to a decrease in the production of uric acid. It is highly well-tolerated but still it is contraindicated in kidney failure and with thiazide diuretics. Another xanthine oxidase inhibitor is Febuxostat which is given where allopurinol is not used. Probenecid causes uric acid to be secreted in urine and maintaining its level as shown in figure 1.6. (Cai *et al.*, 2019).

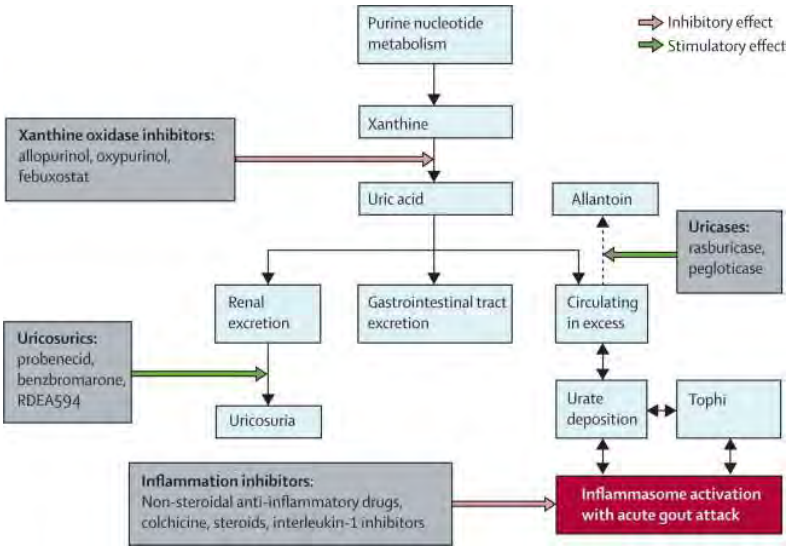


Figure. 1.6. Mechanism of action of available drugs in management of gouty attack (Burns and Wortmann, 2011).

1.11. Controversies and Uncertainties

Gout is complex metabolic disorder and research is continued to offer new targets and development of novel drugs. There are certain aspects of gout which still need attention such as some patients are show deposition of crystals as soon as the level of uric acid raised while the others show crystal formation at the later stages or no formation at all and sometimes even with the crystals the patient is asymptomatic. As goal of long-term treatment with uric acid lowering drug is that the level of uric acid should be decreased to less than 6 mg/dL, stopping recurring attacks and dissolution of MSU crystals. There is still insufficient data to support the use of uric acid lowering drug at the start of this accumulation in the body.

There are guidelines that suggest the use of urate lowering drugs in gout, but this goal is still not achieved because of issues with individual patient variations, underlying diseases, and compliance issues. The most important issue is lack of patient education as some patients think that this pain will subside, and they do not get medical intervention without thinking about the consequences of the disease. Moreover, it is sometimes associated with stigmas such as obesity and alcohol abuse. So, people ignore getting checked and receiving medications. Improper management of gout can also be due to defects in the healthcare system where the providers have little knowledge about the disease and do not consider it seriously running in their own raze of competition and not giving proper time to patients all contribute to poor management. In gout,

individualized care plans should be recommended. For proper treatment, seminars and awareness raising platforms should be used educating healthcare givers and patients about acute gout attacks and long-term management of chronic disease and novel discoveries and techniques. Self-management of the symptoms by patients and putting them in charge of their therapy is still the most effective mode of treatment. Early diagnosis and medical intervention are quite important as it will prevent attacks of gout and the quality of life of patients could be increased.

For the proper management all the healthcare providers come together and finalize the individualized patient care program. For this purpose, doctors, nurses, pathologists, nutritionists, and rheumatologists come up with the plan which is most effective and promotes healing and at the end relieves healthcare burden in the long run.

Additionally, the uric acid lowering drug should be available to all the patients in need of it without discrimination. Everybody should have a basic right to health through health ministry policies. Urate lowering drugs are the drugs of choice in managing gout. So, the issues in management of gout should be resolved worldwide and quality of life should be improved (Keenan, 2017).

1.12. Kaurenoic Acid in Gout—A Novel Approach

Kaurenoic acid is considered as the potential cure of gout because its ability to counter inflammation has been studied. It is a natural compound obtained from a plant called *Aralia continentalis*. This diterpenoid substance can inhibit iNOS and COX-2 by reducing NO, PGE2. It prevents NF-κB to activate and inhibit inflammatory process.

1.13. Problem Statement

The increase in the level of uric acid leads to formation of crystals in synovial region. It causes gout attacks, renal stones development and impaired kidney function. The management of the disease is not effective because of minimal research and less options for treatment such as inflammation lowering and uric acid lowering drugs.

Allopurinol and Febuxostat are the drugs of choice as they inhibit xanthine oxidase and lower the level of uric acid in body by stopping its production. Other drugs work by secreting uric acid and ultimately lowering the level in body. These include Probenecid and Benzbromarone. There are certain limitations in the use of medicines such as drug

interactions with other medicines the patients take, many underlying diseases and due to allergies and hypersensitivity in some patients.

So, there is need of effective drugs that will not only reduce production, promote excretion of uric acid but also lower the risk of hypersensitivity and well-tolerated in other pathological conditions. If inflammatory pathways can be targeted effectively, this problem could be solved.

To control inflammation is the key factor which can lead to development of the novel drugs. By inhibiting inflammatory mediators in cell signaling pathways, the process can be stopped at the very beginning and there will be no attacks and no pain at all.

Novel drugs will not only lower the uric acid level, but their reduced side effects and minimal drug-interactions will be the turning point in treatment of gout and it will relieve a huge burden from healthcare.

In current times drugs that lower urate level and the uricosuric medicines, but the limitations associated with the current medications are making the use difficult. For this purpose, researchers need to develop novel therapies that will address all the issues related to current therapies and improve the overall quality of life in patients.

1.14. Rationale of the Study

The rationale of the study is that Kaurenoic acid possesses anti-inflammatory properties and ameliorates the symptoms of gout in dose-dependent manner.

There is a dire requirement for developing a novel treatment that could ameliorate the gout symptoms and protect against inflammation and joint degeneration induced by oxidative stress markers. The drug under study holds promise in countering inflammation and alleviating the symptoms.

If the drug proves to be effective in clinical trials, then it will be a good treatment option in gout. As this drug attempts to target both symptoms and underlying mechanism of gout, it has potential to provide protective effect to joints, mitigating progression of disease and overall improvement in the quality of life in gout patients.

1.15. Aim

The aim of this study to investigate the anti-inflammatory and urate lowering potential of the Kaurenoic Acid

1.16. Objectives

- The main objective of the study is to evaluate the possible therapeutic role of Kaurenoic acid in treating the symptoms of gout which are induced by MSU crystals. This effect can be investigated by observing the signs of inflammation in joint and serum analysis of uric acid after administration of Kaurenoic acid. If the uric acid is controlled and inflammation subsides showing positive results in quality of life, it will be the potential therapy in the near future for curing acute and chronic gout.
- Hematological analysis will be performed to study the effect of Kaurenoic acid on blood cells. The level of WBCs, RBCs and platelets will be monitored closely. This test is performed to see if the defects occur in immune system and health after administration of Kaurenoic acid further highlighting its safety profile in gout treatment.
- LFTs are performed to study the impact of Kaurenoic acid on liver and its biomarkers. ALT and AST levels are evaluated to observe the hepatotoxic effects if any of the drug under study and compare it with other treatment available and evaluate safety of the drug under study.
- BUN and creatinine levels are also observed to observe the side effects of Kaurenoic acid if any. These studies will predict the safety profile of kaurenoic acid as no side effects will be observed on renal and hepatic functions

CHAPTER 2

MATERIALS AND MATHODS

2. MATERIALS AND METHODS

2.1. Materials

2.1.1. Chemicals

The chemical reagents used in this study are the test drug Kaurenoic Acid (KA) obtained from Prof Eun Kyoung Seo, College of Pharmacy, Ewha Woman University, Seoul, Korea. Monosodium Urate Crystals (MSU), ketamine, dimethyl sulfoxide (DMSO), Normal Saline (Abbot. Pharmaceuticals), acetone, and Phosphate Buffered Saline (PBS). The avidin-biotin complex (ABC), proteinase-K, 3,3-diaminobenzidine (DAB) (Sigma, USA), Xylene (Sigma,USA), ELISA kit for inflammatory cytokines analysis and mounting media were purchased from Santa Cruz (Santa Cruz, Inc). Allopurinol was obtained from the research center. All the drugs and reagents used in this study were of analytical grade.

2.1.2. Animals

Albino BALB/c male mice (5-7) weeks, weighing 25-30 g, were used in the study. Male mice were included in the study to avoid variations in the results due to hormonal cycle in female mice. Fresh animals were procured from National Institute of Health Sciences, Islamabad and were used only once for the current study. Animals were acclimatized for about 1 week in the animal house of the pharmacology lab in a pathogen free environment. During the complete study animals were provided with fresh food and free access to water. The study animals were exposed to light; dark cycle 12:12 and temperature around 23 ± 2˚C, relative humidity was maintained <55 %. The experimental protocol was prior approved by the bio-ethical committee of Faculty of Biological Sciences, Quaid i Azam University, Islamabad (approval # BEC-FBS-QAU 2023-468). The authority expressed no reservations or any such conflict. Vigilant efforts were made to keep the number of animals at a minimum and prevent any harm to the animals.

2.1.3. Apparatus

Cleaned, dried glassware apparatus was used to prepare solutions. Beakers (50 ml, 100 ml, 1000 ml), Volumetric flask, Sterile syringes, Dissection box, thermometer and glass slides, Ultrasonicator (Elmasonic GmbH, Germany),Centrifuge machine (Hermlegmbh Z-326K, Germany) were used. Hot plate, Von Frey, and Randall–Selitto were used for behavioral studies. Eppendorf and Falcon tubes of 5 ml and 50 ml were used to keep PBS and formalin – dipped tissues.

2.1.4. Software's

Image J software was used for quantification of histopathological and immunohistochemistry images. GraphPad prism and Origin were used to plot the graphical results. Bio render (14 days free trial version) with permission was used to graphically illustrate the drug mechanism. End Note was used to add references.

2.2. Methods

2.2.1. Preparation of inducer solution

The 3mg of prepared MSU crystals were resuspended in 70µL of sterile PBS according to reported method (Mariotte *et al.*, 2020).

2.2.2. Preparation of drug solution

Kaurenoic Acid 1 mg, 10 mg and 25 mg dilutions were prepared by dissolving 1 mg, 10 mg, and 25 mg in 2% DMSO followed by final dilution with 0.9% normal saline. The three doses of drug were selected according to literature reported effect of kaurenoic acid (Borghi *et al.*, 2021). Each solution was properly labelled and properly stored.

2.2.3. Induction of gout

Gout induced in seven groups by subcutaneous (Mariotte *et al.*, 2020) and intraarticular injection (Fan *et al.*, 2022) of 3mg MSU crystals resuspended in 70µL of Phosphate Buffered Saline. After MSU crystal administration different behavior analysis were performed (0h, 2h, 4h, 6h, and 24h) to observe the induction of disease.

2.2.4. Study design

Animals were grouped into eight groups, each containing seven mice (n=7/ group). The number of mice in each group was carefully determined considering statistical and bioethical parameters. The grouping was done randomly without any preference. The normal control group placed in suitable conditions without giving any test substance, the other seven groups were injected with MSU crystals. Out of seven groups two groups were negative in which disease was induced by two different routes (S.C),(i.art) .In negative (S.C) group MSU induced subcutaneously in left hind paw and in negative (i.art) MSU crystals injected intra-articularly in left knee joint and these two groups were left untreated, positive (Allopurino10mg/kg), and the three treatment groups injected with MSU (S.C) received test doses of KA (1 mg/kg) , (10mg/kg) and (25 mg/kg).The fourth treatment group that is injected with MSU (i.art) received test dose of KA (25mg/kg).The test doses were given from day two to d seven with exception of

day six to observe the dose dependent effect of KA. The test doses were decided based on the previous studies mentioned in literature. The study design of the experiment was drawn by bio-render (14 days trail version).

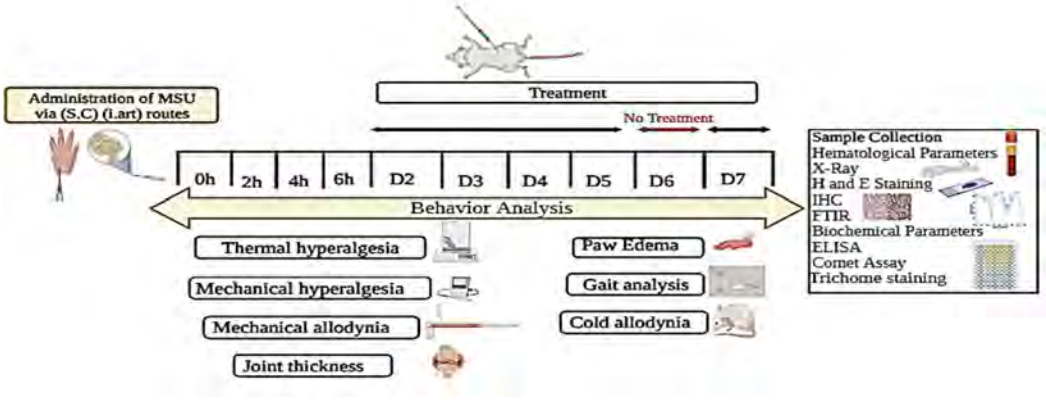


Figure 2.1. Schematic representation of *in vivo* study design. (Software: Biorender-14 days free trial version).

2.3. Behavioral Parameters

2.3.1. Assessment of paw edema

Paw edema is the measure of the increase in the thickness of the paw due to inflammation response as reported (Goo *et al.*, 2021). Paw thickness was measured by peacock dial gauge using reported method (Yu *et al.*, 2021). To evaluate the inhibitory effect of Kaurenoic acid on the MSU-induced paw edema. The MSU injection into left hind paw triggered the marked elevation in the paw edema. Paw edema was measured from day 1 (0h, 2h,4h,6h) to day 7 in each group.

2.3.2. Assessment of joint thickness

Joint is the measure of the increase in the thickness of the joint due to inflammation response as reported (Han *et al.*, 2016). Joint thickness was measured by peacock dial gauge. To evaluate the inhibitory effect of Kaurenoic acid on the MSU-induced joint inflammation. The MSU injection into left knee joint triggers the marked elevation in the joint edema. Joint thickness edema measured from day 1 (0h, 2h,4h,6h) to day 7 in each group.

2.3.3. Assessment of mechanical allodynia

Mechanical allodynia was assessed by using Von Frey as reported (Marcotti *et al.*, 2018). The Von Frey test measures the force necessary to cause a pain response in mice by applying a series of calibrated filaments with varying degrees of stiffness to their

paws. Variations in mechanical sensitivity may be a sign of the disease's advancement or how well gout therapy was working.

