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Metabolite Profiling and Bioactivity Assessment of Antioxidant and Antibacterial Properties in Bulb Fractions of *Eleutherine bulbosa* (Mill.) Urb

Profil Metabolit dan Evaluasi Bioaktivitas Antioksidan serta Antibakteri pada Fraksi Umbi *Eleutherine bulbosa* (Mill.) Urb

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ABSTRACT

Eleutherine bulbosa is known to contain diverse secondary metabolites with pharmacological potential. This study aimed to identify the bioactive compounds in the ethanol, n-hexane, and unsaponified n-hexane fractions of *E. bulbosa* bulbs and evaluate their antioxidant and antibacterial activities. The bulbs were extracted using ethanol, followed by n-hexane fractionation and saponification. Antioxidant activity was determined using the DPPH assay, and antibacterial activity was assessed against *Staphylococcus aureus* and *Escherichia coli* using the disc diffusion method. LC-MS/MS and GC-MS analyses identified compounds such as eleutherol, resveratrol, coniferaldehyde, and stigmasterol. The ethanol fraction exhibited the highest antioxidant activity (IC_{50} = 47.54 ± 0.03 mg/L), while the unsaponified n-hexane fraction showed the strongest antibacterial effect (inhibition zone = 16.12 ± 0.16 mm). These findings confirm the potential of *E. bulbosa*as a natural source of antioxidant and antibacterial agents.

ABSTRAK

Eleutherine bulbosa diketahui mengandung berbagai metabolit sekunder yang berpotensi farmakologis. Penelitian ini bertujuan untuk mengidentifikasi senyawa bioaktif dalam fraksi etanol, n-heksana, dan n-heksana tidak tersabunkan dari umbi *E. bulbosa* serta mengevaluasi aktivitas antioksidan dan antibakterinya. Umbi diekstraksi menggunakan etanol, diikuti fraksinasi n-heksana dan saponifikasi. Aktivitas antioksidan diuji dengan metode DPPH, sedangkan aktivitas antibakteri diuji terhadap *Staphylococcus aureus* dan *Escherichia coli* menggunakan metode difusi cakram. Analisis LC-MS/MS dan GC-MS mengidentifikasi senyawa seperti eleutherol, resveratrol, korniferaldehida, dan stigmasterol. Fraksi etanol menunjukkan aktivitas antioksidan tertinggi (IC_{50} = 47,54 ± 0,03 mg/L), sementara fraksi n-heksana tidak tersabunkan menunjukkan efek antibakteri paling kuat (zona hambat = 16,12 ± 0,16 mm). Hasil ini menegaskan potensi *E. bulbosa* sebagai sumber alami antioksidan dan antibakteri.

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1. INTRODUCTION

Eleutherine bulbosa (Mill.) Urb., commonly known as Dayak onion, belongs to the Iridaceae family. The plant originated in the tropical regions of South America and has been traditionally used in medicine across several tropical countries (Prameela et al., 2018). Numerous studies have shown that *E. bulbosa* possesses a wide range of pharmacological activities, including antioxidant (Lestari et al., 2019; Muthia et al., 2021; Pramiastuti et al., 2021; Shi et al., 2019), antibacterial (Novaryatiin et al., 2019; Padhi & Panda, 2015), anti-inflammatory, antimicrobial (Kamarudin et al., 2020), anticancer (Alamsyah Lubis et al., 2018; Mutiah & Rachmawati, 2024), and antifungal effects (Hayati et al., 2019; Hermawan et al., 2019). Moreover, its phytochemicals have demonstrated promising therapeutic potential in managing metabolic syndrome (Permatasari et al., 2024).

The pharmacological effects of *E. bulbosa* are closely linked to various secondary metabolites, such as flavonoids and alkaloids (Alamsyah Lubis et al., 2018; Haerani et al., 2019; Mutiah et al., 2024a; Pramiastuti et al., 2021), as well as phenolic compounds and other bioactive molecules (Haerani et al., 2019; Pramiastuti et al., 2021). Several compounds were identified in the ethanol extract of *E. bulbosa* bulbs, including 1,8-naphthalenediol-2,7-diacetyl-3,6-dimethyl (Pasanda et al., 2021), p-cresol, 4-fluorophenol, guaiazulene, furaneol, p-cymene, elaeocanin C, flavokawain A, 3-hydroxy-3,4-bis[(4-hydroxy-3-methoxyphenyl)methyl]oxolan-2-one, euparin, and eleutherol (Permatasari et al., 2024). Meanwhile, the chloroform fraction of the 96% ethanol extract contained hexadecanoic acid, 9,12-octadecadienoic acid, linoleic acid, octadecanoic acid, androstan-17-one, and 1-(2,3,4,6-tetramethylphenyl)ethanone (Lestari et al., 2019).

The antioxidant activity of *E. bulbosa* ranged from moderate to strong. The ethanol extract of bulbs from Thailand demonstrated notable antioxidant capacity, with an IC_{50} value of 0.432 ± 0.002 mg/mL (DPPH method) and a FRAP value of 3.73 ± 0.18 mg/mL. This activity correlated positively with total phenolic (70.91 ± 2.30 mg GAE/mL) and flavonoid (25.97 ± 0.66 mg QE/mL) contents (Panyachariwat et al., 2024). Similarly, the chloroform fraction of the ethanol extract displayed antioxidant activity with an IC_{50} value of 19.694 ppm (Lestari et al., 2019), while the n-hexane fraction of the aqueous extract demonstrated stronger activity with an IC_{50} value of 10 ppm. Furthermore, *E. bulbosa* also showed antibacterial properties. The ethanol extracts inhibited *Staphylococcus aureus*, with inhibition zones of 18.0 ± 1.7 mm at 15% concentration (Novaryatiin et al., 2019) and 12.33 ± 1.61 mm at 100% concentration (Warsiti et al., 2018). Other studies observed inhibition zones of 12.3 ± 0.58 mm and 14.5 ± 2.6 mm against *Escherichia coli* and *S. aureus*, respectively (Padhi & Panda, 2015).

