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In Vitro Evaluation of Antioxidant and Anti-Inflammatory Activities of Ethanolic Leaf Extract of *Syzygium polyanthum*

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ABSTRACT

Bay leaf (*Syzygium polyanthum* (Wight) Walp.) has traditionally been used for conditions associated with oxidative stress and inflammation. To date, few studies have evaluated antioxidant and anti-inflammatory activities jointly in vitro. This study examined the antioxidant and anti-inflammatory activities of the ethanolic leaf extract of *Syzygium polyanthum*, prepared by maceration with 96% ethanol. Antioxidant activity was assessed using 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonate) (ABTS), nitric oxide (NO) radical-scavenging, and ferric reducing antioxidant power (FRAP) assays. Anti-inflammatory activity was evaluated using red blood cell membrane stabilization against heat- and hypotonic-induced hemolysis and inhibition of protein denaturation. Total phenolic content (TPC) and total flavonoid content (TFC) were also determined. The extract contained 89.443 ± 1.201 mg GAE/g of total phenolics and 171.637 ± 1.016 mg QE/g of total flavonoids. It exhibited strong to very strong radical-scavenging activity, with IC_{50} values of 57.446 ± 0.394 µg/mL for DPPH, 36.750 ± 0.354 µg/mL for ABTS, and 58.853 ± 0.233 µg/mL for NO. FRAP reducing capacity increased with concentration, reaching 35.826 ± 1.182 mg QE/g at 100 µg/mL. At 100 µg/mL, the extract inhibited heat-induced hemolysis by 75.133%, hypotonic-induced hemolysis by 68.310%, and protein denaturation by 65.839%. The extract exhibited radical-scavenging activity, reducing capacity, and anti-inflammatory effects in vitro, supporting its potential as a natural source of bioactive compounds. Further studies are needed to assess its safety and effects in vivo.

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ABSTRAK

Daun salam (*Syzygium polyanthum* (Wight) Walp.) secara tradisional digunakan untuk kondisi yang berkaitan dengan stres oksidatif dan inflamasi. Masih sedikit penelitian yang menguji aktivitas antioksidan dan antiinflamasi secara bersamaan melalui uji *in vitro*. Penelitian ini mengkaji aktivitas antioksidan dan antiinflamasi ekstrak etanol daun *Syzygium polyanthum*, yang diperoleh melalui maserasi menggunakan etanol 96%. Aktivitas antioksidan diuji menggunakan metode 1,1-difenil-2-pikrilhidrazil (DPPH), 2,2′-azino-bis(3-etilbenzotiazolin-6-sulfonat) (ABTS), penangkapan radikal oksida nitrat (NO), dan *ferric reducing antioxidant power* (FRAP). Aktivitas antiinflamasi dievaluasi melalui stabilisasi membran sel darah merah terhadap hemolisis akibat panas dan kondisi hipotonik serta penghambatan denaturasi protein. Kadar fenolik total (TPC) dan flavonoid total (TFC) juga ditentukan. Ekstrak memiliki kadar fenolik total sebesar 89,443 ± 1,201 mg GAE/g dan kadar flavonoid total sebesar 171,637 ± 1,016 mg QE/g. Ekstrak menunjukkan aktivitas penangkapan radikal kuat hingga sangat kuat, dengan nilai IC_{50} sebesar 57,446 ± 0,394 µg/mL pada DPPH, 36,750 ± 0,354 µg/mL pada ABTS, dan 58,853 ± 0,233 µg/mL pada NO. Kapasitas reduksi FRAP meningkat seiring peningkatan konsentrasi, mencapai 35,826 ± 1,182 mg QE/g pada 100 µg/mL. Pada konsentrasi 100 µg/mL, ekstrak menghambat hemolisis akibat panas sebesar 75,133%, hemolisis akibat kondisi hipotonik sebesar 68,310%, dan denaturasi protein sebesar 65,839%. Ekstrak menunjukkan aktivitas penangkapan radikal, kapasitas reduksi, dan efek antiinflamasi secara *in vitro*, sehingga berpotensi sebagai sumber alami senyawa bioaktif. Penelitian lanjutan diperlukan untuk menilai keamanan dan efeknya secara *in vivo*.
Kata Kunci: *Syzygium polyanthum*; DPPH; ABTS; FRAP; penangkapan *nitric oxide*; stabilisasi membran sel darah merah

1. Introduction

Non-communicable diseases (NCDs) are responsible for approximately 74% of deaths worldwide, with cardiovascular diseases, cancer, chronic respiratory diseases, and diabetes mellitus being the major contributors [1]. Oxidative stress and inflammation are closely linked mechanisms involved in the development of these chronic conditions. Owing to their high reactivity, excessive levels of reactive oxygen species (ROS) and reactive nitrogen species (RNS) can damage proteins, lipids, and DNA. Oxidative stress develops when the generation of these reactive species exceeds the capacity of antioxidant defenses, resulting in redox imbalance and macromolecular damage [2], [3].

Antioxidants protect against oxidative damage through radical scavenging, metal chelation, and enzymatic mechanisms [4]. Therefore, natural antioxidants are widely studied to support endogenous defenses and reduce oxidative stress. Medicinal plants are promising sources due to their phytochemical content, which has also been reported to exhibit other biological activities, including detoxification potential against mycotoxin exposure [5].

Inflammation occurs in response to infection or tissue damage and is characterized by redness, warmth, swelling, and pain. If uncontrolled, it may contribute to chronic diseases such as arthritis, atherosclerosis, and cancer [6]. Although steroidal and non-steroidal anti-inflammatory drugs are commonly used to treat inflammation, prolonged use may cause adverse effects [7]. This has encouraged research into natural products as potential anti-inflammatory agents, particularly in regions where traditional medicine remains widely practiced, including Asia and Africa.