2.3.4. Assessment of mechanical hyperalgesia

In a gout model of mice, the mechanical hyperalgesia test is used to examine the mice's pain response and how sensitive they are to mechanical stimuli (such as pressure or contact). This test aids in the investigation of gout-related pain and the assessment of the efficacy of treatment group. The paw pressure test, also called the Randall-Selitto test, is used to evaluate mechanical hyperalgesia. It involves restraining the animal and applying increasing pressure to their paw with a dome-shaped pusher. The meter records the withdrawal behavior of mice gram, up to a cutoff load of 300 g as reported in literature (Yam *et al.*, 2020).

2.3.5. Assessment of thermal hyperalgesia

Hot plate was performed to access the animal's sensitivity towards thermal pain stimuli as reported. In the hot plate test, an animal was placed on a metal surface that is kept at a constant temperature (50°C), and the amount of time it takes for a withdrawal and licking of paw behavior to be observed were recorded according to the established protocol (Deuis *et al.*, 2017).

2.3.6. Assessment of cold allodynia

To observe the sensitivity of pain towards cold stimuli acetone evaporation test was performed as reported. Individual mice are put in tiny cages with a mesh bottom. After the hind paw is treated with acetone, the withdrawal reaction was timed by using a stopwatch (Deuis *et al.*, 2017).

2.3.7. Assessment of gait pattern

Understanding behavioral changes in acute gout models can be done with the help of gait analysis. Usually, scoring is done by putting the animal in an open space and filming it to be scored afterwards as reported (Patil *et al.*, 2021). Gait analysis was observed from day 1 to day 7. The following inclusion approach would be used to score gait dysfunction: Score 0: A normal walk with even ground contact on both feet. Score 1: The foot is slightly limp, and the toes are not spread apart. Score 2: The foot is twisted and clearly limping, with the toes on the ground. A three-legged gait with the foot elevated off the ground receives a score of 3 (Lakes and Allen, 2016).

2.3.8. Assessment of body weight

During the study period, each mouse in each group was weighed using a digital weighing balance from induction, or days 1 (0h, 2h, 4h, and 6h), to day 7 to note any significant variations in body weights between diseased groups and treatment groups.

2.4. X ray Analysis

To evaluate the disease establishment in the negative group and recovery of disease in treatment group X ray analysis was performed. The radiological assessment of paw and joint were performed as reported in literature (Wang *et al.*, 2019).

2.5. FTIR (Fourier-Transform Infrared Spectroscopy)

To determine the effect of KA on lipids, proteins, and carbohydrates an FTIR analysis was performed. Changes in the band width were determined. The paw and joint tissue cut in a uniform section was observed for absorbance in FTIR.

2.6. Hematological Analysis

2.6.1. Total blood count

To evaluate the effect of inflammation on total blood count hematological parameters was determined. The effect of MSU and KA on blood parameters were evaluated according to the reported procedure (Kiyani *et al.*, 2019).

2.6.2. RFTS and LFTS

To observe the effect of MSU and KA on vital organs RFT and LFTS were evaluated. The renal function test was performed to determine the uric acid accumulation which is an important hallmark in gout disease and level of creatinine was also determined (Tang *et al.*, 2017).

The alternation of liver function and liver enzymes were observed through LFT test as reported in literature. Determination of serum C-reactive protein is essential to evaluate the acute gout symptoms as well as therapy effectiveness (Kiyani *et al.*, 2022).

2.7. Histological Analysis

2.7.1. Hematoxylin and eosin staining

Histological changes in the paw and joint tissues were evaluated by histopathological analysis as reported (Karim *et al.*, 2021). According to a previously described procedure (Mansouri *et al.*, 2015), the paw, joint kidney, and liver tissues were removed, rinsed with normal saline, and then maintained in fixative solution (10% formalin) overnight. Tissue samples were allowed to dehydrate the following day with alcohol and a xylene

replacement. The specimens had paraffin in them and then stained with H & E using the previously described technique.

2.7.2. Masson's trichrome staining

The deposition of collagen in the paw and joint tissues were determined by Masson trichrome staining as reported (Zhao *et al.*, 2021). The slides were first deparaffinized by immersing them in a series of concentrations of 100% xylene and ethanol. Using modified Weigert's hematoxylin to distinguish the nuclei and acid fuchsin to stain the cytoplasm and erythrocytes, respectively. Methyl blue solution was used to stain collagen and fibroblasts, while phosphomolybdic acid solution was utilized as a mordant. The slides were then dehydrated in a series of alcohol solutions with increasing alcohol concentrations after this staining, and then received one final dip in 100% xylene for one minute. Finally, the slides were mounted using mountain media and a cover slip. Pathological changes in the paw and joint collagen fibers were observed under a light microscopy (Suvik and Effendy, 2012).

2.8. Immunohistochemistry (IHC)

The anti-inflammatory activity of KA was further assessed by the immunohistochemical analysis of paw and joint tissue as reported (Qiao *et al.*, 2020). The antibodies corresponding to the relevant markers involved in inflammation were used; TLR4, NF-κB, IκBα, COX2 and iNOS. Following a previously established methodology, tissue sections from the paw and joint were fixed in paraffin and subjected to an avidin-biotin peroxidase complex (ABC) approach to look for inflammatory markers. These tissue sections were deparaffinized with xylene and then hydrated for predetermined amounts of time with various alcohol dilutions (95%, 90%, 80%, and 70% v/v) in distilled water. Enzymatic antigen retrieval was carried out, then PBS treatment. Following this, the tissues were incubated overnight with protein kinase K, primary antibodies, and normal goat serum (5%) to promote the formation of antigen-antibody complexes. A 3x dilution factor was used to dilute the primary antibody at a ratio of 1 part antibody to 1000 parts blocking buffer. Following incubation, PBS was used to wash the primary antibodies, Anti-rabbit secondary antibodies were used, with the tissues were then incubated with ABC for 1 hour in a humid environment. The slides were rinsed with 0.1 M PBS and stained with diaminobenzidine (DAB) to see the generated antigen-antibody combination. To

protect the slides, excess DAB was removed, and mounting medium was used to apply coverslips (Magaki *et al.*, 2019).

2.9. Molecular Docking Analysis

To determine the binding affinity of inflammatory markers molecular docking analysis was performed (Hussain *et al.*, 2020). The three-dimensional (3D) and two-dimensional (2D) structural affinity of Kaurenoic acid with protein targets were analyzed via AutoDock. KA was docked against with TLR4/ NFκB/ IκB-α/COX-2 and iNOS (Khan *et al.*, 2022).

2.10. Biochemical Parameters

2.10.1. Determination of NO

Nitric oxide synthase (iNOS) is the main contributor to inflammatory reactions. Inflamed joints' synoviocytes can also be observed to contain iNOS. iNOS inhibitors as potential anti-inflammatory medications (Sharma *et al.*, 2007). To evaluate the inhibitory effect of Kaurenoic acid on the MSU-induced inflammation. NO assay was performed as reported. The Griess reagent method was used to determine the nitric oxide level in inflamed paw and joint tissue according to established protocols (Costa *et al.*, 2004). Briefly, a tissue sample was taken from the inflamed paw and joint tissue followed by homogenization and centrifuged at 5000 rpm for 10 min. The collected supernatant was mixed with an equal quantity of Griess reagent. In this test, nitrite is transformed into a purple azo dye that can be quantitatively examined using spectrophotometry at a wavelength of about 540 nm. For this reaction, sulphanilamide and N-naphthyl-ethylenediamine are necessary components (Wang *et al.*, 2010).

2.10.2. Lipid peroxidase (LPO) assay

To evaluate the inhibitory effect of Kaurenoic acid on the MSU-induced inflammation. LPO assay was performed as reported in (Riaz *et al.*, 2022). This approach describes how to measure the byproducts of lipid peroxidation to evaluate damage in tissues. First, animal tissues are gathered, cleaned, and weighed. The tissues are then homogenized, and the supernatant is then poured into fresh tubes. To measure the quantities of malondialdehyde (MDA) the supernatant from MSU-treated animal tissues, the mixture, followed by 40 minutes of incubation at 45 °C, cooling on ice, and 10 minutes of centrifugation. The optical density at 536 nm (OD536) is calculated after the supernatant is transferred to a 96-well plate (Yet *et al.*, 2002).

2.10.3. Eosinophil peroxidase (EPO) assay

Eosinophil peroxidases serve as a marker for eosinophil recruitment in the tissue (Tsompos *et al.*, 2014). Using a previously developed technique, the activity of eosinophil peroxidase (EPO) in paw and joint homogenates was measured. In this process, the supernatant from the centrifugation of the lysate was utilized for the EPO assay. The procedure for the assay was to stop the reaction with sulfuric acid after a 30-minute incubation at room temperature, transfer the supernatant to a 96-well plate, add a substrate mixture, and measure the enzyme activity by measuring the absorbance at 492 nm (Enobe *et al.*, 2006).

2.10.4. Myeloperoxidase (MPO) assay

To evaluate the inhibitory effect of Kaurenoic acid on the MSU-induced inflammation. MPO assay was performed as reported in (Stamp *et al.*, 2014). In µ1 ml of PBS (pH 7.4) the tissue samples from the paws and joints were homogenized. Homogenates were centrifuged (10,000 x g) at 4 °C for approximately 10 mins. A sandwich ELISA approach using capture and detection antibodies was used to measure the amounts of MPO in paw and joint tissue samples. On 96-well microtiter plates, all activity assays were run in triplicate, and the results were analyzed using a microplate reader. Peroxidase activity was evaluated using 3,3′,5,5′-Tetramethylbenzidine (TMB, Sigma). A plate was incubated at 37°C for 5 min with 10µl sample, 80 µl 0.75 mM H2O2, 110 µl TMB solution and the plate was incubated. Before introducing each solution, an incubation period of one hour was followed by four cycles of washing with wash solution. After TMB administration, a 25-minute incubation period at room temperature was permitted. By adding 100 µl of stop solution, the MPO activity was calculated from measurements of absorbance at 450 nm (Pulli *et al.*, 2013).

2.10.5. Inflammatory cytokines analysis

ELISA was carried out as described (Lin *et al.*, 2020) to ascertain the inhibitory effect of KA on the production of pro-inflammatory cytokines in MSU-induced. Paw and joint tissue were first removed and homogenized. After homogenizing the excised tissue (100 mg in 1 ml PBS), it was centrifuged at 5000 rpm for 10 min. Pro-inflammatory cytokines were later quantified using the collected supernatant. Using commercially available ELISA kits, the levels of TNF- and IL-1β were quantified.

2.11. Evans Blue Assay

To evaluate vascular permeability and barrier integrity Evans Blue (EB) dye is essential. Due to its ability to bind tightly to albumin, which is typically kept in the circulation by intact barriers, EB can be used to detect barrier breakage (Yao *et al.*, 2018). Evans Blue dye (30 mg/kg in 100 l PBS) was injected into the tail vein of 7 to 8-week-old mice for this vascular permeability assay. Photographs were taken 30 minutes after the Evans Blue dye treatment (Han *et al.*, 2016). Allow it for 30 minutes. To determine the amount of Evan blue that had reached the inflamed site is determined by isolation of the tissue and soaking it in formamide for 48-72 hours. To determine the optical density (OD) at 620 nm, which corresponds to Evans blue's maximum absorbance. First, after 48 to 72 hours of incubation at room temperature (RT), 50 L of the Evans blue-infused formamide is carefully removed from a microfuge tube. It is imperative to make sure that no tissue fragments accidentally transfer with the formamide during this process. Next, empty wells within a 96-well polystyrene plate are filled with 50 L of pure formamide to create blank measurements. For readings of baseline absorbance, these blanks serve as a reference. In the 96-well plate, the OD620 of each well is then measured and noted. Finally, using an absorbance plate reader, the OD620 of each well in the 96-well plate is determined and recorded. 620 nm was selected as the measurement wavelength because that is where Evans blue absorbs the lightest (Wick *et al.*, 2018).

2.12. Statistical Analysis

The data was subjected to statistical analysis using Sigma plot version 10.0 (SYSTAT SOFTWARE, INC. USA). Data obtained from were expressed as mean(n=7) ± S.D run in triplicate. Data from behavioral were subjected to two-way analysis of variance (two-way ANOVA). Immunohistochemical and biochemical data was analyzed using a one-way analysis of variance (one-way ANOVA) followed by Bonferroni's Post hoc test. For the results to be considered statistically significant, the level of significance was kept 0.001 (Zafar *et al.*, 2023).

CHAPTER 3

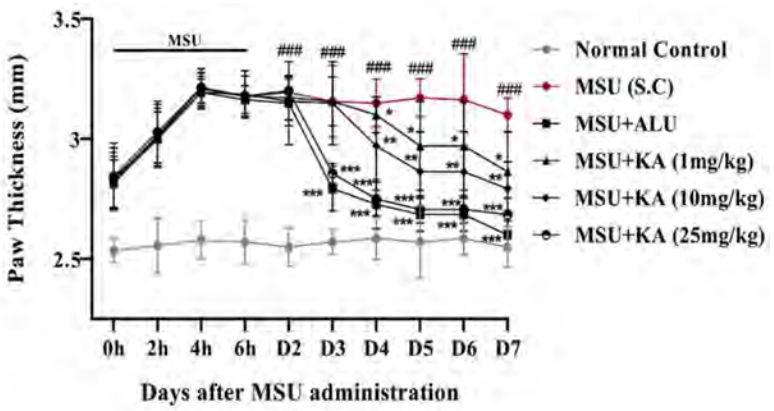
RESULTS

3. RESULTS

3.1. Effect of KA on MSU (S.C) -induced paw edema and joint thickness

The administration of MSU (S.C) injections significantly ($p < 0.001$) increased paw thickness and caused inflammation in the corresponding joint area. Paw edema and joint thickness were measured to assess the inhibitory impact of KA (1 mg/kg, 10 mg/kg, 25 mg/kg). When compared to MSU (S.C)-inflamed hind paw and respective joint, it was shown that KA (25mg/kg) considerably ($p < 0.001$) improved paw edema and joint thickness as shown in Figure 3.1.1. The dose of 25 mg/kg significantly reduces inflammation compared to the doses of 10 mg/kg and 1 mg/kg. Like this, Allopurinol (10 mg/kg) reversed joint and paw edema**.**

A



B

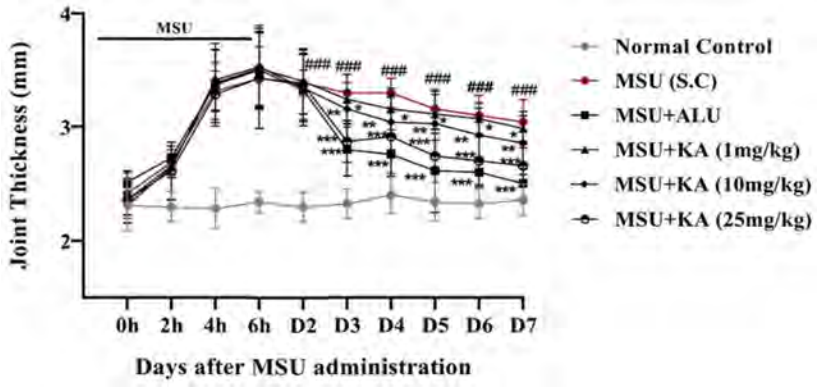


Figure 3.1. The effect of Kaurenoic acid on the MSU-induced paw edema (A) and joint thickness (B) in mice.

