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ORIGINAL ARTICLE

In Vitro Analysis of Anti-Inflammatory Effects of Purple Chrysanthemum (*Chrysanthemum morifolium*) Leaf Extract

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Abstract

Chronic inflammatory diseases are the most significant cause of death in the world and pose the greatest threat to human health. Treatment of inflammation is usually done by taking anti-inflammatory drugs that have dangerous side effects in long-term use. Chrysanthemum is the largest horticultural commodity in North Sulawesi that also holds great potential in the world of health. Based on its phytochemical content, purple chrysanthemum leaves have potential as an anti-inflammatory agent. This study aimed to analyze the anti-inflammatory activity of ethanol extract of purple chrysanthemum leaves using the red blood cell membrane stabilization method. This study used an in vitro laboratory experimental method using rat blood. The anti-inflammatory activity of the extract was tested using the red blood cell membrane stabilization method at 4 concentration series, namely 50 ppm, 100 ppm, 200 ppm, and 400 ppm, then measured on a UV-Vis spectrophotometer. The results of phytochemical screening showed that the ethanol extract of purple chrysanthemum (*Chrysanthemum morifolium)* leaves contains active compounds such as flavonoids, alkaloids, tannins, steroids, and phenolics. The ethanol extract of purple chrysanthemum leaves exhibits anti-inflammatory activity, with higher extract concentrations demonstrating stronger anti-inflammatory effects. The stability percentage values obtained at 50, 100, 200, and 400 ppm were 28.87%, 41.85%, 47.41%, and 64.15% with an IC_{50} of 235.88 ppm and a correlation coefficient of 0.94.

Introduction

Chronic inflammatory diseases are the leading cause of mortality worldwide and represent the most serious threat to human health [1]. More than 50% of all deaths worldwide are attributed to inflammation-related diseases, including ischemic heart disease, stroke, cancer, diabetes mellitus, chronic kidney disease, non-alcoholic fatty liver disease (NAFLD), as well as autoimmune and neurodegenerative disorders [2–4]. Inflammation is a part of the body's defense mechanisms that ideally arises quickly and lasts for a few days to restore balance and repair damaged tissues. However, the dynamics of its role change significantly when inflammation persists and becomes chronic without a clear trigger which can result in tissue damage and be the cause of many chronic health conditions [5].

The treatment of inflammation typically involves the use of steroidal anti-inflammatory drugs (SAIDs) and non-steroidal anti-inflammatory drugs (NSAIDs), which can suppress or reduce inflammation [6]. However, the long-term use of anti-inflammatory drugs is associated with harmful side effects that can affect the gastric mucosa, renal system, hepatic system, cardiovascular system, and hematological system [7]. Consequently, the development of anti-inflammatory agents derived from natural sources, particularly plants, has been extensively explored [8].

Chrysanthemum morifolium is an ornamental flower plant that have been known since decades ago in Indonesia and is a mainstay commodity in the horticultural industry [9]. In North Sulawesi, chrysanthemum production can be found in Tomohon city and is the largest horticultural commodity in North Sulawesi [10]. Chrysanthemums are not only the pride of Tomohon, but also hold great potential for health benefits.

Chrysanthemum contains natural ingredients that can be utilized as herbal medicines for infections such as pneumonia, vertigo, fever, colic, and cancer. The therapeutic effects of chrysanthemum against various diseases are mainly attributed to its antioxidant, anti-inflammatory, antimicrobial, anti-genotoxic, and anticancer activities, as well as its hepatoprotective, neuroprotective, and immune regulatory effects [11,12]. Most previous research has focused on exploring the compounds and biological activities in the flowers of Chrysanthemum species, yet it is evident that the leaves of *Chrysanthemum morifolium* still exhibit considerable biological activity that warrants further investigation [13]. Research conducted by Rawung et al. showed that there are flavonoid, alkaloid, tannin, and saponins compounds in the ethanol extract of chrysanthemum leaves [14].