In Indonesia, *Syzygium polyanthum* is traditionally used to manage diabetes, hypertension, hyperuricemia, gastrointestinal disorders, and inflammation. Its leaves contain phenolics, flavonoids, tannins, alkaloids, and saponins, which have been linked to antioxidant and anti-inflammatory activities [8]. Ethanolic leaf extracts have also shown antifungal activity [9]. Phenolics and flavonoids may exert antioxidant effects by scavenging free radicals, while flavonoids and tannins have been associated with membrane stabilization and inhibition of protein denaturation [10].

However, most previous studies on *S. polyanthum* have evaluated its antioxidant activity using a single or limited number of assays; for example, an earlier study combined phytochemical profiling with antidiabetic and radical-scavenging assays but did not extend the evaluation to anti-inflammatory endpoints [8]. Anti-inflammatory activity has mainly been reported in in vivo studies of a related species, *Syzygium myrtifolium* [7], whereas in vitro studies investigating the underlying mechanisms in *S. polyanthum* remain limited. Furthermore, antioxidant assays are based on different reaction principles, including electron transfer, hydrogen-atom transfer, and reducing capacity, which may lead to different results for the same extract [11], [12]. To date, the antioxidant and anti-inflammatory properties of a single *S. polyanthum* leaf extract have not been evaluated together using four antioxidant assays (DPPH, ABTS, FRAP, and nitric oxide scavenging) and in vitro assessments of red blood cell membrane stabilization and inhibition of protein denaturation.

Accordingly, this study evaluated the ethanolic leaf extract of *S. polyanthum* using four complementary antioxidant assays (DPPH, ABTS, nitric oxide scavenging, and FRAP), together with in vitro anti-inflammatory assessments of red blood cell membrane stabilization against heat- and hypotonic-induced hemolysis and inhibition of protein denaturation. Total phenolic content (TPC) and total flavonoid content (TFC) were also determined. This integrated approach provides a broader assessment of the extract's antioxidant capacity and anti-inflammatory potential, accounts for assay-dependent differences, and enables examination of the relationship between these biological activities.

2. Methods

Sample Collection and Plant Determination

Bay leaf samples (*S. polyanthum* (Wight) Walp.) were collected from Poasia District, Anggoeya Village, Kendari City, Southeast Sulawesi. Plant determination was carried out at the Pharmacy Laboratory, Faculty of Pharmacy, Halu Oleo University, by comparing the morphological characteristics of the collected samples with authenticated reference specimens. The identity of the plant material was confirmed under voucher specimen number 615/UN29.18/PP/2024**.**

Preparation of Simplicia

The leaves were wet-sorted to remove damaged parts and impurities, then washed, cut, and oven-dried at 50 °C for 48 hours. Drying was controlled to minimize thermal degradation of phenolic and flavonoid compounds. Dry sorting was then performed to ensure material quality. The dried leaves were powdered and stored in a closed container prior to extraction . Mature leaves were selected based on dark green color, thick texture, and position below young leaves [13].

Preparation of Ethanolic Extract

Powdered simplicia (1,000 g) was macerated in 5 L of 96% ethanol. This solvent was chosen for its food-grade status, relatively low toxicity, and high polarity, which facilitate the dissolution of phenolic and flavonoid compounds while limiting the degradation of thermolabile constituents compared with heat-based extraction techniques. Maceration was carried out for 3 × 24 hours with intermittent stirring. The resulting filtrate was concentrated using a rotary vacuum evaporator to produce a viscous extract, which was stored at 4 °C until analysis [14].

Phytochemical Screening

Phytochemical screening was performed to preliminarily identify the classes of secondary metabolites present in the extract, following previously reported procedures [8]. Flavonoids were indicated by red, yellow, or brown coloration after addition of magnesium powder and hydrochloric acid. Alkaloids were indicated by white, brown, or orange precipitates using Mayer, Wagner, and Dragendorff reagents. Phenolics and tannins were indicated by dark green coloration with $FeCl_3$, saponins by stable foam formation, terpenoids by red/yellow coloration, and steroids by blue/purple coloration with acetic and sulfuric acid. This qualitative, color-reaction-based screening indicates the possible presence of certain metabolite classes rather than confirming specific active compounds.

Determination of Total Phenolic and Flavonoid Content

Total phenolic and flavonoid contents were determined using the Folin–Ciocalteu and aluminium chloride colorimetric methods. The extract concentration was 100 µg/mL. For phenolic analysis, the sample was treated with Folin–Ciocalteu reagent and sodium carbonate, then incubated in the dark. Absorbance was recorded at 750 nm using a gallic acid standard curve. For flavonoid analysis, the sample was combined with aluminium chloride, potassium acetate, and distilled water. After 30 minutes, absorbance was recorded at 415 nm using a quercetin standard curve. Results were expressed as mg GAE/g extract for phenolics and mg QE/g extract for flavonoids [15].

