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Research Article

Eremostachys laevigata Bunge responses to different extraction solvents and methods: physiological, biochemical, and antibacterial attributes

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Abstract

This study examined the biochemical composition of *Eremostachys laevigata* Bunge's aerial and underground organs using various solvents (methanol, water, N-hexane, chloroform, and acetone) and their impact on antibacterial activity against eight bacterial strains (*Escherichia coli*, *Staphylococcus aureus*, *Salmonella enterica*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Shigella dysenteriae*, *Proteus mirabilis*, and *Bacillus cereus*). Soxhlet and maceration extraction methods were used, and the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined using the tube dilution method. The study found that N-Hexane and water solvents had the highest total phenol content, while methanol, acetone, and chloroform had the lowest. Additionally, N-Hexane and methanol solvents showed the highest and lowest flavonoid content, respectively. The methanol extract from underground organs using the Soxhlet method exhibited the strongest antibacterial activity, whereas the acetone extract showed the weakest antimicrobial effect. Staphylococcus aureus displayed both the MIC and MBC, and Salmonella enterica showed the highest susceptibility to the well diffusion method, while both Escherichia coli and Staphylococcus aureus were highly susceptible to the disk diffusion method. The underground organs contained 6.87 $mg.g^{-1}$ of phenolic compounds and 0.19 $mg.g^{-1}$ of flavonoids. Two iridoid glycosides, pholoyoside and sesamoside, were separated from the methanol extract of the underground organs using reversed-phase HPLC. The research highlights *E. laevigata* Bunge's potential as a natural reservoir of antimicrobial compounds, with phenolic and flavonoid compounds, along with iridoid glycosides, presumed to contribute to its antibacterial properties.

Keywords: extraction; flavonoids; medicinal plants; solvent extraction; Soxhlet

Introduction

In recent years, the emergence of new pathogens and strains resistant to conventional antibiotics has raised public concern and limited the use of these drugs for treating bacterial infections. Therefore, many efforts

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have been made to exploit the potential power of natural antimicrobial compounds such as medicinal plants (Kebede *et al*., 2021). Moreover, antimicrobial agents sourced from plants eliminate bacteria through diverse mechanisms compared to antibiotics, which holds clinical significance in addressing infections caused by microbial-resistant strains (Cheesman *et al*., 2017). Low side effects of chemical drugs are an important reason beyond using herbs and their compounds which have proven over years of consumption in traditional medicine by medical societies (Lim *et al*., 2018). The widespread use of medicinal herbs is attributed to their natural origin, lower risk of adverse effects, availability, and affordability compared to synthetic drugs. In addition, medicinal plants contain effective and natural ingredients that promote biological balance in the body and help prevent drug accumulation (Tadese *et al*., 2022). Many secondary metabolites and plant extracts have been found to possess a wide range of biological activities, including antimicrobial properties (Allemailem, 2021). These natural compounds have been shown to have potential therapeutic benefits in the treatment of infectious diseases caused by bacteria, viruses, and fungi. Additionally, the use of plant extracts as antimicrobial agents may help reduce the development of antibiotic resistance, which is a growing concern in modern medicine (Kebede *et al*., 2021; Mohanasundari *et al*., 2022).

Eremostachys spp with 60 known species is distributed mainly in the Middle East and central and western areas of Asia, including Afghanistan, Pakistan, Turkey, and Iran (Rechinger *et al*., 1982). According to reports, 15 species of this genus have been identified and documented in the Damavand Highlands, as well as in the East and West Azerbaijan regions of Iran (Hadipour *et al*., 2016). *Eremostachys laevigata* Bunge, a member of the Lamiaceae family, is a perennial plant that can grow up to 100-150 cm in height. It bears white or purple flowers and has stalks covered with visible trichomes, particularly on the slopes of Bozgosh, Qaflankooh, and Mahoor hills around Miyaneh city of Iran. The plant typically flowers from April to June (Asnaashari *et al*., 2016; Hadipour *et al*., 2016).

Eremostachys species, such as *E. laciniata* Bunge, are commonly referred to as 'Chelle-Daghi' or 'çilə dağı' in the Azeri language of Iran. Local people typically gather the stems and rhizomes of these plants in August for various purposes. (Jamshidi and Ramezani, 2016). Various scientific reports suggest the use of *E. laciniata* Bunge for its antibacterial, antinociceptive, anti-inflammatory, anti-malarial, antioxidant, liver disease treatment, asthma, allergy, headache, and rheumatoid arthritis (Delazar *et al*., 2009; Asgharian *et al*., 2017). Phytochemical analyses of *Eremostachys* species have revealed the presence of various compounds, including flavonoids, alkaloids, coumarins, tannins, starch, resin, phytosterols, terpenoids, steroids, iridoid glycosides, and mono-, di-, and sesquiterpene saponins. These compounds have been shown to possess antibacterial activity, highlighting the potential of *Eremostachys* species for use in traditional medicine and as a source of natural antimicrobial agents (Hadipour *et al*., 2016; Mohammadhosseini *et al*., 2019)

Numerous studies have demonstrated the antibacterial activity of *E. laciniata* Bunge. For example, Rehman *et al*. (2015) reported that the extracts obtained using chloroform, N-hexane, butanol, and ethyl acetate have been found to possess significant antimicrobial activity against various strains of *Escherichia coli*, *Staphylococcus aureus*, *Staphylococcus typhimurium*, and *Pseudomonas aeruginosa*, with minimum inhibitory concentration (MIC) values of 29. Moreover, Liu *et al*. (2011) reported that the methanol extract of *E. purpurea* possesses significant antibacterial activity against a range of bacterial strains, including *Staphylococcus aureus* and *Escherichia coli*, with MIC values ranging from 0.1 to 1.25 $mg.mL^{-1}$. The results of these studies indicate that *E. laciniata* Bunge has promising potential as a natural source of antimicrobial agents, which could be utilized in the development of novel and effective treatments against bacterial infections.

Analyses of plant chemicals have identified iridoid glycosides, phenylethanoid glycosides, and flavonoid derivatives in *E. azerbaijanica*. Iridoid glycosides and isoflavones have also been identified in *E. moluccelloides* and *E. vicaryi*, respectively. These compounds exhibit diverse biological and pharmacological properties, such as anti-inflammatory, regenerative, and antibacterial effects, underscoring the therapeutic potential of

Eremostachys species as a reservoir of natural compounds with medicinal benefits (Asnaashari *et al*., 2015, 2016).

The choice of solvent for extracting bioactive compounds from plants can have a significant impact on their antimicrobial activity. Different solvents have varying abilities to extract different types of compounds, and the resulting extracts may have different chemical profiles and bioactivities (Rocchetti *et al*., 2020). For example, polar solvents such as methanol and ethanol are often used to extract polar compounds like phenolics and flavonoids, while non-polar solvents like hexane and dichloromethane are often used to extract non-polar compounds like terpenoids and alkaloids (Abd Aziz *et al*., 2021). Therefore, the selection of an appropriate solvent or combination of solvents is crucial for obtaining extracts with optimal antimicrobial activity. According to Delazar *et al*. (2017), the methanol, N-hexane, and dichloromethane extracts of *Scutellaria pinnatifida* did not exhibit any antimicrobial activity against gram-positive and gram-negative bacteria, as well as *Candida albicans* species. A report indicated the antibacterial activity of methanol extract of *E. labiosiformis* against bacteria of *Xanthamonas compestris, Rathayibacterrathyi,* and *Pseudomonas viridiflave* (Nigussie *et al*., 2021). The study had two primary objectives. Firstly, to assess the impact of various solvents on the biochemical compounds present in the *E. laevigata* Bunge plant. Secondly, to evaluate the antibacterial properties of both the aerial and underground parts of the plant extract using different extraction methods.