Although several studies have reported the antioxidant and antibacterial activities of crude *E. bulbosa* extracts, particularly the ethanol and n-hexane fractions, limited information exists on the dominant secondary metabolites in each fraction and their contribution to biological activity. To date, no systematic comparative study has evaluated the antioxidant and antibacterial properties of these fractions based on their bioactive compound profiles. Therefore, this study aims to identify major secondary metabolites in the ethanol, n-hexane, and unsaponified n-hexane fractions of *E. bulbosa* bulbs and to evaluate their antioxidant and antibacterial activities. In addition, the study analyzes the relationship between chemical composition and biological activity. The findings are expected to contribute to the utilisation of *E. bulbosa* as a natural source of antioxidant and antibacterial agents and its potential application in the development of herbal-based health products.

2. METHODS

2.1. Materials and Equipment

Bulbs of *E. bulbosa* were collected from Cipedong Village, RT 03/RW 08, Sumur Bandung Subdistrict, Cipatat District, West Bandung Regency, West Java, Indonesia. Species identification was conducted at the Characterisation Laboratory of InaCC–BRIN, Cibinong Science Centre, LIPI Complex.

The equipment used in this study included standard glassware, a reflux apparatus, a rotary evaporator (IKA RV10), an oven (Memmert), an analytical balance (Ohaus), a laminar air flow cabinet (LAF), and a UV lamp (254 and 366 nm, CAMAG). Analytical instruments consisted of an LC-MS/MS Thermo Scientific Vanquish Flex UHPLC system coupled with a Q Exactive Plus Orbitrap-HRMS (Thermo Scientific, Munich, Germany), a GC-MS QP-Ultra (Shimadzu, Duisburg, Germany), and a UV-Vis spectrophotometer (Shimadzu PharmaSpec UV-1700, Kyoto, Japan). Data processing was performed using MZmine 4.5.20 software (University of Turku, Finland).

The materials used included *E. bulbosa* bulbs; polar solvents (96% ethanol, methanol); non-polar solvents (n-hexane and diethyl ether; SD Fine-Chem Ltd., Mumbai, India); silica gel 60 F254 (0.25 mm, Merck) for thin-layer chromatography (TLC); and phytochemical reagents. For the antioxidant assay, 2,2-diphenyl-1-picrylhydrazyl (DPPH; TCI) was used, with ascorbic acid and α-tocopherol as positive controls. The antibacterial assay used Mueller-Hinton Agar (MHA; HiMedia Laboratories, Mumbai, India), physiological saline (0.9% NaCl), distilled water, and dimethyl sulfoxide (DMSO; Merck). *E. coli* and *S. aureus* were obtained from the Biology Laboratory, IPB University.

2.2. Preparation and Extraction of *E. bulbosa* Bulbs

Fresh and dried bulbs of *E. bulbosa* were sorted and cut into approximately 1 cm pieces (Munaeni et al., 2019). The extraction process involved maceration, during which 100.20 g of bulb material was soaked in 200 mL of technical ethanol for three consecutive 24-hour periods with intermittent agitation. The mixture was then filtered (Hayati et al., 2019), and the filtrate was concentrated using a rotary evaporator to approximately 100 mL. The extract was then partitioned with n-hexane at a ratio of 2:1

(v/v), and the n-hexane and ethanol fractions were separated and evaporated to obtain concentrated fractions.

2.3. Saponification of *n*-Hexane Fraction

Saponification was conducted following a modified method described by Furi et al. (2022). A total of 7.59 g of the n-hexane fraction was dissolved in 50 mL of ethanol and mixed with 9.5 g of KOH and 10 mL of distilled water in a round-bottom flask. The mixture was refluxed for 1 hour at 40 ˚C in a water bath. After cooling, 50 mL of distilled water was added and stirred for 15 minutes using a magnetic stirrer. The solution was transferred to a separatory funnel and extracted three times with 50 mL of diethyl ether. The ether layer was washed with distilled water to remove residual alkali and concentrated using a rotary evaporator to obtain the unsaponified fraction.

2.4. Phytochemical Screening Using Thin-Layer Chromatography (TLC)

Phytochemical screening was performed using TLC following the method described by Pramiastuti et al. (2021). The spots on the silica gel plates were visualised under UV light at wavelengths of 254 nm and 366 nm. Each fraction was analysed employing silica gel F254 as the stationary phase, while the mobile phase and detection reagents were tailored to suit the specific group of target compounds. This screening successfully identified various classes of bioactive compounds, including flavonoids, alkaloids, tannins, saponins, phenolics, and terpenoids-steroids.

