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**Research Article**

# *Anti-inflammatory Activity of Cherry Leaf Extract on TNF-α Levels in Wistar Rats with Acute Gout Arthritis Model*

Aktivitas Antiinflamasi Ekstrak Daun Kersen terhadap Kadar TNF-α pada Tikus Wistar Model Gout Artritis Akut

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***Abstract***

*The inflammatory condition, marked by pain, swelling, and elevated TNF-α levels, prompted the exploration of cherry leaves' flavonoids, known for their anti-inflammatory properties. An in vivo experimental study with a pre-and post-test control group design was conducted to compare the efficacy of cherry leaf extract (Muntingia calabura L.) in the Wistar rat model of acute gout arthritis. Five treatment groups (negative control (Na-CMC 0,5%), positive control (Colchicine), cherry leaf extract dose 200mg/kgBW, cherry leaf extract dose 400mg/kgBW, and cherry leaf extract dose 800mg/kgBW) were administered to Wistar rats to compare their ability in reducing TNF-α levels. Results revealed the extract's significant efficacy in reducing TNF-α levels at all doses compared to negative control. There is no significant difference among the three different doses compared to colchicine as a positive control. In conclusion, cherry leaf extract demonstrated significant efficacy as an anti-inflammatory agent by reducing TNF-α levels in acute gout arthritis rat models with the most efficient dose of 200mg/kg BW.*

***Keywords****: gout arthritis; TNF-α; inflammatory mediator; cherry leaves extract; in vivo*

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**Abstrak**

Kondisi peradangan, ditandai dengan rasa sakit, pembengkakan, dan peningkatan kadar TNF-α, mendorong eksplorasi flavonoid daun ceri, yang dikenal karena sifat anti-inflamasinya. Sebuah studi eksperimental in vivo dengan desain kelompok kontrol pra- dan pos-tes telah dilakukan untuk mengamati efikasi ekstrak daun ceri (Muntingia calabura L.) pada model tikus Wistar dengan artritis gout akut. Lima kelompok perlakuan (kontrol negatif (Na-CMC 0,5%), kontrol positif (Kolkisin), dosis ekstrak daun ceri 200 mg/kgBB, dosis ekstrak daun ceri 400 mg/kgBB, dan dosis ekstrak daun ceri 800 mg/kgBB) diberikan kepada tikus Wistar untuk membandingkan kemampuan mereka dalam menurunkan kadar TNF-α. Hasil penelitian ini menunjukkan efikasi ekstrak dalam menurunkan kadar TNF-α yang signifikan pada semua dosis dibandingkan dengan kontrol negatif. Tidak ada perbedaan yang signifikan di antara ketiga dosis yang berbeda dibandingkan dengan kolkhisin sebagai kontrol positif. Sebagai kesimpulan, ekstrak daun ceri menunjukkan efikasi yang signifikan sebagai agen anti-inflamasi dengan menurunkan kadar TNF-α pada model tikus arthritis gout akut dengan dosis paling efisien adalah 200 mg/kg BB.

**Kata kunci**: Artritis gout; TNF-α; mediator inflamasi; ekstrak daun kersen; *in vivo*

## Introduction

Gout arthritis (GA) is an inflammatory condition resulting from chronic hyperuricemia and the accumulation of monosodium urate (MSU) crystals in joint tissues.[1] Acute GA manifests as sudden joint redness, warmth, pain, and swelling, with distinct features like severe and frequent attacks, multi-joint involvement, interictal pain, and long-term damage, significantly affectingpatients' quality of life.[2,3] Common comorbidities include hypertension, chronic kidney disease, obesity, and cardiovascular disease, which contribute to elevated mortality rates.[4] The prevalence of gout has been rising from 20.2 million in 1990 to 41.2 million in 2017.[4,5]

MSU crystal deposition triggers gout flares through NLRP3 inflammasome activation, and secretion of proinflammatory cytokines, including TNF-α.[6,7] Colchicine is recommended for acute GA with an onset of less than12 hours however, due to its potential side effects, alternative therapies such as medicinal plants, like cherry leaves (*Muntingia calabura L.*), known for their flavonoid content, are promising candidates.[8–11] *Muntingia calabura L.* is a tropical fruit plant that is easy to cultivate and highly adaptable, even in poor soil conditions.[11] Flavonoids have demonstrated antibacterial, antioxidant, and anti-inflammatory properties, including the potential to inhibit TNF-α production.[12,13] Despite these findings, no study has explored the anti-inflammatory activity of cherry leaf extract in acute gout arthritis. This study aims to fill this gap by investigating the effects of cherry leaf extract on TNF-α levels in Wistar rat model of acute gout arthritis.