Note: *Data sets were expressed as the mean ± S.D (n=7). *p < 0.05, **p < 0.01, and ***p < 0.001 indicate significant difference of each treatment compared to negative control group. ### p < 0.001 indicates significant difference from MSU (S.C)-treated group.*

3.2. Effect of KA on MSU (S.C) -induced mechanical allodynia and hyperalgesia

The pain hypersensitivity was significantly ($p < 0.001$) increased when MSU (S.C) injections were administered to the left hind paw, resulting in a decrease in paw withdrawal to mechanical allodynia and mechanical hyperalgesia. Compared to the diseased group, KA treatment significantly ($p < 0.001$) reduces MSU (S.C)-induced mechanical allodynia and thermal hyperalgesia, as depicted in Figure 3.2. The behavioral analysis in this pain assessment used three different doses of KA (1, 10, and 25 mg/kg). It was noticed that KA at a portion of 25 mg/kg fundamentally suppresses gouty nociceptive pain. Like this, the MSU (S.C)-induced group's paw sensitivity was decreased by Allopurinol (10mg/kg).

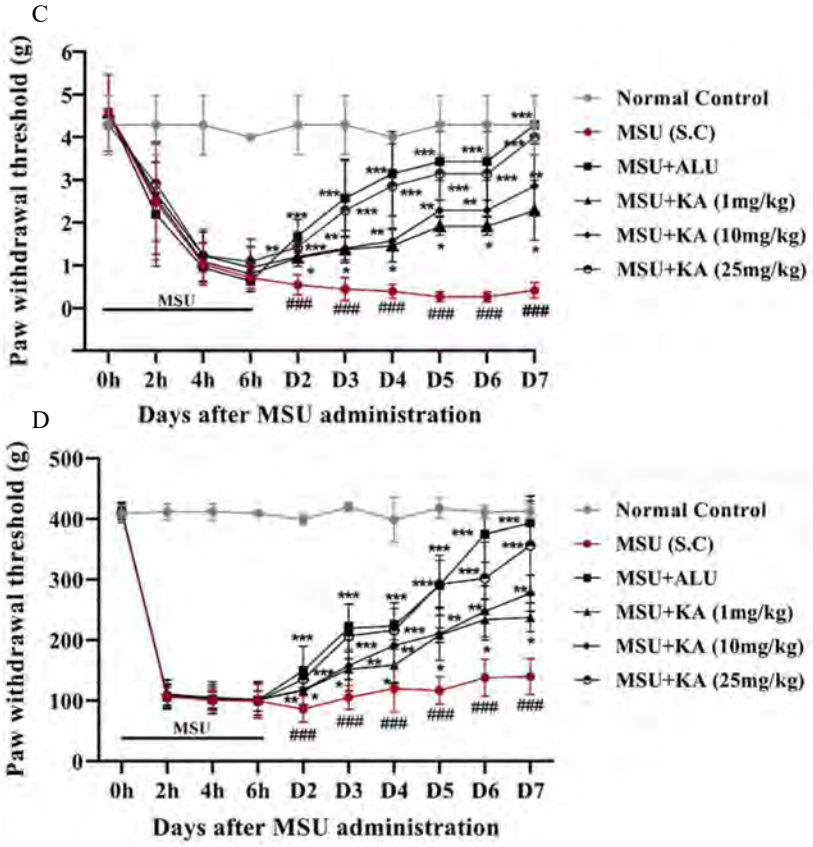


Figure 3.2. The inhibitory effect of Kaurenoic acid on the MSU (S.C) induced mechanical allodynia(C) hyperalgesia (D).
Note: *Data sets were expressed as the mean±S.D (n=7). *p < 0.05, **p < 0.01, and ***p < 0.001 indicate significant difference of each treatment compared to negative control group. ###p < 0.001 indicates significant difference from MSU (S.C)-treated group.*

3.3. Effect of KA on MSU (S.C) -induced thermal hyperalgesia and cold allodynia

The hot plate technique and the acetone test were used to assess the impact of KA (1 mg/kg, 10 mg/kg, and 25 mg/kg) against MSU (S.C)-induced thermal hyperalgesia and cold allodynia. As shown in Figure 3.3 KA treatment significantly ($p < 0.001$) reduced the paw-licking and cold allodynia response compared to the MSU (S.C) group. Allopurinol (10 mg/kg) also significantly decreased the sensitivity to heat and cold.

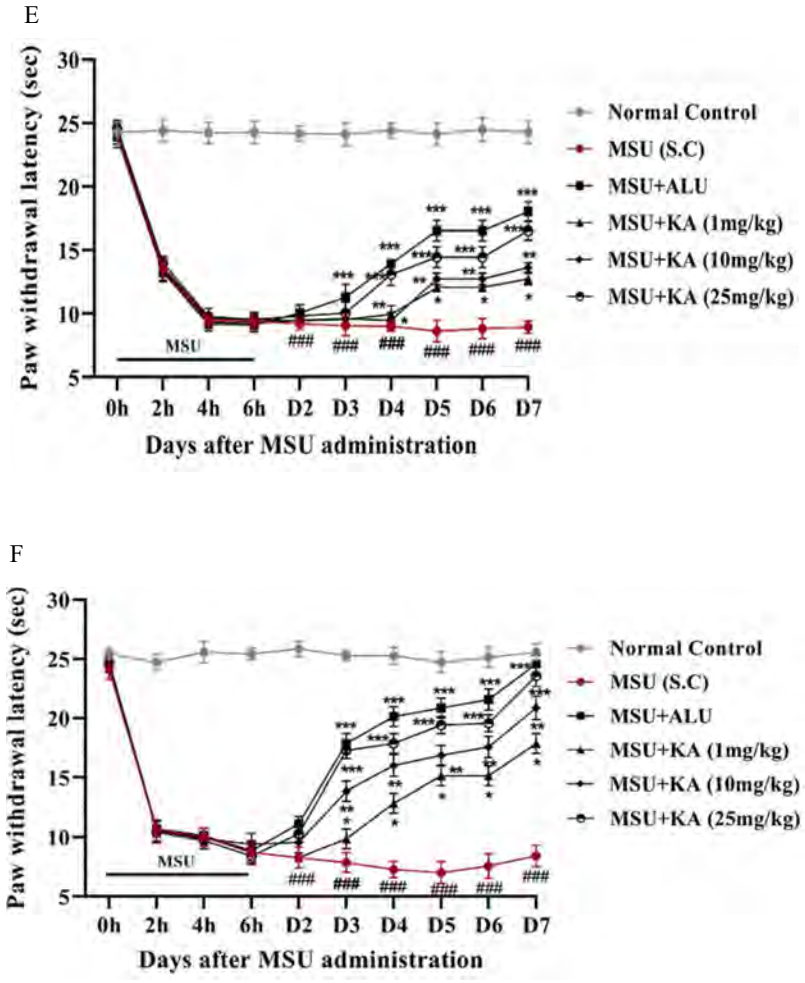


Figure 3.3. The inhibitory effect of the Kaurenoic acid on the MSU (S.C) induced thermal hyperalgesia (E) and cold allodynia (F) pain**.**

***Note**: Data sets were expressed as the mean±S.D (n=7). *p < 0.05, **p < 0.01, and ***p < 0.001 indicate significant difference of each treatment compared to negative control group. ###p < 0.001 indicates significant difference of negative control group compared to MSU-treated group*

3.4. Effect of KA on MSU (S.C) -induced gait analysis and weight assessment

When MSU (S.C) injections were given to the left hind paw, it caused the gait dysfunction to be significantly ($p < 0.001$) enhanced, which led to aberrant walk

patterns in the animals. As shown in Figure 3.4 KA therapy significantly ($p < 0.001$) lowers MSU (S.C)-induced abruption in gait when compared to the diseased group: Three distinct KA dosages were employed in this pain evaluation clinical score: 1, 10, and 25 mg/kg. It was discovered that KA at a dose of 25 mg/kg basically changed the score in gouty nociceptive discomfort from 3 to 0. Like this, Allopurinol (10 mg/kg) lowered the paw score in the MSU (S.C)-induced group. The weight assessment indicated there was a decrease in the weight of animals in negative group due to sickness behavior (loss of appetite, dehydration restricted locomotion) while KA therapy normalized this behavior and effect on weight recovery.

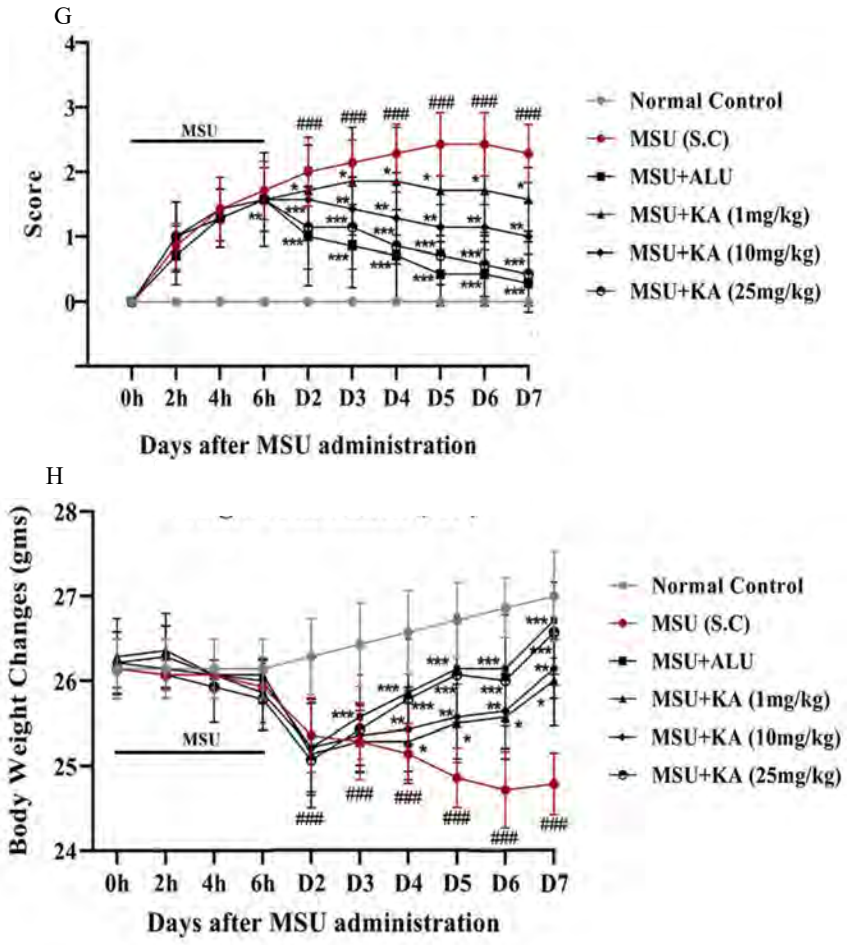


Figure 3.4. The inhibitory effect of the Kaurenoic acid on the MSU (S.C) induced Gait analysis (G) and weight assessment (H).
Note: *Data sets were expressed as the mean±S.D (n=7). *p < 0.05, **p < 0.01, and ***p < 0.001 indicate significant difference of each treatment compared to negative control group. ###p < 0.001 indicates significant difference of negative control group compared to MSU-treated group.*

3.5. Effect of KA on MSU (i.art) -induced paw edema and joint thickness

As a reaction to an actual gout episode, the treatment of MSU (i.art) injections substantially ($p < 0.001$) increased joint thickness and paw edema. To determine the inhibitory effect of KA (25 mg/kg) on MSU intraarticularly generates gout in particular joint region and inflammation in the corresponding paw area, paw edema and joint thickness were assessed. KA (25 mg/kg) significantly ($p < 0.001$) improved joint thickness and paw edema as compared to MSU (i.art)-inflamed joint and related paw as shown in Figure 3.5.

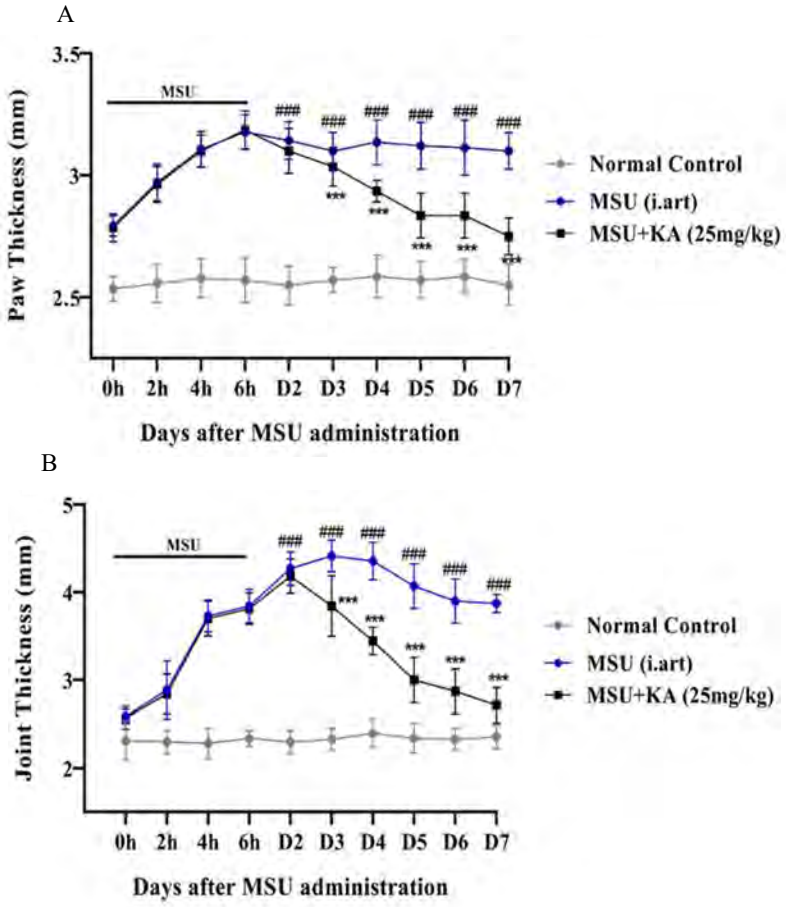


Figure 3.5. The effect of Kaurenoic acid on the MSU-induced paw edema (A) and joint thickness (B) in mice**.**

Note: *All values are expressed as mean ± S.D. (***) p< 0.001 shows significant difference compared with MSU (i.art) group. (###) denotes comparison of Normal control and MSU (i.art) group with KA group.*

3.6. Effect of KA on MSU (i.art) -induced mechanical allodynia and mechanical hyperalgesia

MSU (i.art) injection induced group is used to examine mechanical hyperalgesia and cold allodynia to determine the pain threshold in a localized location caused by a gouty

flare. When MSU (i.art) injections were given to the left joint, the pain hypersensitivity dramatically ($p < 0.001$) increased, causing a reduction in the paw's withdrawal from mechanical allodynia and mechanical hyperalgesia. As shown in Figure 3.6, KA therapy considerably ($p < 0.001$) lowers the mechanical allodynia and thermal hyperalgesia caused by MSU (i.art).