2.5. Identification of Hexane Fraction Metabolite Compounds with GC-MS

The identification of metabolites followed the method described by Dinh et al. (2020), with modifications in the oven temperature program. The analysis was carried out using an Rtx-5MS Low-Bleed capillary GC column (15 m × 0.25 mm × 0.25 µm; Saad et al., 2023). A 0.5% (v/v) solution of the n-hexane fraction (both saponified and unsaponified) in dichloromethane was injected (1.0 µL) into the GC-MS. Mass spectra were recorded in the m/z range of 40–700 using electron impact ionisation (70 eV), with a split ratio of 1:5 and helium as the carrier gas at 1 mL/min. The oven program was: 50 ˚C for 30 seconds, ramped at 5 ˚C/min to 180 ˚C, then at 20 ˚C/min to 325 ˚C, and held for 65 minutes.

2.6. Identification of Ethanol Fraction Metabolite Compounds with LC-MS/MS

Active compounds in the ethanol fraction were identified using an LC-MS/MS system (Munich, Germany) equipped with an Accucore C18 column (100 × 2.1 mm, 1.5 µm) at 30 ˚C (El-Hawary et al., 2022). The mobile phase consisted of solvent A (0.1% formic acid in acetonitrile) and solvent B (0.1% formic acid in water) with a flow rate of 0.2 mL/min. The gradient elution was: 5% B (0–1 min), 5–95% B (1–25 min), 95% B (25–28 min), returning to 5% B (28–33 min). Detection was conducted via electrospray ionisation (ESI) in both positive and negative ion modes using a Q-Orbitrap analyser. Instrument settings: nebuliser gas 40 psi, dry gas 10 L/min, capillary temperature 320 ˚C, spray voltage ±3800 V, cone voltage 100 V, m/z range 100–1500, and ionisation energies of 18, 35, and 53 eV.

2.7. Antioxidant Activity Assay

The antioxidant activity was assessed using the DPPH free radical method, as described by Pramiastuti et al. (2021). A 2 mL aliquot of 0.1 mM DPPH solution was mixed with 2 mL of each sample (ethanol, n-hexane, and unsaponified n-hexane fractions) at concentrations of 10, 30, 50, 70, and 90 mg/L. The mixture was incubated in the dark for 30 minutes at room temperature. Absorbance was measured using a UV–Vis spectrophotometer at the DPPH maximum wavelength (λ = 400–600 nm, error ±0.2–0.8). All tests were conducted in triplicate. Ascorbic acid and α-tocopherol served as positive controls, and a blank was used for accuracy.

2.8. Antibacterial Activity Assay

Antibacterial activity was evaluated using the disc diffusion method described by Sripahco et al. (2022). Each test was performed in duplicate. *E. coli* and *S. aureus* were cultured on MHA and suspended in 0.9% NaCl to match the McFarland standard (1×10^8 CFU/mL). Bacterial suspensions were spread evenly on the agar surface using sterile cotton swabs. Extract fractions (5–25%) in 10% DMSO were dispensed (30 µL) onto 6 mm sterile paper discs and placed on the inoculated plates. Plates were incubated at 37 ˚C for 24 hours. Inhibition zones were measured with a vernier calliper. A 10% clindamycin solution was used as a positive control and 10% DMSO as a negative control.

2.9. Data Analysis

Quantitative data from antioxidant and antibacterial assays were expressed as mean ± standard deviation (SD). Antioxidant activity was calculated based on DPPH inhibition percentage:

$$\text{Inhibition (\%)} = \frac{A_0 - A_s}{A_0} \times 100\%$$

where A_0 is the absorbance of the control and A_s is the absorbance of the sample. IC_{50} values were determined by interpolation from the concentration–inhibition curve at 50% inhibition. Antibacterial data were analysed using the Kruskal–Wallis test (SPSS v27) with significance set at $p < 0.05$. Dunn–Bonferroni post hoc tests were conducted for pairwise comparisons when significant differences were found.

3. RESULTS AND DISCUSSION

3.1. Phytochemical Screening

As shown in **Table 1,** the ethanol fraction contained alkaloids, saponins, phenolics, terpenoids/steroids, tannins, and flavonoids. In contrast, both the n-hexane and unsaponified n-hexane fractions contained alkaloids, phenolics, and terpenoids/steroids. These differences indicate that solvent polarity and fractionation processes have a significant influence on the metabolite profiles of *Eleutherine bulbosa* bulbs. The ethanol fraction was more effective in extracting polar compounds, whereas the n-hexane fraction preferentially extracted non-polar constituents.

Table 1. Phytochemical screening results of ethanol, *n*-hexane, and unsaponified *n*-hexane fractions of *E. bulbosa* bulbs

Sample Type	Metabolite Secondary Type					
	Alkaloid	Saponin	Phenolic	Terpenoid/steroid	Tannin	Flavonoid
Ethanol Fraction	+	+ + +	+ +	+	+ + +	+
n-Hexane Fraction	+ +	-	+	+ + +	-	-
Unsaponified *n*-Hexane Fraction	+ + +	-	+ + +	+	-	-

Note: (-): Not identified; (+): Identified, faint stain colour; (+ +): Identified, slightly visible stain colour; (+ + +): Identified, visible stain colour

A considerable body of research has focused on the ethanol extract of *E. bulbosa*; however, limited information is available regarding the phytochemical screening of its fractions. Haerani et al. (2019) reported that the ethanol extract of *E. bulbosa* bulbs from Manoko Plantation, Lembang, West Java, contained alkaloids, flavonoids, polyphenols, quinones, monoterpenoids, and sesquiterpenoids. Similarly, Mutiah et al. (2024a) identified alkaloids, flavonoids, terpenoids, steroids, and tannins in the 96% ethanol extract of *E. bulbosa* from East Kalimantan.

