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The Effectiveness of Tempuyung Leaves' (*Sonchus arvensis*) Water Fraction in Preventing Inflammation in a Rat Model of Acute Gouty Arthritis

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ABSTRACT

Gouty arthritis is inflammation of the joints that occurs in conditions of chronic hyperuricemia, resulting in the deposition of monosodium urate (MSU) crystals. The management of gouty arthritis emphasizes both the acute and chronic phases, and the initial drug options for gouty arthritis are non-steroidal anti-inflammatory drugs (NSAIDs), corticosteroids, and colchicine. However, administering these drugs can cause multiple risks and side effects. Hence, alternative treatments are needed, one of which is using herbal medicine. Tempuyung (*Sonchus arvensis*) is a plant often found and easily cultivated in Indonesia. The water fraction of tempuyung leaves (WFTL) contains flavonoids, which can inhibit the pathogenesis of gouty arthritis owing to their anti-inflammatory and antioxidant activities. Therefore, this study examined the role of WFTL in preventing gouty arthritis and analyzed the phytochemicals in the fraction. Phytochemical screenings, total flavonoid content (TFC), total phenolic content (TPC), antioxidant activity, and gas chromatography-mass spectrometry (GC-MS) analysis were conducted. Meanwhile, the reduction in inflammation-induced MSU injection was observed through the number of neutrophils in an acute goat arthritis rat model. The TFC and TPC were 14.59% and 10.62%, respectively, with an IC_{50} of 57.20 ppm. All the tested phytochemicals were detected in the screening, and 14 compounds were identified through GC-MS. All WFTL doses (10, 20, and 40 mg/kg BW) reduced the number of neutrophils in synovial tissue to a similar level to colchicine. This result shows that WFTL can prevent acute gouty arthritis in rats. More research on the toxicity test of WFTL should be explored to ascertain the safety of the fraction.

Keywords: gout, prevention, tempuyung leaf, water fraction

INTRODUCTION

Gouty arthritis is inflammation of the joints that occurs in conditions of chronic hyperuricemia, resulting in the deposition of monosodium urate (MSU) crystals (Dehlin et al., 2020). The prevalence of gouty arthritis ranges from 1-4% worldwide, and the incidence ranges from 0.1-0.3%. The prevalence increases with each decade of life (Singh & Gaffo, 2020). According to the World Health Organization (WHO), 3.9% of people worldwide suffer from gouty (Aung et al., 2017). The most frequent clinical manifestations of gouty arthritis are swelling of the joints and burning sensations. In some cases, joint damage and kidney

dysfunction are also found (Xiao et al., 2018). Acute onset of arthritis, chronic joint injuries, joint malformations, and renal calculus formation in patients with gouty arthritis reduce the quality of life and cause disability (Zhang et al., 2018). Thus, the management of gouty arthritis emphasizes both the acute and chronic phases (Richette et al., 2014).

The initial drug options for gouty arthritis are non-steroidal anti-inflammatory drugs (NSAIDs), corticosteroids, and colchicine (Kang et al., 2021). Colchicine, as a therapeutic option, apart from being useful in the management of gouty arthritis, also has a therapeutic effect in the prevention phase. Prevention of gouty arthritis is an important part of controlling the disease and preventing repeated attacks of acute inflammation, which ultimately have a negative impact on tissue integrity (Khanna et al., 2012; Alkadi & Khubeiz, 2017; Ridker, 2016). However, administering chemical drugs can cause various risks and side effects (Gürkan et al., 2018). Thus, alternative treatments are needed, one of which is using herbal medicine. Medicinal plants have a long history of use and benefits. Numerous medicinal plants have been evaluated for gouty arthritis (Kapoor et al., 2017; Singh et al., 2021). For instance, sambiloto (*Andrographis paniculata*) has been shown to have prominent anti-gout activity by suppressing pro-inflammatory mediator production and decreasing uric acid levels in *in vivo* and *in vitro* tests, owing to andrographolide—a labdane diterpenoid (Rahmi et al., 2021). Another example is *Orthosiphon aristatus*, commonly known as cat's whiskers. Its anti-gout properties are primarily attributed to its diuretic, anti-inflammatory, and antioxidant effects. These activities were thought to be mediated by rosmarinic acid, sinensetin, and flavonoids (Ameer et al., 2012; Wahab & Chua, 2023). Recent findings showed that the uric acid-lowering activity is related to gut microbiota regulation (Zhu et al., 2023). Tempuyung (*Sonchus arvensis*) is a medicinal plant widely found and easily grown in Indonesia (Wahyuni et al., 2019). Tempuyung leaves have strong antioxidant and anti-inflammatory activities. The antioxidant activity of petroleum ether, methanol, and water extract of tempuyung leaves has been evaluated, showing excellent antioxidant activity (Ghosal et al., 2019). Extract ethanol of tempuyung leaves was able to suppress the expression of pro-inflammatory cytokines in MSU-induced gouty arthritis rats (Hidayat et al., 2020), while its water fraction can inhibit xanthine oxidase (Trivadila et al., 2022). Water fraction of tempuyung leaves (WFTL) significantly decreased the levels of TNF-α and IL-1β (Parisa et al., 2021). Tempuyung leaves contain flavonoids (Harahap, 2020), which can inhibit the pathogenesis of gouty arthritis by suppressing NLRP3 protein expression and reducing oxidative stress (Zhang et al., 2021; Spano et al., 2022; Al-Khayri et al., 2022). Research on WTFL as a potential treatment for gouty arthritis is still limited. Thus, a detailed description and elucidation of the biological activities of tempuyung leaves are required, particularly concerning their chemical constituents and mechanisms of action. This study examined the role of WFTL in preventing gouty arthritis and analyzed the phytochemicals in the fraction. The findings of this study can serve as a scientific foundation for the development of WTFL as an alternative drug for gouty arthritis with minimal side effects.

