

An Assessment of the Antioxidant, Antimicrobial, and Anti-inflammatory Properties of *Eleutherine bulbosa* (Mill.) Urb. Extract

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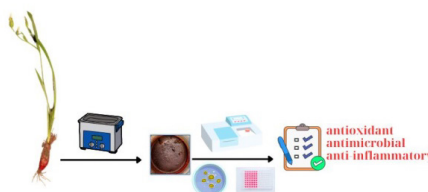
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Abstract: *Eleutherine bulbosa* is a medicinal plant known for its rich content of bioactive compounds, particularly polyphenols and flavonoids, which contribute to its notable antioxidant, antibacterial, and anti-inflammatory properties. In this study, ultrasound-assisted extraction of *E. bulbosa* tubers was performed using 96% ethanol (EtOH₉₆), water (W), and a 1:1 ethanol–water mixture (EtOH₉₆:W). The extracts were assessed for biological activities through 1,1-diphenyl-2-picrylhydrazyl (DPPH) and 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) radical scavenging assays, agar well diffusion, minimum inhibitory concentration (MIC), and anti-inflammatory tests. Among the solvents, EtOH₉₆ yielded the highest antioxidant activity, with IC₅₀ values of 202.2 ± 17.6 µg/mL (DPPH) and 250.8 ± 7.8 µg/mL (ABTS). These effects corresponded to elevated levels of polyphenols (94.9 ± 4.0 mg gallic acid equivalent (GAE)/g dry weight (DW)) and flavonoids (119.3 ± 17.1 mg quercetin equivalent (QE)/g dry weight (DW)). Additionally, the EtOH₉₆ extract displayed antibacterial activity against *Staphylococcus aureus* and *Escherichia coli* with MIC values of 6.25 mg/mL and suppressed heat-induced protein denaturation with an IC₅₀ value of 86.0 µg/mL, highlighting *E. bulbosa* tubers as a credible source of phytotherapeutics for both animal and human use.



Keywords: *Eleutherine bulbosa* (Mill.) Urb., ultrasonic-assisted extraction, antioxidant, antibacterial, anti-inflammatory

1 Introduction

Eleutherine bulbosa (Mill.) Urb., a member of the Iridaceae family, is a herbaceous flowering plant widely distributed in tropical regions such as Vietnam, South America, and the Amazon basin¹⁻³). The plant is morphologically distinguished by its characteristic red roots, from which three major classes of bioactive compounds, naphthalene derivatives, anthraquinones, and naphthoquinones, have been isolated. In recent years, molecular biology techniques have been employed to confirm species identity within the genus⁴⁻⁷). Notably, Goldblatt and Manning utilized *rbcL* and *matK* gene sequences to elucidate phylogenetic relationships within the Iridaceae family, confirming *E. bulbosa* as a monocot species closely related to the genera *Tigridia* and *Sisyrinchium*⁸). The taxonomic classification of *E. bulbosa* has evolved from traditional morphological approaches to molecular-based analyses. However, in Vietnam, such molecular identification remains underutilized, leading to frequent misidentification due to phenotypic similarities with other species an issue that adversely affects the quality and consistency of medicinal plant materials. *E. bulbosa* is well adapted to tropical

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climates and is commonly cultivated in home gardens for medicinal and ornamental purposes in provinces such as Son La, Phu Tho, Hoa Binh, Yen Bai, Ha Noi, Da Nang, and Quang Nam⁹⁾.

E. bulbosa has been increasingly recognized as a promising medicinal plant owing to its rich repertoire of bioactive secondary metabolites, particularly polyphenolic compounds. Previous studies have demonstrated that ethanol extracts of *E. bulbosa* bulbs exhibit a high total phenolic content (70.9 ± 2.3 mg gallic acid equivalent (GAE)/mL), which correlates with substantial antioxidant capacity, while aqueous extracts of different plant parts confirmed a hierarchy of polyphenol abundance and antioxidant activity in the order flowers > bulbs > leaves^{10, 11)}. Beyond its antioxidant properties, *E. bulbosa* has shown considerable anti-inflammatory potential. The ethanol extract of *E. bulbosa*, containing (–)-isoeleutherin, displayed potent inhibition of nitric oxide production with an IC₅₀ of 27.3 ± 3.0 µg/mL, supporting its role as an anti-inflammatory agent relevant to conditions such as rheumatoid arthritis^{12, 13)}. In addition, eleuthalene E, a naphthalene derivative isolated from the bulbs, was identified as a key bioactive compound acting through NF-κB/MAPK suppression and Nrf2/Keap1 pathway activation¹⁴⁾. Consistently, membrane stabilization assays confirmed anti-inflammatory efficacy of the ethanol bulb extract (EC₅₀ = 52.9 ± 2.3 mg/mL), corresponding to approximately half the potency of indomethacin¹⁵⁾. In vivo, administration of 70% ethanol bulb extract (24 mg/200 g BW) significantly ameliorated inflammation in an MIA-induced osteoarthritis rat model¹⁶⁾, while in vitro whole blood assays highlighted selective inhibition of COX-2 (IC₅₀ = 40.6 ng/mL) over COX-1 (IC₅₀ = 364.9 ng/mL), underscoring its potential relevance for chronic inflammatory conditions such as benign prostatic hyperplasia¹⁷⁾. Furthermore, compounds such as eleutherinol A–C and eleutherinoside C isolated from the red bulbs demonstrated moderate antibacterial activity against *Bacillus subtilis* (*B. subtilis*), *Staphylococcus aureus* (*S. aureus*), and *Escherichia coli* (*E. coli*), with MIC values ranging from 15.6 to 250.0 µg/mL¹⁸⁾. Collectively, these findings provide compelling evidence that *E. bulbosa* bulbs represent a valuable source of polyphenols and bioactive constituents with antioxidant, antibacterial, and anti-inflammatory properties, supporting their therapeutic potential in managing oxidative stress and inflammation-related disorders.