Materials and Methods

Plant materials

In August 2019, specimens of the plant referred to as 'Chelle-Daghi' in the local dialect were gathered from the village of Chern, situated near Kendovan in the Miyaneh city of East Azerbaijan province, Iran (37° 40′ 18″ N, 47° 32′ 52″ E, altitude of 2600 MASL). The plants were identified by the Iranian flora (Rechinger *et al*., 1982). A specimen (TUM-ADE 0204) from this collection has been preserved in the herbarium of the Faculty of Pharmacy, Tabriz University of Medical Sciences, Iran. The present study was conducted in 2021 at the Department of Biological Sciences laboratory, Faculty of Agriculture, Islamic Azad University, Mianeh Branch, Iran.

Preparation of rhizome and aerial organ extract

The plant samples were air-dried in the shade at room temperature and subsequently pulverized into a powder using a blender. Fifty grams of the powdered plant material underwent Soxhlet extraction using methanol, distilled water, chloroform, acetone, and N-hexane solvents for 6 h. Additionally, for the maceration method, 50 g of 'Chelle-Daghi' powder were immersed in 200 mL of each solvent in an Erlenmeyer flask and agitated on a shaker for 48 h. The resulting extracts were concentrated using a rotary evaporator, completely dried in a 37 °C oven, and stored at 4 °C for future use.

Total phenol content

The total phenolic content of the extract was determined by the Folin–Ciocalteu method (Kaur and Kapoor, 2002), then calculated using the calibration curve, and the results were calculated in terms of mg of gallic acid on g DW of the extract. The absorption levels of the samples were measured at a wavelength of 760 nm.

Total flavonoid content

The total flavonoid content was assessed using the aluminum chloride method (Chang *et al*., 2002). Subsequently, a calibration curve was constructed, and the results were expressed in terms of mg of rutin per g of dry weight of the extract. The samples' absorption levels were measured at a wavelength of 360 nnm.

DPPH assay

The DPPH (2,2-diphenyl-1-picrylhydrazyl) assay was used to determine the antioxidant activity of the plant sample. Briefly, 100 µL of the sample extract was added to 3.9 mL of a 0.1 mM DPPH solution in methanol. The mixture was vortexed and allowed to stand in the dark at room temperature for 30 min. The absorbance was then measured at 517 nm using a spectrophotometer. A control experiment was also performed by replacing the sample extract with methanol. The antioxidant activity was calculated using the following equation (Eq. 1):

Eq. 1. % inhibition = [(A_control – A_sample) / A_control] × 100

Where A_control is the absorbance of the control, and A_sample is the absorbance of the sample.

Bacterial strains

In this study, the microorganisms of *Escherichia coli* ATCC 1399, *Salmonella enterica* ATCC 1787, *Staphylococcus aureus* ATCC 1431, *Proteus mirabilis* ATCC 1076, *Pseudomonas aeruginosa* ATCC 1074, *Bacillus cereus* ATCC 1015, *Shigella dysenteriae,* and *Klebsiella pneumoniae* ATCC 1188, were prepared lyophilized from the collection center of Iranian Industrial Microorganisms.

Antimicrobial tests

This study investigated the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of the extracts through the tube dilution method and culture in Muller Hinton agar medium (Vanden Berghe and Vlietinck, 1991; Sindambiwe *et al*., 1999). Disk diffusion and Well methods were used in the Muller-Hinton agar (MHA) culture medium to determine the diameter of the non-growth halo (Mangena and Muyima, 1999).

Preparation of bacterial suspension

The lyophilized bacterial ampoules were opened under sterile conditions, and the initial culture was prepared using the nutrient broth medium. Subsequently, the bacteria were cultured on a nutrient agar medium and incubated at 37 °C for 48 h. A fresh culture was then used for the antimicrobial test. To prepare the McFarland standard 0.5 bacterial suspension, a 24-h microbial colony was suspended in normal saline physiological serum. The turbidity of the bacterial suspension was adjusted to match the McFarland standard 0.5 using a spectrophotometer at a wavelength of 625 nm, with an absorbance range of 0.08-0.13, and was compared to the standard McFarland 0.5 sample to ensure accuracy.

Preparation of concentrations of polar and non-polar extracts

The extracts were prepared using 5% DMSO and were concentrated to a high native level of 10 mg/mL for polar extracts and 30 mg/mL for non-polar extracts. To ensure sterility, the extracts were filtered using a 0.22 µm syringe filter and stored in sterile tubes at -18 °C until further use (Das *et al*., 2010).

Separation of iridoid glycoside

To separate the iridoid glycosides, the methanolic extract (2 g) was subjected to solid-phase extraction (SPE) using a C18 Sep-Pak cartridge (Waters, USA) with a gradient elution of methanol/water mixture (10:90, 20:80, 40:60, 60:40, 80:20, and 100:0). The resulting methanol extracts were analyzed using reversed-phase high-performance liquid chromatography (HPLC) with a C18 column system (250 mm length, 4.6 mm), and the other extracts were similarly analyzed (Delazar *et al*., 2017).

Statistical analysis

In this study, both sections were subjected to a completely randomized design (CRD) using SAS software version 9.1, and the data were analyzed using analysis of variance and mean comparison (performed

with Duncan's multiple range test at a 5% significance level). Additionally, the simple correlation between traits was assessed using Minitab software version 18.

Results

Effect of different solvents on the biochemical compounds

The data analysis revealed that the utilization of various solvents significantly influenced the antioxidant activity (DPPH), flavonoid content, and total phenols at a significance level of 1% (Table 1). As depicted in Figure 1, the analysis of the mean data demonstrated that employing solvents apart from water led to the highest average antioxidant activity, whereas utilizing water as a solvent resulted in the lowest average antioxidant activity (40.09%).

