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Antiradical and antibacterial activity of *Echium altissimum* extracts on human infective bacteria and chemical composition analysis

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Abstract

The evolution of bacterial strains with greater resistance to conventional antibiotics has led to the use of new generations of antibiotics. This research aimed to investigate the antiradical and antibacterial properties of a methanol extract from the aerial and underground parts and seeds of *Echium altissimum* (Syn. *Echium italicum*) on pathogenic bacteria. The samples (i.e., aerial and underground parts and seeds) of *E. altissimum* were collected from Urmia, Iran. The antibacterial properties were analyzed by agar well diffusion assay. In addition, the minimum inhibitory and bactericidal concentrations were measured by the serial dilution method. However, the phenolic and flavonoid contents were obtained by the Folin-Ciocalteu and Aluminum chloride methods, respectively. Furthermore, free radical scavenging activity was measured by 2, 2-diphenyl-1-picrylhydrazyl. The most susceptible was obtained for *Micrococcus luteus* on the seed methanol extract of *E. altissimum*. Next, the IC50 of the aerial and underground parts and seeds were determined as 0.1054, 0.1278, and 0.1508 mg mL^{-1}, respectively. The methanol extract of seed exhibited a MIC of 0.75 mg mL^{-1} against *M. luteus*. Lastly, the highest phenol and flavonoid contents were determined as 246.12 (mgGA/g) and 6.29 (mgQ/g) from the seed methanol extract. The major compounds were determined to be rosmarinic acid (28.36%) and myricitrin (12.38%). In general, *E. altissimum* exhibited antibacterial and antioxidant activity and is therefore suggested for producing natural antibiotics and rare drugs, especially anti-infective drugs for the pharmaceutical science.

1. Introduction

The Medicinal plants are considered an important source of biologically active compounds with antibacterial activity and are used for the treatment of pathogenic microorganisms in the medicinal field (Rcid et al. 2005). From ancient times, traditional medicinal plants have been known to possess diverse biological activities like antimicrobial, anticancer, antipyrexial, and antihypertensive activities (Baltisberge & Widmer 2006). In addition, today more than 60% of anti-cancer compounds are obtained from plant, marine, and microorganism sources (Lelpo et al. 2000). Infectious diseases have always threatened human health (Suerbaum & Michetti 2002). Due to increasing pathogenic resistance to synthetic antibiotics, it is necessary to identify and introduce

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new and effective plants to produce natural antibiotics with low side effects (Sharma & Kumar 2009). Bacterial resistance against herbal extracts and essential oils is slower compared to single and synthetic drugs (Hounsome et al. 2008). Antibiotics and medicinal plants with antimicrobial compounds cause bacteriostatic and bactericidal effects (Rice-Evans 2004).

The family *Boraginaceae* contains more than 2,000 species in 100 genera and grows in temperate and tropical regions (Watson & Dallwitz 1992). The presence of secondary metabolites, including phenols, flavonoids, and terpenoids, with antibacterial, antioxidant, antitumor, antifungal (Koca et al. 2012), and anti-inflammatory properties (Chen et al. 2003) have been reported in this family. The hypnotic and anti-anxiety effects of *Boraginaceae* extract have been proven on mice (Hosseinzadeh et al. 2012). *Echium altissimum* (Syn. *Echium italicum*) is a fragrant and biennial plant that reaches 40-100 cm in height (Gibbs 1971) and has been reported in southern Europe and southwest Asia. The laxative, antimicrobial, and antitussive activities of *Echium* spp have also been reported (Ghassemi et al. 2003). *E. italicum* root contains antioxidant compounds including shikonin and pyrrolizidine alkaloid (Koca et al. 2012; Fuyu & Ruifa 2012). *E. italicum* seeds have exhibited anticancer, antiradical, and antimicrobial activities because of the presence of phytosterol and are used in the treatment of diseases such as diabetes (Tanaka et al. 2006).

Microbiologists are always looking for compounds with antioxidant properties to reduce free radical scavenging effects on the body and oxidative stress (Koksal & Gulcin 2008). Antioxidants are mostly polyphenol compounds found in different parts of plants, including the skin, fruit, stems, leaves, seeds, and roots (Stoilova et al. 2007). Polyphenols play a positive role in controlling osteoporosis, diabetes, and neurological diseases (Scalbert et al. 2005). Antioxidant compounds have a positive effect on cardiovascular and cancer diseases and also protect cell membranes (Samy & Gopalakrishnakone 2008). The chemical composition, including carotenoids, fibers, phenolic, flavonoids, isoflavones, and ascorbic acid, removes free radicals (Sharif et al. 2008). Different factors such as environmental conditions, solvent type, extraction method, plant growth stage, type of microorganism, and plant species can affect the antimicrobial and antioxidant activity of plant materials (Tiwari et al. 2011). Compared to gram-positive bacteria, gram-negative bacteria are usually resistant to antimicrobial compounds due to the presence of an impermeable wall (Hounsome et al. 2008). The purpose of this research was to investigate the antioxidant and antibacterial properties of *E. altissimum* methanol extract against human pathogenic bacteria and perform a GC/MS analysis in laboratory conditions.