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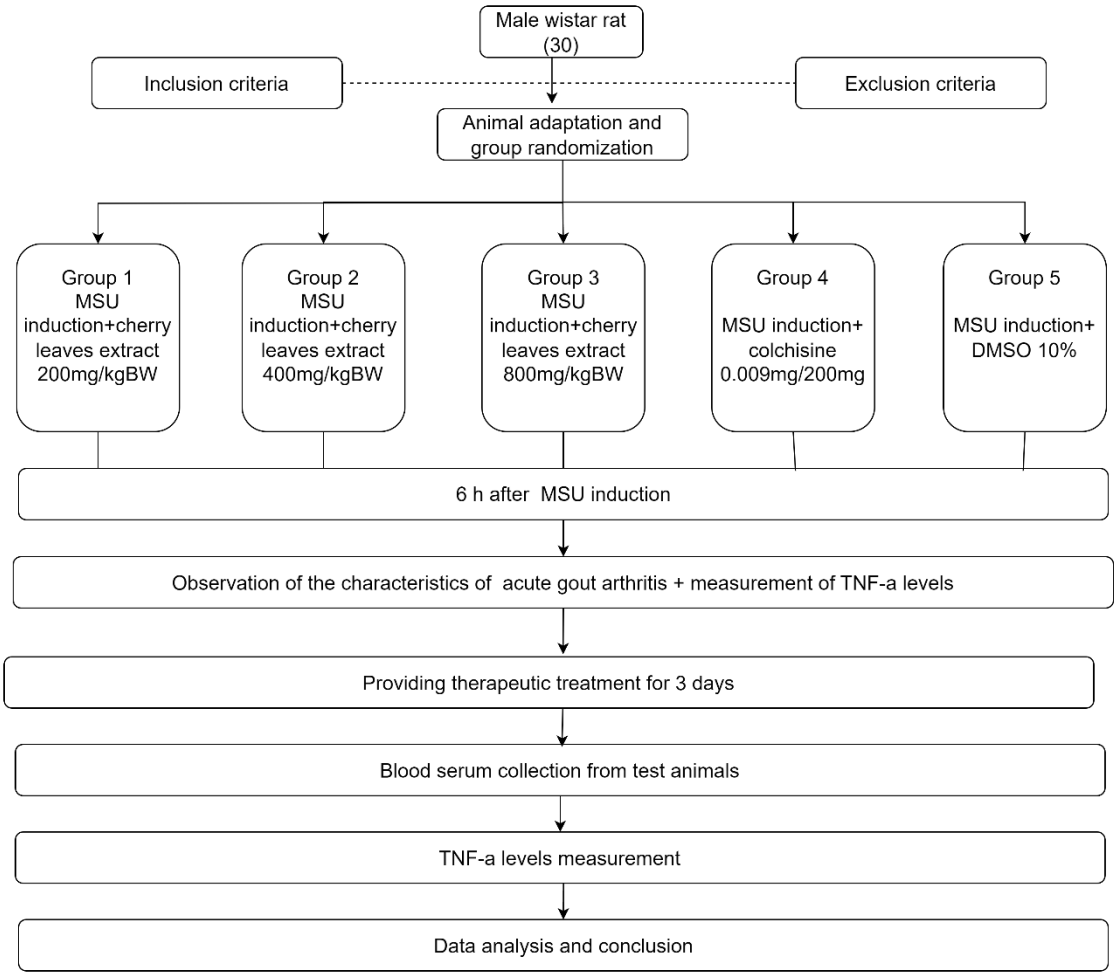
## Methods

### Study design

An in vivo experimental study with a pre- and post-test control group design has been conducted at the Faculty of Medicine, Universitas Sriwijaya, from August 2023 to December 2023. This study has been approved by the ethics committee of the University of Sriwijaya under Protocol No. 377-2023.




