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Antioxidant and Anti-inflammatory Activity of Ethanol Extracts from Sambal Orange Peel (*Citrus microcarpa* Bunge) on Erythrocyte Membrane Stabilization

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Abstract

Inflammation is the body's defense response to various stimuli. Sambal orange (*Citrus microcarpa* Bunge) belongs to the Rutaceae family and is widely cultivated in West Kalimantan. This study uses the erythrocyte membrane stabilization method to test the secondary metabolite content, antioxidant activity, and anti-inflammatory activity of ethanol extracts of sambal orange peel. Antioxidant activity was assessed using the DPPH method, while anti-inflammatory activity was evaluated through erythrocyte membrane stabilization. The results showed that the extract had an antioxidant IC50 value of 17.90 ppm, which is very strong. Membrane stability at concentrations of 50, 100, 150, 200, and 250 ppm showed increases of 32.20%, 38.39%, 47.37%, 55.18%, and 65.52%, respectively. The % stability values of sodium diclofenac at the same concentrations were 50.10%, 58.40%, 66.69%, 78.59%, and 87.70%, respectively. The one-way ANOVA test showed a significance value of 0.000 ($p < 0.05$), indicating a significant difference between treatments. The Tukey post-hoc test showed no significant difference between the 250 ppm extract concentration and the 150 ppm sodium diclofenac concentration. The IC50 value for the extract's anti-inflammatory activity was 163.55 ppm (moderate activity), while sodium diclofenac exhibited stronger activity with an IC50 value of 54.09 ppm.

Keywords: Antioxidant; Anti-inflammatory; Citrus microcarpa Bunge; Ethanol extract; Erythrocyte cells

INTRODUCTION

Inflammation is defined as the body's defensive response to various stimuli. Chemical and physical stimuli can trigger inflammation (Sutiswa et al. 2024). Inflammation is characterized by symptoms such as redness, heat, pain, swelling, and loss of function in the affected area. An uncontrolled and prolonged inflammatory process has the potential to damage cells, thereby causing the pathological effects of various diseases (Wasiaturrahmah and Amalia 2023).

According to data from the 2018 Riskesdas survey, a study conducted by the Indonesian Ministry of Health, the national prevalence of diseases involving inflammation in the body is as follows: asthma (2.4%), cancer (1.79%), diabetes mellitus (1.5%), acute respiratory infections (4.4%), pneumonia (2.0%), pulmonary tuberculosis (0.42%), joint diseases (7.30%), heart disease (1.5%), and chronic kidney disease (0.38%) (Balitbangkes, 2018).

Inflammation is commonly managed using steroids and nonsteroidal anti-inflammatory drugs (NSAIDs). However, prolonged use of these conventional medications has been shown to cause undesirable side effects. Extended NSAID use has been linked to a range of adverse effects, potentially impacting specific organs such as the gastrointestinal tract, cardiovascular system, and kidneys. (Amriani et al., 2024). Consequently, there is an urgent need to develop relatively safe drugs that can effectively relieve pain (analgesics) and treat inflammation, especially those formulated from natural ingredients, which have the advantage of fewer side effects. The skin of the sambal orange fruit is believed to possess anti-inflammatory properties.

The sambal orange (*Citrus microcarpa* Bunge) is a type of citrus fruit from the Rutaceae family widely cultivated in West Kalimantan. The locals commonly consume the fruit and leaves of the sambal orange as juice or an ingredient in specific culinary preparations. Conversely, the peel of the sambal orange is often discarded as a by product (Nurhaliza et al., 2018). The selection of sambal orange peel as the material for further investigation of the benefits of the waste discarded by the community was predicated

on the assumption that it would provide a suitable foundation for the study. This also enhances the potential of local natural resources as an alternative herbal medicine.

Phytochemical compounds such as flavonoids, polyphenols, and alkaloids have been identified in the ethyl acetate and methanol extracts of sambal orange peel, indicating the presence of bioactive constituents (Wulandari et al., 2013). Conversely, the n-hexane extract of orange peel predominantly contains steroid compounds. Notably, the methanolic extract exhibits significant antioxidant potential, with an IC_{50} value of 94.01 µg/mL, classifying it as a strong antioxidant (Wulandari et al., 2013). These findings highlight the potential of sambal orange peel as a source of natural antioxidants and bioactive phytochemicals, warranting further investigation (Wulandari et al. 2013). The secondary metabolites present in the plant have been shown to possess anti-inflammatory properties (Hidayah et al., 2023).