Although numerous studies have examined the total polyphenol content and selected biological activities of *E. bulbosa*, most have been limited to evaluating only one or two parameters. A comprehensive assessment encompassing total polyphenols, flavonoids, and multiple biological activities, such as antioxidant, antibacterial, and anti-inflammatory effects, remains largely unexplored, thereby limiting insights into the correlations between phytochemical composition and biological efficacy. Furthermore, conventional extraction methods, particularly maceration, continue to dominate in prior research, resulting in extended extraction times, excessive solvent consumption, and relatively low yields¹⁹⁾. In addition, taxonomic authentication of plant materials has rarely been addressed, creating challenges in comparing chemical and biological data across different studies. In this study, *E. bulbosa* samples were taxonomically validated, and ultrasound-assisted extraction (UAE) was employed to efficiently obtain bioactive-rich extracts. UAE accelerates mass transfer via acoustic cavitation, thereby shortening extraction time and reducing solvent requirements while increasing yields at lower temperatures, features that preserve thermolabile phytochemicals and enable a more efficient, scalable, and environmentally considerate recovery of natural products²⁰⁾. The extracts were systematically characterized by physicochemical analyses and quantification of key phytochemicals, followed by evaluation of their antioxidant, antibacterial, and anti-inflammatory properties. Protein denaturation assay was applied as a rapid and reliable method for preliminary anti-inflammatory screening. *S. aureus* and *E. coli* were selected as model microorganisms for antimicrobial testing due to their clinical significance, ease of cultivation, and representation of Gram-positive and Gram-negative bacteria^{21, 22)}. The findings of this study provide, for the first time, a comprehensive dataset on the phytochemical composition and biological activities of UAE-derived *E. bulbosa* extracts, contributing valuable evidence to the scientific documentation of medicinal plant resources in Vietnam and beyond.

2 Materials and Methods

2.1 Materials

Eleutherine bulbosa (Mill.) Urb. was transported in Tam Hop commune, Quy Hop district, Nghe An province, Vietnam. Leaves and roots were removed, tubers were retained and washed several times with water before being dried with UF160 Memmert, Germany at 60 °C, ground into fine powder and stored at room temperature before being used for the extraction process.

2.2 Chemicals and reagents

2,2-diphenyl-1-picrylhydrazyl (DPPH, C₁₈H₁₂N₅O₆, ≥ 95%) was provided by Thermo scientific, America. 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS, C₁₈H₂₄N₆O₆S₄, ≥ 98%), L-ascorbic acid (Vitamin C, C₆H₈O₆, ≥ 99%), Folin–Ciocalteu reagent were provided by Sigma-Aldrich, China. Sodium carbonate (Na₂CO₃, ≥ 99.8%),

sodium hydroxide (NaOH, $\geq 96\%$), methanol (CH₃OH, $\geq 99.5\%$), potassium peroxydisulfate (K₂S₂O₈, $\geq 99.5\%$), sodium chloride (NaCl, $\geq 99.5\%$), aluminium chloride hexahydrate (AlCl₃·3H₂O, $\geq 97\%$), lead(II) acetate trihydrate (Pb(CH₃COOH)₂·3H₂O, $\geq 99.5\%$), potassium acetate (CH₃COOK, $\geq 92\%$), sodium acetate trihydrate (CH₃COONa·3H₂O, $\geq 99\%$), acetic acid (CH₃COOH, $\geq 99.5\%$), magnesium ribbon (Mg, $\geq 99.95\%$), hydrochloric acid (HCl, $\geq 35\%$), sulfuric acid (H₂SO₄, $\geq 95\%$), ammonia solution (NH₃, $\geq 25\%$), and chloroform (CHCl₃, $\geq 99\%$) were provided by Xilong, China. Diethylene glycol (O(CH₂CH₂OH)₂, $\geq 98.5\%$), were provided by Oxford, India. Mueller-Hinton agar (99%), resazurin (99%) were provided by Himedia, India. Bovine serum albumin (99%), acetate buffer were provided by Merck, Germany. Chloramphenicol (C₁₁H₁₂Cl₂N₂O₅, 99%) were provided by Bio Basic, Canada.

2.3 Ultrasonic assisted extraction method

The processing of raw materials and the extraction of *E. bulbosa* roots are illustrated in **Fig. 1a** and **1b**. An accurately weighed 5 g portion of *E. bulbosa* root powder was introduced into a glass beaker containing 100 mL of solvent either 96% ethanol (EtOH_96), water (W), or a 1:1 v/v mixture of ethanol and water (EtOH_96:W). The beaker was then subjected to ultrasonic-assisted extraction using a PRO 150S ultrasonic bath (Asonic, China) at 50 °C for 1 hour. Following extraction, the mixture was filtered through a vacuum filtration apparatus (Rocker, Taiwan) to separate the solid residues, and the resulting filtrate was retained. The extraction process was repeated with fresh solvent until the plant material was deemed exhausted. All collected extracts were subsequently concentrated by rotary evaporation (IKA RV10, Germany) to remove the solvent. The dried extracts were stored at ambient temperature for subsequent experimental procedures.

2.4 Qualitative determination of phytochemical composition

A 1-mg portion of the EtOH_96 extract of *E. bulbosa* was solubilized in 10 mL of deionized water to yield a working solution at 0.1 mg/mL, and its phytochemical constituents were qualitatively assessed according to the protocols specified in **Table 1** ²³⁾.

2.5 Evaluation of characteristics of the extract

The physicochemical properties of *E. bulbosa* extracts, specifically pH and viscosity, were measured using a pH meter (P-512, PEAK Instrument, China) and a viscometer (DV1MLVTJ0, BROOKFIELD, USA) to generate data supporting the evaluation of their stability and compatibility with excipients in future product formulations.

2.6 Method for determining total polyphenol and flavonoid content

Various *E. bulbosa* extracts, such as EtOH_96, W, and EtOH_96:W, were dissolved in 96% ethanol, water, and 96% ethanol:water (1:1 v/v), respectively, to prepare extract solutions at a concentration of 1 mg/mL for the determination of total polyphenol and flavonoid contents.

The total polyphenol content of the extract was quantified using the Folin–Ciocalteu colorimetric method ²⁴⁾. Specifically, 0.5 mL of the sample was mixed with 2.5 mL of 10% Folin–Ciocalteu reagent (comprising phosphomolybdate and phosphotungstate) and 2.0 mL of saturated sodium carbonate solution (7% Na₂CO₃). The mixture was thoroughly shaken and incubated at room temperature for 2 hours. Subsequently, it was centrifuged at 7000 rpm for 5 minutes (TG16, Yingtai, China) to remove particulate matter.