Antioxidant Activity Assays

Four antioxidant assays (DPPH, ABTS, nitric oxide scavenging, and FRAP) were performed in triplicate (n = 3). Ascorbic acid (2–10 µg/mL) served as the positive control for DPPH, ABTS, and nitric oxide scavenging, whereas quercetin was used as the reference standard for FRAP. The extract was tested at concentrations of 6.25–100 µg/mL, with a reaction volume of 200 µL per well. Percentage inhibition in the DPPH, ABTS, and nitric oxide scavenging assays was calculated as follows:

$$\% \text{ inhibition} = \frac{A\ control - A\ sample}{A\ control} \times 100,$$

Where:
A control = absorbance of the negative control
A sample = absorbance of the test sample

For each replicate, inhibition (%) was plotted against concentration and fitted using linear regression (y = a + bx). IC_{50} was determined by setting y = 50 in the corresponding equation, and the three replicate-specific values were expressed as mean ± SD.

a) DPPH Radical Scavenging Assay: 0.1 mM DPPH solution in methanol was mixed with sample solutions at different concentrations. The mixtures were incubated in the dark for 30 minutes, and absorbance was measured at 516 nm [15].

b) ABTS Radical Scavenging Assay: Potassium persulfate was used to generate ABTS radical cations. The sample was incubated with the resulting reagent at room temperature for 10 minutes, and absorbance was measured at 734 nm [11].
c) Nitric Oxide Scavenging Assay: Nitric oxide was generated using sodium nitroprusside in a phosphate buffer. The reaction mixture was incubated at 37 °C for 1 hour before being treated with Griess reagent. Absorbance was measured at 540 nm [16].
d) Ferric Reducing Antioxidant Power (FRAP): The FRAP procedure involved combining the sample with phosphate buffer and potassium ferricyanide, followed by incubation and centrifugation. Ferric chloride was then added, and absorbance was recorded at 700 nm. Results were reported as mg quercetin equivalent per gram of extract [17].

Anti-Inflammatory Activity

The anti-inflammatory activity of the extract was evaluated in vitro using red blood cell (RBC) membrane stabilization and protein denaturation inhibition assays, with diclofenac sodium as the positive control [18]. This study was approved by the Health Research Ethics Committee, Faculty of Medicine, Universitas Halu Oleo (No. 032a/UN29.17.1.3/ETIK/2024). All volunteers provided informed consent prior to blood donation, and donor identity was kept confidential throughout the study. This study activity was expressed as percentage inhibition, where values >20% indicate anti-inflammatory potential [19].

a) Red Blood Cell Membrane Stabilization: Anti-inflammatory activity was assessed through heat- and hypotonic-induced hemolysis assays. Following written informed consent, 10 mL of venous blood was collected from one healthy volunteer into sterile EDTA tubes. The volunteer had not taken non-steroidal anti-inflammatory drugs (NSAIDs) for at least 48 h before collection. Blood samples were centrifuged at 2,500 rpm for 5 min, after which the plasma and buffy coat were removed. Erythrocytes were washed three times with isotonic saline and resuspended in phosphate-buffered saline (PBS, pH 7.4) to prepare a 40% (v/v) RBC suspension. The suspension was incubated with the extract at 6.25, 12.5, 25, 50, and 100 µg/mL. Diclofenac sodium at the corresponding concentrations served as the positive control, whereas the negative control contained RBC suspension and all assay reagents except the extract and diclofenac sodium. After heat- or hypotonic-induced hemolysis, the mixtures were centrifuged, and supernatant absorbance was measured at 560 nm using a microplate reader. The percentage inhibition of hemolysis was calculated as follows:

$$\text{Hemolysis inhibition (\%)} = \frac{A\ control - A\ sample}{A\ control} \times 100$$

Where:
*A*control = absorbance of the negative control
*A*sample = absorbance of the test sample

b) Protein Denaturation Inhibition: Egg albumin served as the model protein. The extract (6.25, 12.5, 25, 50, and 100 µg/mL) was combined with egg albumin and phosphate-buffered saline (PBS). Diclofenac sodium at matching concentrations replaced the extract in the positive control, while the negative control contained only egg albumin and PBS. The mixtures were incubated at 37 °C, then heated at 70 °C for 5 min to induce protein denaturation. After cooling to room

temperature, absorbance was measured at 660 nm using a microplate reader. The percentage inhibition of protein denaturation was calculated as follows:

$$\text{Protein denaturation inhibition (\%)} = \frac{A\ control - A\ sample}{A\ control} \times 100$$

Where:
Acontrol = absorbance of the negative control
Asample = absorbance of the test sample

Data Analysis

IC_{50} values for antioxidant activity were obtained from linear regression equations using Microsoft Excel. Anti-inflammatory activity was quantified as percentage inhibition and analyzed with GraphPad Prism 8 (GraphPad Software, San Diego, CA, USA). Results are reported as mean ± standard deviation (SD) from triplicate experiments (n = 3). Data normality was assessed using the Shapiro–Wilk test before statistical analysis. Differences among groups were evaluated by one-way analysis of variance (ANOVA), followed by Tukey's multiple-comparison test. A p-value of less than 0.05 indicated statistical significance.

3. Result and Discussion

Table 1 presents the physical characteristics and extraction yield of the ethanolic extract. The yield was 21%, calculated as the ratio of extract weight to the initial simplicia weight [7].

Table 1. Extraction yield and physical characteristics of the ethanolic leaf extract of *S. polyanthum*.

Simplicia weight (g)	Extract weight (g)	Yield (%)	Extract characteristics		
			Appearance	Color	Odor
1000	210	21%	Thick	Dark green	Typical bay leaf

Phytochemical Screening

Phytochemical analysis of the ethanolic leaf extract of *S. polyanthum* (**Table 2**) revealed flavonoids, alkaloids, phenolics, tannins, saponins, terpenoids, and steroids, consistent with previous reports on its bioactive constituents and anti-inflammatory potential [8]. Identification was based on characteristic color reactions or precipitate formation.

Table 2. Phytochemical screening of the ethanolic leaf extract of *S. polyanthum*.