Table 1. Analysis of variance for the effect of extraction with different solvents on the physiological and biochemical characteristics of *Eremostachys laevigata* Bung

SOV	df	Mean square (MS)		
		DPPH	Flavonoids	Total phenol
Solvents	4	146.3**	339.7**	153.03**
Error	10	13.59	21.31	14.68
CV (%)	-	7.16	7.83	5.27

** Significant at the 1% probability level.

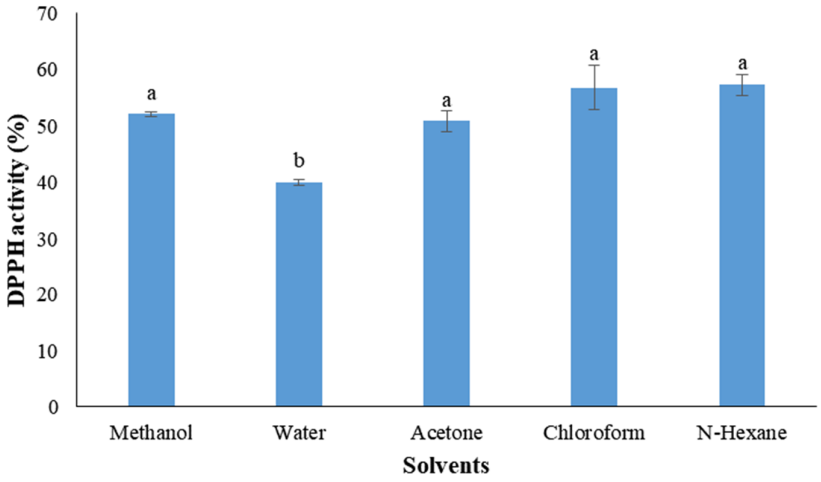


Figure 1. Effect of extraction with different solvents on the DPPH activity of *Eremostachys laevigata* Bung
The mean (±SE) presented with different letters differ significantly in terms of the DMRT.

The results indicated that the utilization of N-hexane and methanol solvents led to the highest and lowest flavonoid content, respectively, with values of 71.98 and 43.04 mg.g^{-1} DW. Conversely, the application of water, acetone, and chloroform solvents yielded an intermediate level of this property on average (Figure 2).

The study findings revealed that the use of N-hexane and water solvents yielded the highest total phenol content, with mean values of 81.96 and 78.35 mg.g^{-1} DW, respectively. Conversely, methanol, acetone, and chloroform solvents resulted in the lowest average values of this property, with values of 65.64, 70.06, and 65.64 mg.g^{-1} DW, respectively (Figure 3).

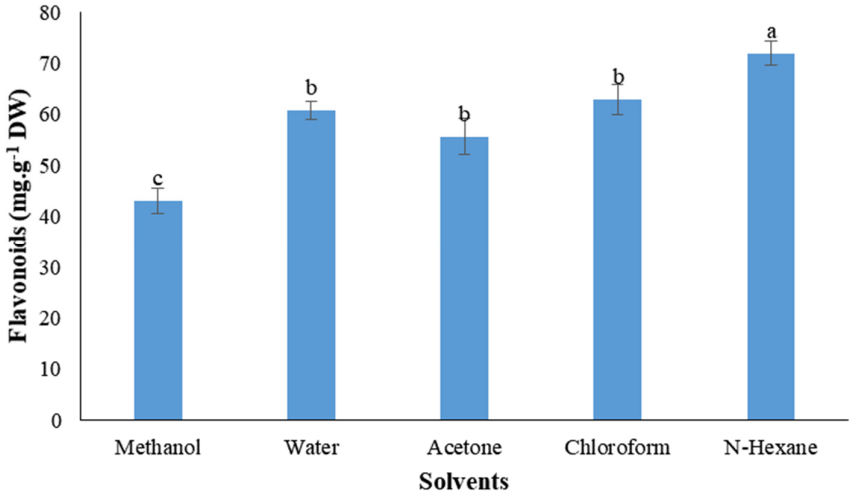


Figure 2. Effect of extraction with different solvents on the flavonoids content of *Eremostachys laevigata* Bung

The mean (±SE) presented with different letters differ significantly in terms of the DMRT.

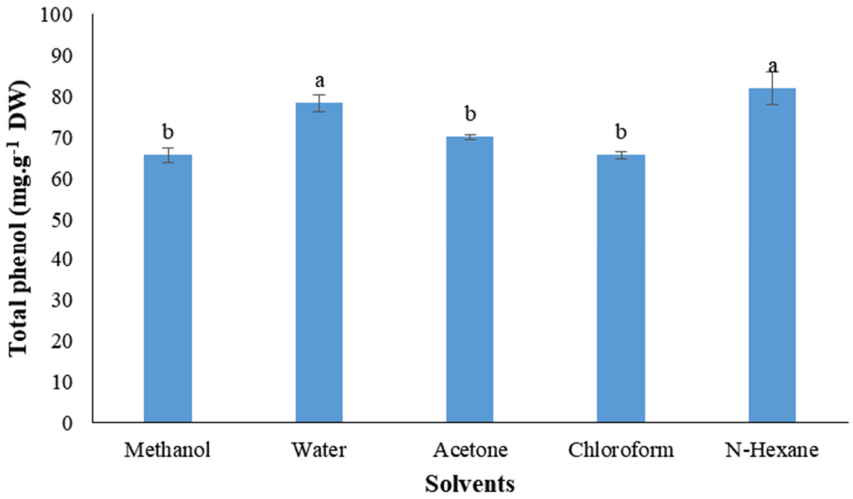


Figure 3. Effect of extraction with different solvents on the total phenol content of *Eremostachys laevigata* Bung

The mean (±SE) presented with different letters differ significantly in terms of the DMRT.

Soxhlet extraction method

The study results revealed that among the bacteria examined, the methanol extract of underground organs exhibited the largest non-growth halo diameter against *Salmonella enterica*, while the water extract showed the highest efficacy against *Shigella*. The chloroform extract exerted the greatest impact on *Proteus mirabilis*, while the N-hexane and acetone extracts demonstrated the most significant effects on *Escherichia coli*, *Proteus mirabilis*, and *Klebsiella pneumoniae*, respectively. Regarding aerial organs, both the methanolic and water extracts displayed the most substantial effects on *Escherichia coli*, while the chloroform extract was most effective against *Shigella*. The N-hexane extract had the highest impact on both *Escherichia coli* and *Klebsiella pneumoniae*, whereas the acetone extract exhibited the greatest efficacy against *Klebsiella pneumoniae* (Table 2).

Table 2. The mean diameter of inhibition zone of methanolic, water, N-hexane, chloroform, and acetone extracts of aerial and underground organs of *Eremostachys laevigata* Bung extracted by Soxhlet and maceration through well diffusion