Total flavonoid content was determined according to the method described by Sitthichai²⁵⁾. 0.5 mL of the sample was placed in a test tube, followed by the addition of 0.1 mL of 10% AlCl₃, 0.1 mL of 1 M CH₃COOK solution, and 4.3 mL of deionized water. The mixture was vortexed and allowed to stand at room temperature for 30 minutes. Absorbance was measured at 760 nm for polyphenols and 415 nm for flavonoids using a UV-Vis spectrophotometer (Evolution 60S, Thermo Scientific, USA). Gallic acid was employed to construct the calibration curve for polyphenols, and results were expressed in mg gallic acid equivalents per gram of herbal material (mg GAE/g DW). Quercetin served as the standard for flavonoids, with concentrations expressed as mg quercetin equivalents per gram of herbal material (mg QE/g DW).

2.7 Evaluation of biological activity

2.7.1 DPPH free radical scavenging ability

The in vitro antioxidant activity of the sample will be assessed using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay, with ascorbic acid employed as the reference standard, as described by Sitthichai et al. (2022)²⁵⁾. In this method, 1 mL of a 0.6 mM DPPH methanolic solution will be thoroughly mixed with 1 mL of the sample solution at varying concentrations (12.5, 25, 50, and 100 µg/mL). The reaction mixture will be incubated in the dark for 60 minutes

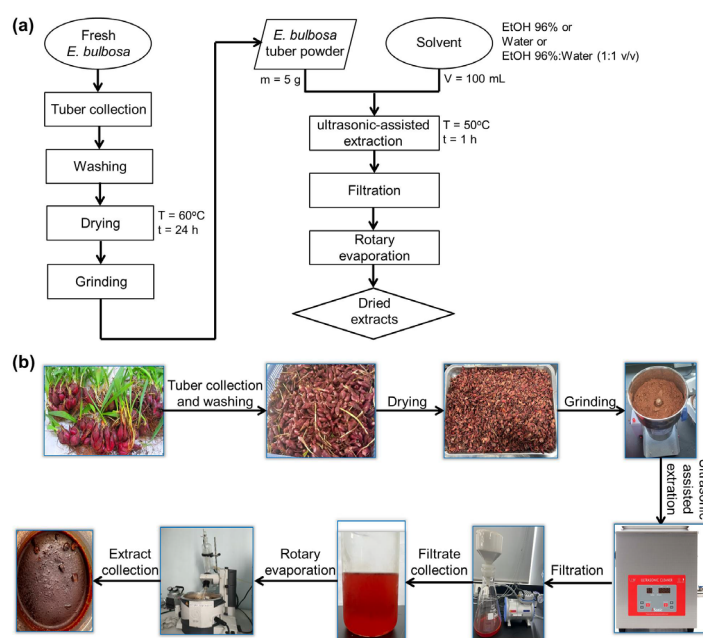


Fig. 1 (a) Flowchart and (b) schematic illustration of the *E. bulbosa* extraction process.

Table 1 Reactions and visual markers for the presence of phytochemicals.

No.	Compounds	Methods	Positive Indicators
1	saponins	1 mL of sample solution was combined with 1 mL of deionized water in a 10-mL conical tube and subsequently shaken for 10 minutes.	Stable foam formation
2	polyphenols	An aliquot of 50 μL of the sample solution was diluted with 500 μL of deionized water and subsequently mixed with three drops of 5% FeCl_3 solution.	Moss green complex
3	flavonoids	A strip of Mg ribbon and 1 mL of concentrated HCl was subsequently added into a 10-mL conical tube containing 1 mL of sample solution.	Red color
4	alkaloids	A few drops of Wagner's reagent were added into a 10-mL conical tube containing 1 mL of sample solution. Wagner's reagent was prepared by dissolving 0.635 g of I_2 and 1 g of KI in distilled water, followed by adjusting the final volume to 50 mL.	Reddish brown precipitate
5	coumarins	A 10-mL conical tube containing 1 mL of the sample solution and 5 drops of 10% NaOH solution was examined under UV illumination at 365 nm.	Blue fluorescence
6	anthraquinones	2 mL of 10% H_2SO_4 were combined with 2 mL of the sample solution, and the mixture was subsequently boiled and filtered. The resulting filtrate (2 mL) was vigorously agitated with 2 mL of chloroform until two distinct phases were formed, after which the organic layer was isolated and treated with five drops of NH_3 solution.	pink color on the top layer

to prevent photodegradation. Subsequently, the absorbance will be measured at 517 nm using a UV-Vis spectrophotometer (Evolution 60S, Thermo Scientific, USA). The half-maximal inhibitory concentration (IC₅₀) was determined by fitting the experimental data to a nonlinear regression curve using Microsoft Excel 2010 software, and then interpolating the extract concentration at which the % inhibition reached 50%.

2.7.2 ABTS^{••} free radical scavenging ability

The in vitro antioxidant capacity will be assessed using the ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) radical scavenging assay, with ascorbic acid serving as the reference standard, as described by Sittichai et al. (2022)²⁵. In brief, 0.5 mL of ABTS methanolic solution and 0.5 mL of deionized water will be combined with 1 mL of the sample solution at various concentrations (12.5, 25, 50, and 100 µg/mL). The mixture will be incubated in the dark for 6 minutes to allow the reaction to proceed. Absorbance will then be measured at 734 nm using a UV-Vis spectrophotometer (Evolution 60S, Thermo Scientific, USA). IC₅₀ values were derived through nonlinear regression fitting using Microsoft Excel 2010 software, and subsequently calculating the extract concentration corresponding to 50% inhibition.