Secondary metabolite	Test result	Indication
Flavonoids	Red color formation	(+)
Alkaloids (Wagner)	Brown precipitate	(+)
Alkaloids (Mayer)	White precipitate	(+)
Alkaloids (Dragendorff)	Orange precipitate	(+)
Phenolics	Dark green coloration	(+)
Saponins	Stable foam formation	(+)
Tannins	Dark green coloration	(+)
Terpenoids	Red coloration	(+)
Steroids	Purple coloration	(+)

Alkaloid tests with Mayer, Wagner, and Dragendorff reagents produced white, brown, and orange precipitates due to insoluble complex formation with metal ions [20]. Flavonoids showed red coloration, while phenolics and tannins produced a dark green

color from Fe^{3+}–phenolate complex formation; terpenoids and steroids showed red and purple color changes due to reactions with specific reagents targeting unsaturated structures [8]. Saponins generated stable foam, indicating surfactant properties [5]. Overall, these metabolites support the antioxidant and anti-inflammatory potential of the extract.

Total phenolic content (TPC) and Total Flavonoid Content (TFC)

TPC was measured using the Folin–Ciocalteu method with gallic acid as the standard, whereas TFC was assessed by the aluminium chloride colorimetric method using quercetin [21]. In the Folin–Ciocalteu assay, phenolic compounds reduce phosphomolybdate–phosphotungstate complexes under alkaline conditions, forming a blue chromophore proportional to phenolic concentration [22]. Calibration curves at 20, 40, 60, 80, and 100 µg/mL showed good linearity, with R^2 values of 0.9992 for gallic acid and 0.9842 for quercetin (**Figure 1**).

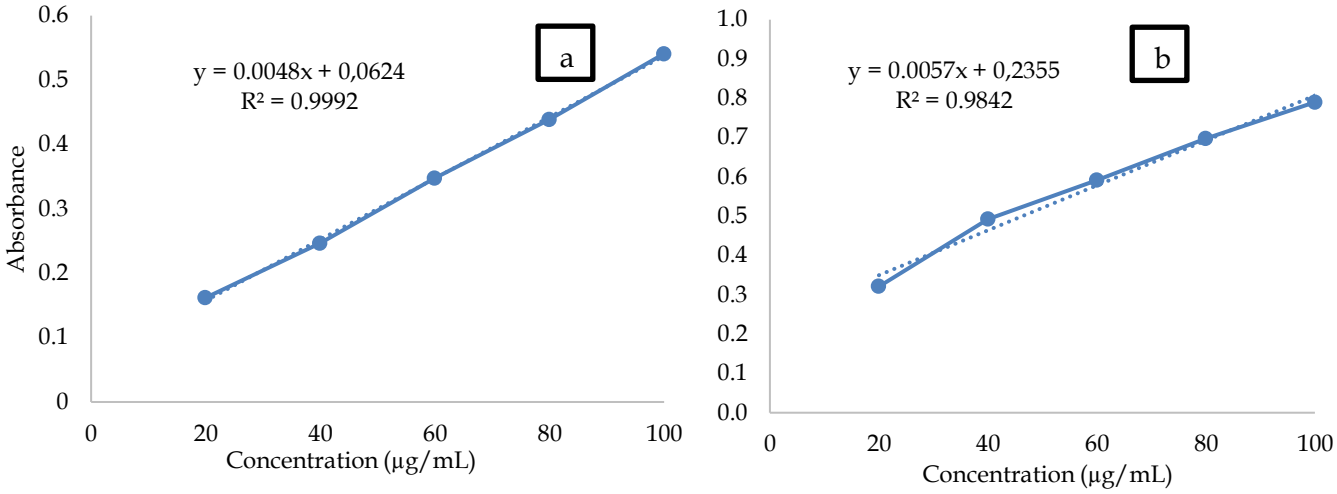


Figure 1. Gallic acid (a) and Quercetin (b) standard calibration curve

The calibration curves were used to calculate TPC and TFC, with the results reported in **Tables 3 and 4.**

Table 3. Total phenolic content of the ethanolic leaf extract of *S. polyanthum*.

Total phenolic content (mg GAE/g)			Mean ± SD (mg GAE/g)	RSD (%)
I	II	III		
88.750	90.830	88.750	89.443 ± 1.201	1.343

The extract had a TPC of 89.443 ± 1.201 mg GAE/g and a TFC of 171.637 ± 1.016 mg QE/g. TPC was determined using the Folin–Ciocalteu assay, which involves an oxidation–reduction reaction that produces a blue chromophore [22]. TFC was determined using the aluminium chloride colorimetric method, which involves complex formation between flavonoids and Al(III) ions. The intensity of the color produced is proportional to flavonoid concentration [21], [22].

Table 4. Total flavonoid content of the ethanolic leaf extract of *S. polyanthum*.

Total flavonoid content (mg QE/g)			Mean ± SD (mg QE/g)	RSD (%)
I	II	III		
171.050	171.050	172.810	171.637 ± 1.016	0.592

Antioxidant activity

These assays provide a broader assessment of antioxidant activity by reflecting distinct radical-scavenging and reducing mechanisms. Antioxidant activity was classified according to IC_{50} values as very strong (<50 µg/mL), strong (50–100 µg/mL), moderate (101–150 µg/mL), or weak (250–500 µg/mL) [23].

DPPH radical scavenging activity

Table 5 presents the IC_{50} values of the ethanolic leaf extract of *S. polyanthum* and ascorbic acid in the DPPH assay.

Table 5. IC_{50} values of ascorbic acid and the ethanolic leaf extract of *S. polyanthum* in the DPPH radical-scavenging assay.

Sample	IC_{50} (µg/mL)			Mean ± SD (µg/mL)	RSD (%)
	I	II	III		
Ascorbic acid (standard)	5.434	5.530	5.467	5.477 ± 0.049	0.891
Extract	57.196	57.901	57.242	57.446 ± 0.394	0.687

Three replicate-specific regression equations were obtained for ascorbic acid: y = 8.4545x + 4.0608 (R^2 = 0.9542), y = 8.6157x + 2.3559 (R^2 = 0.9646), and y = 8.4356x + 3.8815 (R^2 = 0.9627). The mean IC_{50} value was 5.477 ± 0.049 µg/mL. Similarly, three replicate-specific regression equations were obtained for the ethanolic extract: y = 0.7595x + 6.5597 (R^2 = 0.9748), y = 0.7766x + 5.0341 (R^2 = 0.9806), and y = 0.7524x + 6.9312 (R^2 = 0.9806). The mean IC_{50} value was 57.446 ± 0.394 µg/mL. Based on the mean IC_{50} values, ascorbic acid and the ethanolic extract exhibited very strong and strong antioxidant activity, respectively [23].