Method of extraction	Plant organs	Solvent	*Escherich coli*	*Staphylococcus aureus*	*Salmonella antrica*	*Pseudomonas aeruginosa*	*Klebsiella pneumoniae*	*Shigella*	*Preteus mirabilis*	*Bacillus cereus*
Soxhlet	Underground	Methanol	23.3±1.89 c	22.7±1.25 c	24.7±2.49 b	19±2.16 c	19.7±0.47 c	21.3±1.25 b	19.3±0.94 c	15.3±1.2 c
		Water	20±1.41 d	12±0.82 f	19.7±1.7 fg	10±0.82 fg	14.3±0.47 e	21.3±1.25 b	19.3±0.94 c	12±0.82 e
		Chloroform	10±0.82 ghi	11±0.82 fg	11.7±1.25 k	3±0.82 k	3±0.82 j	12±0.82 ef	14.3±0.47 fg	0.5±0 g
		N-hexane	15.7±0.47 e	11±0.82 fg	7±2.16 f	8.3±1.25 gh	13.7±0.47 e	10±0.82 fg	16±0 e	0.5±0 g
		Acetone	0.5±0 k	0.5±0 j	0.5±0 g	9±0 gh	10±0.82 g	0.5±0 i	0.5±0 k	0.5±0 g
	Aerial organ	Methanol	23.3±2.05 c	18±0.82 d	22.3±2.87 bc	16.3±2.87 d	18±0.82 d	18±0.82 c	19±0.82 c	13±1.41 d
		Water	21.7±0.47 cd	14±0.82 e	19.7±0.47 c	5±0.82 jk	12±0.82 f	15.7±0.47 d	17.7±0.47 d	11±1.41 ef
		Chloroform	9±0 hij	8±0.82 i	10±0 d e	0.5±0 l	0.5±0 k	11.7±1.25 ef	10.7±0.47 i	0.5±0 g
		N-hexane	13.7±0.47 ef	8±1.41 i	6±0.82 f	0.5±0 l	13.7±0.47 e	5.7±1.7 h	9.7±0.47 ij	0.5±0 g
		Acetone	0.5±0 k	0.5±0 j	0.5±0 g	5.7±1.7 ij	9±0 g	0.5±0 i	0.5±0 k	0.5±0 g
Maceration	Underground	Methanol	22±0 cd	18±0.82 d	21.3±1.25 c	13.7±0.47 ef	13.7±0.47 e	20.3±1.7 bc	19.7±0.47 c	14.3±0.47 cd
		Water	19.7±0.94 d	10±0.82 fgh	19.7±1.7 c	5±0.82 jk	10±0.82 g	19.3±0.47 bc	15.7±0.47 ef	11±0.82 ef
		Chloroform	8.3±2.49 ij	9.7±0.47 gh	8±1.63 e f	0.5±0 l	0±0 k	18.7±0.94 c	15±0.82 ef	0.5±0 g
		N-hexane	11±1.63 gh	11±0.82 fg	8±1.63 ef	8±1.63 ghi	12±0.82 f	11±2.16 fg	13.7±0.47 gh	0.5±0 g
		Acetone	0.5±0 k	0.5±0 j	0.5±0 g	7±0.82 hij	5±0.82 ij	0.5±0 i	0.5±0 k	0.5±0 g
	Aerial organ	Methanol	20±1.63 d	12±2.16 f	19.3±0.47 c	12±0.82 ef	18±0.82 d	13.3±1.25 e	13±0 h	11±0.82 ef
		Water	15±0.82 e	10±0.82 fgh	19.3±0.47 c	5±0.82 jk	7±2.16 h	12.7±1.7 e	13.7±0.47 gh	10±0 f
		Chloroform	7±0.82 i	7±0 i	5.7±1.7 f	0.5±0 l	0.5±0 k	8±0.82 g	10.7±0.47 i	0.5±0 g
		N-hexane	12±0.82 fg	7.3±0.47 i	6±0 f	0.5±0 l	10±0.82 g	5±0 h	9±0.82 j	0.5±0 g
		Acetone	0.5±0 k	0.5±0 j	0.5±0 g	5.7±1.7 ij	0±0 k	0±0 i	0±0 k	0.5±0 g
Gentamicin			30±1.63 b	28±0.82 b	30±1.63 a	25±0.82 b	27.3±0.47 b	28±0.82 a	26±0.82 b	21.3±1.25 b
Ampicillin			34.7±0.47 a	30±1.63 a	31.3±1.25 a	31.3±1.25 a	33±0.82 a	30±1.63 a	29±0.82 a	27.3±1.89 a
Control			0.5±0 k	0.5±0 j	0.5±0 g	0.5±0 l	0.5±0 k	0.5±0 i	0.5±0 k	0.5±0 g

Means (±SD) followed by the same letter in each column are not significantly different according to the DMRT test at 5% level

Number of *ml**

Maceration extraction method

The results of the study showed that the methanol extract of underground organs had the highest non-growth halo diameter on *Escherichia coli* and *Salmonella enterica*, while the water extract was most effective on *Escherichia coli*, *Salmonella enterica*, and *Shigella*. The chloroform extract had the highest impact on *Shigella*, while the N-hexane and acetone extracts were most effective on *Proteus mirabilis* and *Pseudomonas aeruginosa*, respectively. Regarding aerial organs, the methanolic extract showed the highest effectiveness against *Escherichia coli* and *Salmonella enterica*, while the water extract exhibited the greatest impact specifically on *Salmonella enterica*. The chloroform extract was most effective on *Proteus mirabilis*, and the N-hexane and acetone extracts had the greatest effect on *Escherichia coli* and *Pseudomonas aeruginosa*, respectively. Among all the extracts studied, the methanol extract of underground organs using both Soxhlet and maceration methods

exhibited the strongest inhibitory effect on the tested bacteria, which was significant compared to the control group but not as effective as ampicillin and gentamicin (Table 2).

During the Soxhlet extraction of underground organs, the methanolic extract showed the largest non-growth halo diameter against *Escherichia coli*, whereas the water extract exhibited the most efficacy specifically against *Proteus mirabilis* (Table 3).

Table 3. The mean diameter of inhibition diameter of methanolic, water, N-hexane, chloroform, and acetone extracts of aerial and underground organ of *Eremostachys laevigata* Bung extracted by Soxhlet and maceration by disk diffusion