2.7.3 Evaluation of antibacterial ability

The LB-Miller agar medium was formulated with the following components (per liter): 10 g peptone, 5 g yeast extract, 5 g sodium chloride (NaCl), and 20 g agar, dissolved in 1 L of deionized water. The LB-Miller broth (liquid medium) was similarly prepared without agar, using 10 g peptone, 5 g yeast extract, and 5 g NaCl per liter of deionized water. Both media were thoroughly stirred to ensure complete dissolution of the components, transferred into 2 L experimental flasks, and sterilized by autoclaving using a JEIOTECH ST-50G autoclave system.

The antibacterial activity was evaluated using the agar well diffusion method²⁶. Test bacterial strains were uniformly inoculated onto Mueller-Hinton agar plates and incubated for 24 hours. The bacterial suspension was subsequently adjusted to a concentration of 10⁶ – 10⁷ CFU/mL based on optical density measurements at 660 nm. The extracts were reconstituted in the solvents applied during the extraction process, specifically 96% ethanol, deionized water, and a 1:1 (v/v) mixture of 96% ethanol and deionized water. These solvents functioned as the negative control while chloramphenicol (1 mg/mL) served as the positive control. To assess antimicrobial efficacy, 50 µL of sample solutions at various concentrations were dispensed into wells punched into the agar surface previously inoculated with the test organisms. After 24 hours of incubation, the antimicrobial effect was determined by measuring the diameter of the inhibition zones (in millimeters), and results were documented photographically. The assay was conducted against two representative bacterial strains: *E. coli* (Gram-negative) and *S. aureus* (Gram-positive).

The minimum inhibitory concentration (MIC) is defined as the lowest concentration of a test compound capable of inhibiting visible microbial growth. To determine the MIC, the test substance was serially diluted to obtain a range of decreasing concentrations. The MIC was identified as the lowest concentration at which no visible bacterial growth was observed.

The MIC determination was performed using a broth microdilution method in a 96-well microplate²⁶. Each well was filled with 150 µL of bacterial suspension and 50 µL of the test substance at varying concentrations. The microplates were incubated at 37 °C for 16 – 24 hours. Following incubation, 20 µL of 0.01% resazurin solution was added to each well as a redox indicator. The MIC was recorded based on the absence of color change, indicating inhibited bacterial metabolic activity.

2.8 Evaluation of anti-inflammatory activity

The anti-inflammatory properties of the sample were investigated through its efficacy in inhibiting the thermal denaturation of bovine serum albumin (BSA)²⁷. A reaction mixture was prepared by combining 2 mL of 0.025 M acetate buffer (pH 5.5), 1 mL of 0.16% BSA solution, and 1 mL of the test extract at varying concentrations. The mixture was first incubated at 37 °C for 30 minutes, followed by heat treatment in a water bath at 67 °C for 3 minutes to induce protein denaturation. After cooling to room temperature, the absorbance was measured at 660 nm using a spectrophotometer.

The percentage inhibition of BSA denaturation was calculated using the formula:

$$\text{Inhibition (\%)} = [(OD_0 - OD_{\text{sample}})/OD_0] \times 100$$

where OD_0 represents the optical density of the control (without extract) and OD_{sample} represents the optical density of the test sample.

2.9 Statistical analysis

The results are expressed as the mean ± standard deviation (SD), with data analysis conducted using Microsoft Excel. All experiments were performed in triplicate to ensure reproducibility and reliability of the findings.

3 Results and Discussion

3.1 Scientific identification of *Eleutherine bulbosa* (Mill.) Urb.

Fresh *E. bulbosa* specimens, including all essential morphological parts stems, leaves, roots, flowers, and fruits were submitted to the Research Center of Ginseng and Medicinal Materials (CGMM), under National Institute of Medicinal Materials, Vietnam, for taxonomic identification. Three samples, designated as TBDD-24050604, TBDD-24050604, and TBDD-24050604, have been deposited at 75/4 Phan Van Hon Street, Tan Thoi Nhat Ward, District 12, Ho Chi Minh City, Vietnam. In addition to recording vernacular or local names, scientific nomenclature was determined for each specimen.

The primary method employed for plant identification was morphological comparison, focusing particularly on reproductive structures such as flowers, cones, and sporangia. **Figure 2** illustrates the diagnostic morphological traits of *E. bulbosa* observed in this study. This species is a perennial herb reaching approximately 28 cm in height (**Fig. 2a**). Its ovoid bulb, covered with reddish-brown scales and measuring about 8 cm in length, resembles an elongated onion with a pinkish brown to reddish brown interior. The lanceolate leaves, characterized by longitudinally parallel venation, are reminiscent of young areca palm leaves and extend up to 20 cm (**Fig. 2b**). Emerging from the bulb is a floral stalk bearing floral structures, including petals, stamens, and a pistil, with an overall length of 28 cm (**Fig. 2c**). The flower consists of five white or pale yellow petals, each approximately 1 cm long, and three distinctive orange stamens measuring 5 mm (**Fig. 2d** and **2e**). The ovary is ovoid, approximately 2 mm in length, and when cross-sectioned, appears trilobular, resembling three awl-like divisions (**Fig. 2f** and **2g**). Based on these morphological characteristics, and by consulting dichotomous keys and taxonomic descriptions from specialized literature^{28, 29}, the plant was conclusively identified as *Eleutherine bulbosa* (Mill.) Urb.



Fig. 2 Sample photo of scientific identification of *E. bulbosa* (Mill.) Urb.

3.2 Qualitative phytochemical composition of the extract

The physicochemical properties of *E. bulbosa* root extracts varied depending on the solvent used for extraction. Specifically, the extract obtained with EtOH₉₆ exhibited a yellow hue with a pH of 4.60 ± 0.02 , while the aqueous extract (W) appeared reddish-brown with a pH of 5.56 ± 0.01 . The hydroethanolic extract EtOH₉₆:W displayed a red coloration and had a pH of 5.40 ± 0.01 . In comparison, the native solvents 96% ethanol and deionized water had pH values of 6.09 ± 0.231 and 7.12 ± 0.097 , respectively. Viscosity measurements further revealed that the EtOH₉₆, W, and EtOH₉₆:W extracts exhibited viscosities of 3.67 ± 0.58 , 4.00 ± 0.00 , and 5.17 ± 0.29 mPa·s, respectively, whereas the initial solvents had lower viscosities: 2.67 ± 0.289 mPa·s for ethanol and undetermined for water. The observed decrease in pH and increase in viscosity of the extracts compared to their respective solvents confirm the presence of