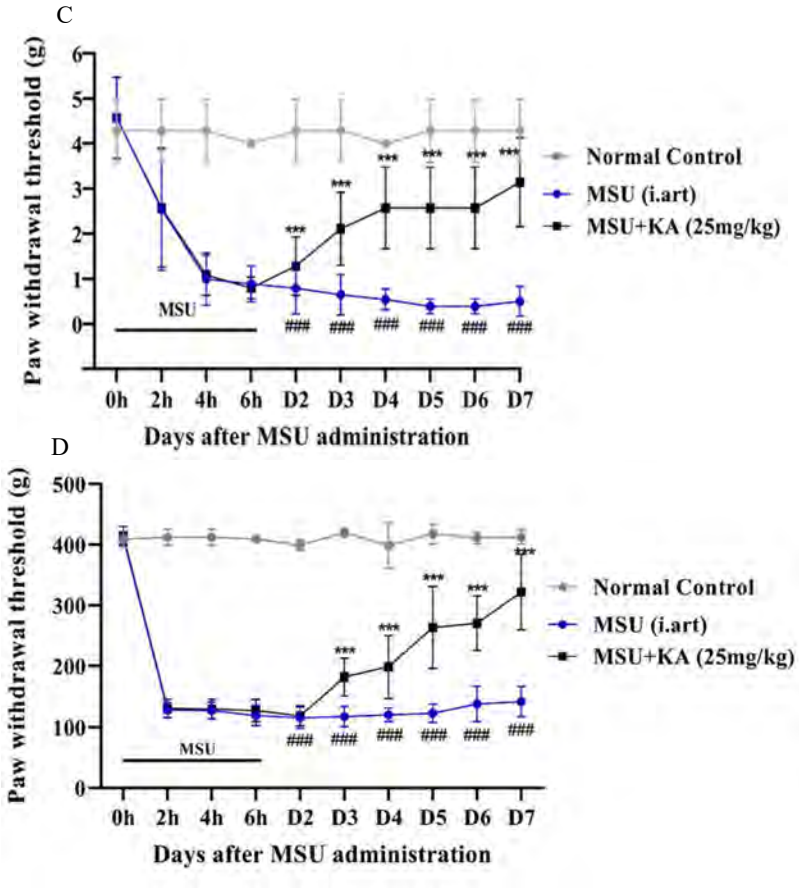


Figure 3.6. The inhibitory effect of Kaurenoic acid on the MSU (i.art) induced mechanical allodynia(C) hyperalgesia (D).

3.7. Effect of KA on MSU(i.art)-induced thermal hyperalgesia and cold allodynia

To evaluate the effect of KA (25 mg/kg) against MSU (i.art)- prompted heat hyperalgesia and cold allodynia. As depicted in Figure 3.7, KA treatment significantly ($p < 0.001$) decreased the paw-licking and cold allodynia reaction contrast with the MSU (i.art) group.

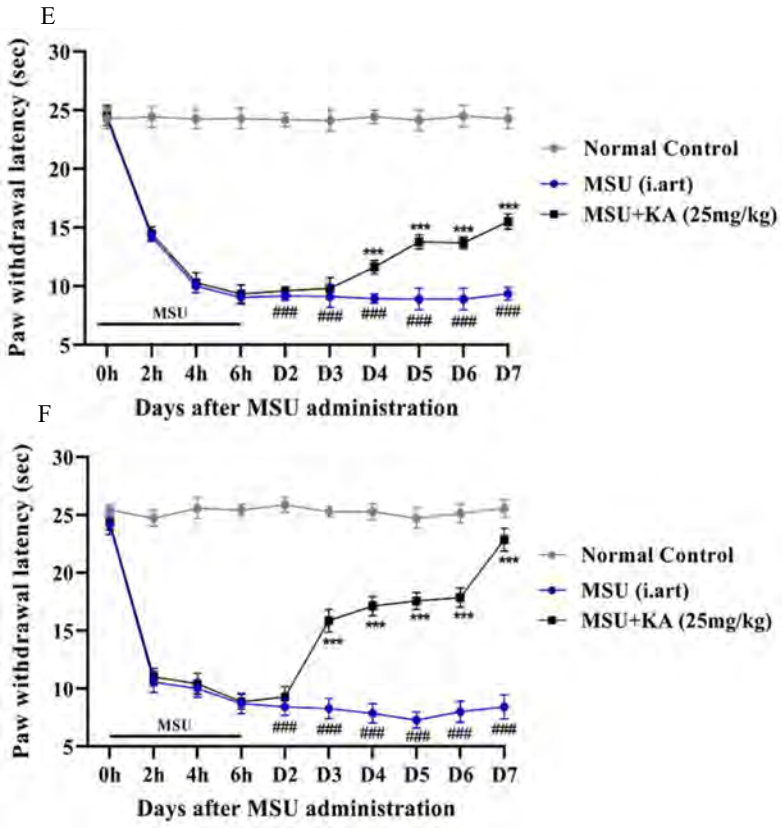


Figure 3.7. The inhibitory effect of the Kaurenoic acid on the MSU (i.art) induced thermal hyperalgesia (E) and cold allodynia (F) pain.
Note: *All values are expressed as mean ± S.D. (***) p < 0.001 shows significant difference compared with MSU (i.art) group. (###) denotes comparison of Normal control and MSU (i.art) group with KA group.*

3.8. Effect of KA on MSU (i.art) -induced gait analysis and weight assessment

When MSU (i.art) injections were given to the left joint caused the gait dysfunction to be significantly ($p < 0.001$) enhanced, which led to aberrant walk patterns in the animals. As shown in Figure 3.8, KA therapy significantly ($p < 0.001$) lowers MSU (i.art)-induced abruption in gait when compared to the diseased group. It was discovered that KA at a dose of 25 mg/kg basically changed the score in gouty nociceptive discomfort from 3 to 0. Similarly, the KA therapy showed significant effect on mice weight recovery.

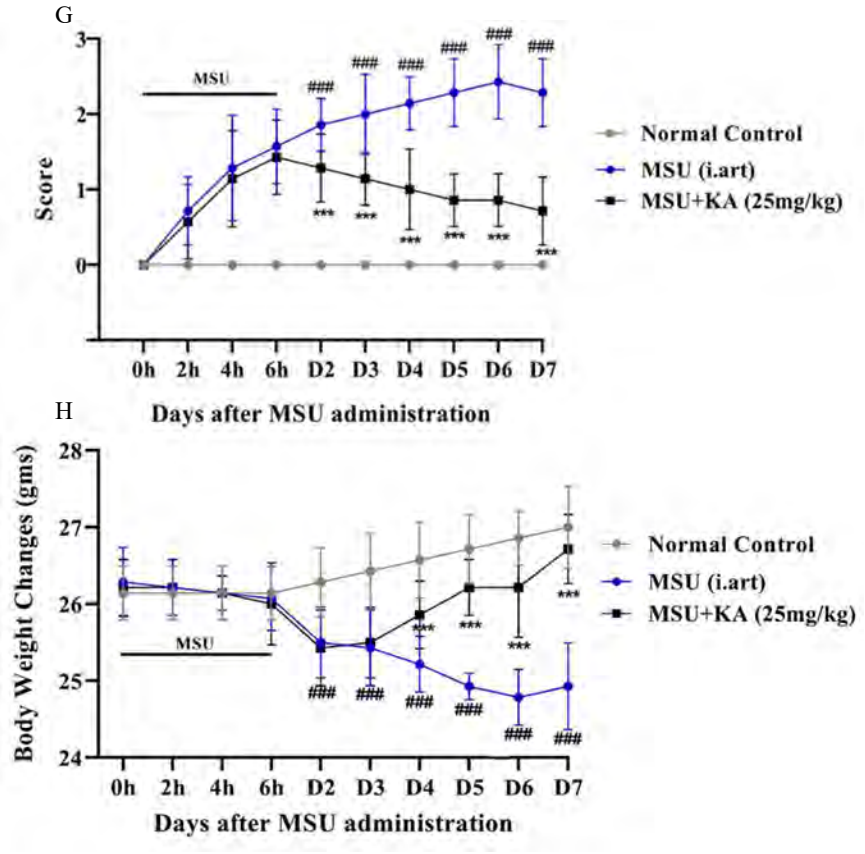


Figure 3.8. The inhibitory effect of the Kaurenoic acid on the MSU (i.art) induced Gait analysis (G).
Note: *All values are expressed as mean ± S.D. (***) $p < 0.001$ shows significant difference compared with MSU (i.art) group. (###) denotes comparison of Normal control and MSU (i.art) group with KA group.*

3.9. Representative Images of Gout

Effect of KA (25 mg/kg i.p.) left knee joint on day 7 in BALB/c mice of each group. These images indicate that both Kaurenoic acid (10 mg/kg i.p.) and Allopurinol (10 mg/kg i.p.) promisingly show anti-inflammatory and urate-lowering effects as compared to the MSU (S.C), (i.art) group.

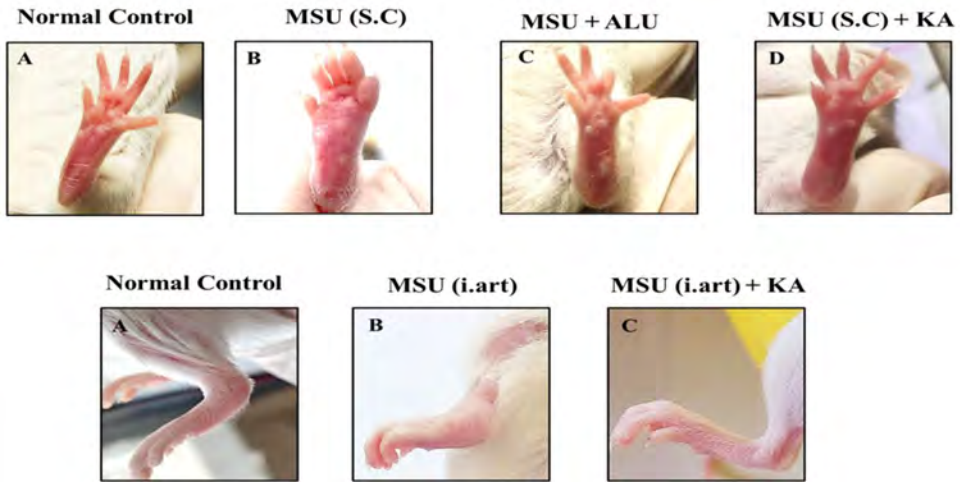


Figure 3.9. Representative images of MSU-induced Gout.(A-D) Effect of KA (1, 10, 25 mg/kg i.p.) and Allopurinol (10 mg/kg, i.p.) on left hind paws on day 7 in BALB/c mice of each group. (A-C).

3.10. Effect of KA on Vascular Permeability of Paw and Joint Tissue

The Evans blue dye assay was used to measure plasma extravasation. The outcomes were noticed both in paw and joint tissues. There was a comparison between the plasma leakage and cell membrane permeation of the Normal control, MSU (S.C), (i.art), Allopurinol, and treatment compound KA (25 mg/kg) based on the optical absorbance of the amount of Evans blue. In the MSU (S.C), (i.art) group, the evans blue dye had a significantly ($p < 0.001$) higher absorbance than in the KA (25 mg/kg) treated group. When compared to the MSU (S.C), (i.art) treated group, the infiltration of Evans blue into the paw tissue and joint was significantly ($p < 0.001$) reduced by the KA (25 mg/kg) group as shown in the Figure 3.10.

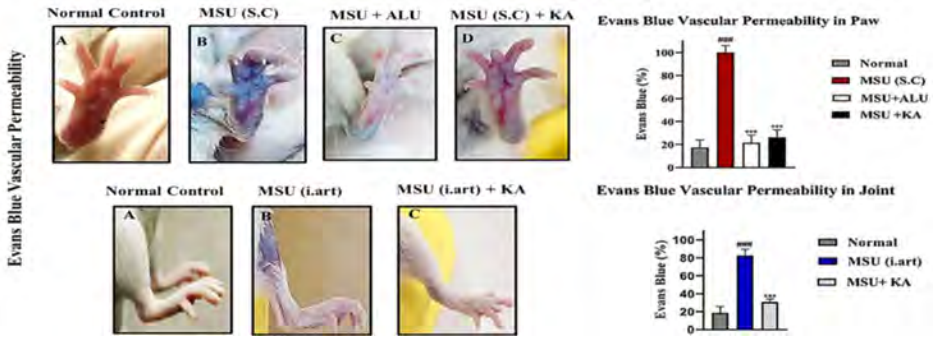


Figure 3.10. The effect of kaurenoic acid (25 mg/kg i.p.) on Paw (A-D) and left knee joint (A-C) pronouncedly decreases the infiltration of the Evans blue.
Note: *Data sets were expressed as the mean±S.D (n=7). *p < 0.05, **p < 0.01, and ***p < 0.001 indicate significant difference of each treatment group compared to negative control group. ###p < 0.001 indicates significant difference from the MSU-treated group.*

3.11. Radiological Assessment of Paw and Joint Tissue

An x-ray of the paw and joint tissue was taken to assess the impact of KA (25 mg/kg) on morphological alterations such as soft tissue edema, bone erosion and joint space narrowing, in the paw and joint after MSU (S.C) (i.art). When paw and joint tissue from the MSU (S.C), (i.art) group was compared to normal, it was found that there was a significant ($p < 0.001$) increase in paw edema and joint damage. However, as shown in Figure 3.11, when given with KA (25 mg/kg), paw and joint thickness was significantly ($p< 0.001$) reduced to a normal level, demonstrating the efficiency of KA in the treatment of gout.