MATERIALS AND METHODS

Tempuyung was from the Tawangmangu Herbal Research Center, Karanganyar, Indonesia, and was botanically identified at the Biological Research Center of the Indonesian Institute of Sciences (No.780/IPH.1.02/If.8/V/2020). GC-MS grade solvent was purchased from Merck. The remaining chemicals used in this research were all analytical grade and purchased from Sigma Aldrich, Bratachem, and Merck.

Preparation of tempuyung leaves water fraction

Tempuyung leaves were dried and ground. Next, the tempuyung leaves powder was macerated using 96% ethanol with a simplicia:solvent ratio of 1:4. The mixture was left with occasional stirring for 3 x 24 hours. The mixture was filtered, and the filtrate was evaporated using a rotary evaporator (Heidolph, Germany) until a thick extract was gained. The thick extract was used in the liquid-liquid fractionation process. After the thick extract was dissolved in distilled water in a ratio of 1:3, liquid-liquid fractionation using n-hexane and ethyl acetate was performed subsequently. The water fraction was collected and freeze-dried (LC-10N-50A, China). The dried water fraction was stored in a desiccator at room temperature.

Analysis of tempuyung leaves water fraction

Qualitative and quantitative analyses were done to determine the presence of phytochemical groups, total levels of flavonoids and phenols, and antioxidant activity, as well as identify phytochemicals of WFTL. Phytochemical screening

was carried out as reported earlier (Maria et al., 2018; Hartiadi et al., 2020), which consisted of tests for tannins, saponins, phenols, flavonoids, terpenoids/steroids, and alkaloids.

The total flavonoid and phenolic compounds were determined using aluminum chloride colorimetric and Folin-Ciocalteu assays, respectively, as reported by Aprilliana et al. (2021) and Phuyal et al. (2020) with minor modifications. Quercetin (QE) was used as the standard for the calibration curve in total flavonoid content (TFC) determination. Ten mg of QE was weighed and mixed with 0.3 ml of 5% sodium nitrite. After 5 minutes, 0.6 ml of 10% $AlCl_3$ was added. Five minutes later, 2 ml of 1 M NaOH was added. A series of QE concentrations was prepared by diluting the prepared solution with aquadest. Fifty mg of the sample was prepared accordingly. The absorbance measurement of the solutions was done at 510 nm. For total phenolic content (TPC), 10 mg of gallic acid was mixed with 0.5 ml of Folin-Ciocalteu reagent and 7.5 ml of aquabidest. The mixture was left for 10 minutes at room temperature, and then 1.5 ml of 20% sodium carbonate was added. Next, the mixture was heated in a water bath at 40°C for 20 minutes and quickly cooled in ice water. The mixture was diluted with aquabidest to make a series of standard gallic acid solutions for the standard curve. Next, the absorbance was measured at 760 nm against a blank. The sample was prepared accordingly, and the concentration was calculated against the gallic acid equivalent (GAE) standard. All the experiments were carried out in triplicate.

The antioxidant activity was determined using the DPPH scavenging method. Briefly, a series of sample concentrations was prepared in aquadest. Five ml of each solution was transferred into a separate test tube. Two ml of DPPH solution was added to the test tube, and incubated in the dark for 30 minutes. The samples' absorbance was measured at 515 nm. Antioxidant activity was calculated based on its inhibition activity.