### Animal model

The preparation and maintenance involved setting up cages and selecting test animals that met inclusion criteria. Thirty male Wistar strain white rats (*Rattus norvegicus*), aged 2-3 months and weighing 200–300 grams, were drawn as test animals, and they were induced by MSU crystal to make an acute gout arthritis model. Before the treatment, the test animals were acclimatized for 7 days to prevent stress in the new environment. The test animals were housed in group cages, each with six rats. They were provided with standard food and had *ad libitum* access to water.[14]

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**Cherry leaf (*Muntingia calabura L.*) extraction**

The preparation of cherry leaves simplicial involved obtaining 2.2 kg of mature cherry leaves from local farmers in Prabumulih. Mature cherry leaves are characterized by their dark green color and small hairs on their upper surface.[15] The leaf processing began with athorough cleaning using running water. Subsequently, the leaves were cut into small pieces and dried either by natural air drying (away from direct sunlight) or in an oven at a temperature below 40°C. The dried cherry leaves were then blended, sieved using a No. 40 mesh, and weighed.

For the extraction process, the maceration method was employed. The simplicial cherry leaves were soaked in 96% ethanol solvent until fully submerged in a maceration container. The container was sealed and left for a minimum of 3 days. Throughout the soaking process, occasional stirring was performed to expedite the solvent penetration into the simplicia, ensuring the dissolution of chemical components. Maceration took place in a location shielded from light to prevent potential degradation of active substance structures, particularly for non-polar compounds that are less stable in light. After the extraction period, the extract was separated from the remaining plant material through filtration. Subsequently, the extract was isolated from the solvent through evaporation, either in an oven or in a water bath.[16]

**Animal model of gout arthritis**

Gout arthritis induction in rats was performed by injecting 50 µl of monosodium urate (MSU) crystal suspension into the synovial fluid of the right knee joint.[14] MSU crystals were prepared by dissolving 4 grams of uric acid in 800 mL of water with 9 mL of 0.5 N NaOH, adjusting the pH to 8.9 at 60°C. The solution was then cooled at a low temperature (4°C) overnight.[14] Subsequently, the crystals were washed, dried at 70°C for 4 hours, and ground into a fine powder. This powder was then filtered through a 200-mesh metal sieve, sterilized at 180°C for 1 hour, and stored in a sterile environment. Before use, the crystals were suspended in a sterile saline solution with a concentration of 20 mg/mL.[14,17] This animal model has been widely utilized, particularly in studies related to gout arthritis.[18]

**Assessment of inflammation**

The assessment of clinical symptoms of gout, such as increased swelling and gait abnormalities, becomes a success parameter for the experimental animal induction. Inflammation process evaluation through swelling is measured by using a vernier calliper to measure the knee circumference of rats at the patella point. Knee diameter measurements are taken before and after MSU crystal injection at intervals of 6, 24, 48, and 72 hours. The data from knee circumference

measurements is processed using the swelling ratio formula: (Ct-C0)/C0 x 100%, while Ct is acircumference at various times and C0 is a circumference at 0 hours.[14]

**Assessment of gait dysfunction**

The development of acute arthritis is evaluated through a macroscopic assessment of the knee joint. Data is recorded at intervals of 6, 24, 48, and 72 hours after the administration of MSU crystals. Inflammation and dysfunction scores in rats are visually determined by two independent observers or observed twice.[18]

Criteria used to assess dysfunction:[14]

- Level 0 (0 points): normal walking style, and both legs are level
- Level 1 (2 points): toes not spread, and legs slightly limp
- Level 2 (4 points): legs bent and clearly limping, and toes on the ground
- Level 3 (6 points): legs completely lifted from the ground, three-legged walking style