Phytochemical screening of the n-hexane and unsaponified n-hexane fractions revealed the presence of alkaloids, phenolics, and terpenoids, consistent with the findings of Pramiastuti et al. (2021), who reported alkaloids, tannins, steroids, and terpenoids in the *n*-hexane fraction of the aqueous extract of *E. bulbosa* bulbs from Tegal, Indonesia. Likewise, Malheiros et al. (2015) identified steroids, triterpenoids, azulene, anthraquinones, and naphthoquinones in the *n*-hexane extract of *E. bulbosa* from Brazil.

The unsaponified n-hexane fraction exhibited higher levels of alkaloids and phenolic compounds compared to the n-hexane fraction. This phenomenon can be attributed to the saponification process, which removes most lipids and saponified fatty acids, thereby concentrating non-lipid bioactive compounds such as alkaloids and phenolics. Consequently, these compounds were observed with greater intensity in the unsaponified fraction.

3.2. Metabolite Characteristics (GC–MS Analysis)

Gas chromatography–mass spectrometry (GC–MS) analysis identified 28 compounds in the *n*-hexane fraction and 18 compounds in the unsaponified *n*-hexane fraction (**Table 2**). In the *n*-hexane fraction (**Figure 1a**), the dominant peak corresponded to methyl palmitate (methyl hexadecanoate) at a retention time of 34.809 minutes, a compound previously reported to exhibit antioxidant potential (Andriana et al., 2022). In contrast, the predominant peak in the unsaponified n-hexane fraction (**Figure 1b**) appeared at 45.894 minutes and was identified as a flavonoid-type compound with potential antioxidant activity (Chaisuwan et al., 2022).

As shown in **Table 2**, the n-hexane fraction primarily contained nonpolar compounds, including long-chain hydrocarbons, methyl ester fatty acids, and various steroids. Conversely, the unsaponified n-hexane fraction exhibited higher concentrations of steroids, coumarins, flavonoids, and terpenoids. These findings are consistent with the phytochemical screening results presented in **Table 1**. The reduction in ester-type peaks after saponification indicates a decrease in lipid content, further supporting the concentration of non-lipid bioactive compounds.

Several metabolites were present in both fractions before and after saponification, including 5,7-dihydroxy-6-methyl-4-oxo-2-phenylchromen-8-carbaldehyde, (3β,22E)-ergosta-5,22-dien-3-ol, cholesterol, and coumarin. The persistence of these compounds indicates their stability during saponification and suggests that they possess nonpolar to semi-polar properties. These differences in metabolite composition between the two fractions may influence their respective pharmacological activities. These results align with previous reports identifying similar compounds in *E. bulbosa* extracts (Fridayanti et al., 2022; Pasanda et al., 2021; Saad et al., 2023).

The increased abundance of bioactive compounds—including steroids, coumarins, flavonoids, and terpenoids—in the unsaponified n-hexane fraction is attributed to the removal of dominant lipophilic compounds (e.g., fatty acid esters) during saponification. This process enhanced the detectability of minor components previously masked in the chromatogram. These compounds were not newly formed but were originally present in low concentrations and became more prominent due to reduced lipid interference.

3.3. Metabolite Characteristics (LC–MS/MS Analysis)

The ethanol fraction was analysed using liquid chromatography–tandem mass spectrometry (LC–MS/MS). Data were processed with MZmine software, employing an in-house database with a mass tolerance threshold of 5 ppm. Compound identification was confirmed using MS/MS (MS^2) spectral data for structural validation. LC–MS/MS analysis revealed six classes of secondary metabolites: naphthalenes, flavonoids, anthraquinones, polyphenols, phenylpropanoids, and sterols (**Table 3**). Among these, eleutherol, resveratrol, coniferaldehyde, and stigmasterol were prominent due to their known antioxidant and antibacterial properties.

As shown in **Figure 1**, eleutherol was detected at a retention time of 8.97 minutes. This compound is a characteristic marker of *E. bulbosa* extract and was previously reported at the same retention time by Permatasari et al. (2024). Eleutherol has demonstrated significant antioxidants (Gomes et al., 2023) and antibacterial (Bone et al., 2019) activities. According to Gomes et al. (2023), eleutherol exhibits superior antioxidant capacity compared to related naphthoquinone derivatives such as eleutherine and isoeleutherine. Bone et al. (2019) also confirmed its antibacterial potential against pathogenic bacteria.

Resveratrol, another compound detected in the ethanol fraction, is well-documented for its anticancer activity, particularly against lung cancer, as reported by Mutiah & Rachmawati (2024). Stigmasterol, also identified in this study and previously found in the chloroform extract of *E. bulbosa* by Saad et al. (2023), is recognised for its antioxidant and antibacterial effects (Bakrim et al., 2022).