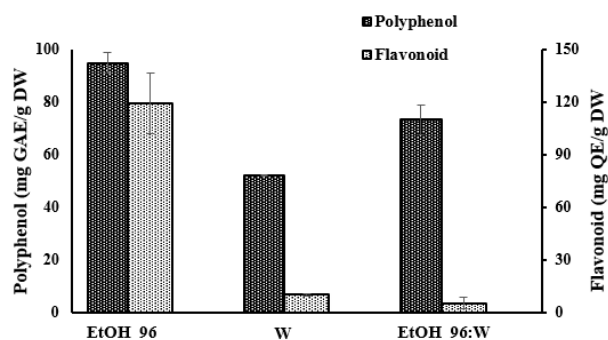


Fig. 3 Polyphenol and flavonoid content in the extract.

phytochemical constituents in the *E. bulbosa* extracts. Furthermore, the measured pH and viscosity values provide valuable reference parameters for the formulation and development of extract-based liquid products, as they influence product stability, compatibility with excipients, sensory characteristics, and overall quality control in pharmaceutical and nutraceutical applications.

Preliminary phytochemical screening confirmed the presence of several major classes of bioactive constituents. The generation of stable foam during the shaking test with water is indicative of saponins, whereas the emergence of a green coloration in the ferric chloride assay denotes the presence of phenolic compounds. The Shinoda, Wagner, and Borntrager tests yielded positive reactions, thereby verifying the occurrence of flavonoids, alkaloids, and anthraquinones, respectively. The appearance of blue fluorescent emission under UV illumination in alkaline conditions confirms the presence of coumarins. Among these, polyphenols and flavonoids are especially notable for their antioxidant properties and are frequently investigated during the initial evaluation of medicinal plant extracts.

Quantitative analysis (**Fig. 3**) demonstrated that the total polyphenol content was highest in the EtOH_96 extract (94.9 ± 4.0 mg GAE/g DW), followed by EtOH_96:W (73.2 ± 5.6 mg GAE/g DW), and lowest in the water extract (52.1 ± 0.3 mg GAE/g DW). This aligns with the known solubilizing capabilities of ethanol, a strong organic solvent, which enhances the extraction of polyphenolic compounds compared to water³⁰. Regarding flavonoid content, EtOH_96 again yielded the highest concentration (119.3 ± 17.1 mg quercetin equivalents QE/g DW), while W and EtOH_96:W extracts showed significantly lower values (10.2 ± 0.7 and 5.4 ± 3.4 mg QE/g DW, respectively). When compared to previous studies, the ultrasonic-assisted extraction method employed in the current work demonstrated promising efficiency. For instance, Almad et al. (2023) utilized microwave-assisted extraction followed by filtration and centrifugation using 50% ethanol, resulting in a total polyphenol content of 28.6 ± 1.2 mg GAE/g sample³¹. Similarly, Ali et al. (2020) investigated various extraction methods including maceration, reflux, and Soxhlet using ethanol and hexane in different ratios. The highest values reported were 8.6 mg GAE/g and 28.2 mg QE/g at a 70:30 ethanol-to-solvent ratio³². Taken together, these comparisons suggest that ultrasonic-assisted extraction with 96% ethanol provides an effective approach for recovering polyphenolic and flavonoid compounds from *E. bulbosa* roots; however, differences in phytochemical yields may also arise from other variables such as plant origin, cultivation conditions, soil quality, climate, and harvesting time.

3.3 Biological properties of *E. bulbosa*

Both the DPPH and ABTS assays are widely employed to evaluate antioxidant activity, relying on the ability of antioxidants to donate electrons or hydrogen atoms to neutralize stable free radicals^{33, 34}. These interactions result in a measurable color change, enabling the quantification of radical scavenging capacity through the IC₅₀ value the concentration of extract required to inhibit 50% of the radical activity. Notably, the DPPH method reflects antioxidant potential primarily via electron or hydrogen transfer mechanisms in organic media, whereas the ABTS method is applicable in both polar and non-polar environments, offering broader solubility compatibility³⁵.

The antioxidant capacity of *E. bulbosa* root extracts was evaluated using the DPPH assay, which demonstrated a concentration-dependent increase in radical scavenging activity across the range of 0.125 to 1.0 mg/mL, irrespective of the solvent employed (**Fig. 4a**). The calculated IC₅₀ values (**Fig. 4b**) varied significantly among solvents, with EtOH_96 yielding the lowest IC₅₀ (202.2 ± 17.6 µg/mL), indicating the highest antioxidant activity, followed EtOH_96:W and W, which had the highest IC₅₀ (962.1 ± 14.0 µg/mL). This order EtOH_96 > EtOH_96:W > W reflects the varying efficiencies

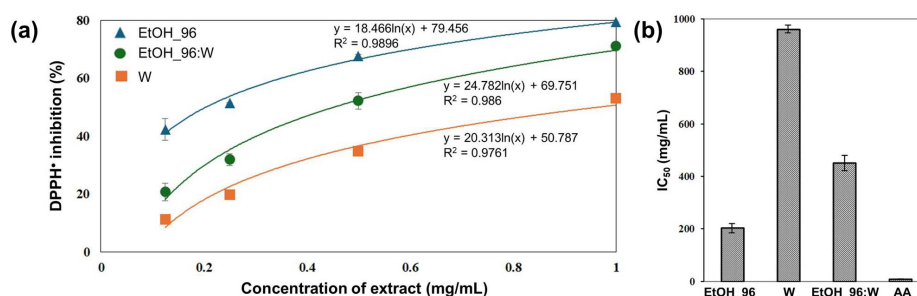


Fig. 4 (a) DPPH inhibition percentage of concentration extract and (b) their IC₅₀ values.

