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Antiinflammatory Activity of Methanolic Extract of *Alpinia monopleura* Rhizome In Vitro Using The HRBC Membrane Stabilization Method

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ABSTRACT

***Intoduction**: Alpinia monopleura, a member of the Zingiberaceae family, is a plant species found in Southeast Sulawesi and has been traditionally used to alleviate muscle soreness. The rhizome contains bioactive compounds, including phenolics and flavonoids, which are believed to contribute to its anti-inflammatory activity. **Aim**: This study aimed to evaluate the anti-inflammatory activity of the methanolic extract of Alpinia monopleura rhizome. The anti-inflammatory activity was assessed in vitro using the Human Red Blood Cell (HRBC) membrane stabilization method, based on the extract's ability to protect erythrocyte membranes against hypotonic solution-induced hemolysis. **Result**: The results demonstrated that the methanolic extract of Alpinia monopleura rhizome exhibited significant anti-inflammatory activity, as evidenced by its ability to stabilize red blood cell membranes. An increase in extract concentration corresponded to an increase in membrane stabilization. At the highest concentration tested, the methanolic extract of Alpinia monopleura rhizome showed 84.352 ± 0.024% membrane stabilization, which was comparable to that of diclofenac sodium 88.164 ± 0.046%.. **Conclusion**: These findings suggest that the methanolic extract of Alpinia monopleura rhizome has promising potential for development as a natural anti-inflammatory agent.*

***KEYWORDS**: Alpinia, anti-inflammatory, human red blood cell, zingiberaceae, in vitro.*

INTRODUCTION

The Zingiberaceae family represents a diverse group of flowering plants comprising more than 150 species distributed across Southeast Asia and other tropical regions, and they play important roles in ecology, culture, and traditional medicine (Boonma et al., 2023). One of the largest genera within this family is *Alpinia*, which includes approximately 500 species and is widely recognised as an ornamental plant, spice, and traditional medicinal resource used in the management of inflammation, hyperlipidaemia, and cancer (Youn et al., 2024). One species found in Southeast Sulawesi is *Alpinia monopleura*.

Alpinia monopleura is an endemic species in Sulawesi and is relatively easy to find (Rugayah, 2019). Local communities have empirically used it to relieve muscular discomfort (Yodha et al., 2023). Muscular discomfort is associated with inflammatory processes in muscle tissue, characterised by the release of inflammatory mediators (Sulistyarto et al., 2022). Uncontrolled inflammatory responses are major contributors to various disorders, including allergies, cardiovascular dysfunction, metabolic syndrome, cancer, and autoimmune diseases; therefore, anti-inflammatory agents are required to suppress or modulate the progression of inflammation (Aidoo et al., 2021; Phull et al., 2022).

Various *Alpinia* species are known to contain bioactive compounds, including diarylheptanoids, terpenoids, flavonoids, and phenolics, which contribute to their pharmacological activities, including anti-inflammatory, antihyperlipidaemic, anticancer, antioxidant, and antimicrobial effects (Youn et al., 2024). *A. monopleura* has been reported to contain flavonoids such as glycitein and nevadensin, as well as phenolic compounds including apocynin and *E*-p-coumaric acid (Wahyuni et al., 2025; Yodha et al., 2024). Apocynin and coumarin derivatives have been shown to exert anti-inflammatory effects by modulating inflammatory signalling pathways (Hwang et al., 2019; Rostom et al., 2022). Furthermore, flavonoid and phenolic compounds are widely recognised for their anti-inflammatory mechanisms, including the inhibition of pro-inflammatory mediators and modulation of oxidative stress (Mir & Masoodi, 2020).

Flavonoid and phenolic compounds are known to exhibit anti-inflammatory activity through the stabilization of lysosomal and erythrocyte membranes, thereby inhibiting the release of inflammatory mediators and tissue damage (Efimova & Ostroumova, 2023). Based on this scientific background, the present study aimed to evaluate the anti-inflammatory activity of the methanolic extract of *A. monopleura* rhizome using the HRBC membrane stabilization method.

MATERIAL AND METHODS

Materials

The in vitro anti-inflammatory activity of the extract was evaluated developed using the HRBC membrane stabilization method according to the procedure described by Gandhidasan *et al* (Yesmin et al., 2020). The materials used in this study included rhizomes of *A. monopleura*, aluminium foil, fresh human red blood cells, methanol, dextrose, sodium chloride (NaCl), disodium hydrogen phosphate dihydrate ($Na_2HPO_4 \cdot 2H_2O$), distilled water, sodium dihydrogen phosphate monohydrate

($NaH_2PO_4 \cdot H_2O$), phosphate buffer (0.15 M, pH 7.4), and diclofenac sodium.