Both samples demonstrated increased percentage inhibition. For ascorbic acid, inhibition rose from 25.619% at 2 µg/mL to 94.926% at 10 µg/mL, while the ethanolic extract exhibited an increase from 8.886% at 6.25 µg/mL to 79.238% at 100 µg/mL.

DPPH exhibits a characteristic deep-purple color because its unpaired electron is delocalized throughout the molecule in its stable radical form [24]. The assay measures radical-scavenging activity by assessing the reduction in DPPH absorbance following hydrogen-atom or electron donation [25].

ABTS radical scavenging activity

The ABTS radical-scavenging results for the ethanolic leaf extract of *S. polyanthum* and ascorbic acid are summarized in **Table 6.**

Table 6. IC_{50} values for ascorbic acid and the ethanolic leaf extract of *S. polyanthum* in the ABTS radical-scavenging assay.

Sample	IC_{50} (µg/mL)			Mean ± SD (µg/mL)	RSD (%)
	I	II	III		
Ascorbic acid (standard)	4.681	4.606	4.614	4.634 ± 0.041	0.889
Extract	36.951	36.341	36.958	36.750 ± 0.354	0.964

Analysis of the three replicate concentration–response datasets for ascorbic acid yielded the regression equations y = 6.9037x + 17.687 (R^2 = 0.9638), y = 6.8449x + 18.474 (R^2 = 0.9678), and y = 6.7913x + 18.666 (R^2 = 0.9693), with a mean IC_{50} of 4.634 ± 0.041 µg/mL. For the ethanolic extract, the equations were y = 0.5888x + 28.243 (R^2 = 0.9631), y = 0.5781x + 28.991 (R^2 = 0.9537), and y = 0.5749x + 28.753 (R^2 = 0.9564), yielding a mean IC_{50} of 36.750 ± 0.354 µg/mL. According to the IC_{50} classification, both ascorbic acid and the ethanolic extract exhibited very strong antioxidant activity [23].

Stronger ABTS radical-scavenging activity was observed at higher test concentrations. Percentage inhibition for ascorbic acid rose from 36.028% at 2 µg/mL to 91.194% at 10 µg/mL; for the ethanolic extract, the corresponding values were 26.421% at 6.25 µg/mL and 83.668% at 100 µg/mL.

Antioxidant capacity is determined by measuring the reduction of the ABTS radical cation ($ABTS^{\bullet+}$), a process that predominantly occurs through single-electron transfer (SET). Phenolic and flavonoid compounds can donate electrons via hydroxyl groups, reducing $ABTS^{\bullet+}$ and decreasing its blue–green color intensity [12], [26]. Although hydrogen atom transfer (HAT) may occur, electron transfer predominates due to the electrophilic nature of $ABTS^{\bullet+}$ [24]. This method is sensitive, reproducible, and suitable for complex plant extracts [11].

Differences between ABTS and DPPH results may arise from radical characteristics and method limitations. DPPH has a bulky structure that may hinder interaction with some antioxidants, whereas $ABTS^{\bullet+}$ is more accessible and reactive toward both hydrophilic and lipophilic compounds [24].

Nitric oxide (NO) scavenging activity

The NO-scavenging results for the ethanolic leaf extract of *S. polyanthum* and ascorbic acid are summarized in **Table 7**.

Table 7. IC_{50} values for ascorbic acid and the ethanolic leaf extract of *S. polyanthum* in the NO radical-scavenging assay.

Sample	IC_{50} (µg/mL)			Mean ± SD (µg/mL)	RSD (%)
	I	II	III		
Ascorbic acid (standard)	5.392	5.404	5.429	5.408 ± 0.019	0.349
Extract	58.906	59.055	58.598	58.853 ± 0.233	0.396

Regression analysis of the three concentration–response datasets for ascorbic acid gave the equations y = 7.6041x + 8.9986 (R^2 = 0.9636), y = 7.7048x + 8.3614 (R^2 = 0.9643), and y = 7.6962x + 8.2179 (R^2 = 0.9638), with a mean IC_{50} value of 5.408 ± 0.019 µg/mL. For the ethanolic extract, the corresponding equations were y = 0.7799x + 4.059 (R^2 = 0.9758), y = 0.7811x + 3.872 (R^2 = 0.9712), and y = 0.7919x + 3.5965 (R^2 = 0.9716),

yielding a mean IC_{50} value of 58.853 ± 0.233 µg/mL. Based on the mean IC_{50} values, ascorbic acid and the ethanolic extract exhibited very strong and strong antioxidant activity, respectively [23].

In the NO assay, percentage inhibition increased with concentration for both samples. Ascorbic acid inhibition rose from 29.005% at 2 µg/mL to 90.269% at 10 µg/mL, while the ethanolic extract showed values of 4.903% at 6.25 µg/mL and 78.817% at 100 µg/mL.

The assay determines the ability of test compounds to scavenge nitric oxide released from sodium nitroprusside under aerobic conditions. Nitric oxide reacts with oxygen to produce nitrite, which reacts with Griess reagent to form a colored azo compound for detection. A decrease in color intensity indicates reduced nitrite formation and greater NO-scavenging activity. Thus, the assay reflects the scavenging of nitric oxide after its release rather than inhibition of its release from the donor compound [11].