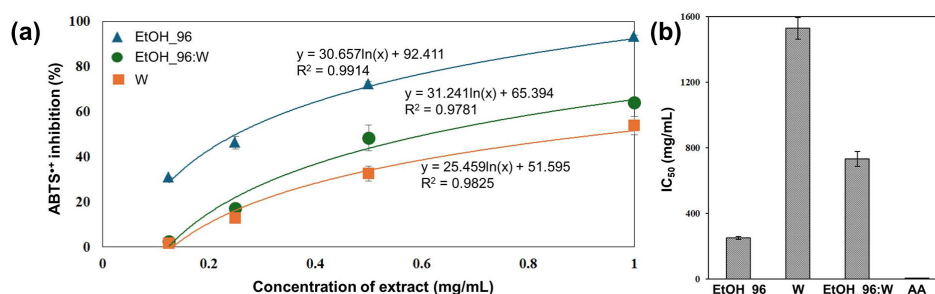


Fig. 5 (a) ABTS inhibition percentage of concentration extract and (b) their IC₅₀.

of these solvents in extracting antioxidant constituents. These findings are consistent with those reported by Ali et al. (2020), which identified a strong correlation between polyphenol content and antioxidant activity as measured by the DPPH method³⁶. Similarly, ABTS assay results (**Fig. 5**) confirmed the same trend: increased extract concentration correlated with increased free radical inhibition. The IC₅₀ values determined via the ABTS method were $250.8 \pm 7.8 \mu\text{g/mL}$ (EtOH_96), $755.3 \pm 12.9 \mu\text{g/mL}$ (EtOH_96:W), and $1529.1 \pm 44.6 \mu\text{g/mL}$ (W), further substantiating the superior extraction efficiency of 96% ethanol, particularly when paired with ultrasonic-assisted extraction. These findings underscore the enhanced performance of ultrasonic extraction using 96% ethanol in isolating antioxidant compounds from *E. bulbosa*, aligning with data from³⁶, who reported an ABTS radical scavenging efficiency of $74.9 \pm 1.3\%$ using ethanol optimized via response surface methodology (RSM)³⁷. Despite exhibiting lower antioxidant activity compared to ascorbic acid (AA) (i.e., IC₅₀ = 8.2 $\mu\text{g/mL}$ for DPPH and 6.0 $\mu\text{g/mL}$ for ABTS), the extracts of *E. bulbosa* still hold considerable promise due to their origin as a natural, renewable, and potentially source of bioactive constituents, which may be further harnessed for applications in pharmaceutical and nutraceutical development.

Polyphenolic compounds are well-documented for their antimicrobial properties and have been extensively investigated for their potential use as natural preservatives or edible coatings in food systems to inhibit microbial proliferation^{38, 39}. In this study, the antibacterial activity of *E. bulbosa* extracts was assessed against two representative bacterial strains: *E. coli* (Gram-negative) and *S. aureus* (Gram-positive). As shown in **Fig 6**, extracts prepared in different solvents were tested at a concentration of 4 mg/well to evaluate their inhibitory efficacy. At this concentration, all three extracts exhibited measurable antibacterial activity against *E. coli*, with inhibition zone diameters of $6.6 \pm 0.3 \text{ mm}$ (EtOH_96), $6.0 \pm 0.0 \text{ mm}$ (W), and $6.0 \pm 0.0 \text{ mm}$ (EtOH_96:W). For *S. aureus*, the inhibition zones were $8.0 \pm 0.4 \text{ mm}$ for EtOH_96, $6.0 \pm 0.0 \text{ mm}$ for W, and $6.0 \pm 0.4 \text{ mm}$ for EtOH_96:W. These inhibition zones were smaller compared to those observed with chloramphenicol, a synthetic antibiotic used as the positive control ($22.0 \pm 0.3 \text{ mm}$ for *E. coli* and $25.5 \pm 0.2 \text{ mm}$ for *S. aureus*). These observations were consistent with the MIC values obtained from the broth dilution assay (**Fig. 7**). Notably, the EtOH_96 extract and the EtOH_96:W extract demonstrated inhibitory activity against both *E. coli* and *S. aureus*, with MIC values of 6.25 mg/mL and 12.5 mg/mL (**Table 2**), respectively. In contrast, the aqueous extract exhibited no detectable inhibitory effects under the tested conditions, likely due to the higher concentration required to achieve measurable MIC values. Taken together, the MIC data indicates that the EtOH_96 extract exerted the strongest antibacterial activity among the tested samples. This enhanced activity is presumably attributable to its higher polyphenol content compared to the other extracts.

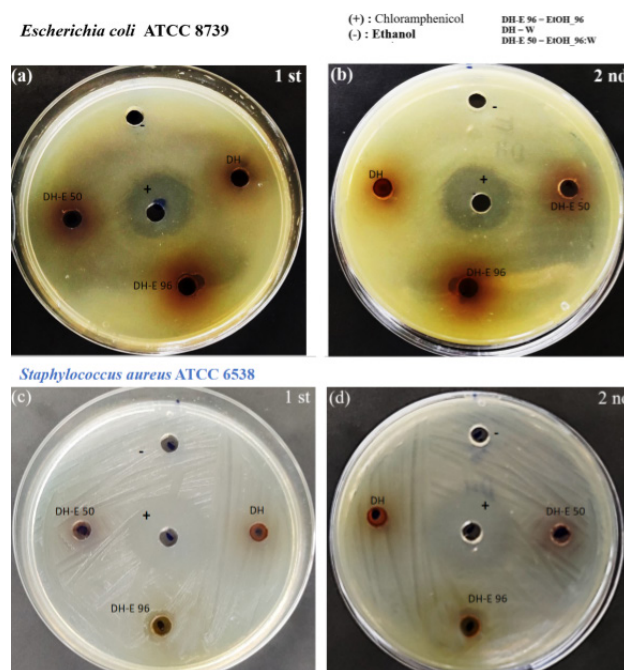


Fig. 6 Antibacterial ability of *E. coli* (a and b) and *S. aureus* (c and d) in the extract by agar well diffusion method.