Preparation of Phosphate Buffer

Phosphate buffer (0.15 M, pH 7.4) was prepared by dissolving 13.35 g of Na_2HPO_4 $2H_2O$ and 4.14 g of $NaH_2PO_4 \cdot H_2O$ separately in distilled water to a final volume of 500 mL. Subsequently, 80.8 mL of Na_2HPO_4 solution and 19.2 mL of NaH_2PO_4 solution were mixed at room temperature. The pH was measured using a pH meter, and the solution was sterilised in an autoclave at 121°C for 15-20 minutes used autoclave (Fristiohady et al., 2020).

Preparation of Isosaline and Hyposaline Solutions

Isosaline solution was prepared by dissolving 0.85 g of NaCl in phosphate buffer (0.15 M, pH 7.4) to a final volume of 250 mL, and the solution was sterilised at 121°C for 15-20 minutes in an autoclave. Hyposaline solution was prepared by dissolving 0.36 g of NaCl in phosphate buffer (0.15 M, pH 7.4) to a final volume of 100 mL, then sterilised under the same conditions (Fristiohady et al., 2020).

Preparation of Red Blood Cell Suspension

Fresh blood was mixed with sterile solution as an anticoagulant. The mixture was centrifuged at 3000 rpm for 10 minutes, and the packed cells were washed three times with isosaline solution. The supernatant was discarded, and the erythrocytes were reconstituted to obtain a 10% v/v suspension in isosaline (Fristiohady et al., 2020).

Preparation of Positive Control

Fifty milligrams of diclofenac sodium were dissolved in phosphate-buffered saline (PBS) and made up to 50 mL to obtain a stock solution of 1000 mg/L. Serial dilutions were prepared to yield concentrations of 6.25, 12.5, 25, 50, and 100 mg/L.

Anti-inflammatory Assay (HRBC Membrane Stabilization)

The test solutions, positive control, and negative control were incubated at 56°C and subsequently centrifuged at 5000 rpm for 15 minutes. The supernatant was collected, and haemoglobin content was measured using a microplate reader at 560 nm (Yesmin et al., 2020).

Data Analysis

The percentage of haemolysis and membrane stabilization was calculated using standard formulae.

$$\%\ Haemolysis\ = \frac{Absorbance\ of\ sample}{Absorbance\ of\ negative\ controle}\ x\ 100\%$$

$$\%Stability = 1 - \frac{Absorbance\ of\ sample}{Absorbance\ of\ negative\ controle} x\ 100\%$$

The result expressed as mean ± SD and the statistical analysis used paired T-test. Result considered to be significant when p-values were less than 0.05 ($p < 0.05$)

RESULTS AND DISCUSSION

The anti-inflammatory activity in this study was evaluated using the HRBC membrane stabilization method. This method measures the ability of a test compound to stabilise erythrocyte membranes and prevent lysis under hypotonic stress conditions. Stabilization of the erythrocyte membrane is considered analogous to lysosomal membrane stabilization in vivo, thereby preventing the release of inflammatory mediators (Pandey, 2024).

In the HRBC assay, isosaline solution serves as the control because it has an osmotic pressure comparable to physiological conditions and does not induce haemolysis, thereby ensuring membrane stability. In contrast, hyposaline solution creates a hypotonic environment, leading to erythrocyte swelling and subsequent haemolysis (Yesmin et al., 2020). The Human Red Blood Cell (HRBC) membrane stabilization assay is widely recognised as a reliable in vitro model for screening potential anti-inflammatory agents, especially natural products. This method is predicated on the concept that erythrocyte membranes are analogous to lysosomal membranes in structure and composition. That stabilization against hypotonic or stress-induced haemolysis reflects a compound's capacity to prevent the release of inflammatory mediators from cellular organelles (Fujiati et al., 2022).

In the HRBC model, exposure to hypotonic solutions induces membrane swelling and haemolysis, measured spectrophotometrically as haemoglobin release; compounds that reduce haemolysis are interpreted as exerting membrane-protective anti-inflammatory effects. Several recent studies have applied this method to evaluate plant extracts and phytochemical that demonstrate significant HRBC membrane stabilization, supporting their anti-inflammatory potential (Pangemanan et al., 2024).