The antioxidant activity was evaluated using the DPPH (1,1-diphenyl-2-picrylhydrazyl) method due to its simplicity, user-friendliness, speed, and high sensitivity. DPPH is a stable free radical, a reagent in assays measuring the ability to neutralize free radicals. This method quantitatively measures antioxidant content. The objective of this study is to evaluate the antioxidant activity of ethanol extract obtained from the peel of sambal orange (*Citrus microcarpa* Bunge). A 70% ethanol solution was chosen as the solvent because it is considered more effective and safer than methanol, known for its toxicity. The extraction process employed maceration to minimize potential damage to the active compounds in the raw material (Kartikasari et al., 2022; Yanti et al., 2023).

Anti-inflammatory activity can be assessed through in vivo and in vitro methodologies. In vitro testing is conducted as an initial screening to evaluate the anti-inflammatory properties of the substance before conducting in vivo testing. The merits of in vitro testing include its capacity to eliminate the need for test animals, minimize the number of samples required, and expedite the testing process. In vitro testing of anti-inflammatory activity can be performed using the protein denaturation inhibition method and the erythrocyte cell membrane stability method. In this study, in vitro testing of anti-inflammatory activity was performed using the erythrocyte cell membrane stability method, or Human Red Blood Cell (HRBC) (Sari et al., 2024).

The erythrocyte membrane stability method is employed due to the erythrocytes' similarity to lysosomal membranes, which have the capacity to influence inflammatory processes. Consequently, if erythrocyte stability is maintained, lysosomal membrane stability will also be preserved, allowing anti-inflammatory activity to be measured (Sari et al. 2024; Kamilla et al. 2021). The HRBC method has been demonstrated to have certain advantages, including the ease of obtaining and isolating erythrocytes from blood samples. Additionally, the membrane structures of erythrocytes are comparable to those of other cell membranes, which renders them suitable for anti-inflammatory activity testing (Sari et al. 2024). It is hypothesized that this study will reveal the potential of sambal orange peel extract as a promising anti-inflammatory therapy due to its content of flavonoids, terpenoids, phenolic acids, and antioxidants. This anti-inflammatory therapy has the potential to serve as an attractive alternative for managing inflammation, both topically and through oral supplementation.

This research is of great significance in developing natural anti-inflammatory therapies. The utilization of sambal orange peel as a source of anti-inflammatory agents has the potential to serve as a safer and more environmentally friendly alternative to synthetic drugs, which frequently result in deleterious long-term side effects.

METHODOLOGY

Materials and Instrumentals

The equipment utilized in this study encompassed a *UV-Visible* Spectrophotometer, vacuum rotary evaporator, centrifuge, autoclave, oven, water bath, desiccator, analytical balance, blender, knife, beaker glass, measuring flask, reaction tube, centrifuge tube, micropipette, porcelain dish, funnel, spatula, dropper pipette, stirring rod, watch glass, glass bottles with lids, pH meter, stopwatch, and filter cloth.

The materials utilized in this study encompassed an ethanol extract of sambal orange peel (*Citrus microcarpa* Bunge), 70% ethanol, distilled water, a 10% red blood cell suspension, sodium diclofenac, a phosphate buffer with a pH of 7.4 (0.15 M), 10% NaOH, dextrose, sodium citrate, citric acid, concentrated H_2SO_4, NaCl, chloroform, DPPH, hydrochloric acid, Mayer's reagent, Dragendorff's reagent, 10% $FeCl_3$, 2 N HCl, magnesium powder, 1% gelatin, acetic acid (CH_3COOH), sulfuric acid (H_2SO_4) and methanol p.a.

Methods

The process of identifying a plant specimen

The plant specimens were identified at the Faculty of Mathematics and Natural Sciences, Biology Laboratory, Tanjungpura University, Pontianak. This identification aimed to ascertain that the plant utilized as the test material was sambal orange peel.

Simplisia Processing

Two kilograms of sambal orange peel were collected, wet sorted, and washed with running water. Subsequently, the orange peel was meticulously sliced into thin, longitudinal or crosswise slices. The slices were then dried using a cabinet dryer at a temperature ranging from 35 to 38°C, until complete desiccation was achieved. After this, the material was subjected to a dry-sorting process, followed by a weighing procedure. Thereafter, the material was blended and sieved using a 60-mesh sieve. The sieved material was subsequently stored in a closed, dry, and clean environment that was shielded from direct sunlight. The plant specimens were identified at the Faculty of Mathematics and Natural Sciences Biology Laboratory, Tanjungpura University, Pontianak. This identification aimed to ascertain that the plant utilized as the test material was sambal orange peel (Sukandiarsyah et al., 2025).