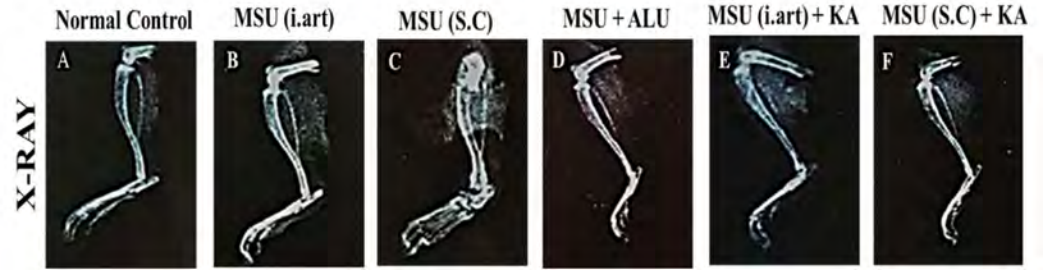


Figure 3.11. X-ray analysis. Illustrate representing (A-F) paw and knee joint x-ray of each group on day7 to indicate morphological changes (paw and joint edema) in the MSU (S.C), (i.art) groups.
Note: *The results indicate that both Kaurenoic acid (10 mg/kg i.p.) and Allopurinol (10 mg.kg i.p.) promisingly reverse MSU (S.C), (i.art) morphological changes.*

3.12. Effect of KA on Biochemical Changes

FTIR spectrum peak trends revealed variations in certain absorption bands that correspond to molecular vibrations at specific wavelengths Table 1. The assesment of FTIR peak trends in the MSU-affected paw and joint tissues revealed significant ($p < 0.001$) alterations in the absorption bands linked to biomolecules such as proteins, lipids, and carbohydrates as shown in Fig.3.12. These modifications occur due to changes in the tissue's molecular makeup, which were linked to inflammation and reversed in KA treated groups.

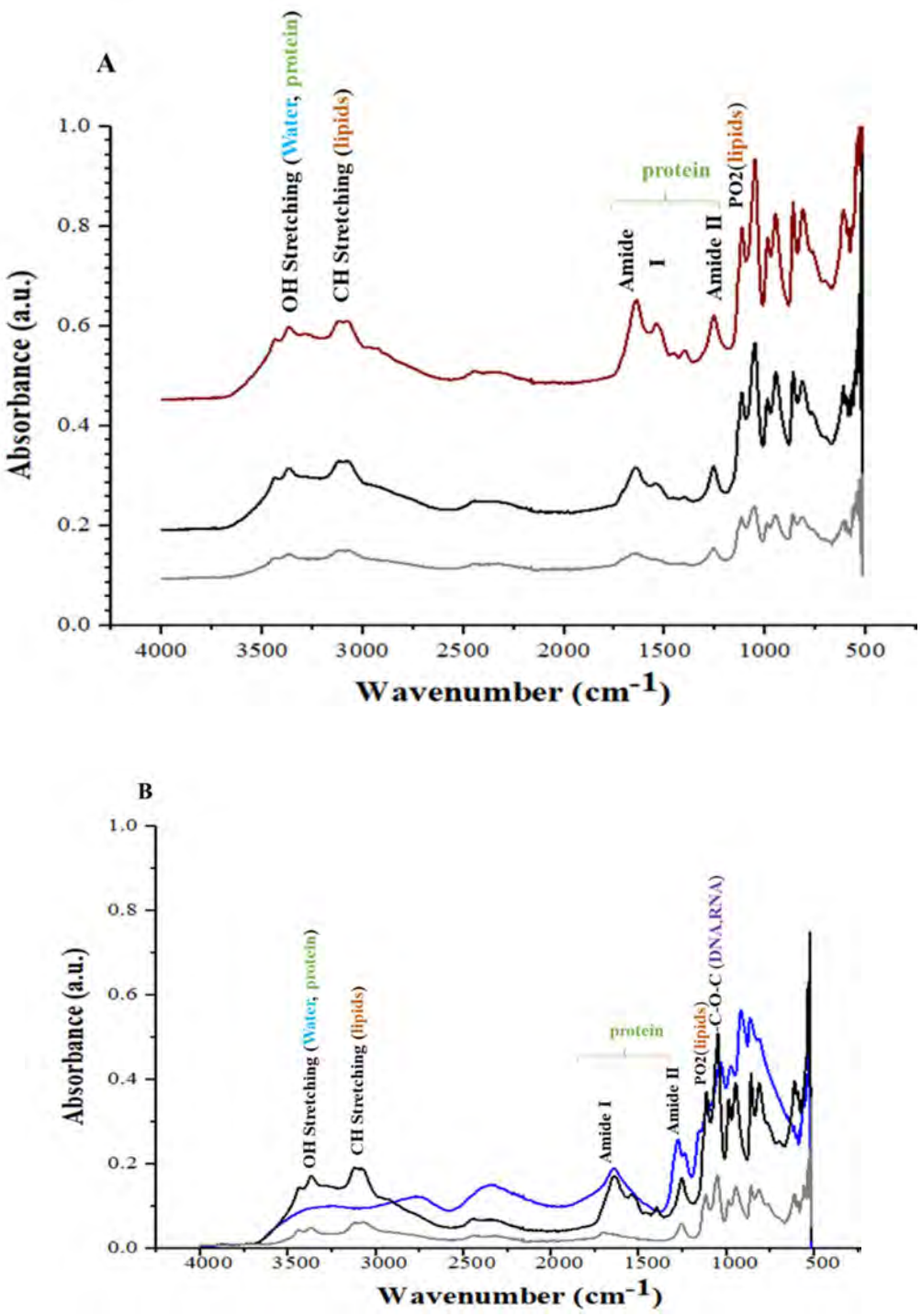


Figure 3.12. FT-IR spectroscopic analysis. The effect of Kaurenoic acid on MSU induces changes in the biochemical composition of the (A) Paw (B) Joint tissues.
Note: *The FTIR spectral absorption indicates that Kaurenoic acid markedly reverse MSU induces changes in the biochemical composition (water, proteins, and lipids) of the paw and joint tissues.*

Table 3.1. The FTIR absorption spectra of the corresponding functional groups, ranging from 1000 to 4000^{-1}.

Wavenumbers(c/m)	Functional Groups
1200-800	-C-O-C Stretching
1270-1000	-PO2
1300-1350	-CH Bending
1700-1500	-Amide I and II
3000-3150	-CH Stretching
3340-3460	-OH Stretching

3.13. Effect of KA on Hematological Parameters

3.13.1. Total blood count

The administration of MSU markedly changed the hematological parameters and significantly ($p < 0.001$) increased the levels of white blood cell count, neutrophils, and platelet count. while lymphocytes were decreased in diseased groups. However, after KA (25 mg/kg) and Allopurinol treatment, the values significantly ($p < 0.001$) showed improved effect as shown in Table 2.

Table 3.2. Effect of KA on hematology of the MSU-induced Gout in mice.

Hematological Parameters	Normal Control (mean ± SD)	MSU (i.art) (mean ± SD)	MSU (S.C) (mean ± SD)	MSU+ ALU (mean ± SD)	MSU (S.C) ± KA (mean ±SD)	MSU (i.art) ±KA (mean ± SD)
WBCs (10^3/µL)	6.2±0.173	12.1±0.18$^{###}$	14.2±0.51$^{###}$	7.1±0.40***	7.8±0.21***	8.5±0.341***
Platelet Count (10^3/µL)	459.2±23.3	705.1±62$^{###}$	985.2±118$^{###}$	503.2±28***	572.7±57.4***	532.8±58.02***
Neutrophils (%)	31.57±0.97	51.5±0.53$^{###}$	71±0.816$^{###}$	36.8±0.89***	42.2±0.78***	48.2±0.75***
Lymphocytes (%)	61.85±0.89	52.2±1.46	47±4.816$^{###}$	59.4±0.89***	56.4±0.53***	54.5±0.53

Note: *Data sets were expressed as the mean±S.D (n=7). *p < 0.05, **p < 0.01, and ***p < 0.001 indicate significant difference of each treatment group compared to negative control group. ###p < 0.001 indicates significant difference from the MSU-treated group.*

3.13.2. RFTs and LFTs

RFTs and LFTs were done to assess the impact of KA (25 mg/kg) on major organs after MSU (S.C), (i.art) as shown in Table 3. When serum values of urea, creatinine, and uric acid from the MSU (S.C), (i.art) group were compared to normal, it was found that there was a significant ($p < 0.001$) increase in values of MSU (S.C) due to its systemic effect. However, when given with KA (25 mg/kg) and Allopurinol, the values significantly ($p < 0.001$) reduced, demonstrating their efficiency in the treatment of

gout. Similarly, when serum values of ALT, AST and CRP from the MSU (S.C), (i.art) group were compared to normal, it was found that there was a significant ($p < 0.001$) increase in values in MSU (S.C). However, when given with KA (25 mg/kg), the values significantly ($p < 0.001$) reduced, demonstrating their efficiency in the treatment of gout. Allopurinol did not significantly lower these values and hence did not improve the hepatic functioning.

Table 3.3. Effect of kaurenoic acid on Biochemical parameters of the MSU-induced Gout in mice.

Biochemical Parameters	**Normal Control (mean ± SD)**	**MSU (i.art) (mean ± SD)**	**MSU (S.C) (mean ± SD)**	**MSU+ ALU (mean ± SD)**	**MSU (S.C) ± KA (mean ±SD)**	**MSU (i.art) ±KA (mean ± SD)**
Serum ALT (µ/L)	26±0.95	39.7±1.9$^{###}$	52.8±1.0$^{###}$	33.7±2.76**	35.5±2.21***	31.7±1.57***
Serum AST(µ/L)	37.2±0.7	48.1±1.8$^{###}$	68.8±0.69$^{###}$	45.4±2.36**	44.4±0.78***	42.5±2.28***
CRP (mg/L)	2.1±10.7	13.5±12$^{###}$	18.3±11.0$^{###}$	3.2±14.06***	2.9±7.74***	2.6±6.8***
Serum Urea (mg/dL)	41±3.51	65.7±5.8$^{###}$	69.4±4.39$^{###}$	44.8±03.3***	54.2±6.11***	47.7±6.10***
Serum Creatinine (mg/dL)	1.3±0.35	1.9±0.11$^{###}$	2.1±0.32$^{###}$	1.57±0.21***	1.77±0.15***	1.62±0.12*
Serum Uric Acid(mg/dL)	5.2±0.65	8.3±0.38$^{###}$	9.1±0.6$^{###}$	5.7±0.637***	6.1±0.553***	6.6±0.36***

Note: *Data sets were expressed as the mean±S.D (n=7). *p < 0.05, **p < 0.01, and ***p < 0.001 indicate significant difference of each treatment group compared to negative control group. ###p < 0.001 indicates significant difference from the MSU-treated group.*

3.14. H and E staining of paw tissues

H & E staining results in inflammatory cell infiltration, tissue destruction, edema, and increased vascularity in paw and MSU treated paw .While treatment with KA significantly ($p < 0.001$) decreased vascularity, healed tissue, inflammation resolution, and the return of normal tissue structure in paw. Like this, Allopurinol (10 mg/kg) significantly ($p < 0.001$) reverse the histopathological changes in the MSU (S.C)-induced group as shown in Figure 3.14.

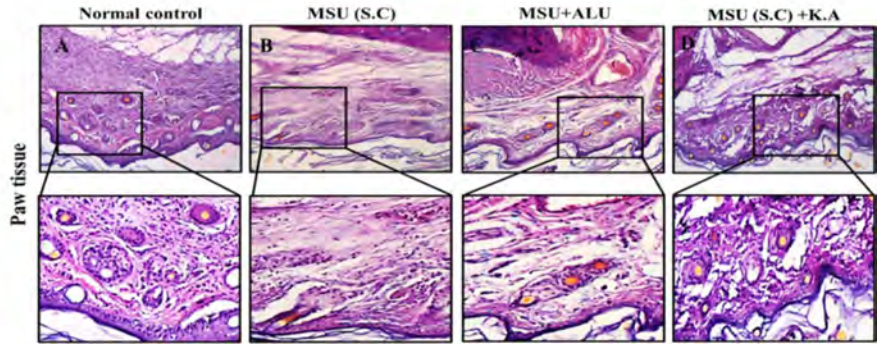


Figure 3.14. Hematoxylin and eosin staining (10X). Impact of 25 mg/kg i.p. KA on the histological alterations in the left hind paw (A–D).
Note: *Histological alterations in the paw show cellular infiltration and modifications to the dermo-epidermal junction.*

3.15. H and E staining of knee joint

H & E staining results in inflammatory cell infiltration, chondrocytes irregularities, edema, and increased vascularity in paw and MSU treated joint tissues .While treatment with KA significantly ($p < 0.001$) decreased vascularity, healed tissue, inflammation resolution, and the return of normal tissue structure in joint as shown in Figure 3.15.

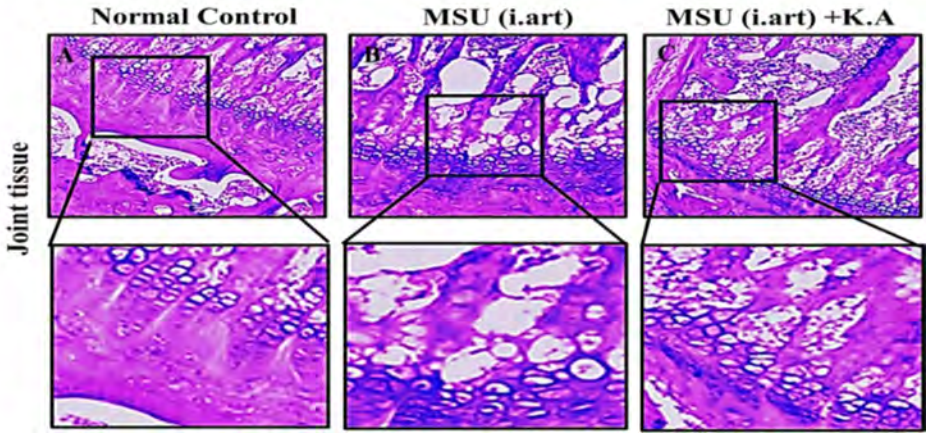


Figure 3.15. Hematoxylin and eosin staining (10X). Impact of 25 mg/kg i.p. H & E-stained joint is represented by illustrations (A–C).
Note: *The presence of inflammatory cells infiltrating the joint and abnormal chondrocytes on the joint surface are indicative of histological alterations in the joint.*

3.16. H and E staining of kidney tissues

The effect of MSU, KA and Allopurinol was observed through H and E Staining. The inflammatory cell infiltration in the glomeruli and interstitial was significantly ($p < 0.001$) increased in the MSU (S.C) group from the control group while MSU (i.art) did not show any significant difference. Treatment with KA showed a significant decrease in the infiltration as compared to negative MSU (S.C) group while Allopurinol failed to reduce the infiltration as shown in Figure 3.16.