Lastly, the active compounds contained in the sample were screened using the GC-MS method (Azifa et al., 2014). The GC-MS system consisted of a Thermo Scientific Trace 1310 gas chromatograph, a Trace ISQ LT Single Quadrupole mass spectrometer detector, and HP-5MS UI (30 m × 0.25 mm × 0.25 μm). The system was set as follows: 230°C for the injection with a 50 split ratio; helium was maintained at a flow rate of 1 ml/min; the MS transfer line and source temperature were set at 250°C and 200°C, respectively; the oven temperature was initially set at 60°C for 2 min, then increased at a rate of 10°C/min until 280°C and held for 8 min. Full scan mode over the m/z range of 40-500 was used to obtain the chromatograms. A mass-selective detector under electron impact ionization mode at a voltage of 70 eV was used to obtain the mass spectra. Chromeleon 7 software was used for data acquisition and processing. Mass spectra library from NIST 2014 and journals were used for identification purposes. The sample was prepared in ethanol, vortexed, and centrifuged at 9500 rpm for 3 minutes. The supernatant was taken and injected into the sample port.

Animal maintenance and treatment

This *in vivo* experiment was carried out from March to December 2023 in the Animal House Laboratory of the Faculty of Medicine, Sriwijaya University, Palembang. Thirty-one male Wistar rats (*Rattus novergicus* L.) weighing 200-300 gr were used in this study. Rats were kept in groups using a polypropylene cage. The animal house temperature was maintained at approximately 25°C, with a relative humidity of 50 ± 10% and a 12-h light-dark cycle. They were fed standard food and water ad libitum. All animal protocols were approved by the Medical and Health Research Ethics Committee, Faculty of Medicine, Sriwijaya University (protocol no. 149-2023).

MSU solution was used to induce inflammation in the knee joints of the rats. To make the MSU, 800 mg uric acid was dissolved in 155 ml boiling distilled water containing 5 mL NaOH. Following pH adjustment to 7.2, the solution was cooled down to room temperature. The crystals were collected by centrifugating the solution at 3,000 g for 2 minutes at 4°C. The product was then evaporated and sterilized at 180 °C for 2 hours. Crystals were stored in sterile microtubes. Prior to administration, MSU was suspended in phosphate-buffered saline, pH 7.2, at 25 mg/ml. In this study, inflammation signs were confirmed by observing the knee size diameter 3 hours after induction, which was confirmed positive if there was an increase in diameter.

After the rats were weighed, they were randomly assigned to five groups, namely negative control (given distilled water), positive control (colchicine 0.28 mg/kg BW/day per oral), WTFL at 10 (WFTL10), 20 (WFTL20), and 40 (WFTL40) mg/kg BW. Treatments were given for 7 days. On day 7, acute gout arthritis was induced by injecting the right knee joint of the rat with MSU solution previously prepared. Before being injected with

MSU solution, mice were anesthetized with ketamine intraperitoneally (i.p.) at 100 mg/kg BW. After the MSU injection, the treatment was continued for 3 days. The dosages and treatment duration in this study were done according to Han et al. (2016), Parisa et al. (2021), and Al-Khayri et al. (2022). At the end of the treatment, the rats were sacrificed by injecting ketamine 3x of the previous dose. The right knee joint from one representative rat of each group was taken and fixed in 4% paraformaldehyde buffer. The joints were dehydrated in alcohol gradients, embedded in paraffin, and cut into 5 μm thick sections. Haematoxylin and eosin (H&E) were used to stain the sections, and the specimens were observed under a light microscope (Olympus Corporation, Japan). Neutrophils, which typically have a lobulated nucleus and finely granular cytoplasm, were counted on 10 different field views at 400x magnification. The density of neutrophils was determined as cellular density by calculating the average of the cell numbers on 10 field views (Suvarna et al., 2018).

Data Analysis

Before the data was analyzed, the data distribution was assessed using Shapiro-Wilk test. Once the data passed the normality test, one-way ANOVA analysis was performed. Tukey's post-hoc test was then applied to determine the significance between groups. Data were presented as the mean ± standard error of mean (s.e.m). All data analyses were done using the IBM SPSS Statistics (version 25).