**The measurement TNF-a level by Enzyme-linked Immunosorbent Assays (ELISA)**

The measurement of TNF-α levels was conducted twice, namely 6 hours after MSU crystal induction and after 3 days of drug treatment, by collecting blood samples from rat orbital eye sinuses. The collected blood samples were then stored in vacuum tubes containing 0.1% EDTA anticoagulant and centrifuged at a speed of 3000 rpm for 25 minutes to separate blood plasma from the residue. The blood plasma samples were examined using the Sandwich ELISA method. On a 96-well plate, standards and test samples were added, followed by specific TNF-α detection antibodies. Subsequently, washing with a buffer was performed to remove unbound antibodies or conjugates. Substrates A and B were added to visualize the enzymatic reaction catalyzed by streptavidin peroxidase, producing a blue-colored product. The plate was then incubated, and an acid-stop solution was added, turning the color to yellow. Optical density measurements were taken using a microplate reader at a wavelength of 450 nm.[19,20]

**Statistical analysis**

The data obtained from the research will be processed and analyzed by using SPSS 22.0 for Windows. Normality tests will be conducted to determine whether the data distribution is normal or not, using the Shapiro-Wilk test. Subsequently, a variance test will be performed to determine the similarity of data using the Levene test. The data will be examined using an analysis based on the results of the normality of data distribution. If the data is normally distributed, a paired T-test will be conducted to assess the efficacy of each treatment between pre- and post-

treatment, and an unpaired T-test will be performed to determine the comparison of the efficacy between the extract and control groups by examining the post-treatment values. On the other hand, if the data is not normally distributed, the Wilcoxon test will be employed to assess efficacy, and the Mann-Whitney test will be used for the comparison of efficacy. A One-way ANOVA test will be conducted to observe the efficacy comparison among more than two groups in the normal data distribution. If the data distribution is not normal, Kruskal-Wallis's test will be applied. Subsequently, a Post Hoc test will be conducted to determine the dosage appropriateness between the treatment and control groups using post-treatment values.

## Results

### Cherry leaf extraction

The production of cherry leaf extract was carried out by collecting 2.2 kg of fresh cherry leaves. Subsequently, the cherry leaves were cleaned with running water and dried to form a crude drug. Drying was performed using an oven to eliminate moisture content. A total of 900 grams of crude drug were soaked in 7.5 liters of 96% ethanol solvent for 3 days. After filtering and drying, the weight of the cherry leaf extract obtained was 150.7 grams. The yield value was 16.74%. Yield is the ratio of the dry weight obtained from the sample to the initial weight of the sample. A good yield value is more than 10%, as a higher yield indicates a higher content of substances extracted from the raw material.[16]

### MSU Crystals Induced Animal Model

Before undergoing induction treatment, all experimental animals were subjected to body weight measurements. The weight data were analyzed by using SPSS to assess the normality and homogeneity of the data. The results of the Shapiro-Wilk normality test indicated a normal distribution of data ($p>0.05$). Based on the Levene test for statistical analysis, the data showed homogeneity ($p>0.05$), meeting the experimental requirements.

Wistar rats were induced with 20mg/mL MSU crystals in a sterile saline solution, which was injected intra-articular into the knee joint. The development of acute gout arthritis was evaluated through a macroscopic assessment of the knee joints, using the swelling ratio and dysfunction gait score. The first measurement was taken 6 hours after the injection of MSU crystals, followed by a second measurement at 24 hours post-induction. The diameter of the knee increased when MSU crystals were injected intra-articular; this increase peaked 24 hours after induction. A decrease in knee circumference and swelling ratio began to be observed in the third

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measurement at 48 hours post-induction and continued to decrease in the fourth measurement at 72 hours post-induction. (Figure 1 and Table 1).