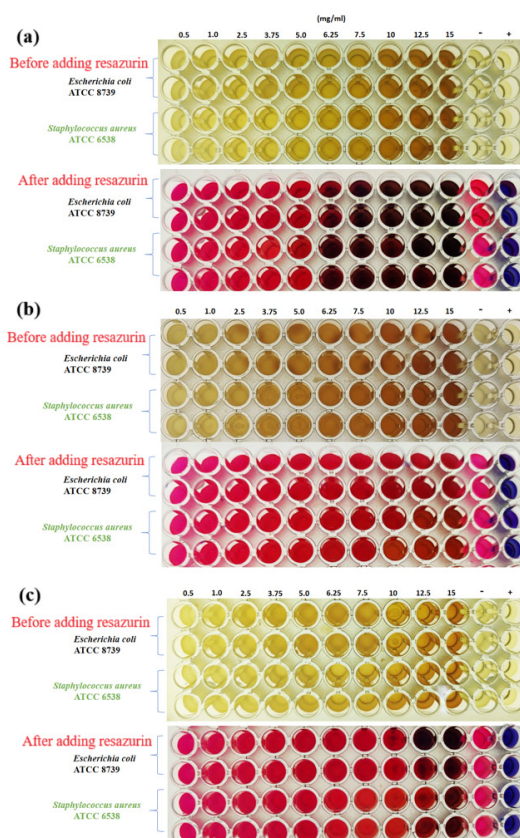
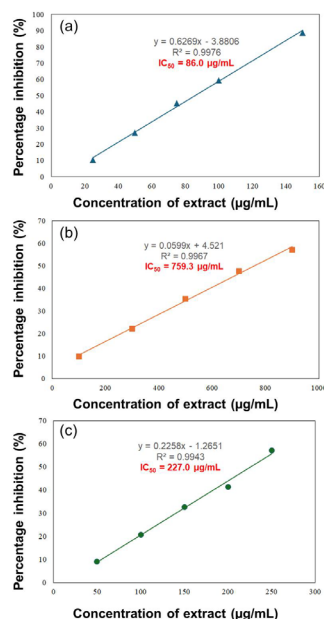


Fig. 7 Antibacterial efficacy of (a) EtOH_96, (b) W, and (c) EtOH_96:W, as evaluated by the resazurin-based assay.

Table 2 MIC values in antibacterial activity of *E. bulbosa* root extracts.

Sample	MIC (mg/mL)	
	<i>Escherichia coli</i>	<i>Staphylococcus aureus</i>
EtOH_96	6.250	6.250
W	-	-
EtOH_96:W	12.500	12.500
chloramphenicol	0.200	0.200

**Fig. 8** Inhibition rate of anti-inflammatory ability of the (a) EtOH_96, (b) W, and (c) EtOH_96:W extracts.

Several compounds present in the plant species, such as polyphenols and essential oils, are known to exert antimicrobial effects through several complementary mechanisms, including the disruption of bacterial cell membrane integrity, binding to surface proteins and enzymes leading to functional inactivation, and promoting the generation of reactive oxygen species (ROS) that can damage cellular components^{32, 40}. These actions may contribute to the moderate antibacterial effects observed in *E. bulbosa* extracts. Although the activity measured in this study was not as pronounced as that of the positive control chloramphenicol, the results are consistent with the traditional use of *E. bulbosa* in folk medicine for wound disinfection and for treating infections in both humans and animals^{41, 42}. This suggests that, while not a strong antibacterial agent, the *E. bulbosa* plant possesses bioactive polyphenols that may support its ethnopharmacological applications, particularly in reducing microbial load and preventing infection.

The anti-inflammatory activity of *E. bulbosa* bulb extracts was investigated through their capacity to prevent heat-induced protein denaturation, a phenomenon involving the loss of the native three-dimensional structure of proteins⁴³. Since protein denaturation has been recognized as a key factor contributing to inflammatory and arthritic conditions, this assay provides a reliable indicator of anti-inflammatory activity, as supported by previous literature⁴⁴. As illustrated in **Fig. 8**, the inhibition of protein denaturation increased proportionally with extract concentration. The IC_{50} values were determined to be 86.0 µg/mL for the EtOH_96 extract, 227.0 µg/mL for the EtOH_96:W extract, and 759.3 µg/mL for the W extract, indicating that the EtOH_96 extract exhibited the strongest anti-inflammatory efficacy. These findings are consistent with the antibacterial and antioxidant activities observed for the same extracts, which may be attributed to the high polyphenol content present in the EtOH_96 extract. Overall, the results suggest that 96% ethanol is the most effective solvent for producing bioactive extracts with significant therapeutic potential.

3.4 Contextual comparison and potential applications

Table 3 summarizes previously published data on the polyphenol and flavonoid composition and the antioxidant, antibacterial, and anti-inflammatory activities of tuber extracts of *E. bulbosa*. Overall, the present study indicates that ultrasound-assisted extraction shortens extraction time while achieving high total concentrations of bioactive constituents. However, cross-study comparisons of bioactivity are inherently difficult due to non-uniform outcome measures, differences in extract concentrations, and variations in experimental protocols. Notably, ultrasound treatment may also degrade certain phytochemicals by free radicals generated via prolonged cavitation and localized heating⁵⁵⁾ and, in some instances, attenuate biological activity; therefore, extraction conditions should be carefully optimized.

Building on the antibacterial, anti-inflammatory, and antioxidant profiles observed for the *E. bulbosa* extracts, several translational avenues are supported by the literature. First, activity against *S. aureus* in *E. bulbosa* extracts indicates potential for topical antiseptic preparations and adjunct wound-care products targeting common skin and soft-tissue pathogens, consistent with its traditional topical use for wounds⁵⁶⁾. Second, inhibition of *E. coli* together with ethnomedicinal antidiarrheal use of *E. bulbosa* suggests the feasibility of standardized oral preparations for short-course management of uncomplicated diarrhea, pending safety and dose-finding studies⁵⁷⁾. Third, the capacity of *E. bulbosa* extracts to inhibit protein denaturation suggests potential for developing agents that address inflammation in cancer development; yet this prospect requires further corroboration⁵⁸⁾. Finally, the demonstrated antioxidant capacity provides a rationale for employing the extract as a natural antioxidant stabilizer in functional food systems and as a free radical inhibitor in skin-protective cosmetic products⁵⁹⁾.

Table 3 Summary of TPC, TFC, and bioactivities of *E. bulbosa* extracts from previous studies.