Ethanol Extraction Process of Sambal Orange Peel

The sambal orange peel was immersed in a 70% ethanol solvent for a period of three days, with the solvent being replaced every 24 hours. The resultant liquid from the soaking process was subjected to evaporation. The concentrated extract was stored in a tightly sealed bottle and underwent qualitative phytochemical screening tests (Sukandiarsyah et al., 2025).

Phytochemical Screening

Alkaloid Test

A concentrated extract of the sample was obtained and acidified by adding 5 mL of 2 N hydrochloric acid. The solution was then filtered to obtain a clear filtrate. For alkaloid detection, 3 drops of Mayer's reagent and 3 drops of Dragendorff's reagent were added separately to aliquots of the filtrate. The formation of a white to yellow precipitate with Mayer's reagent and an orange precipitate with Dragendorff's reagent was used as a qualitative indicator for the presence of alkaloids. (Sukandiarsyah et al., 2023; Fitri et al., 2023)

Phenol Test

The extract is added with 1 ml of 10% $FeCl_3$ solution. If a blue-black or greenish-black solution forms, it indicates the presence of phenolic compounds (Sukandiarsyah et al., 2023; Hasanela et al., 2023).

Flavonoid Test

The extract is added with 2 mL of 2 N HCl and magnesium powder. The formation of an orange to red solution indicates the presence of flavonoids (Fitriyanti et al., 2019)

Tannin Test

The extract is added to 1 ml of 1% gelatin; if a white precipitate forms, it indicates the presence of tannins (Sukandiarsyah et al., 2023).

Saponin Test

Three milliliters of the sample are placed in a test tube, followed by the addition of 10 milliliters of hot water. The mixture is then clarified and vigorously shaken for 10 seconds. If stable foam forms, reaching a height of 1–10 cm and persisting for at least 10 minutes without dissipating after the addition of a drop of 2 M hydrochloric acid, this indicates the presence of saponins (Putri et al., 2023).

Steroid and Triterpenoid Testing

To the extract, 0.5 mL of chloroform was added, followed by 0.5 mL of acetic acid (CH_3COOH). Subsequently, 2 mL of concentrated sulfuric acid (H_2SO_4) was carefully added along the inner wall of the test tube. The development of a blue-green color was used as an indicator for the presence of sterol compounds, while the appearance of a brown or violet ring indicated the presence of triterpenoids. (Sukandiarsyah et al., 2023).

Antioxidant Assay

Each test solution with each concentration (20 ppm, 40 ppm, 60 ppm, 80 ppm, and 100 ppm) was placed in a 2 ml test tube. A 2-ml solution of 0.1 mM DPPH was added, then covered with aluminum foil, homogenized, and incubated for 30 minutes. The measurement of the absorbances was conducted at the maximum wavelength of 0.1 mM DPPH. The measurement was repeated thrice for each concentration of the test solution. The calculation of the IC_{50} value necessitates the procurement of inhibition percentage data from the test (Purwaningsih et al., 2018).

Ethical Clearance Test

The research code test was conducted at the Faculty of Health and Nursing, Muhammadiyah University Semarang.

Preparation of Solutions

The Alsever solution combines 2 grams of dextrose, 0.8 grams of sodium citrate, 0.05 grams of citric acid, and 0.42 grams of NaCl. The mixture is then dissolved in distilled water, yielding a total volume of 100 milliliters. The solution is then sterilized using an autoclave at 115°C for 30 minutes (Wahdaniah et al., 2023). The isosaline solution is prepared by first weighing 4.25 grams of NaCl and subsequently dissolving it in 500 milliliters of phosphate buffer solution with a pH of 7.4 (0.15 M). The solution is then sterilized using an autoclave at 115°C for a duration of 30 minutes (Armadany et al., 2020). A hyposaline solution was formulated by weighing 0.25 grams of NaCl and dissolving it in 100 milliliters of phosphate buffer solution (0.15 M) with a pH of 7.4. The resulting solution was sterilized by autoclaving at 115°C for 30 minutes (Armadany et al., 2020).

The ethanol extract of sambal orange peel was diluted to concentrations of 50 ppm, 100 ppm, 150 ppm, 200 ppm, and 250 ppm, respectively, in 100 ml of isosaline solution. The concentration of sodium diclofenac was utilized as a reference point, with adjustments made to align with the concentrations of the ethanol extract of sambal orange peel, ranging from 50 ppm to 250 ppm.