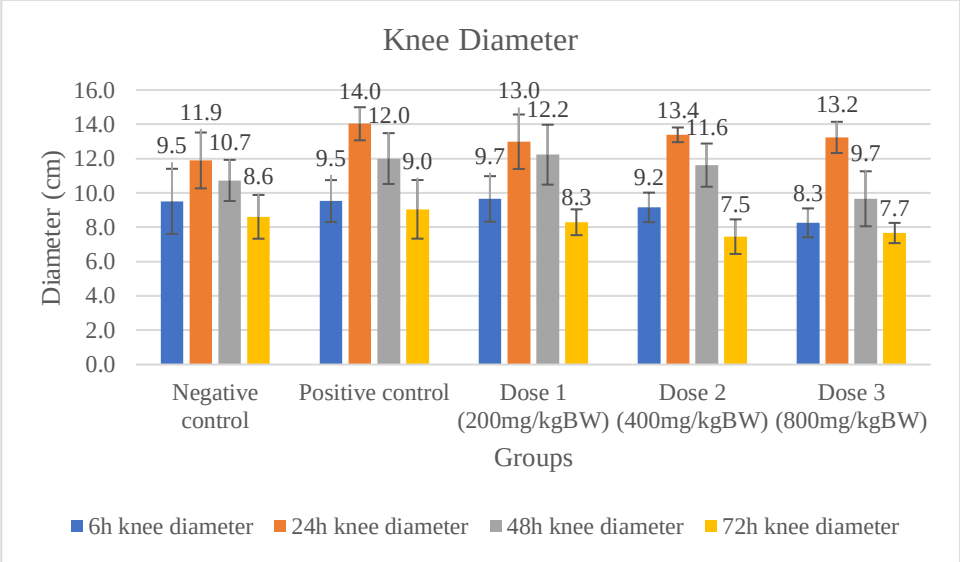


**Figure 1 Knee Diameter of Rats**

There was dysfunction peaking at 24 hours and gait improvement after 24 hours post-induction. The dysfunction gait score in rats 6 hours after induction, with a score of 2 points, indicated that the swelling was not too severe and only caused weakness in the lower extremities. The highest increase in gait score occurred at 24 hours after induction, with the highest dysfunction score being 4 points, signifying that the swelling caused gait disturbances in the observed rats. Improvement began at 48 and 72 hours after induction (Figure 2).

### TNF-a Levels

The examination of serum TNF-α levels was conducted twice, namely after the induction of MSU crystals (pretest) and after administering treatment for acute inflammation in Wistar rat test subjects (post-test). Both pretest and post-test TNF-α data were normally distributed (p>0,05) and homogenous (p>0,05) based on Shapiro-Wilk and Levene test.

**Table 1 The Swelling Index of the Induction Group**

| Groups | *Swelling index* | | |
|---|---|---|---|
| | **6-24 Hours** | **24-48 Hours** | **48-72 Hours** |
| Negative control | 16.10±18.11 | -9.58±10.59 | -18.56±10.30 |
| Positive control | 48.16±9.07 | -14.50±7.97 | -24.90±8.71 |
| Extract dose 200 mg/kgBW | 35.25±10.00 | -5.68±10.69 | -31.22±13.98 |
| Extract dose 400 mg/kgBW | 47.00±14.91 | -13.258±9.868 | -35.34±4.76 |
| Extract dose 800 mg/kgBW | 60.032±10.302 | -26.43±8.53 | -20.23±10.06 |

A positive value indicates an increase in diameter
A negative value indicates a decrease in diameter

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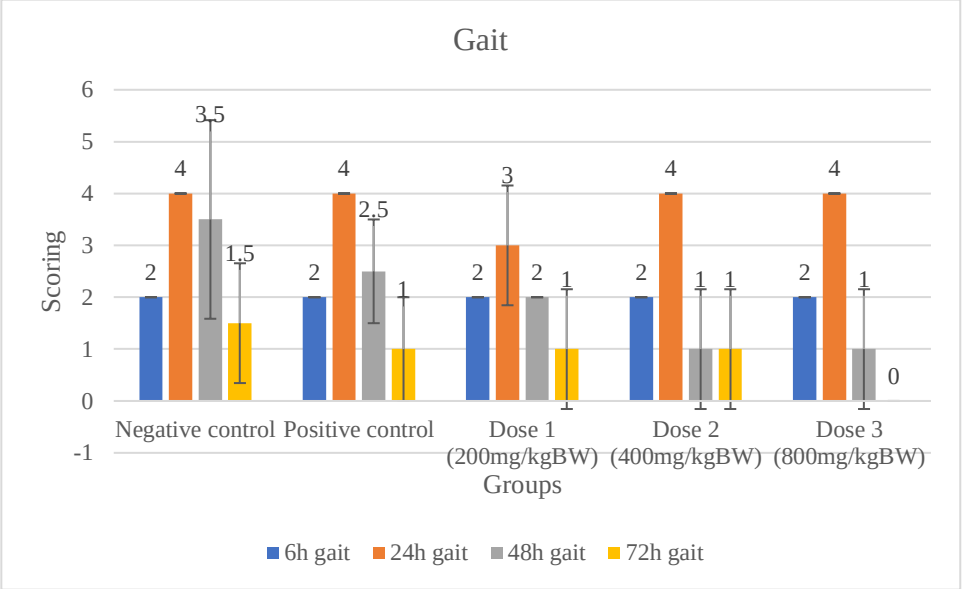