The purple chrysanthemum has potential as an anti-inflammatory agent based on its phytochemical content; however, the effectiveness of purple chrysanthemum leaves as an anti-inflammatory agent has not been thoroughly studied. The anti-inflammatory testing method can be conducted using the in vitro red blood cell (RBC) membrane stabilization model. The RBC membrane stabilization method is applicable for this anti-inflammatory assay due to the structural similarity between red blood cells and lysosomes, whose membranes play a crucial role in the inflammatory process [15]. This assay model is relatively easier to implement in the laboratory to evaluate the anti-inflammatory potential of natural compounds in vitro [16]. This study aims to analyze the anti-inflammatory activity of ethanol extract of purple chrysanthemum leaves using the red blood cell membrane stabilization method.

Materials and Methods

Materials

The materials used are purple chrysanthemum leaves, ethanol 96%, concentrated HCl, magnesium powder, chloroform, ammonia, H_2SO_4, Mayer's, Wagner's, and Dragendorff's reagents, $FeCl_3$, glacial CH_3COOH, red blood cells, ether, disodium hydrogen phosphate dihydrate ($Na_2HPO_4 \cdot 2H_2O$), distilled water (aquadest), sodium dihydrogen phosphate monohydrate ($NaH_2PO_4 \cdot H_2O$), NaCl, EDTA, and diclofenac sodium.

Sample Preparation

The samples used in this study were purple chrysanthemum leaves (*Chrysanthemum morifolium*) obtained from Kakaskasen Tiga, Tomohon City, North Sulawesi Province, located at an altitude of 798 meters above sea level, with coordinates 1°20'37.1" north latitude (LU) and 124°49'14.8" east longitude (BT) (Figure 1). Purple chrysanthemum leaf samples were cleaned of impurities or foreign objects, then washed with running water and dried by aeration and oven at 60°C for 24 hours. The dried leaves were blended to obtain leaf powder, then sieved using a mesh sieve and stored in a closed container.

Extract Preparation

A total of 100 g of purple chrysanthemum leaf powder was extracted with 96% ethanol using 100 mL, employing the ultrasonication method for 30 minutes at 40 °C. The results obtained were then filtered and evaporated using a rotary vacuum evaporator and concentrated again with an oven for 2 days to obtain a thick extract [17].