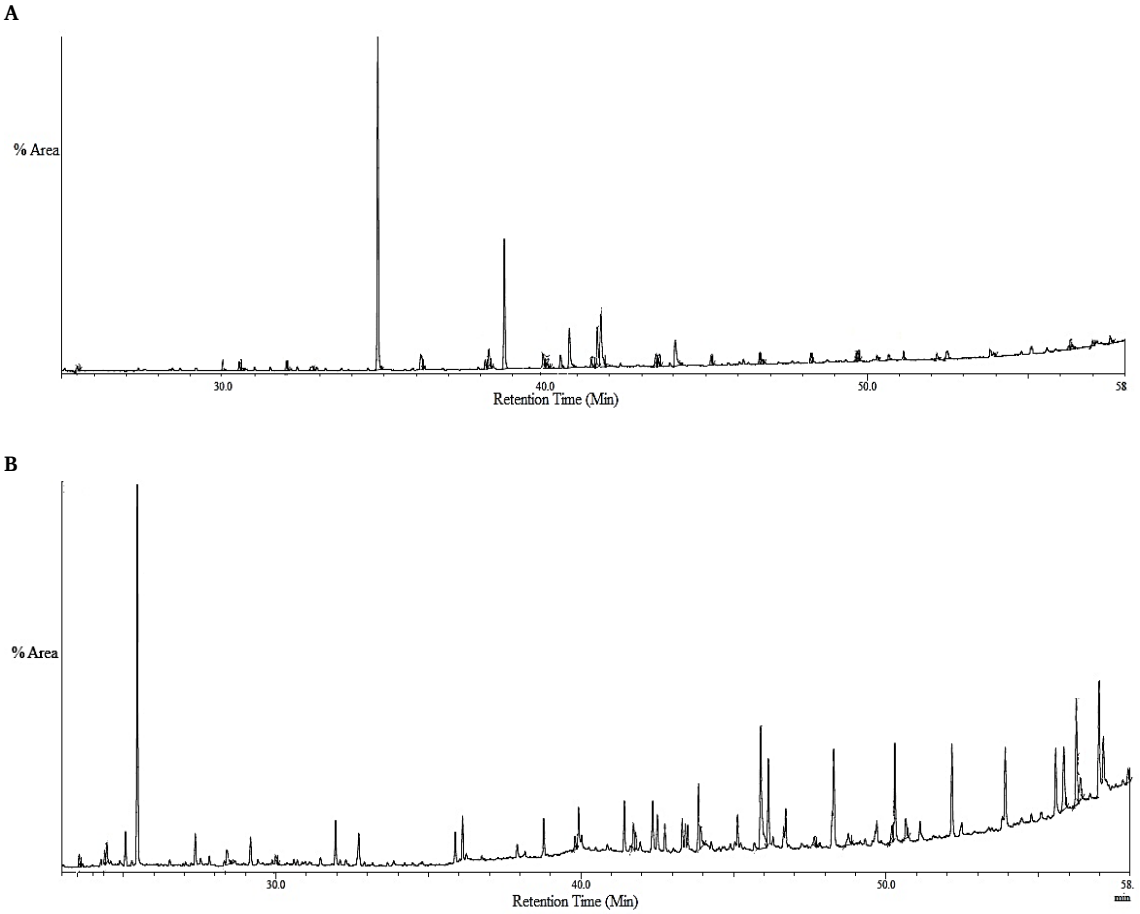

Figure 1. GC-MS chromatograms of (A) *n*-hexane fraction and (B) unsaponified *n*-hexane fraction of *E. bulbosa* bulbs.

Table 2. GC–MS-based identification of metabolite compounds from the *n*-hexane and unsaponified *n*-hexane fractions of *E. bulbosa* bulbs, classified by metabolite group, compound name, retention time (RT), and molecular formula.

Metabolite Group	n-Hexane Fraction			Unsaponified n-Hexane Fraction		
	Compound Name	RT (min)	Molecular Formula	Compound Name	RT (min)	Molecular Formula
Flavonoid	5,7-Dihydroxy-6-methyl-4-oxo-2-phenylchromen-8-carbaldehyde	50.673	$C_{17}H_{12}O_5$	5,7-Dihydroxy-6-methyl-4-oxo-2-phenylchromen-8-carbaldehyde	50.673	$C_{17}H_{12}O_5$
				3-Hydroxy-5-methoxyflavone	45.894	$C_{16}H_{12}O$
				5-Methoxyflavone	43.923	$C_{16}H_{12}O_3$
Fatty acid methyl ester	7-Hexadecanoic acid, methyl ester (Z)	38.256	$C_{17}H_{32}O_2$	Hexadecanoic acid, butyl ester	39.809	$C_{20}H_{40}O$
	8,11,14-Docosatrienoic acid, methyl ester	41.927	$C_{23}H_{40}O_2$			
	9,12-Octadecadienoic acid, methyl ester	38.144	$C_{19}H_{34}O$			
	Hexadecanoic acid, methyl ester	34.809	$C_{17}H_{34}O_2$			
	Methyl stearate	38.736	$C_{19}H_{38}O_2$			
Steroid	Ergosta-5,22-dien-3-ol (3β,22E)	56.263	$C_{28}H_{46}O$	Ergosta-5,22-dien-3-ol (3β,22E)	56.239	$C_{28}H_{46}O$
	Cholesterol	57.000	$C_{27}H_{46}O$	Cholesterol	55.828	$C_{27}H_{46}O$
				Ergost-5,8(14)-dien-3-ol	56.983	$C_{28}H_{46}O$
				4α,5-Cyclo-A-homo-5α-cholestane-6-one	57.936	$C_{28}H_{46}O$