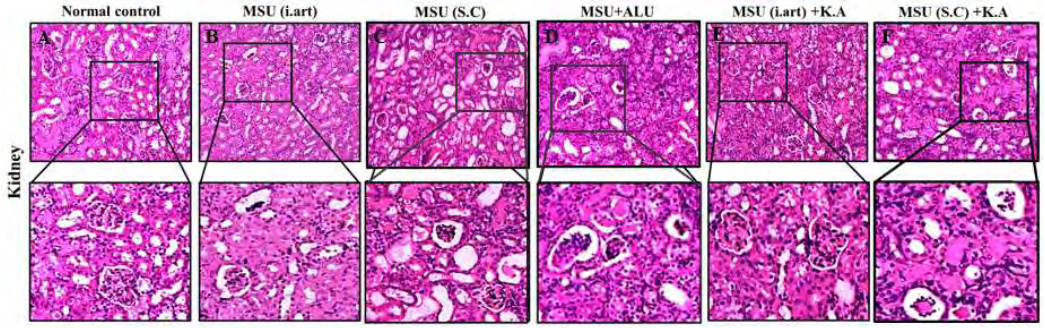


Figure 3.16. Hematoxylin-eosin staining (10X). Effect of Kaurenoic acid (25 mg/kg i.p.) on MSU-induced histopathological changes in kidney **(A-F)**.

3.17. H and E staining of liver tissues

The effect of MSU, KA and Allopurinol was observed through H and E Staining. The inflammatory cell infiltration specifically hepatocytes infiltration and necrosis were significantly ($p < 0.001$) increased in the MSU (S.C) group from the control group while MSU (i.art) did not show any significant difference. Treatment with KA showed a significant decrease in the infiltration as compared to negative MSU (S.C) group while Allopurinol failed to reduce the infiltration as shown in Figure 3.17.

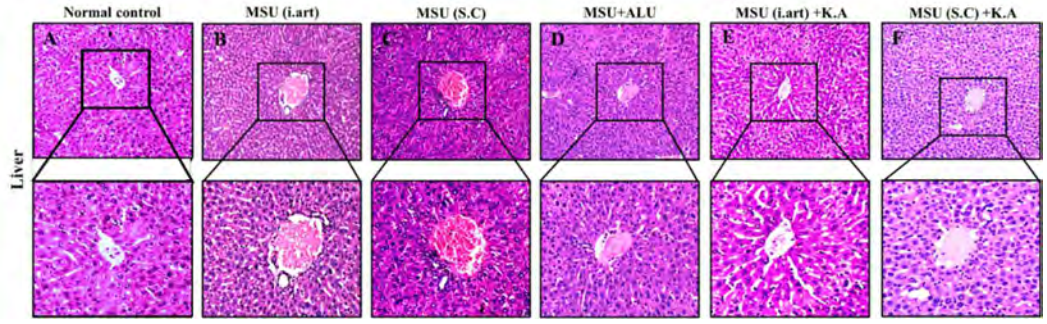


Figure 3.17. Hematoxylin-eosin staining (10X). Effect of Kaurenoic acid (25 mg/kg i.p.) on MSU-induced histopathological changes in liver.
Note: *Illustrate representative images (A–F) of H & E-stained liver. Histological changes indicate hepatocytes infiltration and necrosis.*

3.18. Trichrome staining of paw tissues

In inflamed and treated paw Masson's Trichrome staining results can offer important information about the degree of tissue damage and the efficacy of treatment. Masson's Trichrome staining results in enhanced collagen deposition fibrosis, inflammatory cell infiltration, and tissue integrity in diseased paw tissues. On the other hand, the KA (25 mg/kg) groups result in significantly ($p < 0.001$) less collagen deposition with suppression of inflammation and tissue repair in paw and joint tissue of all treated

groups. Similarly, the Allopurinol (10 mg/kg) also showed significant ($p < 0.001$) changes in the paw histology of the treated group as shown in Figure 3.18.

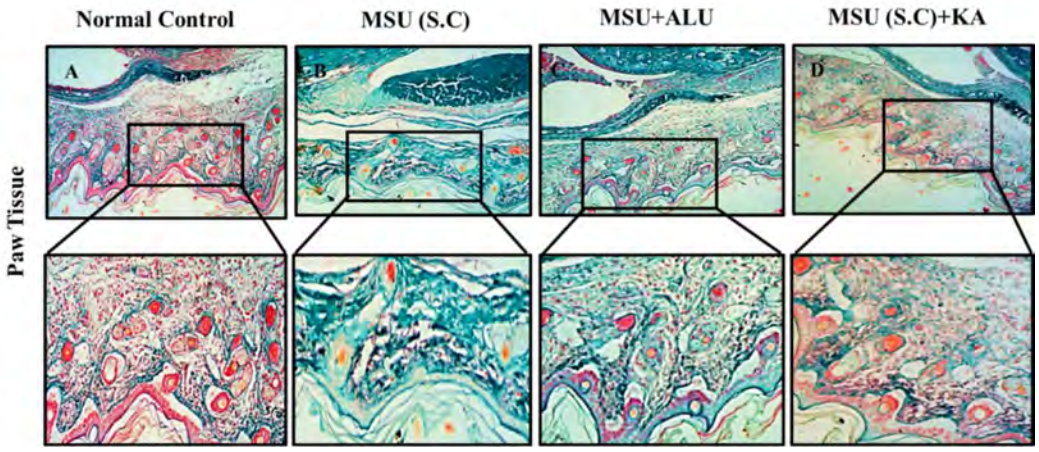


Figure.3.18. Trichrome staining (10X). Effect of Kaurenoic acid (25 mg/kg i.p.) on MSU (S.C)-induced histopathological changes in the left hind paw (A-D).

3.19. Trichrome staining of joint tissues

Masson's Trichrome staining of knee joint tissues results can offer important information about the degree of tissue damage and the efficacy of treatment. Masson's Trichrome staining results in enhanced collagen deposition and pattern, fibrosis, inflammatory cell infiltration, and tissue integrity in diseased tissues**.** On the other hand, the KA (25 mg/kg) groups result in significantly ($p < 0.001$) less collagen deposition with suppression of inflammation and tissue repair of joint tissue of all treated groups as shown in Figure 3.19.

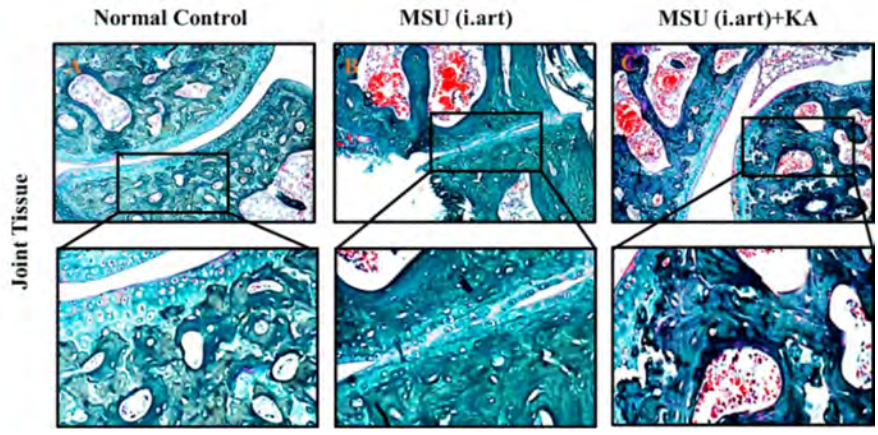


Figure 3.19. Trichrome staining (10X). Effect of Kaurenoic acid (25 mg/kg i.p.) on MSU (i.art)-induced histopathological changes in left knee joint.
Note: *Illustrate representative images (A–C) of trichome stained joint. Histological changes indicate changes in collagen distribution or density due to assess fibrosis.*

3.20. Effect of KA on immunohistochemistry of paw tissues

Immunohistochemistry was used to examine the expression levels of TLR4, NF-κB, IκB-α, COX-2, and iNOS to assess the inhibitory effect of KA on MSU (S.C)-induced paw inflammation. The expression of TLR4, NF-kB, iNOS, and COX-2 is significantly ($p < 0.001$) elevated in the inflamed paw following induction, but IκB-α was significantly ($p < 0.001$) inhibited. But as compared to the diseased group, the KA treatment (25 mg/kg i.p.) significantly ($p < 0.001$) down-regulated the expression of TLR4, NF-kB, iNOS, and COX-2, while the treatment showed a promising ($p < 0.001$) up-regulation of IκB-α expression in paw tissues.

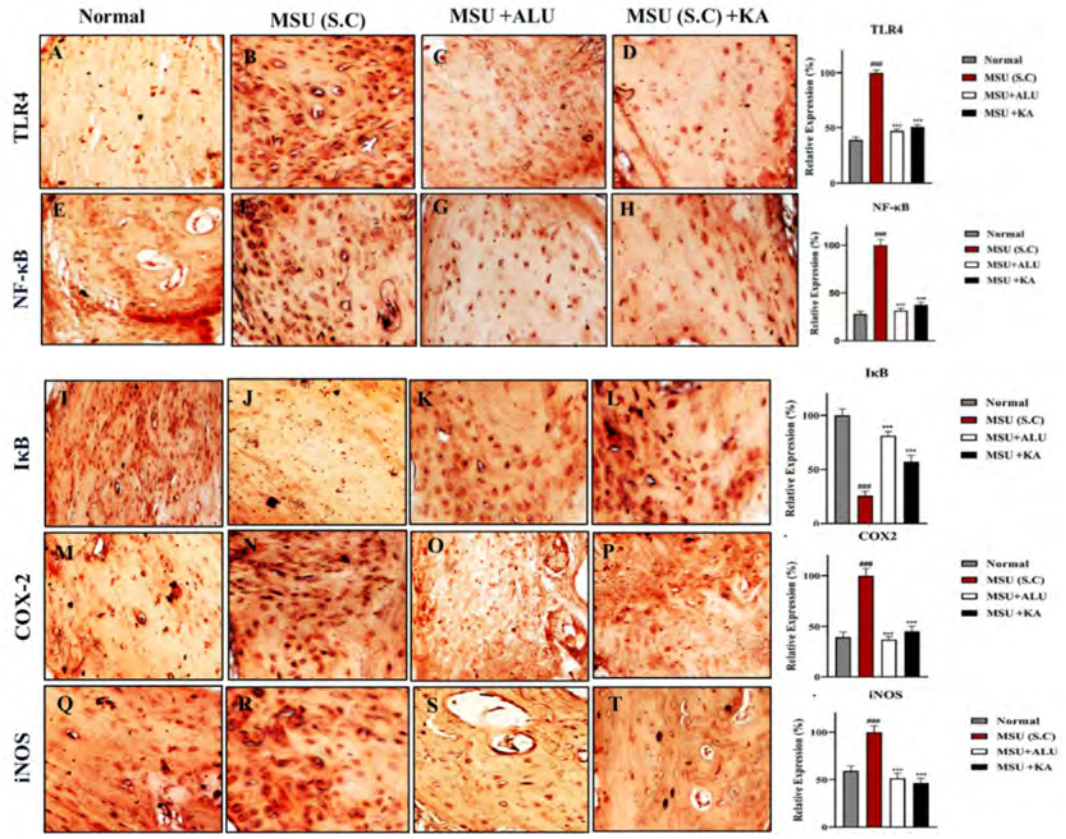


Figure 3.20. Effect of KA on the relative expression of TLR4/NF-κB /IκB-α /COX-2/ iNOS in Paw (10X).
Note: *All values are expressed as mean±S.D. (*) $p < 0.05$, (**) $p < 0.01$ and (***) $p< 0.001$ shows significant difference compared with MSU (S.C) group. (###) denotes comparison of Normal control and MSU (S.C) group with MSU+KA group.*

3.21. Effect of KA on immunohistochemistry of joint tissues

The expression of TLR4, NF- κB, iNOS, and COX-2 is significantly ($p < 0.001$) elevated in the inflamed joint following induction, but IκB-α was significantly ($p < 0.001$) inhibited. But as compared to the diseased group, the KA treatment (25 mg/kg i.p.) significantly ($p < 0.001$) down-regulated the expression of TLR4, NF-kB, iNOS, and COX-2, while treatment showed a promising ($p < 0.001$) up-regulation of IκB-α.

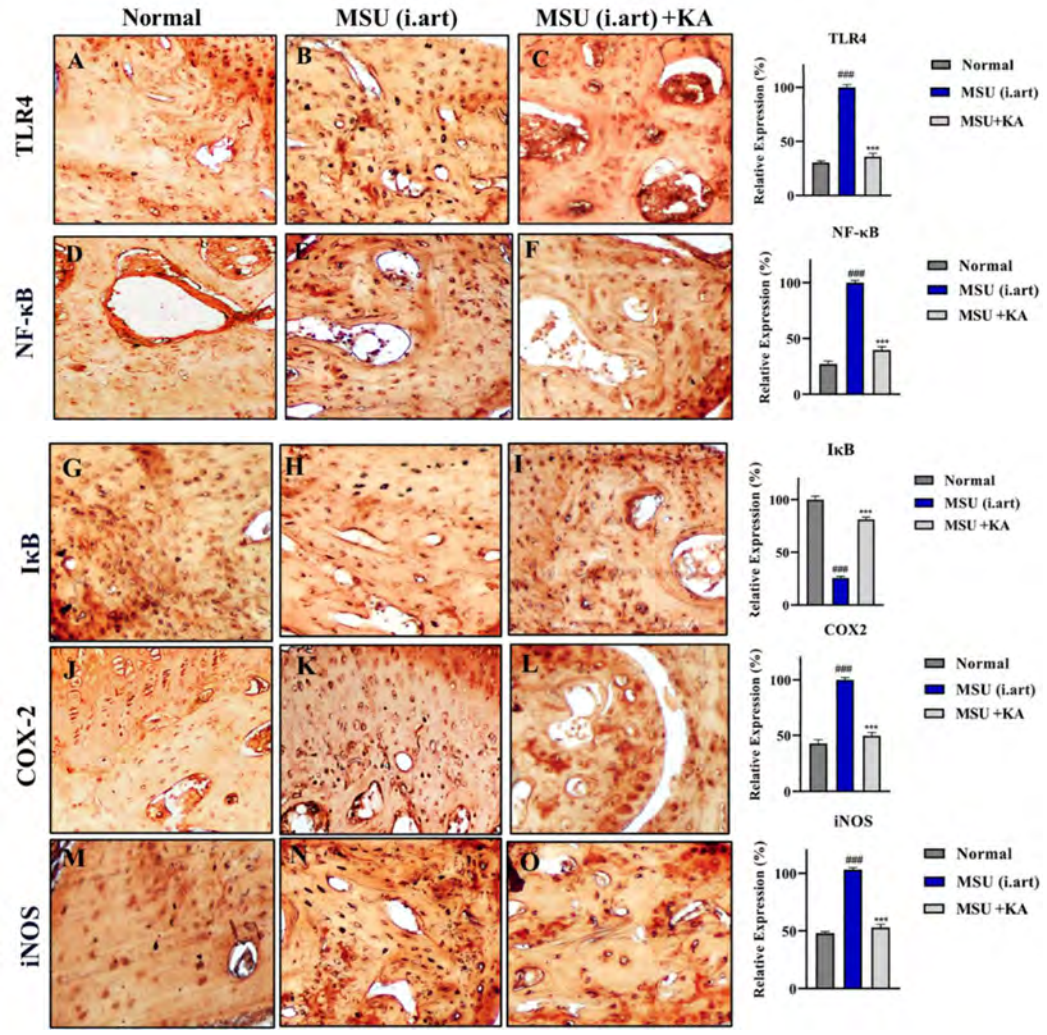


Figure 3.21. Effect of KA on the relative expression of TLR4/NF-κB/ IκB /COX-2/ iNOS in knee joint. KA downregulate relative expression TLR4/NF-κB/COX-2/ iNOS while upregulate the relative expression of IκB.