Method of extraction	Plant organs	Solvent	*Escherich coli*	*Staphylococcus aureus*	*Salmonella antrica*	*Pseudomonas aeruginosa*	*Klebsiella pneumoniae*	*Shigella*	*Preteus mirabilis*	*Bacillus cereus*
Soxhlet	Underground	Methanol	24±22.67 bc	21.3±1.25 c	20±1.41 c	15±0.82 c	15±0.82 c	18±0.82 c	19±2.16 bc	18±0.82 b
		Water	20±16.33 e	14.3±0.47 d	14.3±0.47 e	13.7±0.47 cd	12.7±1.7 de	12.7±1.7 fg	18±0.82 c	11±0 d
		Chloroform	11±8.33 ij	10±0 f	11±0.82 f	0.5±0 i	0.5±0 k	0.5±0 j	0.5±0 g	0.5±0 h
		N-hexane	14±13 fg	10±0 f	8±0.82 g	13.7±0.47 cd	7±0 hi	12±0.82 fg	0.5±0 g	0.5±0 h
		Acetone	0.5±0 k	0.5±0 h	0.5±0 i	10±0.82 g	11±0.82 ef	0.5±0 j	0.5±0 g	11±0.82 d
	Aerial organ	Methanol	23±21.33 c	22.3±2.87 bc	20±1.41 c	13.7±0.47 cd	8.3±2.49 gh	16.3±2.87 cd	14.3±0.47 de	12.3±0.94 c
		Water	17±17 e	19.7±0.47 c	20±1.41 c	12±0.82 ef	10±0.82 fg	11±0.82 gh	14.3±0.47 de	4.3±1.25 g
		Chloroform	7±8 ij	7±2.16 g	8.3±1.25 g	0.5±0 i	0.5±0 k	0.5±0 j	0.5±0 g	0.5±0 h
		N-hexane	12±11.67 gh	7±0 g	8.3±1.25 g	12±0.82 ef	3±0 j	8±0.82 h	0.5±0 g	0.5±0 h
		Acetone	0.5±0 k	0.5±0 h	0.5±0 i	0.5±0 i	5±0.82 j	0.5±0 j	0.5±0 g	0.5±0 h
Maceration	Underground	Methanol	21±20 d	21±0.82 c	18±0.82 d	14.3±0.47 cd	13.7±0.47 cd	18±0.82 c	15.7±0.47 d	13.3±0.47 c
		Water	15±15.33 ef	12.7±1.7 de	20±1.41 c	12±0.82 ef	12±0.82 def	15.7±0.47 de	13.7±0.47 ef	9±0.82 ef
		Chloroform	10±7 j	8.3±2.49 fg	6±0.82 h	0.5±0 i	0.5±0 k	0.5±0 j	0.5±0 g	0.5±0 h
		N-hexane	10±10 hi	10.3±0.47 ef	6±0.82 h	13±0 de	7±2.16 hi	8.3±2.49 h	0.5±0 g	0.5±0 h
		Acetone	0.5±0 k	0.5±0 h	0.5±0 i	8.3±1.25 h	8±0.82 gh	0.5±0 j	0.5±0 g	11±0 d
	Aerial organ	Methanol	16±15.67 ef	21.3±1.25 c	18±0.82 d	12±0.82 ef	11±0 ef	13.7±0.47 ef	13.7±0.47 ef	10±0.82 de
		Water	15±14.33 ef	20±1.41 c	15.7±0.47 e	10±0 g	8±0.82 gh	12.7±1.7 fg	12.7±1.7 f	5±0 g
		Chloroform	8±5.67 j	5.7±1.7 g	5±0 h	0.5±0 i	0.5±0 k	0.5±0 j	0.5±0 g	0.5±0 h
		N-hexane	9±10 hi	6±0 g	5±0.82 h	11±0.82 fg	3±0 j	5±0 i	0.5±0 g	0.5±0 h
		Acetone	0.5±0 k	0.5±0 h	0.5±0 i	0.5±0 i	3.3±1.25 j	0.5±0 j	0.5±0 g	8±0.82 f
Gentamicin			26±24.67 b	24.7±0.94 b	24±0 b	18±1.63 b	24.7±0.94 b	24±0 b	20±0.82 b	19±0.82 b
Ampicillin			30±29.67 a	31.3±1.25 a	27.3±0.47 a	21.3±0.94 a	29.7±0.47 a	27.3±0.47 a	24.3±0.47 a	21.3±1.25 a
Control			0.5±0 k	0.5±0 h	0.5±0 i	0.5±0 i	0.5±0 k	0.5±0 j	0.5±0 g	0.5±0 h

Means (±SD) followed by the same letter in each column are not significantly different according to the DMRT test at 5% level

Number of *ml**

The chloroform extract had the highest impact on *Salmonella enterica*, while the N-hexane and acetone extracts were most effective on *Pseudomonas aeruginosa*, *Klebsiella pneumoniae,* and *Bacillus cereus*, respectively. In terms of Soxhlet extraction of aerial organs, the methanolic extract had the greatest effect on *Staphylococcus*

aureus, while the water extract had the highest impact on both *Staphylococcus aureus* and *Salmonella enterica*. The chloroform extract showed the highest efficacy against *Salmonella enterica*, while the N-hexane and acetone extracts exhibited the most effectiveness against *Escherichia coli*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae*.

Regarding the maceration method for underground organs, the methanol extract showed the highest non-growth halo diameter on *Staphylococcus aureus*, while the water extract had the highest impact on *Salmonella enterica*. The chloroform extract demonstrated the highest effectiveness against *Staphylococcus aureus*, while the N-hexane and acetone extracts exhibited the most effectiveness against *Pseudomonas aeruginosa* and *Bacillus cereus*, respectively. Ultimately, during the maceration extraction of aerial organs, the methanol and water extracts exhibited the highest effectiveness against *Staphylococcus aureus*, whereas the chloroform extract displayed the most efficacy against *Escherichia coli*, *Staphylococcus aureus*, and *Salmonella enterica*. The N-hexane extract showed the greatest impact on *Pseudomonas aeruginosa*, and the acetone extract demonstrated the most effectiveness against *Bacillus cereus*.

Based on the findings provided in Table 4, during the Soxhlet extraction of underground organs, the methanolic extract exhibited the lowest MIC against *Staphylococcus aureus*, whereas the water extract demonstrated the highest efficacy specifically against *Pseudomonas aeruginosa*. The chloroform extract had the lowest MIC on *Staphylococcus aureus*, while the N-hexane and acetone extracts were most effective on *Escherichia coli*, *Pseudomonas aeruginosa,* and *Bacillus cereus*, respectively. Regarding the Soxhlet extraction of aerial organs, the methanolic, water, and chloroform extracts exhibited notable efficacy against *Staphylococcus aureus*, whereas the N-hexane extract displayed optimal effectiveness against *Escherichia coli* and *Staphylococcus aureus*, and the acetone extract showed remarkable effectiveness against *Bacillus cereus*.

Among all the extracts studied, the methanolic and N-hexane extracts of underground organs and the water extract of aerial organs had the greatest effect on *Escherichia coli*. The methanol extract of underground organs and the methanol and water extracts of aerial organs using the Soxhlet method had the greatest effect on *Staphylococcus aureus*. The methanol and water extracts of underground organs using the Soxhlet method had the greatest effect on *Shigella dysenteriae*. The methanol extract from underground organs and the water extract from underground and aerial organs using the maceration method had the greatest effect on *Bacillus cereus*.

According to the findings presented in Table 5, among the extracts obtained from underground organs using the Soxhlet method, the methanol extract had the highest bactericidal effect on *Staphylococcus aureus*, while the water extract was most effective on *Pseudomonas aeruginosa*, *Shigella dysenteriae*, and *Proteus mirabilis*. The chloroform extract exhibited the most potent bactericidal effect against *Staphylococcus aureus* and *Salmonella enterica*, whereas the N-hexane and acetone extracts demonstrated optimal effectiveness against *Escherichia coli* and *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and *Bacillus cereus*. In terms of Soxhlet extraction of aerial organs, the methanol and water extracts had the highest bactericidal effect on *Staphylococcus aureus*, while the chloroform extract was most effective on *Staphylococcus aureus* and *Salmonella enterica*. The N-hexane extract was most effective on *Escherichia coli* and *Staphylococcus aureus*, and the acetone extract was most effective on *Bacillus cereus*.

Regarding the maceration method for underground organs, the methanol extract had the highest bactericidal effect on *Salmonella enterica*, while the water extract was most effective on *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Proteus mirabilis*, and *Bacillus cereus*. The chloroform extract had the highest bactericidal effect on Staphylococcus aureus and *Salmonella enterica*, while the N-hexane and acetone extracts were most effective on *Escherichia coli* and *Pseudomonas aeruginosa*, and *Bacillus cereus*, respectively. Finally, in the maceration extraction of aerial organs, the methanol, water, and chloroform extracts were most effective

on *Staphylococcus aureus*, while the N-hexane extract was most effective on *Escherichia coli* and *Staphylococcus aureus*, and the acetone extract was most effective on *Pseudomonas aeruginosa* and *Bacillus cereus*.