Preparation of Erythrocyte Suspension

Erythrocyte suspension was prepared using 10 ml of venous blood and placed in a centrifuge tube containing Alsever's solution. The mixture was then subjected to a centrifugal process at 3000 rpm for 10 minutes. The upper layer, or the "supernatant," was separated from the lower layer, which contained the residual cell sediment. The latter was washed with an isotonic solution and subjected to a second centrifugal process. This process is repeated three times until the mixture is clear. Subsequently, a 2-ml portion of red blood cells should be amalgamated with 18 ml of isosalin solution, yielding a 10% red blood cell suspension (Armadany et al., 2020).

Maximum wavelength

A reaction mixture was prepared by combining 1 mL of phosphate buffer solution (pH 7.4, 0.15 M), 0.5 mL red blood cell suspension, 1 mL of 0.005% sodium diclofenac solution, and 2 mL of hyposaline solution. The mixture was incubated at 37°C for 30 minutes. Subsequently, the samples were centrifuged at 4,000 rpm for 10 minutes. The absorbance of the resulting supernatant was measured using a UV-Visible spectrophotometer within the wavelength range of 550 to 560 nm (Wahdaniah et al., 2023).

Erythrocyte Membrane Stabilization Test

The test solution, with a total volume of 4.5 mL, was prepared by mixing 1 mL of phosphate buffer (pH 7.4, 0.15 M), 0.5 mL of red blood cell suspension, 1 mL of the sample solution, and 2 mL of hyposaline solution. The positive control consisted of 1 mL phosphate buffer (pH 7.4, 0.15 M), 0.5 mL red blood cell suspension, 1 mL sodium diclofenac solution, and 2 mL hyposaline. For the test control, 0.5 mL of red blood cell suspension was replaced with isosaline solution, combined with 1 mL phosphate buffer, 1 mL sample solution, and 2 mL hyposaline. The negative control solution was prepared by substituting the sample solution with 1 mL of isosaline solution, mixed with 1 mL phosphate buffer, 0.5 mL red blood cell suspension, and 2 mL hyposaline. All prepared solutions were incubated at 37°C for 30 minutes, followed by centrifugation at 4,000 rpm for 10 minutes. The absorbance of the supernatant was then measured at the maximum wavelength using a UV-Visible spectrophotometer (Septian et al., 2019).

Data Analysis

The calculation of the percentage value of antioxidant inhibition is determined by the following formula (Purwaningsih et al., 2018) :

$$\%\ Inhibition = \frac{Abs\ Blanko - Abs\ Sample}{Abs\ Blanko} \times\ 100\%$$

The calculation of the percentage value of anti-inflammatory stabilization is determined by the following formula (Septian et al. 2019) :

% extraction stabilization =

$$100 - \frac{Abs\ Extract - Abs\ Extract\ Control}{Abs\ Negative\ Control} \times\ 100\%$$

% Natrium Declofenac (ND) stabilization =

$$100 - \frac{Abs\ ND - Abs\ ND\ Control}{Abs\ Negative\ Control} \times\ 100\%$$

The dataset, represented as stabilization percentages (%), was subjected to statistical analysis using SPSS software. Normality of the data distribution was verified via the Shapiro-Wilk test,

and homogeneity of variances was assessed using Levene's test. Upon confirmation that these assumptions were satisfied, one-way analysis of variance (*ANOVA*) was conducted to evaluate differences among groups. For comparisons yielding p-values below the significance threshold of 0.05, multiple pairwise comparisons were performed using Tukey's Honestly Significant Difference (HSD) post hoc test to ascertain statistically significant differences.

RESULTS AND DISCUSSION

Extraction And Phytochemical Screening

The study commenced with the preparation of ethanol extracts, subsequently followed by phytochemical screening. Subsequently, the extracts were evaluated for their antioxidant and anti-inflammatory properties using erythrocyte cell membranes as the model system. The sambal orange peel was extracted through maceration with 70% ethanol solvent. Before the maceration process, the samples dried to reduce the water content. This step was implemented to mitigate the risk of degradation or damage to the crude drug substance (Agung et al., 2024). The objective of maceration is to extract the active compounds present in the crude drug. The extraction process yielded a concentrated extract of 55.75 grams. The concentrated extract was subjected to phytochemical screening, a methodical evaluation of the secondary metabolites. In this study, the analysis of secondary metabolites was exclusively qualitative; no checks were performed on the concentration and types of compounds contained, and the extract used was not fractionated, resulting in the detection of numerous compounds (Sukandiarsyah et al. 2023). The yield of the ethanol extract of sambal orange peel was as follows in Table 1.