Note: *All the values are stated as mean±S.D. When compared to the MSU (i.art) groups, there is a significant difference (*) $p < 0.05$, (**) $p < 0.01$ and (***) $p < 0.001$. A significant difference from the MSU (i.art)-treated group is indicated by (###) $p < 0.001$.*

3.22. Molecular Docking of Kaurenoic Acid Against Protein Targets

The three-dimensional (3D) and two-dimensional (2D) structural affinity of Kaurenoic acid with protein targets were analyzed via AutoDock. KA was docked against with TLR4/ NF-κB/ IκB-α/COX-2 and iNOS. The result describes the three-dimensional arrangement of the ligand within the protein's binding site, and the binding affinity score, which measures the strength of the interaction. The precise forces such as hydrogen bonding, van der Waals forces, and electrostatic interactions were also examined.

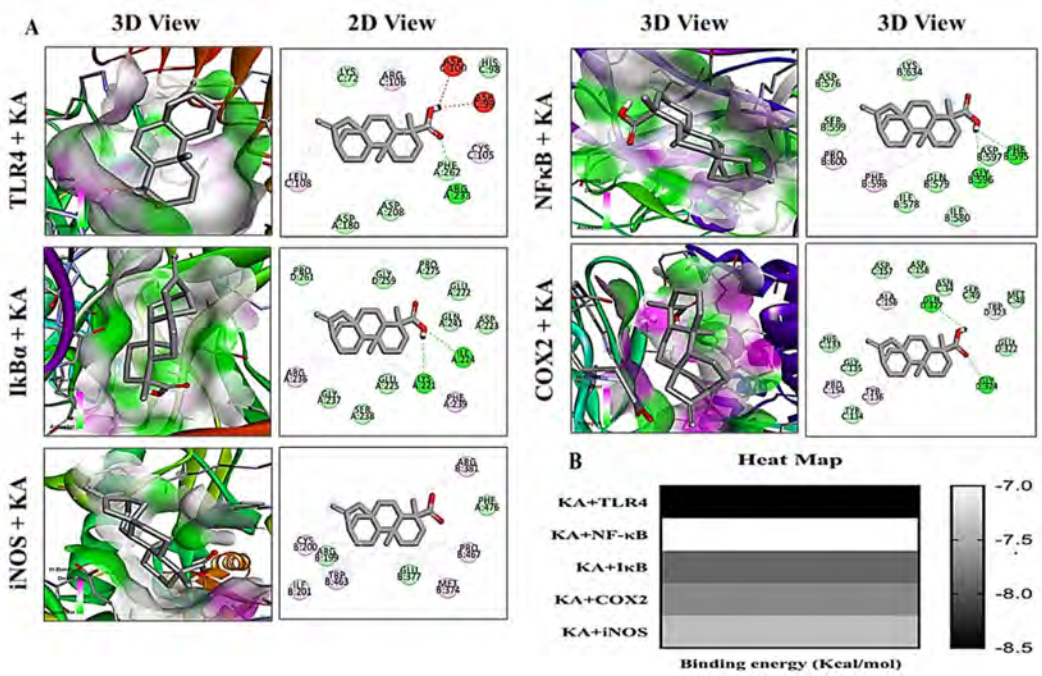


Figure 3.22. Molecular docking of Kaurenoic acid against protein targets. Kaurenoic acid with TLR4/ NF-κB/ IκB-α/COX-2 iNOS.
***Note**: (A) The three-dimensional (3D) and two-dimensional (2D) structural affinity of Kaurenoic acid with protein targets are shown. Kaurenoic acid shows a strong binding affinity towards the above protein targets. (B) Heat map indicating binding energies of different protein targets.*

Table 3.4. Computational (docking) evaluation of Kaurenoic acid against TLR4/NF-κB/ IκB-α/COX-2 / iNOS

KA-Protein interaction	Binding energy (kcal/mol)	Hydrogen bond	Hydrophobic interaction
KA-TLR4	-8.4	ARG A:233	CYS C:105, ARG C:106, LEU C: 108
KA-NFκB	-7	GLY B:596, PHE B:595	PHE B:598, PRO B:600
KA-IkBα	-7.9	LYS A:221, ILE A:224	ARG A:236, PHE A:239

KA-COX2	-7.7	GLY D:324, GLN D:327	TYR C:136, PRO C:154, ALA C:156
KA-iNOS	-7.4	-	CYS B:200, ILE B:201, MET B:374, ARG B:381, TRP B:463, PRO B:467

3.23. Level of oxidative stress markers in paw

Compared to the MSU (S.C) group, the effect of KA on the production of oxidative stress markers like NO, EPO, LPO, and MPO in the paw tissue was assessed. In MSU (S.C) groups raised the NO, EPO, LPO, and MPO, levels while these levels were significantly ($p < 0.001$) diminished by the administration of KA. The production of oxidative stress markers also decreased significantly ($p < 0.001$) in the Allopurinol group as shown in Figure 3.23.

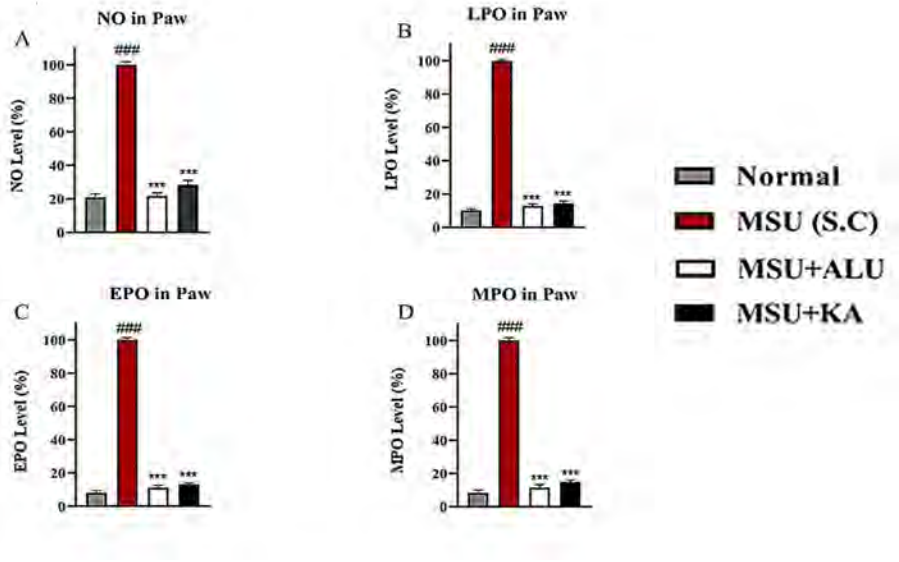


Figure 3.23. Effect of KA on the level of oxidative stress markers. (A) NO production in paw (B) lipid peroxidase (LPO) level in paw (C) Eosinophil peroxidase (EPO) level in paw (D) Myeloperoxidase (MPO) level in paw.
Note: *All values are expressed as mean±S.D. (*) $p < 0.05$, (**) $p < 0.01$ and (***) $p < 0.001$ shows significant difference compared with MSU (S.C) group. (###) denotes comparison of Normal control and MSU (S.C) group with KA group*

3.24. Level of oxidative stress markers in joint

Compared to the MSU (i.art) group, the effect of KA on the production of oxidative stress markers like NO, EPO, LPO, and MPO in the joint tissue was assessed. In MSU (i.art) groups raised the NO, EPO, LPO, and MPO, levels while these levels were

significantly ($p < 0.001$) diminished by the administration of KA as shown in Figure 3.24.

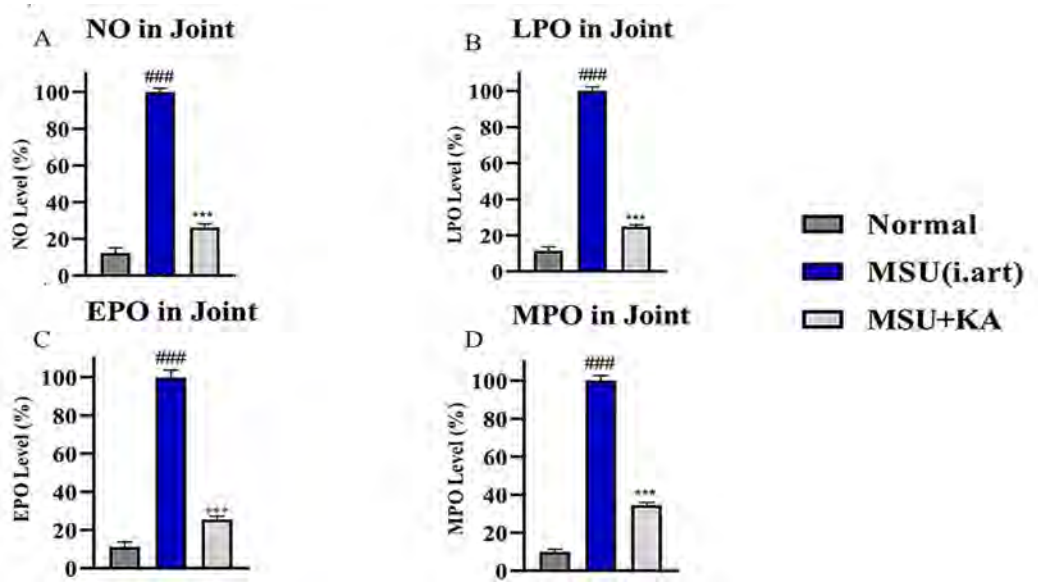


Figure 3.24. Effect of KA on the level of oxidative stress markers. (A) NO production in paw (B) lipid peroxidase (LPO) level in joint (C) Eosinophil peroxidase (EPO) level in joint (D) Myeloperoxidase (MPO) level in joint.
Note: *All values are expressed as mean±S.D. (*) $p < 0.05$, (**) $p < 0.01$ and (***) $p < 0.001$ shows significant difference compared with MSU (S.C) group. (###) denotes comparison of Normal control and MSU (S.C) group with KA group.*

3.25. Effect of KA on MSU-induced Inflammatory Cytokines Production

In the paw and joint tissue, the impact of KA (25 mg/kg) on the release of cytokines against MSU (S.C), (i.art), was assessed. MSU (S.C), (i.art) groups produced more inflammatory cytokines, such as IL-1β and TNF-α and , than other groups. In contrast to the MSU (S.C), (i.art) group, a significant ($p < 0.001$) decrease in inflammation was seen after KA (25 mg/kg) delivery. Furthermore, production of cytokines was inhibited by allopurinol (10 mg/kg).

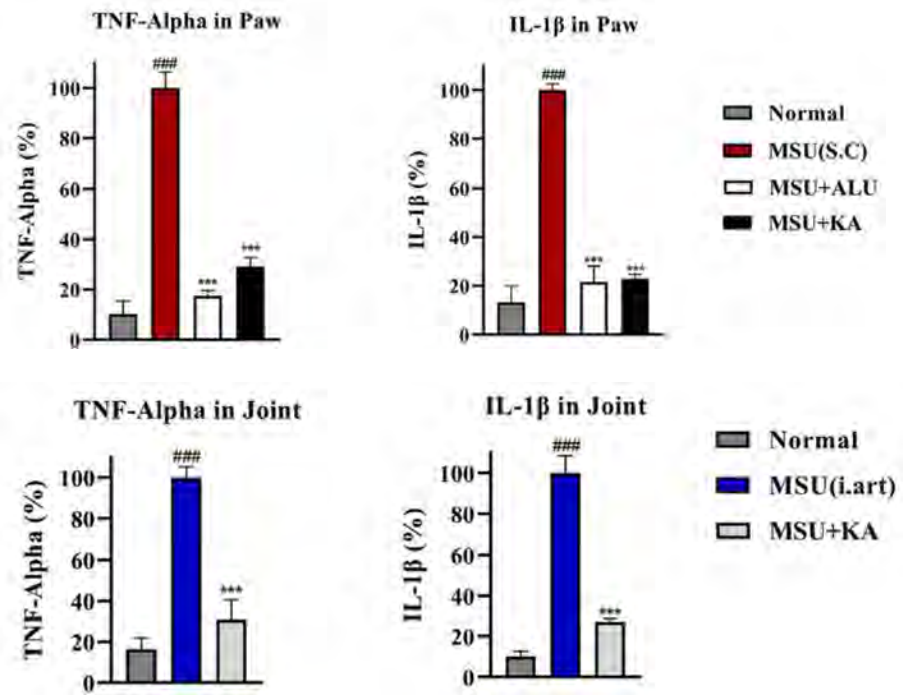


Figure 3.25. Effect of KA in MSU-induced changes in the production of inflammatory mediators in joint (A) TNF-α (B) IL-1β.
Note: *All values are expressed as mean±S.D. (*) $p < 0.05$, (**) $p < 0.01$ and (***) $p < 0.001$ shows significant difference compared with MSU group. (###) denotes comparison of Normal control and MSU group with KA group.*

CHAPTER 4

DISCUSSION

4. DISCUSSION

The present study was aimed to assess the anti-inflammatory and urate-lowering potential of Kaurenoic acid against MSU-induced gout model. Gout has become more common and complex in recent years, and the course of treatment has been slow. There is a need for novel treatments based on the new understandings regarding the function of the inflammasome in MSU crystal inflammation with the introduction of new urate-lowering and anti-inflammatory medication (Pascart and Richette, 2017).