No	Extraction methods	Solvent	Time (h)	TPC (mgG AE/g)	TFC (mg QE/g)	Antioxidant activities	Antimicrobial activities	Anti-inflammatory activities	Refs
1	heating at 90°C	water	0.25	0.5	1.2	n.d.	n.d.	n.d.	45)
	maceration	ethanol	72	1.2	1.3	n.d.	n.d.	n.d.	
2	maceration	ethanol:hexane (7:3)	n.m.	8.6	28.2	IC ₅₀ = 17.3 ppm for DPPH	n.d.	n.d.	36)
	reflux	ethanol:hexane (7:3)		7.9	27.3	IC ₅₀ = 36.2 ppm for DPPH			
	soxhletation	ethanol:hexane (7:3)		5.7	24.0	IC ₅₀ = 41.7 ppm for DPPH			
3	maceration	ethanol 70%	24	n.d.	7.6	n.d.	n.d.	n.d.	46)
4	ultrasonication	ethanol 95%		70.9	26.0	IC ₅₀ = 9700 ppm for DPPH			10)
5	microwave	ethanol 60%	0.16	35.3	n.d.	n.d.	n.d.	n.d.	31)
6	microwave	citric acid-glucose (1:1 g/g)	0.25	61.6	n.d.	n.d.	n.d.	n.d.	47)
7	microwave	citric acid-glycerol (1:1 g/g)	0.25	84.6	n.d.	n.d.	n.d.	n.d.	48)
8	maceration	ethanol ^a	24	n.d.	n.d.	IC ₅₀ = 157.6 ppm for DPPH	n.d.	n.d.	49)
		<i>n</i> -hexane ^a				IC ₅₀ = 408.2 ppm for DPPH			
		chloroform ^a				IC ₅₀ = 19.6 ppm for DPPH			
		water ^a				IC ₅₀ = 218.5 ppm for DPPH			
9	heat-assisted extraction	ethanol 90%	1.16	80.5	32.0 ^b	75.2% ^c for DPPH 74.9% ^c for ABTS	n.d.	n.d.	37)
10	maceration	ethanol	31	5.1	0.9	4.1 mgAAE/g ^d	16.4 mm for at 5% extract	n.d.	50)
11	maceration	ethanol	24	n.d.	n.d.	n.d.	22 mm for <i>V</i>	n.d.	51)

		96%					<i>harveyi</i> at 40 mg/mL extract		
12	maceration	water	24	n.d.	n.d.	n.d.	16 mm for <i>E. coli</i> at 50% extract	n.d.	52)
13	maceration	ethanol 95%	168	n.d.	n.d.	n.d.	MIC = 250 µg/mL for <i>Campylobacter</i> spp.	n.d.	53)
14	maceration	ethanol 95%	n.m.	n.d.	n.d.	n.d.	n.d.	inhibition of nitric oxide production with IC ₅₀ = 27.3 µg/mL	12)
15	maceration	ethanol	24	n.d.	n.d.	n.d.	n.d.	membrane stabilization activity with EC ₅₀ = 52.9 µg/mL	15)
16	maceration	<i>n</i> -hexane	n.m.	n.d.	n.d.	n.d.	MIC = 156.3 µg/mL for <i>S. aureus</i>	inhibition of nitric oxide production with IC ₅₀ = 9.7 µg/mL	54)
		ethyl acetate					MIC = 19.5 µg/mL for <i>S. aureus</i>	inhibition of nitric oxide production with IC ₅₀ = 19.3 µg/mL	
		ethanol					MIC = 39.1 µg/mL for <i>S. aureus</i>	inhibition of nitric oxide production with IC ₅₀ = 43.0 µg/mL	
17	ultrasonication	ethanol 96%	1	94.9	119.3	IC ₅₀ = 202.2 and 250.8 ppm for DPPH and ABTS	MIC = 6.25 mg/mL for <i>S. aureus</i> and <i>E. coli</i>	inhibition of protein denaturation with IC ₅₀ = 86.0 µg/mL	This study
		ethanol 96%:water (v/v 1:1)	1	73.2	10.2	IC ₅₀ = 450.5 and 733.1 ppm for DPPH and ABTS	MIC = 12.5 mg/mL for <i>S. aureus</i> and <i>E. coli</i>	inhibition of protein denaturation with IC ₅₀ = 227.0 µg/mL	
		water	1	52.1	5.4	IC ₅₀ = 962.1 and 1529.1 ppm for DPPH and ABTS		inhibition of protein denaturation with IC ₅₀ = 759.3 µg/mL	

n.d.: not determined

n.m.: not mentioned

^a Data are for fractions, not crude extracts.^b Expressed as mg rutin equivalent per g dry weight.^c Extract concentrations not reported.^d AAE stands for ascorbic acid equivalent.

4 Conclusions

The findings of this study demonstrated that the EtOH₉₆ extract of *E. bulbosa* exhibited significant antioxidant activity, as evidenced by low IC₅₀ values in both the DPPH (202.2 ± 17.6 µg/mL) and ABTS (250.8 ± 7.8 µg/mL) radical scavenging assays. These results align with the high concentrations of two major classes of antioxidant compounds

polyphenols and flavonoids quantified in the extract at 94.9 ± 4.0 mg GAE/g DW and 119.3 ± 17.1 mg QE/g DW, respectively.

Moreover, the EtOH₉₆ extract exhibited antibacterial activity against *S. aureus* and *E. coli* (MIC = 6.25 mg/mL) and inhibited heat-induced protein denaturation (IC₅₀ = 86.0 µg/mL). Collectively, these findings highlight *E. bulbosa* tuber as a promising source of natural bioactive compounds with potential applications in topical antiseptics/wound care, oral antidiarrheals, anti-inflammatory adjuncts in carcinogenesis, antioxidant in cosmeceuticals, and antioxidant food stabilizers.

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Author Contributions

Huong Thi Thanh Pham: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation.

Diep Thi Thanh Le: Methodology, Investigation, Formal analysis, Data curation.

Anh Quoc Khuong Nguyen: Writing – review & editing, Writing – original draft, Visualization, Supervision, Project administration, Funding acquisition, Conceptualization.

Data Availability

All data generated or analyzed during this study are included in this published article.

Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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