**Figure 2 Overview of Dysfunction Gait in Rats**

The analysis of TNF-α levels before and after treatment using the Paired T-test hypothesis test indicated a significant difference between TNF-α levels before and after drug administration in dose groups 1, 2, 3, and the positive control. No statistically significant differences were found in the negative control group (Table 2).

Subsequently, an independent T-test was conducted to determine the mean difference in TNF-α levels after treatment in each group. It was found that there was a difference in TNF-α levels after treatment with cherry leaf extract doses 1, 2, and 3 compared to the negative control (Table 3). Meanwhile, the treatment between dose 1 and the positive control showed that they had the same efficacy in reducing TNF-α levels (Table 3). Because the hypothesis test indicated a statistically significant difference in the reduction of TNF-α levels between treatment groups, further post-hoc tests were needed to determine the potential of cherry leaf extract as a medicine (flat dose-response curve or steep dose-response curve).

**Table 2 Efficacy of Cherry Leaves Extract (*Muntingia calabura L.*)**

| Groups | TNF-α Levels-Pre (pg/ml) | TNF-α Levels-Post (pg/ml) | P value (*) |
|---|---|---|---|
| Negative control | 86.967±5.108 | 81.316 ± 3.026 | 0.126 |
| Positive control | 87.281±7.532 | 70.518 ± 3.794 | 0.006* |
| Extract dose 200 mg/kgBW | 96.333±12.556 | 72.685 ± 4.325 | 0.018* |
| Extract dose 400 mg/kgBW | 69.859±1.290 | 64.966 ± 1.237 | 0.000* |
| Extract dose 800 mg/kgBW | 73.911±3.105 | 65.480 ± 3.002 | 0.004* |

*set max response as 100%, (*) paired T test* p=0,05

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The results of the post-hoc test showed a statistically significant difference between the negative control group and all dose groups and the positive control (drug of choice) (Table 4). This means that the administration of treatment, both in doses 1, 2, 3, and the positive control, is more effective in reducing TNF-α levels compared to the negative control. No significant differences were found in the reduction of TNF-α levels ($p>0.05$) for dose groups 2 and 3. There was no significant difference between the positive group and each of the dosage groups of cherry leaves in reducing TNF- α levels (Table 4).

Overall, this study shows a significant reduction in TNF-α levels at the most efficient dose of 200 mg/kg BW (group 1), with efficacy comparable to the positive control group with a flat dose-response curve nature (Table 4).