Under hypotonic conditions, erythrocytes undergo osmotic swelling, leading to membrane rupture and haemoglobin release. The degree of haemolysis can be quantitatively measured spectrophotometrically and serves as an indicator of membrane integrity. Anti-inflammatory agents reduce haemolysis by protecting membrane proteins and phospholipids from osmotic and oxidative damage. Non-steroidal anti-inflammatory drugs (NSAIDs), such as diclofenac sodium, have been reported to inhibit hypotonicity-induced haemolysis, supporting the validity of this model as a screening tool for anti-inflammatory compounds (Aidoo *et al*., 2021).

The protective as a screening tool for anti-inflammatory compounds (Aidoo *et al*., 2021). The protective effect is believed to involve interactions with membrane

Table 1. Percentage stability of diclofenac sodium and methanol extract *of A. monopleura* rhizome

Sample	Conc. (mg/L)	Abs			%Stabilization			%Stabilization (Mean ± SD)
Alpinia	6.25	0.465	0.472	0.473	67.330	66.866	66.732	66.976 ± 0.256
	12.5	0.451	0.458	0.459	68.335	67.836	67.737	67.969 ± 0.261
	25	0.387	0.393	0.393	72.825	72.417	72.417	72.553 ± 0.192
	50	0.248	0.247	0.248	82.586	82.614	82.572	82.591 ± 0.018
	100	0.222	0.223	0.223	84.385	84.343	84.329	84.352 ± 0.024
Sodium	6.25	0.332	0.335	0.335	76.683	76.444	76.451	76.526 ± 0.111
Diclofenac	12.5	0.280	0.273	0.272	80.274	80.752	80.885	80.637 ± 0.262
	25	0.177	0.179	0.178	87.554	87.421	87.484	87.487 ± 0.055
	50	0.170	0.172	0.177	88.018	87.878	87.505	87.800 ± 0.216
	100	0.167	0.169	0.168	88.222	88.110	88.159	88.164 ± 0.046

Table 2. Percentage haemolysis of diclofenac sodium and methanol extract *of A. monopleura* rhizome

Sample	Conc. (mg/L)	Abs			%Hemolysis			%Hemolysis (Mean ± SD)
Alpinia	6.25	0.465	0.472	0.473	32.670	33.134	33.268	33.024 ± 0.256
	12.5	0.451	0.458	0.459	31.665	32.164	32.263	32.031 ± 0.261
	25	0.387	0.393	0.393	27.175	27.583	27.583	27.447 ± 0.192
	50	0.248	0.247	0.248	17.414	17.386	17.428	17.409 ± 0.018
	100	0.222	0.223	0.223	15.615	15.657	15.671	15.648 ± 0.024
Sodium	6.25	0.332	0.335	0.335	23.317	23.556	23.549	23.474 ± 0.111
Diclofenac	12.5	0.280	0.273	0.272	19.726	19.248	19.115	19.363 ± 0.262
	25	0.177	0.179	0.178	12.446	12.579	12.516	12.513 ± 0.055
	50	0.170	0.172	0.177	11.982	12.122	12.495	12.200 ± 0.216
	100	0.167	0.169	0.168	11.778	11.890	11.841	11.836 ± 0.046

surface charges, inhibition of calcium influx, and prevention of protein denaturation.

Furthermore, plant-derived polyphenols and flavonoids have demonstrated membrane-stabilising activity in the HRBC model. These compounds may exert their effects through antioxidant mechanisms, including scavenging reactive oxygen species, chelating metal ions, and preserving membrane-bound enzymes, thereby reducing erythrocyte fragility (Mir & Masoodi, 2020). Since oxidative stress is closely linked to the progression of inflammation, the membrane-protective effect observed in the HRBC assay is considered mechanistically relevant. Consequently, a concentration-dependent increase in membrane stabilization, accompanied by reduced haemolysis, is interpreted as evidence of potential anti-inflammatory activity. The percentage of membrane stabilization and percentage of haemolysis for diclofenac sodium and the methanol extract of *A. Monopleura* rhizomes are presented in Table 1 and Table 2. Increasing