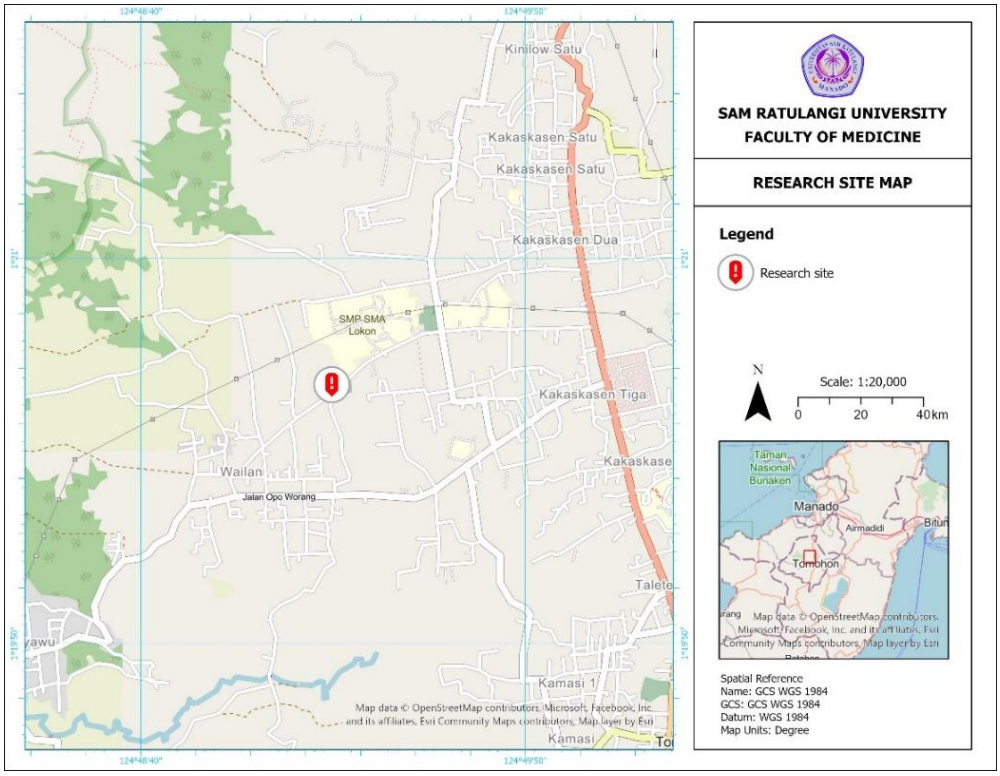

Figure 1. Map of sampling sites.

Qualitative Phytochemical Screening

Phytochemical screening was analyzed qualitatively to determine the secondary metabolite compounds in purple chrysanthemum leaf extract [18]. The ethanol extract of purple chrysanthemum leaves was weighed 2 g and then dissolved with 20 mL of 96% ethanol solvent to obtain an extract concentration of 100 mg/mL. The diluted solution will be used in the phytochemical screening test, included flavonoids, alkaloids, tannins, triterpenoids/steroids, saponins, phenolics [8,19].

Preparation of Phosphate, Isosaline, and Hyposaline Dilutions

A total of 13.35 g $Na_2HPO_4.2H_2O$ and 4.14 g $NaH_2PO_4.H_2O$ were each dissolved with 500 mL of distilled water (0.15 M). Phosphate solution pH 7.4 (0.15 M) was prepared by mixing 80.8 mL of $Na_2HPO_4.2H_2O$ solution with 19.2 mL of $NaH_2PO_4.H_2O$ solution at room temperature and homogenized. Then sterilized with an autoclave. Isosaline solution was prepared by dissolving 0.85 g NaCl with phosphate-buffered solution, to a volume of 250 mL. Hyposaline solution was prepared by dissolving 0.36 g NaCl with phosphate-buffered solution, to a volume of 100 mL. Then each solution was sterilized by autoclaving [19].

Preparation of Red Blood Cell Suspension

Blood samples were collected from Wistar rats previously used in an anti-inflammatory test [20]. A total of 10 mL was mixed with anticoagulants, then centrifuged at 3000 rpm for 10 minutes. The supernatant formed was separated and the precipitate was washed with isosaline solution, centrifuged several times until the supernatant was clear. The volume of blood cells was then resuspended with isosaline to obtain a suspension of red blood cells with a concentration of 10% v/v [15].

Sample Solution Preparation

Concentrations of extracts and diclofenac sodium were prepared by dissolving 0.1 g of ethanol extract of purple chrysanthemum leaves and 0.1 g of diclofenac sodium each with 100 mL of isosaline to obtain a 1000 ppm mother solution. Then the two solutions were diluted into 4

concentration series, 50 ppm, 100 ppm, 200 ppm, and 400 ppm. The concentration range used is based on several studies, where the concentration intervals are doubled [21,22].

Anti-inflammatory Activity Test Using Red Blood Cell Membrane Stabilization Method

The measurement of anti-inflammatory activity against red blood cell membrane stabilization uses 3 solutions, namely negative control solution, sample, and positive control. The negative control solution was made by mixing 1 mL of phosphate-buffered solution, 0.5 mL of red blood cell suspension, and 1 mL of isosaline solution. After that, 2 mL of hyposaline was added. Sample solution of ethanol extract of purple chrysanthemum leaves was made by mixing 1 mL phosphate-buffered solution, 0.5 mL red blood cell suspension, and 0.5 mL sample solution at various concentrations. After that, 2 mL of hyposaline was added. Positive control solution was made by mixing 1 mL of phosphate-buffered solution, 0.5 mL of red blood cell suspension, and 0.5 mL of Na diclofenac solution at various concentrations. After that, each solution was added with 2 mL of hyposaline. The three solutions were incubated at 56°C for 30 minutes, then centrifuged at 3000 rpm for 10 minutes. The supernatant was then measured using a UV-Vis spectrophotometer at a wavelength of 576 nm [19,23].