Table 4. MIC of methanol, water, N-hexane, acetone, and chloroform solvents of underground and aerial organs of *Eremostachys laevigata* Bung extracted by Soxhlet and maceration methods

Method of extraction	Plant organs	Solvent	*Escherich coli*	*Staphylococcus aureus*	*Salmonella antrica*	*Pseudomonas aeruginosa*	*Klebsiella pneumonia*	*Shigella*	*Preteus mirabilis*	*Bacillus cereus*
Soxhlet	Underground	Methanol	625	156.25	312.5	625	625	1875	625	1250
		Water	1250	625	625	312.5	1250	1875	625	1250
		Chloroform	5000	1250	2500	7500	-	3750	3750	3750
		N-hexane	625	2500	2500	1875	3750	3750	3750	15000
		Acetone	-	-	-	1875	3750	7500	3750	1875
	Aerial organ	Methanol	1250	156.25	625	625	1250	1875	1250	1250
		Water	625	156.25	625	625	1250	1875	1250	2500
		Chloroform	2500	1250	2500	7500	-	7500	7500	7500
		N-hexane	1250	1250	5000	3750	3750	3750	7500	15000
		Acetone	-	-	-	7500	7500	15000	15000	3750
Maceration	Underground	Methanol	1250	312.5	625	1250	1250	3750	1250	2500
		Water	2500	625	1250	625	1250	3750	1250	1250
		Chloroform	5000	2500	5000	7500	-	7500	7500	7500
		N-hexane	1250	2500	5000	3750	7500	7500	7500	15000
		Acetone	-	-	-	3750	15000	7500	7500	3750
	Aerial organ	Methanol	1250	312.5	625	1250	2500	7500	7500	15000
		Water	1250	312.5	1250	1250	2500	7500	2500	2500
		Chloroform	2500	2500	5000	7500	-	15000	15000	7500
		N-hexane	2500	2500	10000	7500	3750	7500	15000	15000
		Acetone	-	-	-	7500	15000	15000	15000	7500

Number of ml*

Table 5. MBC of methanol, water, N-hexane, acetone, and chloroform solvents of underground and aerial organs of *Eremostachys laevigata* Bung extracted by Soxhlet and maceration methods

Method of extraction	Plant organs	Solvent	*Escherich coli*	*Staphylococcus aureus*	*Salmonella antrica*	*Pseudomonas aeruginosa*	*Klebsiella pneumoniae*	*Shigella*	*Preteus mirabilis*	*Bacillus cereus*
Soxhlet	Underground	Methanol	2500	625	1250	2500	2500	2500	1250	2500
		Water	5000	2500	2500	1250	5000	1250	1250	2500
		Chloroform	10000	5000	5000	15000	-	7500	7500	7500
		N-hexane	2500	5000	10000	3750	7500	7500	7500	-
		Acetone	-	-	-	7500	7500	15000	15000	7500
	Aerial organ	Methanol	2500	625	2500	1250	5000	2500	5000	2500
		Water	2500	625	2500	2500	2500	1250	5000	5000
		Chloroform	10000	5000	5000	15000	-	15000	15000	15000
		N-hexane	5000	5000	10000	15000	15000	15000	15000	-
		Acetone	-	-	-	15000	15000	-	-	7500
Maceration	Underground	Methanol	5000	2500	1250	2500	5000	2500	2500	5000
		Water	5000	2500	5000	2500	5000	5000	2500	2500
		Chloroform	-	10000	10000	15000	-	15000	15000	15000
		N-hexane	5000	5000	10000	7500	15000	15000	15000	-
		Acetone	-	-	-	15000	-	15000	15000	7500
	Aerial organ	Methanol	2500	1250	5000	2500	5000	5000	5000	5000
		Water	5000	1250	5000	5000	5000	5000	5000	5000
		Chloroform	10000	5000	10000	15000	-	-	-	15000
		N-hexane	5000	5000	10000	15000	15000	-	-	-
		Acetone	-	-	-	15000	-	-	-	15000

Number of ml*

Among all the extracts studied, the methanol and N-hexane extracts of underground organs, methanol and water extracts of aerial organs using the Soxhlet method, and the methanol extract of aerial organs using the maceration method had the most bactericidal effect on *Escherichia coli*. The methanol extract of underground organs and the methanol and water extracts of aerial organs using the Soxhlet method had the greatest bactericidal effect on *Staphylococcus aureus*. The methanol extract of underground organs using the Soxhlet method and maceration method had the greatest bactericidal effect on *Salmonella enterica*. The water extract from underground organs and the methanol extract from aerial organs using the Soxhlet method had

the greatest bactericidal effect on *Pseudomonas aeruginosa*. The methanol extract from underground organs and the water extract from aerial organs using the Soxhlet method had the greatest bactericidal effect on *Klebsiella pneumoniae*. The water extracts of underground and aerial organs using the Soxhlet method had the greatest bactericidal effect on *Shigella dysenteriae*. The methanol and water extracts obtained from underground organs using the Soxhlet method exhibited the most significant bactericidal effect against *Proteus mirabilis*. Additionally, the methanol and water extracts from both underground and aerial organs obtained using the Soxhlet method, along with the water extract from underground organs obtained using the maceration method, displayed the most substantial bactericidal effect against *Bacillus cereus*.

According to the results of reversed-phase HPLC analysis, the methanol extract of the underground organ of *E. laevigata* contained two iridoid glycosides, namely phloyoside I and sesamoside. Phloyoside I was detected at an inhibitory time (Rt) of 3.33 min, while sesamoside was detected at an inhibitory time (Rt) of 100.73 min. It is worth noting that iridoid glycoside compounds were not detected in the distilled water, N-hexane, chloroform, and acetone extracts of *E. laevigata* Bung underground organs, as shown in Figure 4.

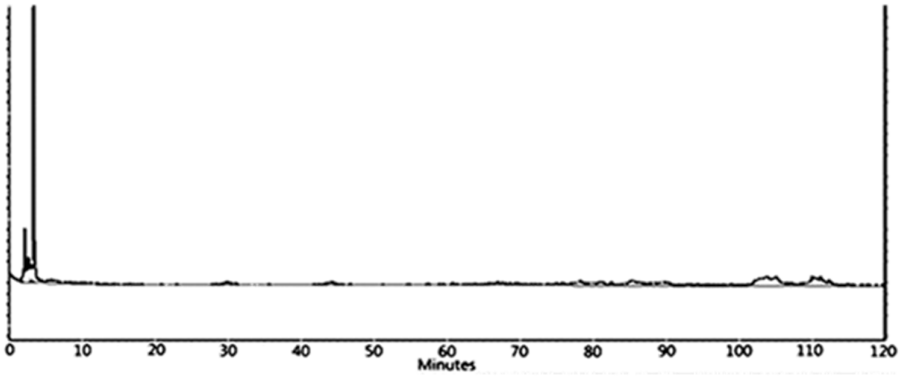


Figure 4. The chromatogram obtained by reversed-phase of methanol extract of the underground organ of *Eremostachys laevigata* Bung

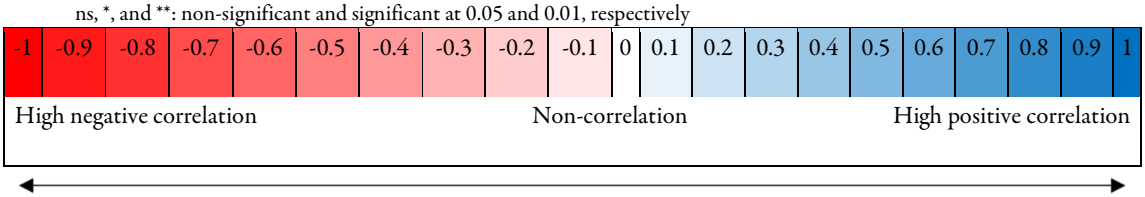
Correlation analysis

To investigate the associations between the biochemical properties and MIC and MBC, a correlation analysis was conducted and the findings are presented in Table 6. The findings indicated a positive correlation between MIC and MBC with flavonoid content and antioxidant activity (DPPH). In other words, an elevation in MIC and MBC was observed to coincide with an increase in flavonoid content and antioxidant activity.