RESULTS AND DISCUSSION

Qualitative and quantitative analyses of tempuyung leaf water fraction

Tannins, saponins, phenols, flavonoids, terpenoids/steroids, and alkaloids were all detected in the phytochemical screenings, aligning with a previous report (Parisa et al., 2021). These compounds have biological activities that may play a role in alleviating gouty arthritis. Flavonoids, which are antioxidants, can protect cellular membranes from oxidative damage due to free radicals and have anti-inflammatory properties (Asgary et al., 2005; Serafini et al., 2010). Their antioxidant activities prevent oxidative stress, dampening the inflammatory cascade on synovial tissue. Flavonoids can also influence inflammatory responses by blocking a number of pathways, including the production of nitric oxide (NO) by macrophages. This prevents vasodilation and increases blood vessel permeability (Fadilaturahmah et al., 2022). Alkaloids inhibit the inflammatory mediators release so they do not affect the peripheral nervous system and produce inflammatory symptoms. This phytochemical group also has antioxidant and anti-inflammatory properties (Li et al., 2020). Similarly, saponins also influence inflammatory reactions through several mechanisms, such as inhibiting the lipooxygenase pathway, which leads to vasoconstriction prevention and increased blood vessel permeability (Wijesekara et al., 2024). Meanwhile, tannins influence inflammatory reactions by inhibiting macrophages from producing ROS (Song et al., 2018). As a result, cell damage can be prevented, and inflammation symptoms disappear, leading to reduced inflammation symptoms. Terpenoids were also reported to be responsible for several pharmacological activities of *S. arvensis*, including anti-inflammatory, antihyperuricemic, and antioxidant activities (Suwartiny et al., 2022).

The TFC and TPC for WFTL were 14.59% and 10.62%, respectively (Figure 1A). The TFC value was equivalent to 145.9 mg QE/g. Total flavonoids from various fractions and extracts of tempuyung from several studies were found to be lower than the total flavonoids found in this study. Kurniati et al. (2018) reported 10.24 mg flavonoids/g WFTL (Kurniati et al., 2018). Another study conducted by Khan et al. (2012) reported total flavonoids in various fractions, including the chloroform fraction of 1.0%, ethyl acetate fraction of 0.8%, and n-hexane fraction of 0.5%. Khan et al. (2012) also stated that the flavonoid content in tempuyung is hydrophilic, so it is most likely present in the water fraction. In addition, research by Syarifuddin and Dewi (2022) reported that the TFC of 70% ethanol extract tempuyung was only 3.3671 mg QE/g extract (Syarifuddin & Dewi, 2022). On the other hand, the TPC value was equivalent to 106.2 mg GAE/g. Khan et al. (2012) reported different TPC in various fractions, including chloroform fraction (5.2%), ethyl acetate fraction (3.2%), and n-hexane fraction (1.3%).

The difference in flavonoid and phenolic levels in plants is influenced by environmental conditions and nutrients in the place where the plant grows (Xu et al., 2019). Although the content of the primary and secondary metabolites is qualitatively similar, the levels of compounds in plants can vary depending on environmental and plant factors. In addition, differences in the fractions used can also affect the total flavonoids contained in a plant.

Following DPPH assay, the IC_{50} of WFTL was 57.20 ppm (Figure 1B). A compound is considered to have a very strong antioxidant if the IC_{50} value is < 50 ppm. Meanwhile, If the IC_{50} values are 50-100 ppm, 100-150 ppm, and 151-200 ppm, the antioxidant activities are considered strong, moderate, and weak, respectively (Sukweenadhi et al., 2020). Based on the IC_{50} classification, the WFTL had a relatively strong antioxidant activity. Research by Khan et al. (2012) reported the IC_{50} values of chloroform, ethyl acetate, and n-hexane fractions were 90.7, 110, and 1322 ppm, respectively. These results indicate that other fractions of tempuyung leaves have moderate to weak antioxidant activity.

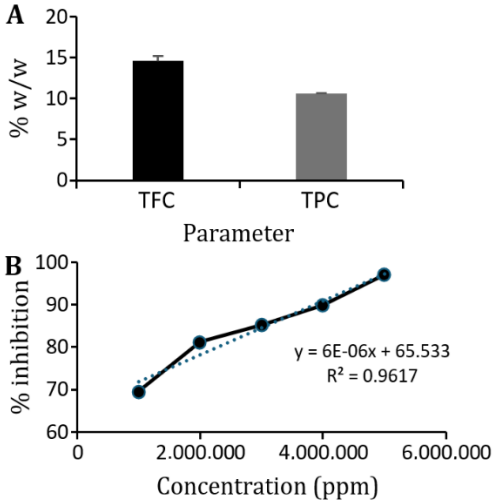

Figure 1. Results of total flavonoids content, total phenolic content, and DPPH assay.

Next, WFTL was analyzed using GC-MS. GC-MS is a tool used to identify compounds that are volatile or easily evaporated (Gilber et al., 2013). The chromatogram results were then analyzed through library (NIST, 2014) and journal compound search analysis (Figure 2). Based on the analysis results, 14 compounds were identified from WFTL, namely 2 phenolic, 1 coumarin, 4 flavonoid, 4 terpenoid, 2 steroid, and 1 lipid group compounds (Table I).