The absorbance value was used to calculate the percentage of membrane stabilization (Equation 1). The IC_{50} value was obtained from the equation y = ax + b which was determined from the linear regression between concentration as x-axis and % membrane stability as y-axis. The data on the percentage of membrane stabilization were expressed as means ± standard deviation. Statistical analyses were performed using Kruskall Wallis test because the data were not normally distributed and followed by Dunn's post hoc test [24]. A p - value of < 0.05 was considered statistically significant.

$$\%\ Membrane\ Stability = 100 - \frac{Abs\ of\ Test\ Solution}{Abs\ of\ Negative\ Control\ Solution} x\ 100\% \quad (1)$$

Results and Discussion

Purple Chrysanthemum Leaf Extraction Results

A total of 100 g of purple chrysanthemum leaf powder extracted using the ultrasonication method produced 11 g of thick extract. Ultrasonic-assisted solvent extraction of plant secondary metabolites is an environmentally friendly method characterized by a high extraction yield, minimal energy usage, and a brief extraction time [25]. This method uses ultrasonic waves that will produce vibrations and can break down cell walls so that metabolite compounds can mix with the solvent used, namely 96% ethanol. Extraction optimization is influenced by the ratio of materials and solvents, as well as the length of extraction [26]. The percentage value of the yield obtained is 11%. The study conducted by Barba et al., which analyzed the anti-inflammatory response of *Erodium glaucophyllum* extracts obtained through conventional extraction or UAE combined with binary mixtures of ethanol and water, stated that the hydroethanolic extract obtained by ultrasound extraction exhibited the greatest inhibition (92%) of the inflammatory response [27].

Phytochemical Screening Test Results

The results of phytochemical screening can be seen in Table 1. In the flavonoid test, the addition of concentrated HCl and Mg powder. Concentrated HCl will reduce the glycoside which will be replaced by H^+ from HCl to form luteolin flavonoid aglycone which is then reacted with Mg powder. Mg powder will bind to carbonyl groups on flavonoids, resulting in an orange-red color complex [19].

Table 1. Phytochemical screening results.

Phytochemicals	Results
Flavonoids	Positive
Alkaloids	Positive
Taninn	Positive
Triterpenoids/Steroids	Positive
Saponins	Negative
Phenols	Positive

The alkaloid test begins with the preparation of a test solution to collect alkaloid compounds contained in the extract which is done by adding chloroform, NaOH, and H_2SO_4 and then reacted with 3 types of reagents (Mayer's, Wagner's, and Dragendorff's reagents) which aim to emphasize the positive results of alkaloids obtained. In addition, the reagents used can be related to differences in sensitivity to different alkaloid groups. Basically, the principle of alkaloid testing is a precipitation reaction. Nitrogen atoms that have free electron pairs on alkaloids can replace iodo ions in all three reagents. Testing with Dragendorff's reagent yielded a positive result, indicated by an orange precipitate. Testing with Wagner's reagent also showed a positive result, marked by the formation of a reddish-brown precipitate. Testing with Mayer's reagent yielded a negative result, as no precipitate was formed. Two positive results from the three types of tests indicate that purple chrysanthemum leaf extract contains alkaloids [19].

Testing of tannins and phenolics is done by adding $FeCl_3$ to the extract. The blackish green color formed is caused by the formation of complex compounds between Fe_3^+ ions from $FeCl_3$ with compounds in the extract. In testing steroids/triterpenoids, the addition of acetic acid reagents and H_2SO_4. Acetic acid serves to form acetyl derivatives and H_2SO_4 serves to hydrolyze water that reacts with acetyl derivatives so that a color solution is formed [19].