The literature study revealed that KA has diverse applications such as anti-inflammatory, antinociceptive, antiulcerogenic, antitumor, antioxidant and antimicrobial properties (Paiva *et al.*, 2002). It is reported that after the MSU crystals administration the urate crystals are taken up in the synovial joint region which activates the monocytes which further increased the level of pro-inflammatory cytokines such as IL-1, IL-6, IL-8 and TNF-α and mast cells (Shi *et al.*, 2010). The administration of MSU both subcutaneously and intraarticularly triggers the activation of several pro-inflammatory mediators via interacting with several intracellular signaling such TLR4/NF-κB (Mariotte *et al.*, 2020). However, the administration of KA markedly reduced the paw edema and joint thickness in MSU-induced (S.C), (i.art) groups. Several behavioral parameters were used to determine the effectiveness of the response to the treatment. The behavioral parameters include paw edema, joint thickness, mechanical hyperalgesia, mechanical allodynia, thermal and cold allodynia, and gait analysis (Zhang *et al.*, 2020). In the current study, the behavioral parameters were changed markedly in both subcutaneously and intraarticularly MSU-induced groups. The Allopurinol and KA-treated group showed effective treatment of inflammation by reducing paw edema and joint thickness. The X-ray assessment (Wang *et al.*, 2019) showed that KA treated groups reverse the morphological alterations such as soft tissue edema, bone erosion and joint space narrowing, in the paw and joint after MSU (S.C), (i.art) administration, demonstrating the efficiency of KA in the treatment of gout. The Evans blue dye was used to measure plasma extravasation (Nidavani *et al.*, 2014).

The outcomes were noticed both in paw and joint tissues. In the MSU (S.C), (i.art) group, the Evans blue dye showed a higher optical absorbance due to plasma leakage and cell membrane extravasation. When compared to the MSU (S.C), (i.art) groups, the infiltration of Evans blue into the paw tissue and joint was reduced by the KA-treated

group and allopurinol group. The hematological analysis evidenced by a significant rise in white blood cell count (WBC), raised neutrophil count, and platelet count due to enhanced immunological response. Moreover, a reduction in the lymphocyte count was noted (Kiyani *et al.*, 2022). While the KA treatment showed a remedial effect on the blood profile, reversed the white blood cells count, standardizing neutrophil levels, and restoring lymphocyte counts. The hepatic and renal function tests (Kiyani *et al.*, 2019) showed that the MSU (S.C), (i.art) markedly altered the liver enzymes, CRP, uric acid level and creatinine level in liver and kidney respectively. However, the treatment control showed marked improvement in hepatic and renal parameters compared to MSU-treated group. While Allopurinol did not significantly lower the liver enzyme. The chemical composition was investigated using the Fourier Transform Infrared (FTIR) technique which showed variations resulting from MSU-induced inflammation in certain bands of the spectra. Edema can lead to higher OH stretching in FTIR spectra because there are more water molecules present, which intensify the OH stretching vibrations. Altered protein composition, where amides I and II are more expressed in the inflammatory state, due to the elevated collagen content in paw and joint tissues. Increased lipid peaks are indicative of lipid accumulation associated to inflammation or modifications in lipid composition in diseased groups while the peaks intensity was decreased by KA therapy in paw and joint tissues (Croxford *et al.*, 2011).

The histological study by performing H and E staining provides insight into the histological details of the tissue such as changes in the bone, joints architecture, and the infiltration of the immune cells in paw and joint (Zhao *et al.*, 2021). Additionally, MSU (S.C) showed the systemic effect by altering the histopathology of kidney and liver. while MSU(i.art) showed the localized effect after the deposition of urate crystals on knee joint .(supplementary data) KA-treated groups improved the changes in all organs while alopurinol group showed markedly improve effect on paw and joint but less significant in kidney and liver tissues as compare to KA treated groups .Masson trichrome staining showed (Zhao *et al.*, 2021) the enhanced collagen deposition fibrosis, inflammatory cell infiltration, and tissue integrity in diseased paw and joint. On the other hand, the KA groups result in improved collagen deposition with suppression of inflammation and tissue repair in paw and joint tissue of all treated groups. The TLR4 /NF-κB signaling have significantly implicated in the pathogenesis of acute gout attack (Dhanasekar and Rasool, 2016). Many studies reported the inhibition of these signaling pathways improved the symptoms of gout, and

inflammation associated with gout attack. The MSU (S.C), (i.art) administration has markedly enhanced the expression level of the TLR4, and NF-κB, COX2, iNOS and decrease the expression of IκB-α proteins. These results were analyzed by using immunohistochemistry of paw and joint. KA significantly reduced the expression level of the TLR4, NF-κB, COX-2, iNOS and enhanced the expression of IκB-α and these are important markers of inflammation, and its expression is directly related with the extent of the inflammation. Similarly, Allopurinol showed the same results.

Furthermore, the MSU administration significantly increased the level of oxidative stress and compromised the antioxidant status of the body (Sagor *et al.*, 2015). The effect of KA was evaluated on the production of the NO. The KA-treated groups showed a marked reduction in the production of the NO compared to the MSU-treated group. The oxidative stress played a critical role in the regulation of inflammatory process, and its extensive role has been reported in the initiation of acute inflammatory processes. The MSU (S.C), (i.art) induction significantly reduced the antioxidants and raised the level of oxidative stress markers such as LPO, MPO and EPO in paw and joint. However, the intervention of KA significantly improved the oxidative and antioxidant balance (Yin *et al.*, 2020). The MSU-treated groups showed significant expression of the IL1β, TNF-α, proteins using ELISA assay (Lu *et al.*, 2019). However, the expression of these proinflammatory mediators such as IL-1β, TNF-α, was marginally reduced by the KA-treated and allopurinol groups. Additionally, the computational analysis was performed to assess the interaction of KA with the pro-inflammatory cytokine proteins. The KA showed great docking interaction and binding affinity with TLR4, and NF-κB, COX2, iNOS and IκB-α (Hussain *et al.*, 2020). Hence, based on its ability to target inflammatory signaling pathways, kaurenoic acid has emerged as a potential therapeutic drug to alleviate gout symptoms. The findings indicated that the naturally occurring compound may have more efficacy in clinical settings due to its preventive effects on different phases of pathogenesis of gout. The usage of kaurenoic acid demonstrated a relative safety profile by successfully treating liver and kidney impairment caused by MSU, in contrast to urate-lowering drugs that are frequently linked to renal and hepatic dysfunctions. When treated with kaurenoic acid, gout was observed to be cured and blood levels of creatinine, urea, uric acid, and CRP returned to normal. Briefly, as inflammation plays a major part in the pathophysiology of gout, kaurenoic acid reverse this effect due to anti-inflammatory action by inhibiting inflammatory signaling pathway and urate-lowering action by

reducing the level of serum uric acid and CRP level and hence can serve as an effective therapy for the treatment of gout by inhibiting inflammatory signaling pathway and urate-lowering by reducing the level of serum uric acid and CRP level.

CONCLUSIONS

- The study found that Kaurenoic acid (KA) can attenuate gouty inflammation in MSU-induced model of gout. KA reversed biochemical, histopathological, and structural changes induced by the MSU. Treatment groups showed significant improvements in behavioral parameters, X-ray analysis, vascular permeability and biochemical parameters.
- KA also showed a dose-dependent effect in inhibiting inflammatory mediators by targeting TLR4/NF-κB pathway, decreasing cytokines like IL-1β and TNF-α, and reducing oxidative stress markers including NO, LPO, EPO, and MPO, thereby reducing inflammation.
- The current findings make it clear that KA is an effective anti-inflammatory and urate-lowering agent used to treat MSU-induced gouty inflammation Figure 4.1

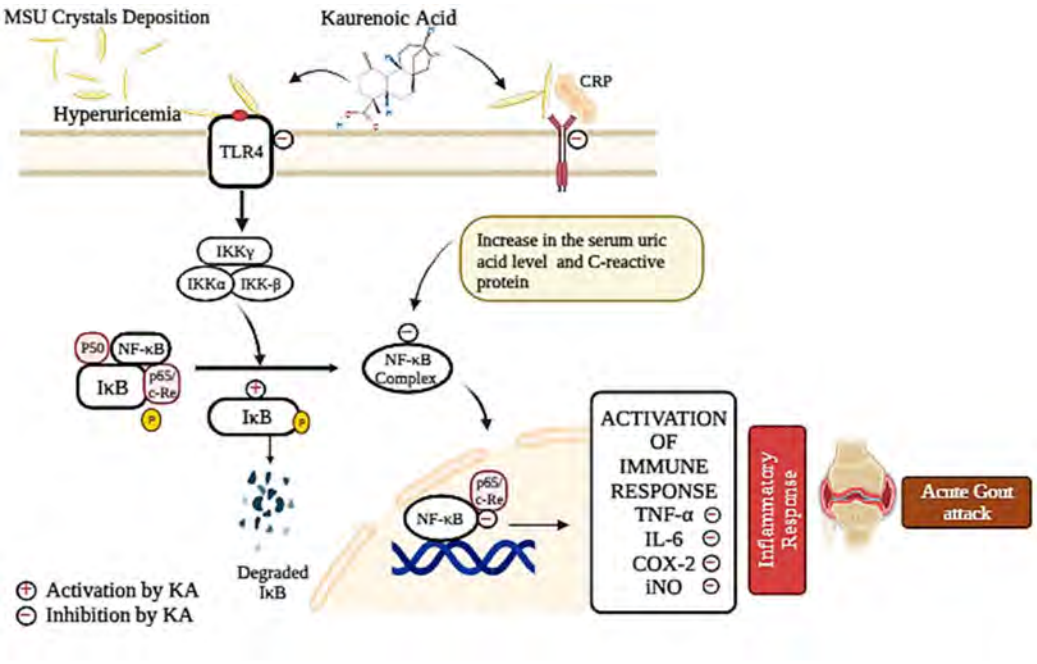


Figure 4.1 An illustration of the proposed molecular mechanism of kaurenoic acid against gout induced by MSU (Biorender 14 days trial).

FUTURE PROSPECTIVES

- Further research is needed to understand underlying mechanisms of MSU-induced Gout that allow us to design individual and rational treatment strategies.
- Positive findings from this study may prompt additional research on the long-term impacts of natural substances, human clinical trials, and in-depth analyses of the possible mechanisms behind Gout.
- If the drug proves to be effective in further studies and clinical trials, it may offer a new therapeutic option for gout patients. By targeting both the symptoms and the underlying mechanisms of gout, this compound has the potential to provide comprehensive protection to the joint tissues, mitigating the progression of the disease and improving the overall quality of life for individuals with gout.

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Annexure I: Approval from Bioethics Committee

قائداعظم یونیورسٹی
QUAID-I-AZAM UNIVERSITY
Faculty of Biological Sciences
Bioethics Committee

No. #BEC-FBS-QAU2023-468 Dated: 27-01-2023

Ms. Maryam Jamil
C/O Dr. Salman Khan,
Department of Pharmacy,
Faculty of Biological Sciences,
Quaid-i-Azam University, Islamabad
45320, Pakistan

Subject: - **"The study of Pharmacological effect of Kaurenoic acid in MSU induced animal model."**

Dear Ms. Maryam Jamil,

We wish to inform you that your subject research study has been reviewed and is hereby granted approval for implementation by Bio-Ethical Committee (BEC) of Quaid-i-Azam University, Your study has been assigned protocol **#BEC-FBS-QAU2023-468.**

While the study is in progress, please inform us of any adverse events or new, relevant information about risks associated with the research. In case changes have to be made to the study procedure, the informed consent from and or informed consent process, the BEC must review and approve any of these changes prior to implementation.

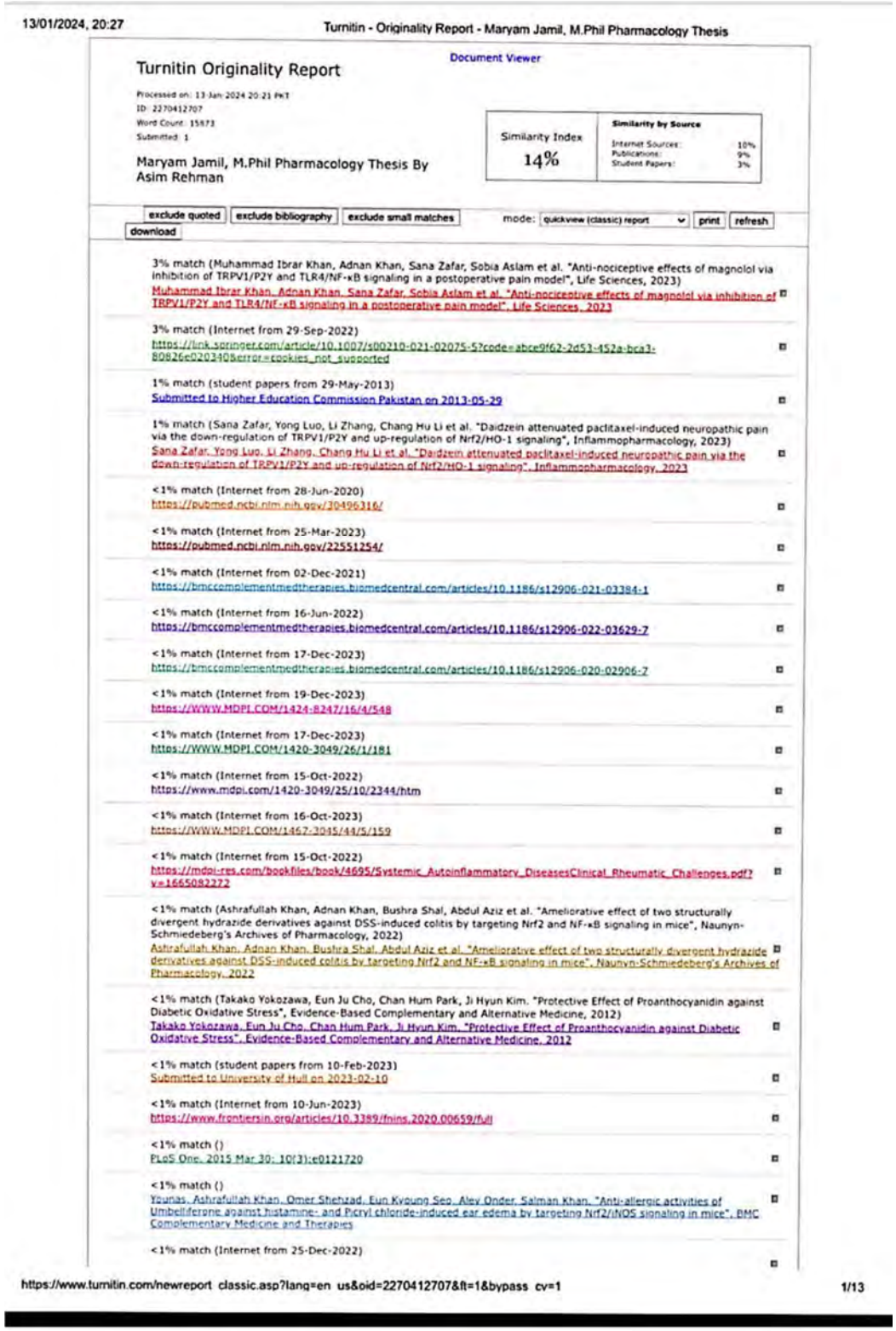
Sincerely,

Sarwat Jahan

Prof. Dr. Sarwat Jahan
Department of Zoology

cc:
Dean, F.B.S

Annexure II: Turnitin Similarity Index Report



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