There were several compounds found in high quantities during GC-MS examination, i.e., pentacosan at 75 mg/kg, 1,2-benzenedicarboxylic acid-di-n-butyl ester at 55 mg/kg, 6,10,14-trimethyl-2-pentadecanone at 38 mg/kg, and n-tricosan at 24 mg/kg (Jayakumar et al., 2017). In addition, in the GC-MS examination of the n-hexane extract of *S. arvensis* leaves, it was found that the highest peak was obtained at a retention time of 21.117 minutes with the largest peak area percentage (40.69%), which is thought to be a triterpenoid compound. However, no one has yet elucidated the structure of these triterpenoids. Thus, further analysis is needed to identify the structure of the compound (Rafi et al., 2022).

In this research, the flavonoid compounds contained in WFTL included those from the flavon (luteolin 7-o-glucoside) and flavanone (2-hydroxyisoflavanone, 7-acetoaminoflavanone, and 4'-acetoxy-5,7-dimethyl flavanone) classes. Among these compounds, luteolin 7-glucoside has been demonstrated to affect inflammatory pathway activation, leading to the promotion of inflammation resolution. Luteolin 7-glucoside was reported to downregulate TNF-α, IL-1β, and IL-6, directly opposing NF-κB, MAPK, and JAK/STAT inflammatory pathways (Caporali et al., 2022). Meanwhile, the most frequently identified volatile compounds were the fatty acid methyl ester group 14-methylpentadecanoic acid methyl ester (23.8%), the sesquiterpene lactone group [(3r,3ar,5as,6s,9ar,9br)-3,5a,9-trimethyl -2-oxo-3h,3ah,4h,5h,6h,7h,9ah,9bhnaphtho[1,2-b]furan 6-yl] oxidanesulfonicacid (11.1%), esculetin coumarin group (9.7%), and steroid group cholesterol acetate (9.7%).

Pentadecanoic acid (C15:0) is a saturated fat, which is an essential fatty acid in the diet to maintain long-term heart and metabolic health. Pentadecanoid acid has broad anti-inflammatory and anti-proliferative activity with dose-dependent properties (Venn-Watson & Butterworth, 2022). Pentadecanoic acid can inhibit IL-6 production via the JAK2/STAT3 pathway (To et al., 2020). In the form of methyl ester and 4-methyl pentadecanoic acid methyl ester, these lipid compound derivatives are volatile, so they are easily detected in GC screening (Ramis-Ramos et al., 2016). Several studies have reported that the compounds have antioxidant, antifungal, and antimicrobial activities (Alla et al., 2019; Manurung et al., 2022; Rizvi et al., 2023). Substituted hydroxyl groups (OH) in ester compounds can increase the antioxidant activity of ester compounds (Widiyarti et al., 2019).

14-Methyl pentadecanoic acid methyl ester is also known to have activity as a catechol-O-methyltransferase (COMT) inhibitor and a methylguanidine (MG) inhibitor. The NFκB transcription factor activation and pro-inflammatory cytokine TNF-α release during inflammation cause a decrease in COMT activity.

Table I. Results of GC-MS analysis of tempuyung (*Sonchus arvensis*) leaves water fraction

No	Retention time	BM Count	Molecular weight MS	Molecular Formula	Compound Name	% Content	Group
1	16.920	270	270	$C_{17}H_{34}O_2$	14-methylpentadecanoic acid methyl ester	23.8	Fatty acid
2	11.060	330	331	$C_{15}H_{22}O_6S$	[(3r,3ar,5as,6s,9ar,9br)-3,5a,9-trimethyl-2-oxo-3h,3ah,4h,5h,6h,7h,9ah,9bh-naphtho[1,2-b]furan-6-yl]oxidanesulfonic acid	11.1	Terpenoid
3	18.590	264	264	$C_{15}H_{20}O_4$	6-hydroxy-3,5a,9-trimethyl-3h,3ah,4h,5h,6h,7h,9bh-naphtho[1,2-b]furan-2,8-dione	6.4	Terpenoid
4	5.620	264	264	$C_{15}H_{20}O_4$	(3s,3as,5ar,6r,9bs)-6-hydroxy-3,5a,9-trimethyl-3h,3ah,4h,5h,6h,7h,9bh-naphtho[1,2-b]furan-2,8-dione	6.3	Terpenoid
5	6.870	268	268	$C_{15}H_{24}O_4$	6-hydroxy-9-(hydroxymethyl)-3,5a-dimethyl-decahydronaphtho[1,2-b]furan-2-one	5.7	Terpenoid
6	15.630	178	178	$C_9H_6O_4$	Esculetin	9.7	Coumarine
7	21.680	428	428	$C_{29}H_{48}O_2$	cholesterol acetate	9.7	Steroid
8	14.620	346	346	$C_{21}H_{30}O_4$	Corticosterone	6.1	Steroid
9	16.240	232	232	$C_{15}H_{20}O_2$	2-ethenyl-6-hydroxy-2,5,7,8-tetramethyl-3,4-dihydro-2h-1-benzopyran	7.3	Phenol
10	17.340	256	256	$C_{13}H_{20}O_5$	3-methoxy-3-(3,4,5-trimethoxyphenyl)propan-1-ol	5.1	Phenol
11	20.200	448	448	$C_{21}H_{20}O_{11}$	luteolin 7-o-glucoside	3.6	Flavonoid
12	16.600	281	281	$C_{17}H_{15}NO_3$	7-acetoaminoflavanone	2.0	Flavonoid
13	19.420	310	310	$C_{19}H_{18}O_4$	4'-acetoxy-5,7-dimethylflavanone	1.9	Flavonoid
14	12.070	240	240	$C_{15}H_{12}O_3$	2-hydroxyisoflavanone	1.2	Flavonoid