Table 1. Results of Ethanol Extraction from Orange Peels in Sambal Research

Simplisia Orange Peel Sambal (g)	Orange Peel Sambal Extract (g)	Yield (%)
370.35	55.75	15.05

The extraction yield is the ratio between the quantity of metabolites obtained following the extraction process and the weight of the raw sample utilized. A yield is generally considered acceptable when it exceeds 10%. This benchmark aligns with the findings of Fajrin et al., (2024), who employed a maceration technique using 70% ethanol as the solvent over a 72-hour extraction period. Several factors may influence the extraction yield, including the extraction method employed, the type and concentration of the solvent, the duration of extraction, and the particle size or fineness of the powdered simplicia used. The results of the phytochemical screening of ethanol extracts from sambal orange peel are presented in Table 2.

Table 2. Phytochemical Screening of Ethanol Extracts from Sambal Orange Peel

No	Test	Reagent	Result	Description
1	Flavonoids	HCl 2N, magnesium powder	An orange solution is formed	Positive
2	Tannins	Gelatin 1%	A white precipitate is formed	Positive
3	Alkaloids	Mayer's solution Dragendorff 's solution	A white precipitate is formed An orange precipitate is formed	Positive
4	Phenols	$FeCl_3$ 10%	A blue-black solution is formed	Positive
5	Saponins	Hot distilled water	A constant foam is formed.	Positive
6	Steroids/Triterpenoids	CH_3COOH, H_2SO_4 Chloroform	A brown ring is formed	Positive Triterpenoids

As illustrated in Table 2, the ethanol extract of sambal orange peel contains various secondary metabolites, including alkaloids, flavonoids, triterpenoids, saponins, tannins, and phenols. These secondary metabolites are natural antioxidants found in sambal orange peel. These compounds have been demonstrated to function as natural antioxidants (Agung et al., 2024).

Antioxidant Activity Test

The measurement of antioxidant activity is typically conducted using the concentration inhibition (IC_{50}) method. This method determines the concentration of antioxidants required to achieve a 50% reduction in DPPH. A smaller IC_{50} value is indicative of stronger antioxidant activity (Irfayanti et al., 2023). The maximum absorption of DPPH occurs at a wavelength of 515 nanometers (nm). Figure 1 illustrates the relationship between extract

concentration and inhibition percentage, demonstrating the effectiveness of ethanol extract from sambal orange peel in neutralizing DPPH free radicals.

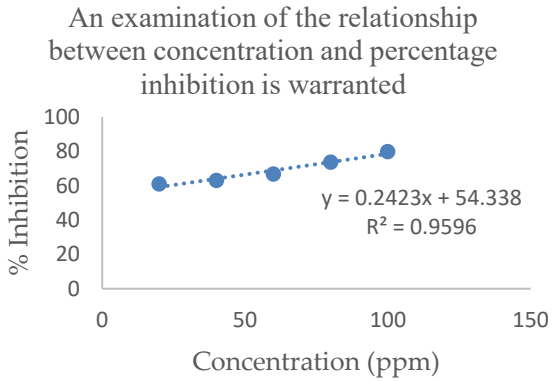

Figure 1. Linear Regression Curve of Antioxidant

In the linear regression equation, the y-intercept represents the IC_{50} coefficient, valued at 50, while the x variable corresponds to the tested extract concentration. Based on this equation, the IC_{50} value for the ethanol extract was calculated to be 17.90 ppm. The coefficient of determination (R^2) indicates the degree of linear correlation between the extract concentration and the percentage of inhibition. IC_{50} values are categorized as follows: values below 50 ppm denote very strong antioxidant activity; values between 50 and 100 ppm indicate strong activity; values from 100 to 150 ppm suggest moderate activity; 150 to 200 ppm represent weak activity; and values exceeding 200 ppm correspond to very weak antioxidant activity (Souhoka et al., 2019). Accordingly, the ethanol extract of sambal orange peel demonstrates strong antioxidant properties (Fithriani et al., 2015). The mechanism of action of DPPH in reducing free radicals involves reacting by donating a hydrogen atom (H^+) to the DPPH radical, converting it into a stable diamagnetic molecule (Aryanti et al., 2021; Hanum et al., 2021). The underlying principle of this technique is predicated on color transformation. The transformation of dark purple to pale yellow is attributable to antioxidant activity, which functions to reduce free radicals. The purple color disappears when the DPPH solution is combined with a solution capable of donating hydrogen atoms, resulting in a pale yellow color obtained from the reduction of radicals that have been donated hydrogen atoms by antioxidants (Aryanti et al., 2021; Rosawanti et al., 2018).