Table 6. Simple correlation coefficients (Pearson) between MIC and MBC with physiological and biochemical traits of *Eremostachys laevigata* Bung under extraction with different solvents

	MIC	MBC	Total phenol	Flavonoids	DPPH
MIC	1				
MBC	0.98**	1			
Total phenol	-0.05ns	-0.07ns	1		
Flavonoids	0.38*	0.48*	0.70**	1	
DPPH	0.57**	0.58**	-0.25ns	0.23ns	1

ns, *, and **: non-significant and significant at 0.05 and 0.01, respectively



Discussion

The objective of this study was to examine the impact of different solvents on the biochemical composition of the *E. laevigata* Bung plant, as well as to investigate the antibacterial properties of its aerial and underground organs using various extraction systems. The influence of several solvents, including methanol, water, chloroform, acetone, and N-hexane, on the biochemical content and antibacterial attributes of the *E. laevigata* Bung plant's aerial and underground organs was also evaluated. The initial phase of the experiment yielded noteworthy outcomes, demonstrating that the impact of various solvents on the biochemical makeup, including total phenols and flavonoids, as well as the level of antioxidant activity (DPPH), was significant. The utilization of N-hexane as a solvent resulted in the highest mean values of these three compounds when compared to the other solvents (Figures 1, 2, and 3). In this case, N-hexane may have a higher affinity for extracting phenols, flavonoids, and antioxidants compared to other solvents used in the experiment. This can result in higher concentrations of these compounds in the resulting extract obtained using N-hexane as a solvent. Additionally, the chemical properties of N-hexane may also play a role in its ability to selectively extract these specific biochemical compounds.

Due to its relatively low polarity, N-hexane is capable of dissolving non-polar and slightly polar compounds, such as phenols, flavonoids, and antioxidants, more effectively than other solvents. This characteristic makes N-hexane an advantageous solvent for extracting specific biochemical compounds from a mixture. As a result, the use of N-hexane as a solvent in the study likely led to a higher concentration of phenols, flavonoids, and antioxidants in the final extract. The selection of the appropriate solvent can significantly impact the composition and properties of the substance being studied, and in this particular case, N-hexane played a crucial role in selectively extracting the desired biochemical compounds (Kua *et al*., 2016; Rocchetti *et al*., 2020).

In recent years, there have been many studies conducted to evaluate the antimicrobial properties of a range of essential oils and plant extracts. In general, plant extracts and their components demonstrate a broad spectrum of biological functions, including antimicrobial and antioxidant properties (Cheesman *et al*., 2017; Gedikoğlu *et al*., 2019). The presence of phenolic compounds, tannins, and flavonoids within the structure of plants is primarily responsible for their antimicrobial properties (Lobiuc *et al*., 2023). Lamiaceae plants are renowned for their antimicrobial properties, attributed to the presence of terpenoids and phenolic compounds, particularly flavonoids (Rahmouni *et al*., 2021). The methanol extract of cirsimarin flavonoid from *Salvia palaestina* leaves was found to exhibit potent antimicrobial activity against *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus vulgaris*, and *Pseudomonas aeruginosa* (Miski *et al*.,

1983). Previous studies on the *Eremostachys* genus have demonstrated its potential for antibacterial, antioxidant, and free radical scavenging activities, as documented by Erdemoglu *et al*. (2006). *E. laevigata* Bunge, which is an endemic species within this genus, is utilized as a medicinal plant for wound healing, snakebite, inflammation, and pain (Mohammadhosseini *et al*., 2019). Phytochemical analyses of the *Eremostachys* genus have revealed the presence of iridoid glycosides, flavonoids, alkaloids, phenols, coumarin, and monoterpene glycosides (Hadipour *et al*., 2016). Both the N-hexane extract from *E. macrophylla* stems and the methanol extract from *E. biosseriana* roots exhibit antibacterial activity (Duffy and Power, 2001).

The well diffusion method was utilized to assess the antibacterial activity of extracts obtained through Soxhlet and maceration extraction methods. Among the study groups, the methanol extract of underground organs obtained through the Soxhlet extraction method exhibited the largest non-growth diameter against *Salmonella enterica*, a gram-negative bacterium. Conversely, the smallest non-growth diameter was observed in the acetone extract of aerial organs obtained through the maceration extraction method. The inhibitory effects of the extracts obtained through both Soxhlet and maceration methods of aerial and underground organs of *E. laevigata* were not as significant as those of ampicillin and gentamicin antibiotics. However, the methanolic extract of underground organs obtained through the Soxhlet extraction method showed the most promising inhibitory effect among all the groups studied (Table 2). The disk diffusion method was employed to compare the inhibitory effects of extracts obtained through Soxhlet and maceration extractions among the study groups. The results indicated that the methanol extract of underground organs obtained through the Soxhlet extraction method exhibited the largest non-growth halo against gram-negative *Escherichia coli*, while the extract obtained from the aerial organs showed the largest non-growth halo against gram-positive *Staphylococcus aureus*. These results were significant when compared to the effects of gentamicin. Conversely, the smallest non-growth halo diameter was observed in the acetone extract (Table 3).

The MIC and MBC of extracts obtained through Soxhlet and maceration extractions were compared in gram-positive *Staphylococcus aureus*, which are presented in Tables 4 and 5, respectively. The varying effects of these extracts on gram-positive and gram-negative bacteria can be attributed to the structural differences in the cell walls of these two types of bacteria. Gram-positive bacteria have a thicker peptidoglycan layer in their cell walls, which makes them more susceptible to certain types of antimicrobial agents, while gram-negative bacteria have an additional outer membrane that provides them with additional protection against certain types of antimicrobial agents. In the case of gram-positive bacteria, antimicrobial agents can easily penetrate and damage the cell wall and cytoplasmic membrane, resulting in cytoplasmic leakage and coagulation. This is likely due to the absence of an outer membrane layer, which provides additional protection against antimicrobial agents, as is found in gram-negative bacteria (Duffy and Power, 2001).

According to a report, the dichloromethane extract of *E. laciniata* demonstrated antimicrobial effects against *S. aureus*, with a MIC of 3 $mg.ml^{-1}$. Additionally, the hydroalcoholic extract of peppermint showed MIC and MBC effects against gram-positive *Staphylococcus aureus* (Zandi *et al*., 2016). In our findings, we observed a non-growth halo diameter of 23 mm in *Escherichia coli* using the well diffusion method, which is in agreement with previous results. An investigation into the methanol extract of *Scutellaria pinnatifida* demonstrated its notable non-growth halo diameter against *Staphylococcus aureus* in both the disk diffusion and well diffusion techniques, albeit showing insignificant impact on gram-negative bacteria. These findings differ from our results, which showed antimicrobial activity against both gram-positive and gram-negative bacteria, indicating that the antimicrobial effects of plant extracts can vary depending on the specific plant species and the extraction method used (Mohammadi *et al*., 2015). The inconsistency between the results of MIC and MBC with the results of well and disk can be due to the more diffusion of extracts into the culture medium in the well method and the technical problem of complete impregnation of the discs with plant extracts (Bubonja-Šonje *et al*., 2020).