Metabolite Group	n-Hexane Fraction			Unsaponified n-Hexane Fraction		
	Compound Name	RT (min)	Molecular Formula	Compound Name	RT (min)	Molecular Formula
Terpenoid				Isosteviol methyl ester	58.665	$C_{21}H_{32}O_3$
				Uvidin C	56.378	$C_{15}H_{26}O_3$
				2,6-Di-tert-butyl-4-hydroxy-4-methylcyclohexa-2,5-dien-1-one	24.392	$C_{15}H_{24}O_2$
Phenolic	Coumarin, 8-allyl-7-hydroxy-6-ethyl-4-methyl	41.728	$C_{15}H_{16}O_3$	Coumarin, 8-allyl-7-hydroxy-6-ethyl-4-methyl	41.724	$C_{15}H_{16}O_3$
	Phenol, 3,5-bis(1,1-dimethylethyl)-	25.479	$C_{14}H_{22}O$			
Quinone				Decahydroisoquinoline-3-carboxylic acid, methyl ester	48.766	$C_{11}H_{19}NO_2$
Hydrocarbon	3-Ethyl-2,6,10-trimethylundecane	49.715	$C_{16}H_{34}$	Docosa-2,6,10,14,18-pentaene-22-al, 2,6,10,15,18-pentamethyl-, all-trans	50.193	$C_{27}H_{44}O$
	7-Isopropyl-1,4a-dimethyl-1,2,3,4,4a,9,10,10a-octahydro-1-phenanthrenol	40.755	$C_{20}H_{31}O$	Benzene, 1,3-dimethoxy-5-pentadecyl	47.652	$C_{23}H_{40}O_2$
	9-Eicosene (E)	36.139	$C_{20}H_{40}$			
	Benzene, (1-pentylheptyl)-	30.607	$C_{12}H_{18}$			
	Benzene, (pentyloctyl)-	32.770	$C_{19}H_{32}$			
	Dodecane, 2,7,10-trimethyl-	43.515	$C_{15}H_{32}$			
	Heptadecane, 2,6,10,15-tetramethyl-	46.730	$C_{21}H_{44}$			
	Pentacosane	52.498	$C_{25}H_{52}$			
Primary fatty alcohol	1-Heneicosanol	39.941	$C_{21}H_{44}O$			
	n-Tetracosanol-1	46.664	$C_{24}H_{50}O$			
Naphthoquinone	2,3-Dimethoxy-1,4-naphthoquinone	40.481	$C_{12}H_{10}O_4$			
Carboxylic acid	2,6-Dihydroxybenzoic acid, 3 TMS derivative	52.173	$C_{16}H_{30}O_4Si$	Malonic acid, isobutyl tetradecyl ester	29.995	$C_{11}H_{20}O_4$
	Methyl tetradecanoate	30.504	$C_{15}H_{30}O_2$			
Aromatic ketone				Benzophenone	28.395	$C_{13}H_{10}O$
Ester	Carbonic acid, octadecyl vinyl ester	57.556	$C_{21}H_{40}O_3$	Oxalic acid, cyclohexylmethyl tridecyl ester	30.006	$C_{22}H_{40}O_4$
Alkyl halide	Propanedinitryl, [(3,4,5-trimethoxyphenyl)methylene]-	44.039	$C_{13}H_{12}N_2O_3$			
Unknown	t-Butyldimethyl(2-styryl[1.3]dithian-2-yl)silane	41.436	$C_{18}H_{28}S_2Si$	Amberone (isomer 2)	23.558	$C_{16}H_{26}O$

Table 3. LC–MS/MS-based identification of secondary metabolites in the ethanol fraction of *E. bulbosa* bulbs, including molecular characteristics, compound class, biological activity, and literature references.

No	Compound Name	Formula	MW	RT (min)	Error (ppm)	Mode Ion	MS/MS	Compound Class	Activity	Reference
1	Eleutherol	$C_{14}H_{12}O_4$	244	8.97	-1.25	[M+H]+	245, 227, 119, 174	Naphthalene	Antioxidant and antibacterial	(Bone et al., 2019; Gomes et al., 2023)
2	Resveratrol	$C_{14}H_{12}O_3$	229	9.31	-1.04	[M+H]+	229, 201, 183, 163	Flavonoid	Antioxidant, anti-inflammatory, antibacterial	(Meng et al., 2020; Mutiah et al., 2024b)
3	Methyl 8-hydroxy-3,4-dimethoxy-1-	$C_{19}H_{16}O_7$	357	10.76	-1.46	[M+H]+	357, 325, 267	Anthraquinone	–	-

No	Compound Name	Formula	MW	RT (min)	Error (ppm)	Mode Ion	MS/MS	Compound Class	Activity	Reference
	methylanthra-9,10-quinone-2-carboxylate									
4	Coniferaldehyde	$C_{10}H_{10}O_3$	179	25.16	-0.98	[M+H]+	179, 161, 133	Phenylpropanoid	Antioxidant and anti-inflammatory	(Park et al., 2024)
5	Stigmasterol	$C_{29}H_{48}O$	413	26.60	-0.34	[M+H]+	413, 159, 57	Sterol	Antioxidant and antibacterial	(Bakrim et al., 2022; Saad et al., 2023)
6	3-Hydroxyirisquinone	$C_{24}H_{38}O_4$	391	29.18	-0.40	[M+H]+	391, 149, 71	Anthraquinone	–	–

3.4. Antioxidant Activity

As presented in **Table 4**, the ethanol fraction of *E. bulbosa* bulbs exhibited the highest antioxidant activity, with an IC_{50} value of 47.54 ± 0.03 mg/L. The major bioactive compounds contributing to this activity include eleutherol, resveratrol, stigmasterol, and coniferaldehyde. These compounds contain hydroxyl (–OH) functional groups that enable the donation of hydrogen atoms or electrons to DPPH radicals, thereby neutralising free radicals (Theafelicia & Wulan, 2023).