It has been demonstrated that several chemical substances exhibit a propensity to react with DPPH radicals through electron transfer or by donating hydrogen atoms. Phenolic compounds have been identified as the most reactive and significant compounds, exhibiting a high degree of reactivity with DPPH (Gulcin and Alwasel 2023). Hydrogen atom abstraction is a reaction that occurs through the transfer of electrons, which can be preceded or followed by the transfer of protons. This reaction is formally classified as a hydrogen atom transfer reaction. Consequently, the DPPH assay provides an estimate of the total reducing agent content present in the plant extract solution (Gulcin and Alwasel 2023).

Anti-Inflammatory Activity Test

The University of Muhamadiyah Semarang has formally endorsed the in vitro treatment of human erythrocyte cell membranes, a procedure that bears the institutional code number No. 0069/KEPK-FKM/UNIMUS/2025. The anti-inflammatory activity of the ethanol extract from sambal orange peel was assessed by monitoring the reduction in absorbance within the test solution mixture. It is understood that the absorbance level correlates positively with the degree of hemolysis, reflecting the sample's anti-inflammatory efficacy. Absorbance readings were taken at 557 nanometers (nm). Sodium diclofenac, a non-steroidal anti-inflammatory drug (NSAID) known to inhibit the release of inflammatory mediators by suppressing prostaglandin and cyclooxygenase synthesis, served as the positive control in this study (Sukmawati et al. 2022). Table 3 shows the average percentage of erythrocyte stability inhibition caused by the Ethanol extract of orange peel Sambal (EKJS) and Sodium Declofenac (ND).

Table 3. Average (%) Ethanol extract of orange peel Sambal and Sodium Declofenac

No	Consentration (ppm)	Average ± SD	
		EKJS (%)	ND (%)
1	50	32.20±1.51	50.10±0.86
2	100	38.39±0.83	58.40±1.08
3	150	47.37±0.68	66.69±0.88
4	200	55.18±0.78	78.59±0.80
5	250	65.52±1.45	87.70±0.57

Table 3 presents the results of observations and calculations of % stability. These results indicate that the ethanol extract of sambal orange peel exhibits anti-inflammatory effects, attributable to its capacity

to preserve red blood cell stability, as evidenced by the % stability value. The mean percentage stability values of the extract at concentrations of 50 ppm, 100 ppm, 150 ppm, 200 ppm, and 250 ppm are 32.20%, 38.39%, 47.37%, 55.18%, and 65.52%, respectively. The percentage stability values of sodium diclofenac at equivalent concentrations were 50.10%, 58.40%, 66.69%, 78.59%, and 87.70%, respectively. The results demonstrate that increasing concentrations of the ethanol extract from sambal orange peel enhance red blood cell stability, indicating its anti-inflammatory potential. Although sodium diclofenac at 50 ppm showed higher inhibition of hemolysis (50.10%) compared to the extract (32.20%). This study is consistent with the findings of Wahdaniah et al., (2023), who reported that variations in the concentration of jackfruit seed ethanol extract showed no statistically significant difference compared to sodium diclofenac. A concentration of 0.005% sodium diclofenac exhibited an anti-inflammatory inhibition of 55.60%, while the extract demonstrated an inhibition of 41.70%. To analyze the results obtained, statistics were used with a *One-Way ANOVA* test after verifying the assumptions of normality and homogeneity of the data beforehand.

Table 4. Normality Test Results

Concentration	Ethanol extract of orange peel Sambal	Sodium Declofenac
50 ppm	0.595	0.991
100 ppm	0.770	0.848
150 ppm	0.441	0.519
200 ppm	0.825	0.633
250 ppm	0.236	0.934

The statistical analysis of the data presented in the table indicates that all p-values are greater than 0.05, suggests that the data is normally distributed. The homogeneity test data are presented in the subsequent results.

According to the results presented in Table 5, the obtained p values are greater than or equal to 0.05. This indicates that the data obtained are homogeneous. The results of the normality and homogeneity tests indicate that a one-way ANOVA test can be performed.