Analysis of Anti-inflammatory Activity Using Red Blood Cell Membrane Stabilization Method

Anti-inflammatory activity test uses red blood cells as a testing medium which is analogous to the lysosomal membrane because it has a similar structure. Damage to the lysosomal membrane can induce arachidonic acid metabolites that produce inflammatory mediators, namely prostaglandins, leukotrins, and lipoxins. Therefore, anti-inflammatory activity is measured by assessing the stability of red blood cells induced with hyposaline solution resulting in hemolysis. Membrane stability is measured by looking at the amount of hemoglobin in the solution which can be read using a UV-ViS spectrophotometer [28].

Table 2. Absorbance value of purple chrysanthemum leaf extract.

Concentration (ppm)	%Stability (Mean ± SD)
50	28.87 ± 0.40
100	41.86 ± 0.07
200	47.41 ± 0.11
400	64.15 ± 0.17

Table 3. Absorbance value of diclofenac sodium.

Concentration (ppm)	%Stability (Mean ± SD)
50	18.81 ± 0.11
100	24.81 ± 0.07
200	56.23 ± 0.40
400	75.71 ± 0.07

Table 2 shows the % stability of the ethanol extract of purple chrysanthemum leaves, while Table 3 presents the % stability of diclofenac sodium as a positive control. Based on the

measurement results for both samples, it was found that the higher the concentration of the test solution, the greater the percent stability, indicating its anti-inflammatory activity.

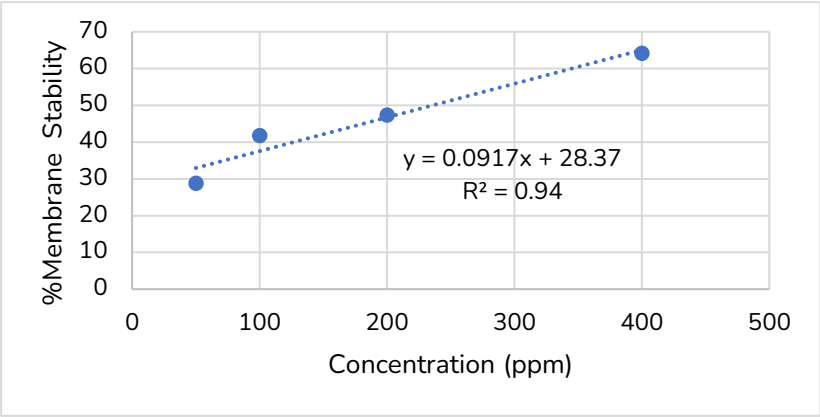

Figure 2. Graph of anti-inflammatory activity test results of purple chrysanthemum leaf extract.

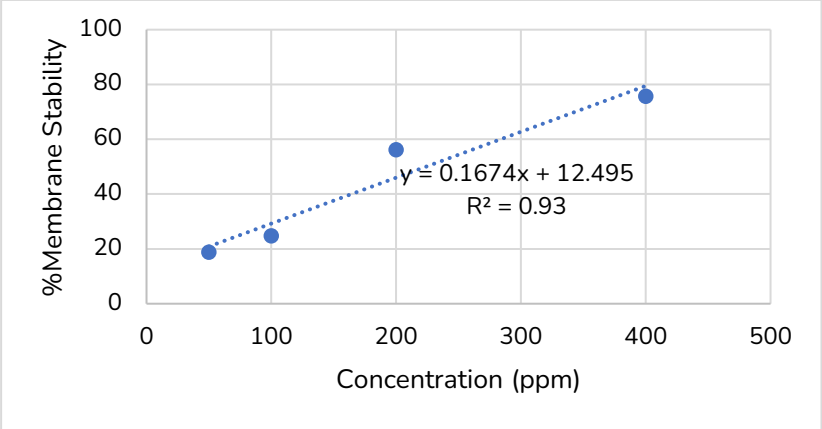

Figure 3. Graph of anti-inflammatory activity test results of diclofenac sodium.

Figure 2 shows the linear regression equation of ethanol extract of purple chrysanthemum leaves, namely y = 0.0917x + 28.37 with a correlation coefficient value of 0.94 and Figure 3 shows the linear regression equation of diclofenac sodium, namely y = 0.1674x + 12.495 with a correlation coefficient value of 0.93. Based on the correlation coefficient value, the two variables in purple chrysanthemum leaf extract and diclofenac sodium have a very strong correlation with a unidirectional relationship, where anti-inflammatory activity will increase with increasing concentration.