The *n*-hexane fraction showed the lowest antioxidant activity, with an IC_{50} value of 75.31 ± 1.59 mg/L, classified as strong. This result differs from that of Pramiastuti et al. (2021), who reported very strong activity (IC_{50} = 10.7 mg/L) in the *n*-hexane fraction. Such discrepancies likely stem from differences in secondary metabolite profiles, particularly tannin and phenolic content, which may vary due to differences in extraction methods and sampling locations.

The lower antioxidant activity of the *n*-hexane fraction may be attributed to the dominance of methyl ester fatty acids, sesquiterpene alcohols, and phenolic compounds. The main compound, methyl hexadecanoate (methyl palmitate), accounted for 31.28% of the total chromatographic area. According to Andriana et al. (2022), methyl palmitate exhibits moderate to strong antioxidant activity in the ethyl acetate extract of *Alpinia galanga*, with IC_{50} values of 127.67 ± 1.41 mg/L using the DPPH method and 54.82 ± 1.18 mg/L using the ABTS method. The variation between these results suggests that DPPH may underestimate the antioxidant potential of lipophilic compounds due to its limited reactivity with such molecules Aryanti et al. (2021). In contrast, the ABTS method is more responsive to lipophilic antioxidants like methyl palmitate, offering a more accurate measure of their activity.

The antioxidant activity of the unsaponified *n*-hexane fraction improved significantly compared to the original *n*-hexane fraction, with IC_{50} values decreasing from 75.31 ± 1.59 mg/L to 50.54 ± 0.81 mg/L—an improvement that reclassifies the activity from strong to very strong. This suggests that saponification enhances antioxidant potential by removing non-active lipid components and enriching active constituents, such as flavonoids and terpenoid/steroid derivatives.

As shown in **Table 2**, the unsaponified *n*-hexane fraction exhibited higher levels of flavonoids, terpenoids, and steroids, along with newly identified quinones. Notably, 3-hydroxy-5-methoxyflavone and 5-methoxyflavone—members of the flavone subclass—were detected. Chaisuwan et al. (2022) demonstrated that methoxyflavone-rich ethanol extracts of *Kaempferia parviflora* showed very strong antioxidant activity, with an IC_{50} value of 12.5 ± 0.86 mg/L. The antioxidant activity of these compounds is attributed to the presence of methoxy ($–OCH_3$) and hydroxyl (–OH) groups, which effectively scavenge free radicals by donating electrons (Chen et al., 2020).

These findings are consistent with those of Jing et al. (2023), who reported that saponification increased antioxidant activity in natural sources such as apple peel (30.26%), turnip peel (91.74%), turnip flesh (425.3%), and corn (242.88%). These data support the hypothesis that fractionation, combined with chemical modifications like saponification, enhances the concentration of bioactive compounds responsible for antioxidant effects.

3.5. Antibacterial Activity

The unsaponified *n*-hexane fraction exhibited the strongest antibacterial activity at concentrations of 20% and 25%, producing clear inhibition zones against both test bacteria. In contrast, the ethanol fraction showed negligible antibacterial activity. Clindamycin was used as the positive control, following the method described by Novaryatiin et al. (2019). The results are summarized in **Table 5**. Overall, the unsaponified *n*-hexane fraction demonstrated the highest inhibitory effect against both *E. coli* and *S. aureus* (**Figure 2**).

The *n*-hexane fraction produced visible inhibition zones at concentrations of 20% and 25% against *E. coli* and *S. aureus*, respectively, although its antibacterial activity remained weak. The unsaponified *n*-hexane fraction showed inhibition zones at 15–25% concentrations for *E. coli* (weak activity), and at all tested concentrations (5–25%) for *S. aureus*, with strong inhibition observed at 20% and 25%. Conversely, the ethanol fraction displayed minimal antibacterial effects, with limited inhibition noted only at 20% against *E. coli* and at 25% against *S. aureus*. The antibacterial activity was categorised by inhibition zone diameter as follows: weak (<5 mm), moderate (5–10 mm), strong (10–20 mm), and very strong (>20 mm), according to Kurniawati et al. (2023).

a b

Figure 2. Inhibition zones of unsaponified *n*-hexane fraction extract against (a) *E. coli* and (b) *S. aureus*.

Table 4. Antioxidant activity of *E. bulbosa* bulb fractions against DPPH radicals.

No.	Sample Type	IC_{50} (mg/L)	Category*
1	Ethanol fraction	47.54 ± 0.03	Very strong
2	*n*-Hexane fraction	75.31 ± 1.59	Strong
3	Unsaponified *n*-hexane fraction	50.54 ± 0.81	Very strong
4	α-Tocopherol	7.81 ± 0.24	Very strong
5	Ascorbic acid	3.06 ± 0.23	Very strong

*Classification based on Souhoka et al. (2021): IC_{50} < 50 ppm = very strong; 50–100 ppm = strong; 100–150 ppm = moderate; 150–200 ppm = weak; >200 ppm = very weak.

Table 5. Antibacterial activity of *E. bulbosa* bulb fractions against *E. coli* and *S. aureus*.