2. Materials and methods

2.1. Chemical materials

2,2-diphenyl-1-picrylhydrazyl (DPPH), Mueller-Hinton agar (MHA), nutrient broth (NB), quercetin, and gallic acid were obtained from the Merck Company (Germany), and clindamycin and levofloxacin antibiotics were prepared by the Padten Tab Company (Iran).

2.2. Plant samples

The plant samples, including aerial and underground parts and seeds of *Echium altissimum*, were collected from Urmia, Iran. The samples were dried under shadow at Bu-Ali Sina University. In addition, the methanol (80%) extract was obtained from dried powder (Fuselli et al. 2008). To complete the drying, the crude extract was transferred to an oven at 37˚C (Shojaemehr & Alamholo 2019). Finally, the residue was stored in a freezer at -22˚C until used (Alamhulu & Nazeri 2016).

2.3. Bacterial strains

The antibacterial properties of *E. altissimum* extracts were tested against pathogenic bacteria, including *Enterococcus faecalis* (PTCC1195), *Micrococcus luteus* (ATCC10987), *Staphylococcus epidermidis* (ATCC1054), *Streptococcus pneumonia* (ATCC1243), *Arcanobacterium haemolyticum* (ATCC3389), *Staphylococcus saprophyticus* (ATCC7791),

Proteus mirabilis (PTCC1287) *Neisseria meningitides* (PTCC4578), *Neisseria gonorrhoeae (*PTCC4579*), Acinetobacter baumannii (*PTCC4413*), Escherichia coli* (ATCC25922), and *Klebsiella pneumoniae* (PTCC1129). A bacterial colony was transferred to MHA medium, and then a loop of the bacterial colony, equivalent to 0.5 McFarland standard (ca. 1.5×10^8 CFU), was transferred to 1 mL of the NB medium for 24h at 37˚C (Alamhulu & Nazeri 2015).

2.4. Agar well diffusion assay

The antibacterial activity of the methanol extract was tested by the agar well diffusion method (Tayoub et al. 2012). The methanol (80%) extract (25 and 50 mg mL^{-1}) was obtained from the aerial and underground parts and seeds of *E. altissimum.* First, a volume of 200 mL of bacterial suspension (1.5×10^8 CFU) was poured on MHA medium. Next, a volume of 50 µL of extract solution was poured into wells with 5 mm diameter, and then the plates were placed for 24h at 37˚C (Alamhulu & Nazeri 2016). Clindamycin and levofloxacin, as positive controls, and methanol, as a negative control, were used (Ayoola et al. 2008). Finally, the results were analyzed by SAS software in three replications as cm.

2.5. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

The MIC and MBC of the methanol extract were measured by serial dilution assay (Shojaemehr et al. 2020). A concentration of 50 mg mL^{-1} of methanol extract was first used to test the MIC and MBC. Next, a dilution series of 25, 12.5, 6.25, 3.125, 1.56, and 0.75 mg mL^{-1} were used for the MIC test. Then, a volume of 385 µL of NB was transferred into all tubes, and 400 µL of the extract was added to the first tube (50 mg mL^{-1}). Finally, a volume of 5 µL of bacterial suspension was transferred to all tested tubes and was incubated for 24h at 37 ˚C. To measure MIC, the lowest dilution with no growth was considered. A volume of 5 µL of the plates with no bacterial growth was transferred to the MHA culture medium and incubated for 24h at 37 ˚C to measure MBC.

2.6. Total phenolic and flavonoid contents

Using the Folin–Ciocalteu method, the total phenolic content of mgGA/gDW at 765 nm (Pourmorad et al. 2006) was measured. A spectrophotometer was used to measure the total flavonoid content of mgQ/gDW at 415 nm (Chang et al. 2002).

2.7. DPPH

The antiradical scavenging property of *E. altissimum* methanol extract was analyzed (Alamhulu & Nazeri 2015). The concentrations of 0.2, 0.4, 0.6, 0.8, and 1 mg mL^{-1} were prepared from the aerial and underground parts and seeds, and ascorbic acid was considered the standard. The absorbance of the sample was calculated at 517 nm using a spectrophotometer.

2.8. GC-MS

Gas chromatography connected to mass spectrometry (GC/MS) (Kermanshah University, Iran) to analyze the chemical compositions of *E. altissimum* (full plant) methanol extract. The instrument was set at an initial temperature of 275 ^{0}C and maintained for 2 min. The temperature was increased to 120 ^{0}C at the rate of 8^{0}C /min and then further to 285^{0}C at the rate of 3.5^{0}C /min. Injection port temperature was ensured at 350^{0}C and a helium flow rate of 0.9 ml/min. The samples were injected in the split/splitless mode. The solvent delay was adjusted for 5 min and 1 µl volume injected.