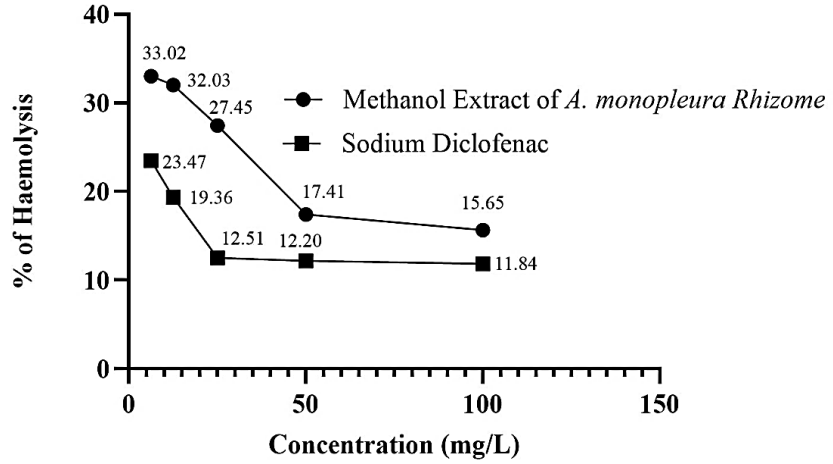

Figure 1. Percentage of stability

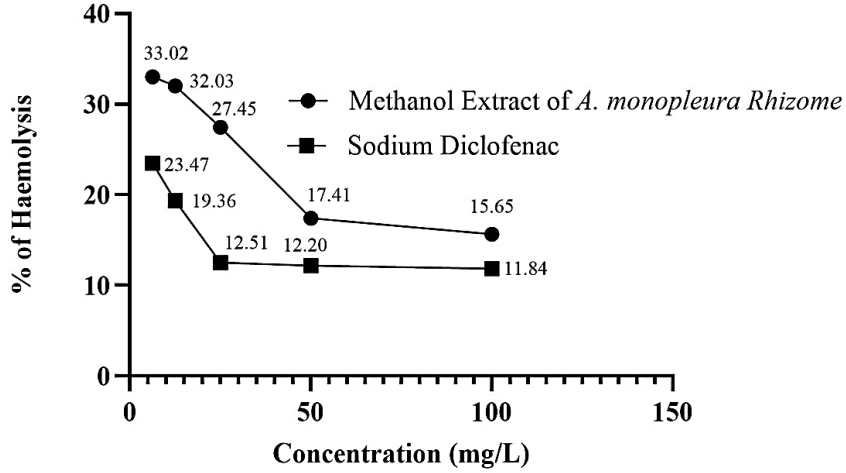

Figure 2. Percentage of haemolysis

The percentage of membrane stabilization and percentage of haemolysis for diclofenac sodium and the methanol extract of *A. Monopleura* rhizomes are presented in Table 1 and Table 2. Increasing concentrations led to greater membrane stabilization and less haemolysis in both groups. At concentrations of 12.5 mg/L and 25 mg/L, diclofenac sodium demonstrated membrane stabilization values of 80.637% and 87.487%, respectively, whereas the methanol extract of *A. monopleura* rhizome showed values of 67.969% and 72.553%. At higher concentrations (50 mg/L and 100 mg/L), the difference between the two groups narrowed, with diclofenac sodium reaching 87.800% and 88.164%, while the methanol extract of *A. monopleura* rhizome achieved 82.591% and 84.352%. The comparison of the percentage stability between Diclofenac sodium and the methanol extract of *A. Monopleura* rhizome is presented in Figure 1.

The haemolysis percentage decreased progressively with increasing concentration. At 6.25 mg/L, diclofenac sodium produced 23.474% haemolysis, while the methanol extract of *A. monopleura* rhizome showed 33.024%. At 100 mg/L, haemolysis decreased to 11.836% for

diclofenac sodium and 15.648% for the methanol extract of *A. monopleura* rhizome. The comparison of the percentage stability between Diclofenac sodium and the methanol extract of *A. Monopleura* rhizome is presented in Figure 2. Lower haemolysis values at higher concentrations indicate improved membrane protection and reduced erythrocyte damage. This effect may be associated with membrane saturation mechanisms or molecular aggregation at higher concentrations, thereby reducing direct interaction with the erythrocyte membrane (Pandey, 2024).

Collectively, the HRBC membrane stabilization method provides mechanistic insight into the ability of test compounds to prevent membrane disruption and subsequent release of inflammatory mediators. Although it does not fully replicate the complexity of in vivo inflammation, it remains a reliable and widely accepted preliminary model for evaluating anti-inflammatory properties of synthetic drugs and natural products.

CONCLUSION

The methanolic extract of *A. monopleura* rhizome exhibited in vitro anti-inflammatory activity by stabilising erythrocyte membranes. At the highest tested concentration, the methanolic extract of *A. monopleura* rhizome achieved 84.352 ± 0.024% membrane stabilization, approaching that of diclofenac sodium 88.164 ± 0.046%. These findings suggest that the methanolic extract of *A. monopleura* rhizome has promising anti-inflammatory potential.

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