Low COMT activity is correlated with the development of musculoskeletal pain and increased pain perception in humans and mice (Manurung et al., 2022). MG is known as a nephrotoxin and neurotoxin formed from creatinine via ROS, especially hydroxyl radicals. Inhibition of MG synthesis is an indication of the antioxidant activity of the compound (Jayakumar et al., 2017). However, pentadecanoid acid does not follow Lipinski's and Veber's rules in the Swiss ADME examination (Reza et al., 2021). Therefore, it is possible that 14-methyl pentadecanoic acid methyl ester has a low absorption rate when administered orally.

[(3r,3ar,5as,6s,9ar,9br)-3,5a,9-trimethyl-2-oxo-3h,3ah,4h,5h,6h,7h,9ah,9b h-naphtho [1,2-b] furan-6-yl] Oxydanesulfonic acid, also known as 1β-sulfate-5α, 6βH-eudesma-3-en-12, or 6α-olide, is a sesquiterpene lactone (SL) compound isolated from tempuyung (Xia Z et al., 2010; Mohammed, 2020). Secondary metabolites are most often found in plants in the Asteraceae family. At the molecular level, SL impacts several inflammatory signaling pathways, namely JAK-STAT, MAPK, and NF-kB (Paco et al., 2022). Testing of the anti-inflammatory activity of SL isolates from the halophyte *Sonchus brachyotus* on LPS-activated RAW264 macrophages showed the most potent anti-inflammatory activity (Kang et al., 2021). This compound decreases the production of TNF-α, cytokines, IL-6, and IL-10 and the expression of COX-2, iNOS, and pro-inflammatory proteins.

In addition, it also decreases the phosphorylation of ERK1/2, MAPK, JNK, and p-38 pathways and regulates Nrf2/HO-1 signaling. These results suggest that bioactive sesquiterpenes are useful therapeutic agents for inflammation (Lee et al., 2023). Esculetin is a coumarin that belongs to the benzopyrone class and is often found in the *Sonchus grandifolius* plant. Previous research shows that esculetin has several biological activities, such as anti-inflammatory, anti-cancer, antioxidant, anti-diabetic, anti-arthritic, and anti-hepatic. The anti-inflammatory effect of esculetin was proven through its action in suppressing levels of NO, PGE2, IL-1β, and TNF-α and inhibiting the formation of ROS in RAW 264.7 cells treated with LPS. Esculetin has two hydroxyl groups, which increases its antioxidant capacity in pathological conditions. Esculetin can also reduce the expression of MPO, IL6, TNF-α, and IL-1β, inhibit neutrophil infiltration, and suppress NF-κB activity in mice induced by acute lung injury (Cai & Cai, 2023; Garg et al., 2022).

Esculetin also has an anti-osteoarthritis effect by reducing inflammatory and pro-inflammatory cytokines such as IL6, TNF-α, and IL-1β in mice induced by osteoarthritis. Treatment with esculetin alleviates the main causes of bone and joint destruction associated with arthritis, which are activation of the NF-κB and MPAK pathways, leukotriene B4 synthesis, and generation of inflammatory cytokines. In addition, a single oral dose of 10 mg/kg BW of esculetin in mice was absorbed relatively quickly, with peak drug concentrations approximately 30 minutes after administration. Oral bioavailability is approximately 19%, and elimination time is relatively fast, with a T1/2 of approximately 2 hours (Chen et al., 2021; Kwak et al., 2021; Garg et al., 2022). Therefore, esculetin could be an option in therapeutic strategies to alleviate arthritis by targeting pro-inflammatory cytokines, oxidative stress, and NF-κB.