**Table 3 Differences in Post-Treatment Mean TNF-α Levels Between Groups**

| Comparison Group | Compared Groups | P *value(*)* |
|---|---|---|
| Negative control (81.316 ± 3.026) | Positive control (70.518 ± 3.794) | 0.000* |
| | Extract dose 200 mg/kgBW (72.685 ± 4.325) | 0.002* |
| | Extract dose 400 mg/kgBW (64.966 ± 1.237) | 0.000* |
| | Extract dose 800 mg/kgBW (65.480 ± 3.002) | 0.000* |
| Positive control (70.518 ± 3.794) | Negative control (81.316 ± 3.026) | 0.000* |
| | Extract dose 200 mg/kgBW (72.685 ± 4.325) | 0.378 |
| | Extract dose 400 mg/kgBW (64.966 ± 1.237) | 0.007* |
| | Extract dose 800 mg/kgBW (65.480 ± 3.002) | 0.029* |
| Extract dose 200 mg/kgBW (72.685 ± 4.325) | Negative control (81.316 ± 3.026) | 0.002* |
| | Positive control (70.518 ± 3.794) | 0.378 |
| | Extract dose 400 mg/kgBW (64.966 ± 1.237) | 0.002* |
| | Extract dose 800 mg/kgBW (65.480 ± 3.002) | 0.007* |
| Extract dose 400 mg/kgBW (64.966 ± 1.237) | Negative control (81.316 ± 3.026) | 0.000* |
| | Positive control (70.518 ± 3.794) | 0.007* |
| | Extract dose 200 mg/kgBW (72.685 ± 4.325) | 0.002* |
| | Extract dose 800 mg/kgBW (65.480 ± 3.002) | 0.706 |
| Extract dose 800 mg/kgBW (65.480 ± 3.002) | Negative control (81.316 ± 3.026) | 0.000* |
| | Positive control (70.518 ± 3.794) | 0.029* |
| | Extract dose 200 mg/kgBW (72.685 ± 4.325) | 0.007* |
| | Extract dose 400 mg/kgBW (64.966 ± 1.237) | 0.706 |

* Uji *independent T test* (p =0,05)

**Table 4 Significance Test of Cherry Leaves Extract Among Treatment Groups**

| Variable | Extract dose 200 mg/kgBW | Extract dose 400 mg/kgBW | Extract dose 800 mg/kgBW | Positive control | Negative control |
|---|---|---|---|---|---|
| Extract dose 200 mg/kgBW | | 0.004* | 0.007* | 1.000 | 0.001* |
| Extract dose 400 mg/kgBW | 0.004* | | 1.000 | 0.067 | 0.000* |
| Extract dose 800 mg/kgBW | 0.007* | 1.000 | | 0.127 | 0.000* |
| Positive control | 1.000 | 0.067 | 0.127 | | 0.000* |
| Negative control | 0.001* | 0.000* | 0.000* | 0.000* | |

* Bonferroni p=0,05

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## Discussion

An in vivo experimental study with a pre- and post-test control group design, involving 30 Wistar rats induced with monosodium urate (MSU) crystals to create an acute gout arthritis model. The rats, aged 2-3 months and weighing 200-300 grams, were selected based on their optimal age and weight, which have been shown to provide consistent and reliable results in previous studies by previous reports.[18,21].

The clinical manifestations of early gout arthritis, including redness, warmth, pain, and sudden joint swelling due to MSU accumulation wereused to determine the success of induction.[1,2,14] Clinical symptoms, such as increased swelling and gait abnormalities, were assessed for all treatment groups, with the highest intensity observed at the 24-hour mark. This aligns with a previous study, which showed spontaneous resolution in rats after 24 hours, influenced by uricase enzymes breaking down MSU crystals.[22] A decrease in symptoms was also observed in the placebo group, likely due to the metabolic processes of the rats. However, the reduction in symptoms in the negative control group was not supported by the significant decrease in TNF-α levels.

Inflammation in gout involves the interaction between MSU crystals and macrophages, activating the NLRP3 inflammasome and IL-1β production. Two signals are required for inflammasome activation: (1) maturation signals from MSU crystals, free fatty acids, and lipopolysaccharides, which induce Toll-like receptor (TLR) 2 or TLR4 signals, and (2) activation signals from MSU crystals initiate NLRP3 inflammasome assembly, leading to caspase-1 activation. Caspase-1 cleaves pro-IL-1β into its active form, IL-1β, triggering the production of inflammatory cytokines like TNF-α, IL-6, and IL-8, accelerating inflammation. The inhibition of the NLRP3 inflammasome-IL-1β pathway is recommended as a therapeutic target for gout inflammation.[23]

Treatment was administered for three days post-MSU crystal induction, considering the self-healing process within 7–14 days.[24] Acute gout attacks can cause severe pain, comparable to childbirth and visceral colic, emphasizing the need for immediate treatment. Colchicine is used as the drug of choice for acute gout arthritis that acts on microtubules and the NLRP3 pathway.[25] The efficacy of cherry leaf extract was evaluated in 3 escalating dose: dose 1 (200 mg/kgBW), dose 2 (400 mg/kgBW), and dose 3 (800 mg/kgBW). Comparing TNF-α levels before and after treatment showed a decrease in all treatment groups, with the most significant reduction in the 200 mg/kgBW dose, resembling the efficacy of colchicine.