Ferric reducing antioxidant power (FRAP)

The FRAP results for the ethanolic leaf extract of *S. polyanthum* are summarized in **Table 8**.

Table 8. FRAP reducing capacity of the ethanolic leaf extract of *S. polyanthum*.

Sample Concentration (µg/mL)	Reducing Capacity (mg QE/g)			Mean Reducing Capacity (mg QE/g)	SD
	I	II	III		
6.25	2.250	3.818	1.636	2.568	1.125
12.5	8.917	6.545	6.182	7.215	1.485
25	13.083	14.727	10.727	12.486	2.011
50	27.250	29.273	26.182	27.568	1.570
100	34.750	35.636	37.091	35.826	1.182

Both the extract and quercetin standard were tested at concentrations of 6.25–100 µg/mL. The extract's reducing capacity increased from 2.568 ± 1.125 to 35.826 ± 1.182 mg QE/g extract, indicating enhanced electron-donating ability at higher concentrations. This capacity reflects the ability of antioxidant compounds to reduce Fe^{3+} to Fe^{2+} through electron transfer and may be associated with the extract's phenolic and flavonoid constituents [17].

Reducing capacity was reported in terms of quercetin equivalents (QE), where 35.826 mg QE/g extract represents activity equivalent to that of quercetin under the same conditions rather than its actual content [17]. The FRAP assay measures total reducing capacity without identifying individual antioxidant compounds [25].

Direct quantitative comparison with previous studies is limited by differences in extraction methods and antioxidant assay conditions [11], [12], [27]. Nevertheless, the present results corroborate earlier reports of antioxidant activity in *S. polyanthum* leaf extracts [8], [27].

Anti-inflammatory activity

The ethanolic leaf extract of *S. polyanthum* was evaluated for in vitro anti-inflammatory activity using red blood cell (RBC) membrane stabilization and protein denaturation inhibition assays.

Red blood cell membrane stabilization assay

The ethanolic leaf extract of *S. polyanthum* was evaluated in vitro for anti-inflammatory activity using an RBC membrane stabilization assay involving heat- and hypotonic-induced hemolysis. Because erythrocyte and lysosomal membranes are analogous, RBC membrane stabilization reflects protection against lysosomal membrane disruption during inflammation. This stabilization prevents the release of lysosomal enzymes, including proteases and phospholipase A_2, which can promote the formation of inflammatory mediators [28]. Concentrations of 6.25, 12.5, 25, 50, and 100 µg/mL were used for both the extract and diclofenac sodium, which served as the positive control. Diclofenac sodium was selected due to its known ability to stabilize lysosomal membranes and inhibit inflammatory enzyme activity [29].

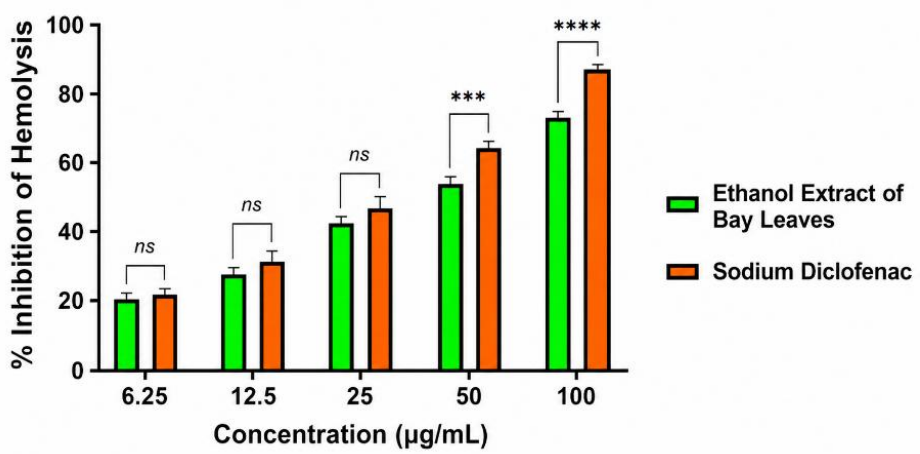
The degree of hemolysis in red blood cell membranes induced by hypotonic solution or heat is used as a parameter to determine anti-inflammatory activity [32]. **Table 9** presents the results of the heat-induced erythrocyte hemolysis assay.

Table 9. Percentage inhibition of heat-induced erythrocyte hemolysis by the ethanolic leaf extract of *S. polyanthum* and diclofenac sodium at various concentrations.

Group	Concentration (µg/mL)	Absorbance (Mean ± SD)	% Inhibition (Mean ± SD)
Negative Control	-	0.752 ± 0.001	0.000 ± 0.132
Extract	6.25	0.596 ± 0.010	20.789 ± 0.334****
	12.5	0.530 ± 0.002	29.477 ± 0.503****
	25	0.436 ± 0.002	42.066 ± 0.427****
	50	0.324 ± 0.009	56.915 ± 0.703****
	100	0.187 ± 0.001	75.133 ± 0.579****
Diclofenac Sodium	6.25	0.590 ± 0.004	21.498 ± 0.203****
	12.5	0.510 ± 0.005	32.137 ± 1.199****
	25	0.410 ± 0.003	45.523 ± 0.276****
	50	0.270 ± 0.003	64.096 ± 0.351****
	100	0.082 ± 0.002	89.140 ± 0.203****

*Note: Data were analyzed using one-way ANOVA followed by Tukey's multiple comparisons test. Values of $p < 0.05$ were considered statistically significant. ** $p < 0.0001$ compared with the negative control.*

Percentage inhibition of erythrocyte hemolysis by the ethanolic leaf extract of *S. polyanthum* increased from 20.789% at 6.25 µg/mL to 75.133% at 100 µg/mL. One-way ANOVA followed by Tukey's multiple-comparisons test revealed significant differences between extract-treated groups and the negative control at all concentrations ($p < 0.05$), supporting membrane-stabilizing activity. Diclofenac sodium produced greater inhibition, particularly at the highest concentration; however, the extract demonstrated comparable activity at various concentrations (**Figure 2**).