Cholesteryl acetate is obtained through the formal acylation of the hydroxy group of cholesterol by acetic acid. Oxidation of cholesteryl acetate to 7-ketocholesteryl is a crucial phase in the production of vitamin D3, which is essential for overall health. By controlling the generation of immune cells and inflammatory cytokines, vitamin D is considered to be vital in modulating the inflammatory system, which plays an essential role in the pathophysiology of numerous immune-related disorders. According to mechanistic studies, immune cells, MAP kinase phosphatase 5 (MKP5), prostaglandins, cytokines, and the NF-κB pathways are among the inflammatory processes that vitamin D influences and contributes to the development of cancer (Liu et al., 2018). Gout arthritis patients have low levels of VD-R and 1,25(OH) 2 VD3, while 1,25(OH) 2 VD3 levels were negatively correlated with C-reactive protein (Chen et al., 2021).

Pretreatment Effect of Water Fraction of Tempuyung Leaf (WFTL) on the Synovium Tissue Neutrophils in the Acute Gout Arthritis Inflammation Model

To evaluate the pretreatment effect of WTFL on the number of neutrophils as one of the inflammation markers, one-way ANOVA followed by Tukey's post-hoc test was performed. The infiltration of neutrophils into the synovium tissue of the rats (Figure 2A), while the number of neutrophils in each group (Figure 2B). The negative control group had the highest number of neutrophils (12.2 ± 1.65), indicating that MSU injection is able to induce inflammation. The positive control (colchicine) group had a significantly lower number of neutrophils compared to the negative control group, with a neutrophil number of 6.4 ± 1.19 ($p < 0.05$). Similarly, pretreatment with WTFL at 10 mg/kg for 7 days resulted in a significantly lower number of neutrophils compared to the negative control group (6.6 ± 1.38; $p < 0.05$). There was no significant difference between the positive control and the WTFL10 group, suggesting that WTFL at 10 mg/kg BW is as effective as colchicine in preventing MSU-induced neutrophil infiltration. Although WTFL20 and WTFL40 groups had a neutrophil count that were not significantly different from the positive control group, the number of neutrophils in both groups were also not statistically different from the negative control group. These findings demonstrate that WTFL10 has the best activity, and its activity is dose-independent. The dose-independent activity of plant extracts and their fractions has been commonly reported elsewhere (Blin et al., 2021; Stepien et al., 2018; Balan et al., 2015; Fentahun et al., 2017). It is plausible that the dose-independent is related to therapeutic window (Balan et al., 2015). The presence of a high concentration of an active compound can decrease its pharmacological activity if the concentration is beyond the therapeutic window.

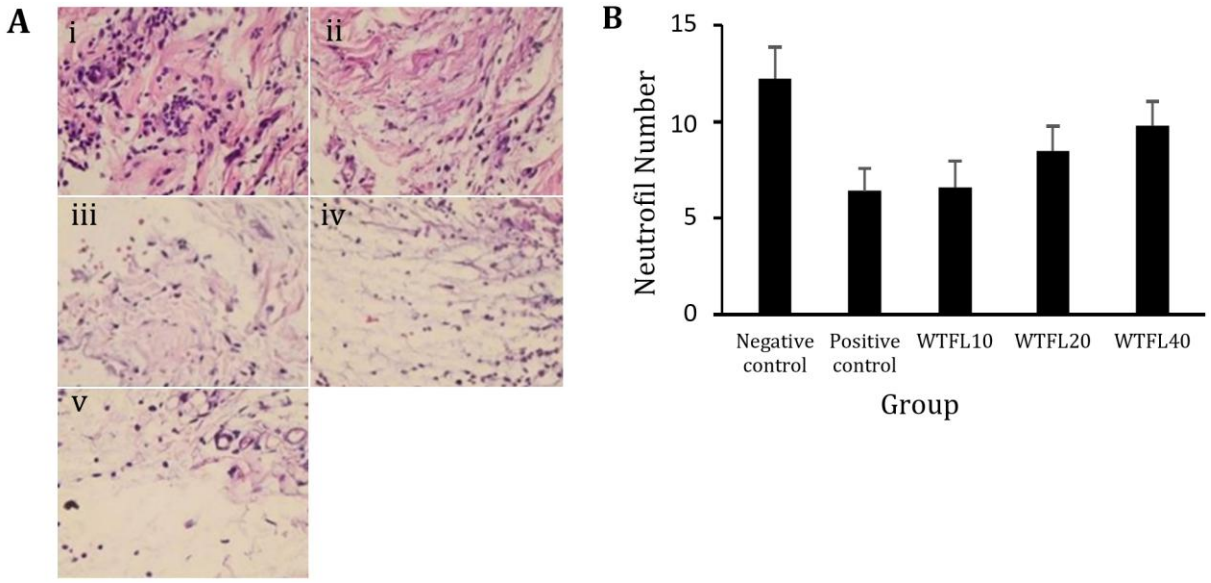

Figure 2. Histology section of synovium tissue depicting neutrophil infiltration (A) and a bar graph showing the number of neutrophils (B) of negative control (i), positive control (ii), WFTL10 (iii), WFTL20 (iv), and WTFL40 (v) groups. Data is presented as mean ± SEM (* $p < 0.05$ from one-way ANOVA analysis, followed by Tukey's post-hoc test)