The results indicated a higher antibacterial effect of the methanolic extract of *E. Laevigata* than other tested extracts, which can result from the presence of more antimicrobial components in the methanol extract. The methanolic extract contains a wide range of polar and relatively polar compounds and extracts components such as tannins, anthocyanins, flavones, polyphenols, and iridoid glycosides through its unique physical properties such as high polarity, low viscosity, and vapor pressure (Song *et al*., 2015).

Based on our results and previous research, it appears that phenolic compounds play an important role in the antibacterial activity of extracts from medicinal plants. Phenolic compounds are commonly found in various plant organs, including roots, leaves, buds, seedlings, and plant epidermis, and have been shown to possess potent antibacterial properties. These compounds are often highly extractable through plant extracts, making them a valuable target for the development of natural antimicrobial agents (Karak, 2019). According to Asgharian *et al*. (2017), the methanol extract of the aerial organs of *E. macrophylla* contained 6.48 mg gallic acid per 100 g DW of total phenol content and 7.8 mg quercetin per 100 g DW of flavonoid content. The results also suggested that phenol and flavonoid in methanolic extract of *E. laevigata* were 6.87 $mg.g^{-1}$ DW and 0.19 $mg.g^{-1}$ DW respectively, the antibacterial effects can be probably attributed to these compounds. The methanol extract of *E. laciniata* rhizomes contains three iridoid glycosides named phlomiol, phloyoside I, and pulchelloside I, which have been found to possess antibacterial properties against *Bacillus cereus*, *Escherichia coli*, *Proteus mirabilis*, and *Staphylococcus aureus*. The MIC of these glycosides is 0.05 $mg.ml^{-1}$, which means that they can effectively inhibit the growth of these bacteria by up to 100% (Modaressi *et al*., 2009).

Our findings have also verified that the *E. laevigata* extract contains two iridoid glycosides, namely phloyoside I and sesamoside, which are likely responsible for its antimicrobial properties. The antibacterial properties of the acetone extract are relatively weaker compared to those of other extracts because acetone is a nonpolar solvent that may not be efficient in extracting phenols from plant tissues. Phenolic compounds can inhibit the growth of microorganisms through various mechanisms, such as surface adsorption and disruption of the cell membrane, interference with cytoplasmic membrane and membrane proteins, enzymatic reactions, and reduction of metal ions required by bacteria. These actions can ultimately lead to the death of bacterial cells caused by phenolic compounds (Tejirian and Xu, 2011).

The methanol extract of the underground organ of *E. laevigata* obtained by the Soxhlet method had a phenol content of 6.87 $mg.g^{-1}$ DW and a flavonoid content of 0.19 $mg.g^{-1}$ DW. It is noteworthy that no phenol or flavonoid compounds were found in other extracts of *E. laevigata*. For comparison, the total phenol content in the methanolic extract of *Coleus blumei* Benth was reported to be 1.1 $mg.g^{-1}$ gallic acid, and the flavonoid content was 7.8 $mg.kg^{-1}$ quercetin. The total phenol content in the methanol extract of *Salvia officinalis* leaf was 1.9 $mg.g^{-1}$ DW, and the flavonoid content was 1.5 $mg.kg^{-1}$ DW. The total phenol content in the methanol extract of *Lavandula officinalis* leaf was 0.9 $mg.g^{-1}$ DW, and the flavonoid content was 2.2 $mg.g^{-1}$ quercetin DW. The total phenol content in the methanol extract of *Rosmarinus officinalis* was 1.7 $mg.g^{-1}$ DW, and the flavonoid content was 1 $mg.g^{-1}$ quercetin DW. Finally, the total phenol content of the methanol extract from the leaves of *Melissa officinalis* was reported to be 1.6 $mg.g^{-1}$ DW, and the flavonoid content was 0.2 $mg.kg^{-1}$ quercetin. These values indicate that the methanol extract of *E. laevigata* underground organ obtained by the Soxhlet method had a higher phenol content compared to the mentioned plants, while its flavonoid content was relatively low (Sytar *et al*., 2018).

Flavonoids, which are a diverse group of phenolic compounds, are produced by plants in response to microbial infections and have been found to have broad-spectrum antimicrobial activity. The mechanism of action of flavonoids involves the formation of complexes with the outer membrane of bacteria and soluble proteins attached to the membrane. Additionally, these compounds can penetrate and disrupt the cell membrane of microorganisms, contributing to their antimicrobial effects (Karak, 2019). It is important to note that experimental results regarding phenolic and flavonoid compounds can be influenced by various factors, such as climatic conditions, the method of extraction and preparation, the techniques used to measure the

compounds, and the drying methods employed. Therefore, it is crucial to carefully consider and control these variables to ensure accurate and reproducible results in experiments involving these compounds.

Conclusions

The results of the first part of the experiment showed that using different solvents led to differences in the content of biochemical compounds, such as total phenols, flavonoids, and antioxidant activity (DPPH). The use of N-hexane and water as solvents resulted in the highest content of these compounds. Furthermore, the antibacterial properties of the acetone, chloroform, and N-hexane extracts were inferior to the methanol and distilled water extracts, possibly due to their lack of polarity. The methanol extract obtained from underground organs displayed greater antibacterial activity compared to the extract obtained from aerial organs, which could be attributed to the existence of iridoid glycoside compounds. Additionally, Soxhlet extraction yielded stronger antibacterial effects compared to maceration, possibly due to the application of heat during the extraction process. The study also identified the MIC and MBC in the gram-positive bacterium *Staphylococcus aureus*. The results also indicated that the well diffusion method exhibited the highest susceptibility in the gram-negative bacterium *Salmonella enterica*, while the disk diffusion method showed the most susceptibility in both *Escherichia coli* and the gram-positive bacterium *Staphylococcus aureus*. The observed differences in the effects of these extracts and the higher MIC and MBC on the growth of gram-positive and gram-negative bacteria can be attributed to the structural differences in the cell walls of these two bacterial groups. This is likely due to the absence of an outer membrane layer in gram-positive bacteria, which makes the cell wall and cytoplasmic membrane more vulnerable to destruction by antimicrobial agents, resulting in the leakage and coagulation of the cytoplasm. The presence of phenolic and flavonoid compounds, as well as iridoid glycosides, in the methanol extract, can be attributed to the polarity of the solvent used for extraction.

Authors' Contributions

Conceptualization, PA, HN, AA, and AF; Data curation, HN and AA; Formal analysis, PA and HA; Methodology, PA, AA, and AF; Writing – original draft, PA; Writing – review & editing, PA, HN, AA, and AF. All authors read and approved the final manuscript.

Ethical approval (for researches involving animals or humans)

Not applicable.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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