Fraction	Inhibition Zone Diameter (mm)						
	5%	10%	15%	20%	25%	K^+	K^-
E. coli							
F1	–	–	–	–	–	4.20 ± 0.22	0
F2	–	–	–	2.13 ± 0.10	2.05 ± 0.15	4.28 ± 0.23	0
F3	–	–	2.20 ± 0.10	4.39 ± 0.11	3.64 ± 0.34	4.22 ± 0.21	0
S. aureus							
F1	–	–	–	–	4.99 ± 0.69	5.95 ± 0.15	0
F2	–	–	–	2.96 ± 0.54	3.96 ± 0.29	5.19 ± 0.68	0
F3	2.17 ± 0.10	3.65 ± 0.62	4.78 ± 0.55	8.40 ± 0.10	16.12 ± 0.16	5.30 ± 0.50	0

Note: F1 = ethanol fraction; F2 = n-hexane fraction; F3 = unsaponified n-hexane fraction; K^+ = positive control (10% Clindamycin); K^- = negative control (10% DMSO); (–) = no inhibition detected.

The differences in antibacterial activity can be partly explained by structural variations in bacterial cell walls. *S. aureus*(Gram-positive) possesses a thick peptidoglycan layer that facilitates compound diffusion, while *E. coli* (Gram-negative) has an outer membrane that restricts penetration of antibacterial agents (Naibaho et al., 2023).

The strong antibacterial activity of the unsaponified *n*-hexane fraction is associated with its elevated content of alkaloids, terpenoids, and phenolic compounds (**Table 1**). Compounds such as 3-hydroxy-5-methoxyflavone, benzophenone, and ergosta-5,22-dien-3-ol identified in this fraction have previously been reported to exhibit potent antibacterial properties (Nguyen et al., 2021; Wilis et al., 2018). These metabolites likely act by disrupting bacterial membranes, denaturing proteins, or interfering with enzymatic pathways, thereby inhibiting microbial growth.

The observed differences in antibacterial activity may be attributed to variations in bacterial cell wall structure. *S. aureus* (Gram-positive) possesses a thick peptidoglycan layer that facilitates compound diffusion, whereas *E. coli* (Gram-negative) has an outer membrane that limits the penetration of antibacterial agents (Naibaho et al., 2023). The strong antibacterial activity of the unsaponified *n*-hexane fraction is associated with its higher content of alkaloids, terpenoids, and phenolic compounds (**Table 1**). Compounds such as 3-hydroxy-5-methoxyflavone, benzophenone, and ergosta-5,22-dien-3-ol detected in this fraction have been reported to possess potent antimicrobial activity (Nguyen et al., 2021; Wilis et al., 2018). These bioactive compounds likely act by disrupting bacterial membranes, denaturing proteins, or interfering with enzymatic systems, thereby inhibiting microbial growth.

These findings clearly demonstrate the correlation between chemical composition and biological activity in *E. bulbosa* bulb fractions. The ethanol fraction, rich in phenolics and flavonoids, displayed strong antioxidant activity through mechanisms involving hydrogen or electron donation. In contrast, the unsaponified *n*-hexane fraction, which contained higher concentrations of terpenoids, steroids, and flavonoids, exhibited enhanced antibacterial and antioxidant activities. This

enhancement is attributed to the removal of non-active lipids during saponification, resulting in the concentration of active metabolites. Overall, these results underscore the influence of solvent polarity and chemical treatment on metabolite composition and biological potential, supporting the application of targeted extraction and fractionation to optimize the recovery of therapeutically valuable bioactive compounds.

4. CONCLUSION

This study demonstrated that the ethanol fraction of *E. bulbosa* bulbs exhibited the highest antioxidant activity (IC_{50} = 47.54 ± 0.03 mg/L), attributed to the presence of compounds such as eleutherol, resveratrol, stigmasterol, and coniferaldehyde. Although the *n*-hexane fraction showed lower antioxidant potential (IC_{50} = 75.31 ± 1.59 mg/L), its activity improved after saponification (IC_{50} = 50.54 ± 0.81 mg/L). In addition, the unsaponified *n*-hexane fraction demonstrated the highest antibacterial activity against *Staphylococcus aureus* and *Escherichia coli*, with an inhibition zone of 16.12 ± 0.16 mm observed at 25% concentration.

These findings indicate that variations in solvent polarity and chemical treatment influenced the composition and activity of the resulting fractions. The results support the potential of *E. bulbosa* bulbs as a natural source of antioxidant and antibacterial compounds. However, further investigation—including correlation and mechanistic studies—is needed to establish causal relationships between specific metabolites and biological activities.

AUTHOR CONTRIBUTIONS

Conseptualization, Gustini Syahbirin, Purwantiningsih Sugita, and Laksmi Ambarsari; methodology, Nurma Angeliani Komalasari; validation, Gustini Syahbirin, Purwantiningsih Sugita, and Laksmi Ambarsari; formal analysis, Nurma Angeliani Komalasari and Natalia Marbun; investigation, Nurma Angeliani Komalasari; writing-original draft preparation, Nurma Angeliani Komalasari; writing-review and editing, Gustini Syahbirin, Purwantiningsih Sugita, and Lakmi Ambarsari; visualization, Nurma Angeliani Komalasari; supervision, Gustini Syahbirin, Purwantiningsih Sugita, and Laksmi Ambarsari. All authors must confirm their agreement with the contribution statement before submission.

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Data supporting the findings of this study are available upon reasonable request form the corresponding author.

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CONFLICTS OF INTEREST

The authors declare there are no competing interests.

DECLARATION OF GENERATIVE ARTIFICIAL INTELLIGENCE (AI) USE

During the preparation of this manuscript, the author(s) used ChatGPT (OpenAI) to assist in improving the clarity, structure, or readability of the text. After using this tool, the author(s) thoroughly reviewed, edited, and verified the entire content to ensure it accurately represents their own ideas and interpretations. The author(s) take full responsibility for the integrity and originality of the published work.

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