As such, higher doses of WTFL did not produce a significant reduction of neutrophil counts. Interestingly, Parisi et al. (2021) found that WTFL at 20 mg/kg BW effectively reduced TNF-α and IL-1β levels. The distinct effective dose in these two separate studies may occur due to the presence of different phytochemical levels of the fractions, which are influenced by environmental factors (Xu et al., 2019). In addition, the parameters evaluated were also different, and thus, the higher doses were found to be effective, but not for neutrophil counts.

Neutrophils are the most prominent white blood cells in circulation and function as the first line of defense in the innate immune system (Tamassia et al., 2018). The presence of MSU crystals reduces the expression of myeloid inhibitory C-type lectin (MICL), which is an inhibitor of the neutrophil activation process. When there is a decrease in MICL, there is an increase in the inflammatory response by neutrophils, which can increase IL-8 production by neutrophils. IL-8 is the main neutrophil chemoattractant, which can cause the neutrophil migration process.

When tissue damage occurs, neutrophils migrate to the tissue involved with the stimulus of several signaling agents, including N-formyl peptide (such as toll-like receptors (TLRs), formylmethionyl-leucyl-phenylalanine (fMLP)), and G protein-related receptors (Selders *et al.*, 2017). fMLP is produced from bacterial proteins or tissue damage. Despite the significance of neutrophil reduction in WTFL10, this study only focuses on neutrophil count as the evaluation parameter. Indeed, this approach is not comprehensive, and additional studies involving other inflammatory markers are required. At the time this paper was written, a study was being conducted to assess WTFL in the same animal model utilizing ELISA to measure pro-inflammatory markers.

The anti-inflammation properties of tempuyung have been shown in previous studies. Extract ethanol of tempuyung leaves suppressed the expression of pro-inflammatory cytokines in MSU-crystal-induced gouty arthritis rats (Hidayat et al., 2020), while its WFTL significantly decreased the levels of TNF-α and IL-1β after the WTFL was given seven days before the MSU induction (Parisa et al., 2021). These inflammatory mediators are responsible for attracting neutrophils to the location where MSU crystal is deposited. As such, the reduction of the inflammatory mediators caused by WTFL leads to a decreased number of neutrophils. The decreased neutrophil counts found in this study further corroborate the role of WTFL in preventing gouty arthritis.

The mechanism by which tempuyung leaves exert their action is not only limited to one mechanism, considering the variety of compounds contained in the leaves, including flavonoids. In addition to their anti-inflammation activities, flavonoids have excellent antioxidant activities, which reduce oxidative stress. Oxidative stress is crucial in the early development of gout, which is triggered by reactive oxygen species (ROS) and pro-inflammatory cytokines. Oxidative stress is one of the early events associated with gouty arthritis. In the purine metabolism pathway, ROS, reactive nitrogen species (RNS), and hydroxyl radicals are generated. Additionally, MSU crystals induce mitochondrial ROS production, trigger the release of ROS and RNS in human fibroblast-like synoviocytes, and activate NLRP3 inflammasome. The excess production of ROS and RNS causes oxidative stress, which results in inflammation and joint destruction (Wahnou et al., 2024). Consequently, phytochemicals with antioxidant properties can reduce oxidative stress, dampening the inflammation. Other compounds identified through GC-MS, such as 14-methylpentadecanoic acid methyl ester, esculatin, and cholesterol acetate, also contribute to the biological actions of WTFL through various inflammation pathways and their antioxidant activities. Additionally, Trivadila et al. (2022) showed the benefit of tempuyung leaves fraction in gouty arthritis since the fraction inhibited xanthine oxidase, the enzyme responsible for the disease.

CONCLUSION

The water fraction of tempuyung leaves at 10 mg/kg BW given as a pretreatment for 7 days reduced neutrophil count increase in the MSU-induced rat model. This inflammation reduction activity is thought to occur due to anti-inflammatory and antioxidant activities of the phytochemicals, including flavonoids. The study results enrich the research related to the development of water fraction of tempuyung leaves for gouty arthritis, providing stronger scientific evidence for the effectiveness of the plant.

Research related to novel drug development must be carried out comprehensively. Therefore, toxicity test should be conducted to ensure the safety of tempuyung leaf water fraction. To determine the ideal dose, research on the comparative efficiency of tempuyung leaves water fractions must be conducted at smaller dosage variations. Future studies could concentrate on isolating anti-inflammatory compounds derived from the plant, such as luteolin 7-glucoside.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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