*Note: Data are presented as mean ± SD (n = 3). Analysis was performed using one-way ANOVA followed by Tukey's multiple comparisons test. Note: ns = not significant; *** p < 0.001; **** p < 0.0001 (same concentration).*

Figure 2. Average percentage inhibition of heat-induced erythrocyte hemolysis by the ethanolic leaf extract of *S. polyanthum* and diclofenac sodium at various concentrations.

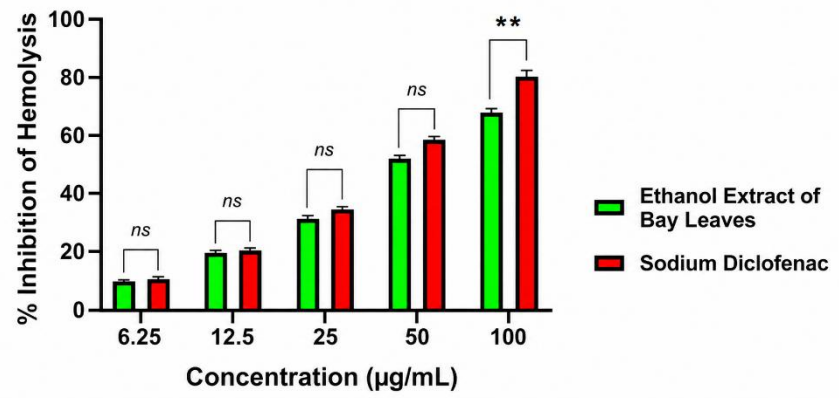
Results of the hypotonicity-induced erythrocyte hemolysis assay are presented in **Table 10**.

Table 10. Percentage inhibition of hypotonicity-induced erythrocyte hemolysis by the ethanolic leaf extract of *S. polyanthum* and diclofenac sodium at various concentrations.

Group	Concentration (µg/mL)	Absorbance (Mean ± SD)	% Inhibition (Mean ± SD)
Negative Control	-	0.520 ± 0.001	0.000 ± 0.221
Extract	6.25	0.467 ± 0.001	10.243 ± 0.293**
	12.5	0.416 ± 0.004	20.166 ± 0.800****
	25	0.353 ± 0.003	32.202 ± 0.692****
	50	0.246 ± 0.004	52.817 ± 0.948****
	100	0.165 ± 0.007	68.310 ± 1.450****
Diclofenac Sodium	6.25	0.463 ± 0.004	11.140 ± 0.947***
	12.5	0.412 ± 0.003	20.935 ± 0.679****
	25	0.337 ± 0.002	35.339 ± 0.588****
	50	0.212 ± 0.005	59.347 ± 1.065****
	100	0.101 ± 0.010	80.538 ± 1.983****

*Note: Data were analyzed using one-way ANOVA followed by Tukey's multiple comparisons test (p < 0.05). Note: ** p < 0.01; *** p < 0.001; **** p < 0.0001 vs negative control.*

Under hypotonic conditions, the ethanolic leaf extract of *S. polyanthum* inhibited erythrocyte hemolysis, with inhibition rising from 10.243% at 6.25 µg/mL to 68.310% at 100 µg/mL. At all tested concentrations, the results differed significantly from the negative control ($p < 0.05$), supporting the extract's membrane-stabilizing anti-inflammatory activity. Hypotonic stress induces osmotic imbalance and water influx, causing cell swelling and membrane rupture. Accordingly, reduced hemolysis indicates improved membrane integrity under osmotic stress [30]. The comparison with diclofenac sodium is presented in **Figure 3**.



Note: *Data are presented as mean ± SD (n = 3). Analysis was performed using one-way ANOVA followed by Tukey's multiple comparisons test. Note: ns = not significant; ** p < 0.01; **** p < 0.0001 (same concentration).*

Figure 3. Average percentage inhibition of hypotonicity-induced erythrocyte hemolysis by the ethanolic leaf extract of *S. polyanthum* and diclofenac sodium at various concentrations.

Differences in hemolysis inhibition between heat- and hypotonic-induced models are related to distinct mechanisms of membrane damage. Heat increases membrane permeability and induces oxidative stress, whereas hypotonic conditions cause osmotic swelling leading to rupture [31], [32]. The observed membrane stabilization may be attributed to secondary metabolites such as flavonoids, tannins, and saponins. Flavonoids stabilize lysosomal membranes and inhibit inflammatory enzymes, tannins bind macromolecules and metal ions, and saponins interact with membrane components, contributing to stabilization [29], [33].

Inflammation is associated with oxidative stress resulting from excessive reactive oxygen species (ROS). Antioxidants can neutralize ROS and protect cellular components, including erythrocyte membranes [34]. When ROS production exceeds endogenous defenses, lipid peroxidation and protein damage occur, increasing membrane fragility and leading to hemolysis [34].

Protein denaturation inhibition assay

The extract's anti-inflammatory activity was examined by measuring its ability to inhibit protein denaturation. Protein denaturation involves loss of secondary and tertiary structures due to factors such as heat, pH, or solvents; heat increases kinetic energy, disrupting hydrogen bonds and hydrophobic interactions, leading to unfolding. Denatured proteins may act as antigens and trigger inflammation, making inhibition of this process an important anti-inflammatory mechanism [35].