Table 5. Homogeneity Test Results

Statistical Test	Sig.
Levene Test	0,862

Table 6. One-way ANOVA test results

Statistical Test	Sig.
One-way ANOVA	0,000

The one-way analysis of variance (ANOVA) test yielded a significant p-value of 0.000 < 0.05, thereby indicating a difference between the ethanol extracts of sambal orange peel and sodium declofenac with varying concentrations on the stability of red blood cell membranes. Subsequently, the data obtained were subjected to a post hoc Tukey test to ascertain significant variations among the concentration treatment groups. The Tukey test aims to identify significant differences in concentrations (Table 7).

The Tukey test results showed significant differences between treatment groups with a significance value (p>0.05) except at a concentration of 250 ppm of sambal orange peel ethanol extract and 150 ppm of sodium diclofenac. These findings suggest that the ethanol extract of sambal orange peel possesses potential as an anti-inflammatory agent, as its stability percentage does not differ significantly from that of the positive control, sodium diclofenac. This assertion is corroborated by statistical analysis, wherein the extract at a concentration of 250 ppm (65.52%) exhibited a significance value of 0.686 (p > 0.05) in comparison to sodium diclofenac at a concentration of 150 ppm (66.69%). This phenomenon can be attributed to the presence of chemical compounds, including flavonoids, alkaloids, tannins, saponins, steroids, and essential oils, within the ethanol extract of sambal orange peel. These compounds act synergistically, contributing to the maintenance of red blood cell membrane stability.

The stability percentage was subsequently determined, and the IC_{50} of the extract and sodium diclofenac was calculated to facilitate a comparison of the anti-inflammatory activity of the ethanol extract of sambal orange peel and sodium diclofenac. The concentration required to inhibit 50% of inflammation is called the IC_{50}. The IC_{50} value was obtained by plotting the stability percentage results against

Table 7. Results of Tukey's Post Hoc Test

(I) Treatment	(J) Treatment	Mean Differeance	Std. Error	Sig.	Lower Bound	Upper Bound
EKJS 250 ppm	ND 150 ppm	-1.17200	0.62557	0.686	-3.2663	0.9223

concentration to obtain a linear equation, which was then used to calculate the IC_{50} value. The ensuing results of the curve relationship are displayed.

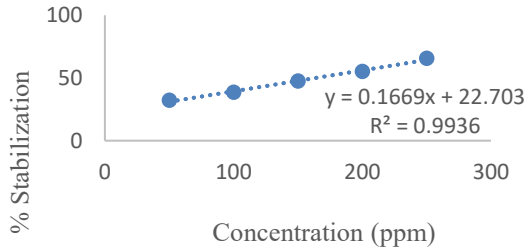

Figure 2: Linear Regression Curve of Sambal Orange Peel Extract

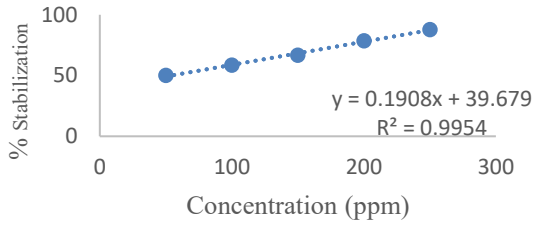

Figure 3. Linear Regression Curve of Sodium Declofenac

The findings of the linear regression equation for Sambal Orange Peel ethanol extract, as depicted in Figure 2, yield the following equation: y = 0.1669x + 22.703, with an R^2 value of 0.9936. Similarly, the linear regression equation results for sodium diclofenac, as illustrated in Figure 3, yield the following equation: y = 0.1908x + 39.679, with an R^2 value of 0.9954. The R^2 values for the extract and sodium diclofenac, which approximate +1, suggest a direct correlation between increasing concentrations of the extract/sodium diclofenac and enhanced anti-inflammatory activity.

Table 8. IC_{50} values for erythrocyte stability of ethanol extract of sambal orange peel and sodium diclofenac.

Inhibision Concentration	Extract (ppm)	Sodium Declofenac (ppm)
IC_{50}	163.55	54.09

Table 8, the IC_{50} values for the extract and sodium declofenac were determined to be 163.55 ppm and 54.09 ppm, respectively. These results are consistent with the findings of Kurnia et al., (2019), who reported that the IC_{50} value of diclofenac sodium was 54.149 ppm. The IC_{50} value is inversely proportional to its anti-inflammatory activity, meaning lower IC_{50} value corresponds to greater anti-inflammatory activity. The IC_{50} values are then classified into five categories: highly active (<50 ppm), active (50–100 ppm), moderate (101–250 ppm), weak (250–500 ppm), and inactive (>500 ppm) (Kurnia et al. 2019). According to the values enumerated in Table 8, the ethanol extract of sambal orange peel is classified as moderate, while sodium diclofenac is designated as active. The ethanol extract of sambal orange peel has been shown to possess anti-inflammatory potential, although its effects are within the moderate range. This phenomenon may be attributed to the low relative efficacy of its active compounds, limited bioavailability, or the concentration used not being optimal to produce a significant anti-inflammatory effect (Desmiaty et al., 2024; Rosida et al., 2024).