Based on the linear regression equation, the IC_{50} value is calculated, representing the concentration required to inhibit 50% of inflammation formation. The IC_{50} value of purple chrysanthemum leaf extract is 235.88 ppm, indicating that the extract can inhibit 50% of the inflammatory process at this concentration. Meanwhile, the IC_{50} value of diclofenac sodium is 224.04 ppm, meaning that 50% inhibition of inflammation occurs at this concentration. A lower IC_{50} value corresponds to higher anti-inflammatory activity. Based on these values, diclofenac sodium, as the positive control, exhibits greater anti-inflammatory activity than purple chrysanthemum leaf extract [29].

This finding is consistent with the study conducted by Pangemanan et al. on the anti-inflammatory activity of pasote leaf extract, which reported that the IC_{50} value of diclofenac sodium (49.98 ppm) was lower than that of pasote leaf extract (58.25 ppm). Nevertheless, the study confirmed that pasote leaf extract exhibited anti-inflammatory activity [30]. Similarly,

another study by Wasiaturrahmah & Amalia on kecapi sentul leaf extract reported an IC_{50} value of 492.31 ppm, which was also found to have anti-inflammatory potential [22].

Statistical Test Results

The results of the normality test using the Saphiro-Wilk test showed that the data were not normally distributed (p-value <0.05). Therefore, the data analysis was continued with a non-parametric test, namely the Kruskal-Wallis test.

Table 4. Results of the Kruskal-Wallis test.

Solution	Concentration (ppm)	*p-value*
Purple chrysanthemum leaf extract	50	0.002
	100	
	200	
	400	
Diclofenac sodium	50	
	100	
	200	
	400	

Table 4 shows the results of the Kruskal-Wallis test with a p-value of 0.002, which means that there is a significant difference between purple chrysanthemum leaves and diclofenac sodium with varying concentrations on red blood cell membrane stability. Data analysis continued with the post hoc test.

Table 5. Results of the post hoc test.

	KU1	KU2	KU3	KU4	KP1	KP2	KP3	KP4
KU1		0.603	0.298	0.038	0.298	0.603	0.119	0.009
KU2	0.603		0.603	0.119	0.119	0.298	0.298	0.038
KU3	0.298	0.603		0.298	0.038	0.119	0.603	0.119
KU4	0.038	0.119	0.298		0.002	0.009	0.603	0.603
KP1	0.298	0.119	0.038	0.002		0.603	0.009	0.000
KP2	0.603	0.298	0.119	0.009	0.603		0.038	0.002
KP3	0.119	0.298	0.603	0.603	0.009	0.038		0.298
KP4	0.009	0.038	0.119	0.603	0.000	0.002	0.298	

Description: KU (Test concentration/purple chrysanthemum leaf extract); KP (Positive control concentration/diclofenac sodium)

Post hoc tests were conducted to determine significant differences between all concentrations which can be seen in Table 5. The test results between the concentrations of purple chrysanthemum leaf extract and diclofenac sodium showed a significant difference between the 50 ppm extract concentration and the 400 ppm positive control concentration, indicating that the 50 ppm concentration of purple chrysanthemum leaf extract has an anti-inflammatory effect identical to diclofenac sodium concentrations of 50 ppm, 100 ppm, and 200 ppm. The 100 ppm extract concentration showed differences with the 400 ppm positive control concentration indicating that the 100 ppm purple chrysanthemum leaf extract concentration had an anti-inflammatory effect identical to that of diclofenac sodium concentrations of 50 ppm, 100 ppm, and 200 ppm. The 200 ppm extract concentration showed a difference with the 50 ppm positive control concentration indicating that the 200 ppm purple chrysanthemum leaf extract concentration had an anti-inflammatory effect identical to that of diclofenac sodium concentrations of 100 ppm, 200 ppm, and 500 ppm. The 400 ppm extract concentration showed a difference with the 50 ppm and 100 ppm positive control concentrations indicating that the 400 ppm purple chrysanthemum leaf extract concentration had an anti-inflammatory effect identical to the 200 ppm and 400 ppm concentrations of diclofenac sodium. Statistically, the purple chrysanthemum leaf extract at each concentration demonstrated anti-inflammatory

effects identical to those of diclofenac sodium, the positive control, at specific concentration variations.