Egg white albumin was used as the model protein due to its susceptibility to heat-induced denaturation and its composition of proteins such as ovalbumin, clusterin, ovoinhibitor, ovotransferrin, and prostaglandin D_2 synthase. Anti-inflammatory compounds can stabilize protein structures through hydrogen bonding and hydrophobic interactions, preventing denaturation [36]. Diclofenac sodium was used as the positive control, as it can interact with albumin and inhibit thermal denaturation through hydrogen bond formation with amino acid residues such as lysine, arginine, and serine [37].

Normality and variance homogeneity were assessed before statistical analysis. The Shapiro–Wilk test indicated a normal distribution, while Levene's test indicated

homogeneous variances ($p > 0.05$). These findings supported the use of one-way ANOVA followed by Tukey's multiple-comparison test. **Table 11** presents the results of protein denaturation inhibition.

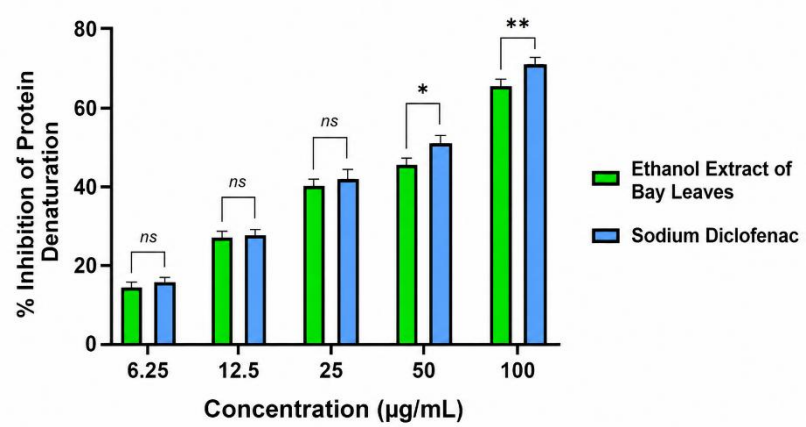
Table 11. Percentage inhibition of protein denaturation by the ethanolic leaf extract of *S. polyanthum* and diclofenac sodium at various concentrations.

Group	Concentration (µg/mL)	Absorbance (Mean ± SD)	% Inhibition (Mean ± SD)
Negative Control	-	0.860 ± 0.015	0.000 ± 1.776
Extract	6.25	0.725 ± 0.010	15.704 ± 1.244****
	12.5	0.620 ± 0.008	27.840 ± 1.033****
	25	0.510 ± 0.015	40.713 ± 1.803****
	50	0.463 ± 0.020	46.142 ± 2.356****
	100	0.294 ± 0.003	65.839 ± 0.374****
Diclofenac Sodium	6.25	0.718 ± 0.006	16.518 ± 0.710****
	12.5	0.616 ± 0.005	28.306 ± 0.681****
	25	0.503 ± 0.013	41.528 ± 1.535****
	50	0.423 ± 0.003	50.756 ± 0.440****
	100	0.246 ± 0.024	71.384 ± 2.856****

*Note: Data were analyzed using one-way ANOVA followed by Tukey's multiple comparisons test ($p < 0.05$). Note: **** $p < 0.0001$ compared with the negative control.*

The ethanolic extract inhibited protein denaturation, with inhibition rising from 15.704% at 6.25 µg/mL to 65.839% at 100 µg/mL. Diclofenac sodium produced inhibition values ranging from 16.518% to 71.384% at the same concentrations. Anti-inflammatory activity is considered significant at inhibition >20% [19], a threshold the extract reached at 12.5 µg/mL, marking the onset of activity. At 6.25–25 µg/mL, inhibition by the extract did not differ significantly from that of diclofenac sodium, whereas at 50 and 100 µg/mL, diclofenac sodium produced slightly higher inhibition. Nevertheless, the extract exhibited comparable activity, as shown in **Figure 4**.

The anti-inflammatory activity is associated with secondary metabolites detected through phytochemical screening, including flavonoids, tannins, saponins, alkaloids, steroids, and terpenoids. Phenolics and flavonoids stabilize proteins through hydrogen bonding and hydrophobic interactions, preventing heat-induced denaturation [38]. Tannins act as antioxidants, protecting proteins against oxidative damage, while saponins and steroids help stabilize membranes and inhibit the release of inflammatory mediators [29].



*Note: Data are presented as mean ± SD (n = 3). Analysis was performed using one-way ANOVA followed by Tukey's multiple comparisons test. Note: ns = not significant; * $p < 0.05$; ** $p < 0.01$; **** $p < 0.0001$ (same concentration).*

Figure 4. Average percentage inhibition of protein denaturation by the ethanolic leaf extract of *S. polyanthum* and diclofenac sodium at various concentrations.

Experimental work was confined to in vitro assays using a crude ethanolic extract. Toxicity was not evaluated, active compounds were not isolated or identified, and in vivo efficacy was not confirmed. The RBC membrane stabilization assays used blood obtained from one donor, potentially restricting the broader applicability of the findings. Mechanistic evaluation was limited to indirect in vitro models and did not identify specific molecular targets or pathways. Therefore, the findings do not establish the safety, therapeutic efficacy, or contribution of individual constituents of the extract.

4. Conclusion

The ethanolic leaf extract of *S. polyanthum* exhibited radical-scavenging activity in the DPPH, ABTS, and nitric oxide assays, while the FRAP assay demonstrated its reducing capacity. In vitro anti-inflammatory activity was also observed through erythrocyte membrane stabilization and protein denaturation assays. Phenolic and flavonoid constituents detected in the extract may have contributed to these activities. Overall, the findings support the potential of *S. polyanthum* leaves as a natural source of bioactive compounds with antioxidant and anti-inflammatory properties. Further safety and in vivo studies are required.

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Conflict of Interest:
The authors declare that there are no conflicts of interest related to this study.

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