Cherry leaves (*Muntingia calabura L.*) are known for their medicinal properties, containing chemical compounds and pharmacological activities, including anti-inflammatory

effects. Flavonoids, alkaloids, saponins, tannins, and terpenoids were found in the leaves, with flavonoids, especially quercetin, as the major constituents.[26] Quercetin belongs to the subgroup of flavanols' within the flavonoid category, targeting the NLRP3 inflammasome pathway and IL-1β production to reduce inflammation.[23] Some polyphenols such as catechin and quercetin influence the balance between the development of pro- and anti-inflammatory cytokines, thereby enhancing the release of IL-10 while inhibiting TNF-α and IL-1β.[27] By modulating the MAPK pathway at various levels of signal transduction, polyphenols can inhibit the release of TNF-α.

Tumor necrosis factor-alpha (TNF-α) is one of the proinflammatory cytokines that can stimulate the production of cytokines and other inflammatory mediators to repair damaged tissues. Elevated and uncontrolled levels of TNF-α can lead to chronic inflammation and damage healthy tissues.[28] Previous research by Zha successfully demonstrated that TNF-α levels increase in acute gout arthritis and can enhance the accuracy of gout diagnosis.[7] Generally, the release of TNF-α is a natural response of the body to combat infection and tissue damage. This cytokine is produced by various types of cells, particularly immune cells such as macrophages, dendritic cells, and lymphocytes, to strengthen the immune response, increase blood vessel permeability, and direct immune cells to the site of inflammation. Through its interaction with specific receptors, TNF-α triggers intracellular signaling pathways that promote leukocyte recruitment, NLRP3 inflammasome activation, and the release of additional inflammatory mediators.[29]

Considering the central role of TNF-α in the pathogenesis of gout, the use of TNF-α inhibitor therapy in the management of gout arthritis has become a topic of significant research and clinical interest. The flavonoid content in cherry leaf extract can inhibit the production of TNF-α by disrupting the NF-kB signaling pathway involved in the regulation of cytokine production. Flavonoids can hinder the binding of TNF-α to its receptors or interfere with signal transduction, thereby reducing TNF-α activity. A study by Cui explained the impact of flavonoids on macrophage activity by inhibiting the production of nitric oxide (NO), migration, and macrophage adhesion. Inhibiting TNF-α has been proven effective in reducing symptoms and preventing acute attacks in patients with gout arthritis. Clinical studies and practical experience have provided strong evidence regarding the efficacy of this therapy in reducing joint pain, swelling, and other inflammatory symptoms associated with gout arthritis.[27] Flavonoids with anti-inflammatory properties can interact with many molecules involved in inflammatory pathways, reducing the activity of cytokines, chemokines, and inflammatory enzymes.

This study demonstrates the potential of cherry leaf extract as an anti-inflammatory agent by reducing TNF-α levels in acute gout arthritis rat models through the NF-κB pathway and NLRP3 inflammasome, both pivotal in the pathogenesis of acute gout arthritis. The decrease in

inflammation observed in this study, indicated by the reduction in TNF-α levels, a key proinflammatory cytokine, underscores the promising anti-inflammatory properties of cherry leaf extract in the management of acute gout arthritis.

## Conclusion

Muntingia calabura leaf extract at doses of 200mg/kgBW, 400mg/kgBW, and 800mg/kgBW is effective in reducing serum TNF-α levels in rats with acute gout arthritis. The 200mg/kgBW dose shows efficacy comparable to that of colchicine. The response curve to TNF-α levels exhibits a flat dose-response pattern compared to the positive control (colchicine).

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