The mechanism of action of extracts and standard anti-inflammatory drugs is believed to be associated with their ability to bind to erythrocyte membranes, leading to alterations in the surface charge of the cells. This effect is achieved by combining agents or promoting the spread of charge repulsion reactions, as in red blood cell hemolysis. The anti-inflammatory mechanism of ethanol extracts from sambal orange peel involves the activity of flavonoids, tannins, alkaloids, triterpenoids, and saponins (Khotimah and Muhtadi 2016). It has been documented that the anti-inflammatory properties of flavonoids may manifest through various mechanisms, including the suppression of cyclooxygenase (COX) or lipoxygenase enzyme activity. Inhibition of the COX and lipoxygenase pathways directly inhibits the biosynthesis of eicosanoids and leukotrienes, which are the end products of the COX and lipoxygenase pathways (Suriyeni et al., 2024). Furthermore, it has been demonstrated to impede the accumulation of leukocytes in inflamed regions, thereby diminishing leukocyte adhesion to the endothelium and consequently attenuating the body's inflammatory response. Flavonoids have also been shown to inhibit neutrophil degranulation, thereby directly reducing these cells release of arachidonic acid. The anti-inflammatory properties of flavonoids are supported by their capacity to act as antihistamines (Jakimiuk et al., 2021). Flavonoids have been demonstrated to possess the capacity to impede the release of histamine from mast cells. Flavonoids have been shown to inhibit the enzyme c-AMP phosphodiesterase, thereby increasing c-AMP levels

in mast cells. This, in turn, prevents calcium from entering the cells and consequently prevents histamine release (Khotimah and Muhtadi 2016).

Polyphenols have been extensively documented to possess anti-inflammatory properties mediated through multiple mechanisms (Randenia et al., 2024). These mechanisms include scavenging reactive oxygen species (free radicals) that contribute to oxidative stress, regulating cellular signaling pathways within inflammatory cells, and modulating key enzymes involved in the synthesis of inflammatory mediators (Yahfoufi et al., 2018) (Hussain et al., 2016). Specifically, polyphenols influence enzymes related to arachidonic acid metabolism, such as phospholipase A2—which releases arachidonic acid from membrane phospholipids—and cyclooxygenase (COX), which is responsible for prostaglandin synthesis. They also affect enzymes involved in arginine metabolism, notably nitric oxide synthase (NOS), which regulates nitric oxide production (Hussain et al., 2016). Moreover, polyphenols modulate the biosynthesis and release of various proinflammatory molecules, attenuating the inflammatory response (Hussain et al., 2016).

The mechanism of alkaloids as anti-inflammatory agents involves inhibiting the release of histamine mediators and suppressing the formation of prostaglandins and leukotrienes (Nugraha et al., 2022). The following compound is tannin, inhibiting endothelial cells and nitric oxide (NO) to maintain vascular tone and prevent vasodilation (Sari et al. 2024). A multitude of studies have indicated an association between triterpenoids and membrane stability. Triterpenoids from Ligustrum have been shown to possess the capacity to impede the catalytic activity of cyclooxygenase enzymes, which are responsible for the conversion of arachidonic acid into prostaglandins, a class of inflammatory mediators (Hasim et al., 2019).

CONCLUSION

The results showed that the extract had an antioxidant IC_{50} value of 17.90 ppm, which is very strong. Membrane stability at concentrations of 50, 100, 150, 200, and 250 ppm showed increases of 32.20%, 38.39%, 47.37%, 55.18%, and 65.52%, respectively. The % stability values of sodium diclofenac at the same concentrations were 50.10%, 58.40%, 66.69%, 78.59%, 87.70%, respectively. The one-way ANOVA test showed a significance value of 0.000 ($p < 0.05$), indicating a significant difference between treatments. The Tukey post-hoc test showed no significant difference between the 250 ppm extract concentration and the 150 ppm sodium diclofenac concentration. The IC_{50} value for the extract's anti-inflammatory activity was 163.55 ppm (moderate activity), while sodium diclofenac exhibited stronger activity with an IC_{50} value of 54.09 ppm.

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