The anti-inflammatory activity of purple chrysanthemum leaves can be attributed to the phytochemical compounds present within them, such as flavonoids, alkaloids, tannins, steroids, and phenolics. Flavonoids help protect lipid membranes from damaging reductions. They act as exogenous antioxidants through four mechanisms: inhibition of nitric oxide synthase activity, inhibition of xanthine oxidase activity, regulation of ion channels, and modulation of enzymes involved in oxidative processes. The anti-inflammatory effect of flavonoids is also linked to the inhibition of COX and lipoxygenase enzyme activities, which affect the production of prostaglandins and leukotrienes, key inflammatory mediators. Furthermore, flavonoids can also inhibit leukocyte accumulation and neutrophil degranulation, thereby reducing the release of arachidonic acid by neutrophils [31–33].

Alkaloids play an anti-inflammatory role by preventing the synthesis of pro-inflammatory cytokines, suppressing histamine release by mast cells, and nitric oxide production [22]. Tannins exhibit antioxidant activity that contributes to their anti-inflammatory effects by inhibiting the production of oxidants (O_2) in neutrophils, monocytes, and macrophages. This inhibition of O_2 production reduces hydrogen peroxide (H_2O_2) formation, which further prevents the generation of hypochlorous acid (HOCl) and hydroxyl radicals (OH). Tannins also directly neutralize reactive oxidants such as hydroxyl radicals (OH) and hypochlorous acid (HOCl) [33]. Additionally, tannins can inhibit endothelial cells and nitric oxide (NO), helping to preserve vascular tone and prevent excessive blood vessel dilation [19]. Phenolics have the ability to scavenge free radicals that can cause tissue damage, thereby inhibiting the biosynthesis of arachidonic acid [33]. Steroids also exhibit anti-inflammatory potential by inhibiting phospholipase enzymes through the arachidonic acid pathway [22].

Nevertheless, the anti-inflammatory activity of ethanol extracts from purple chrysanthemum leaves may be influenced by several factors. In this study, the thick extract was not applied directly, but it is recommended that it be used in its concentrated form for more effective results. Furthermore, attention should be given to the maximum storage time of the prepared red blood cell suspension to maintain the stability of red blood cell morphology.

The anti-inflammatory potential of purple chrysanthemum leaf extract is attributed to its bioactive compounds; however, further studies are necessary to identify the specific compounds or groups responsible for this activity. This requires isolating and purifying individual compounds from the extract and evaluating their anti-inflammatory properties. Furthermore, in vivo studies are necessary to confirm these findings and provide a more comprehensive understanding of the extract's therapeutic potential.

Conclusions

This study focused on identifying secondary metabolites and analyzing the anti-inflammatory activity of the ethanol extract from purple chrysanthemum leaves. The results revealed that the extract contains several secondary metabolites, including flavonoids, alkaloids, tannins, steroids, and phenolics. The ethanol extract demonstrated significant anti-inflammatory activity, which increased with higher concentrations. The highest anti-inflammatory activity was observed at 400 ppm, with a stability percentage of 64.15%. The IC_{50} value of the extract was determined to be 235.88 ppm, while the IC_{50} value of diclofenac sodium, used as a positive control, was 224.04 ppm. Statistically, the anti-inflammatory effects of the extract at various concentrations were comparable to those of diclofenac sodium. However, further research is necessary to confirm these findings and thoroughly evaluate the therapeutic potential of the extract.

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Informed Consent Statement: Not applicable.

Data Availability Statement: The data used in this study are available upon request from the corresponding author in accordance with applicable data protection and privacy regulations.

Conflicts of Interest: All the authors declare no conflicts of interest.

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