2.9. Statistical analysis

Data were analyzed in triplicate with a completely randomized design, and average comparisons were made by an SAS software Duncan test at ($p<0.01$).

3. Results and Discussion

3.1. Antibacterial activity

The growth inhibition zone of bacteria against extracts was calculated (mm). The inhibitory effects of the methanol extract of *E. altissimum* aerial and underground parts and seeds against human infective bacteria are represented in Table 1. Next, antibiotics, including clindamycin and levofloxacin as positive controls, were used. The

levofloxacin showed the highest inhibitory effect on *N.meningitides*, while methanol, as a negative control, didn't show any inhibitory effect. The methanol extract of *E. altissimum* seed exhibited the most potent activity against *M. luteus*. Conversely, *A. baumannii* showed resistance against the methanol extract. Moreover, the aerial part extracts impact on the growth of *N. gonorrhoeae* and *S.pneumonia* revealed no inhibitory effects; the underground part extract on *K.pneumoniae* also revealed no inhibitory effects. However, *E. coli* showed sensitivity to the seed methanol extract. In addition, the seed methanol extracts exhibited a better inhibitory effect compared to other samples. However, the Gram-negative bacteria exhibited more resistance compared to the Gram-positive bacteria in vitro. Based on the findings, the seed methanol extract (50 mg mL^{-1}) showed better antibacterial activity against *M. luteus* and *S. epidermidis* compared to clindamycin.

Table1. Antibacterial activity of methanol extract (mg mL^{-}) obtained from different parts of *E. altissimum* against human infective bacteria (mm).

Bacteria	Aerial part (mg mL^{-})^{c}		Underground part (mg mL^{-})^{b}		Seed (mg mL^{-})^{a}		Clindamycin	Levofloxacin
	25	50	25	50	25	50		
N.meningitides ^{e}	10±0.66^{ij}	11±0.33^{i}	9±0.33^{k}	10.5±0.66^{i}	10.5±1.2^{i}	12±0.57^{hi}	24±0.57^{b}	26.5±0.33^{a}
A. baumannii ^{j}	-	-	-	-	-	-	19±0.33^{e}	25.5±0.66^{a}
K.pneumoniae ^{g}	9.5±0.57^{j}	11±0.66^{i}	-	-	12±00^{hi}	14±0.33^{gh}	18±1^{ef}	23.5±0.66^{b}
P. mirabilis^{d}	10±0.88^{ij}	11±0.88^{i}	10.5±00^{i}	12±0.33^{hi}	13±0.33^{h}	14.5±0.66^{g}	18.5±0.57^{e}	21.5±0.33^{d}
N. gonorrhoeae ^{h}	-	-	8±0.66^{l}	9.5±0.57^{j}	12±0.88^{hi}	12.5±1.2^{h}	22±0.88^{c}	25±0.33^{a}
E. coli ^{i}	-	-	-	-	9±0.33^{k}	11.5±0.66^{i}	16±0.33^{fg}	17.5±0.88^{f}
M. luteus ^{a}	12.5±0.33^{h}	13.5±0.57^{h}	14±0.33^{gh}	15.2±0.33^{g}	16±0.66^{fg}	20.5±0.33^{d}	19±0.57^{e}	24.5±0.66^{b}
S. epidermidis^{b}	13±0.88^{h}	13±0.88^{h}	14.5±0.66^{g}	15±0.33^{g}	15.5±0.33^{g}	17±0.33^{f}	16±0.33^{fg}	22.5±0.33^{c}
S.pneumonia ^{f}	-	-	12±0.33^{hi}	14±0.66^{gh}	15±0.33^{g}	18.2±0.66^{ef}	20.5±0.66^{d}	19.5±0.57^{e}
A. haemolyticum ^{c}	10±0.33^{ij}	11.5±0.33^{i}	10.5±0.88^{i}	13±0.33^{h}	14.5±0.33^{g}	16±0.88^{fg}	19±0.33^{e}	21±0.66^{dc}
S.saprophyticus ^{c}	12±0.33^{hi}	15±0.88^{g}	13±0.55^{h}	16.5±0.66^{f}	12±0.66^{hi}	15±0.66^{g}	15±0.88^{g}	16.5±0.57^{f}
E. faecalis ^{d}	9.5.5±0.33^{j}	12±0.33^{hi}	10±0.33^{ij}	13±0.88^{h}	10±0.33^{ij}	14±0.88^{gh}	16±0.88^{fg}	17.5±0.88^{f}

The same letters are not significantly different at $p<0.01$

As seen in Table 2, the methanol extracts of the seed and underground part of *E. altissimum* against *M. luteus* exhibited a MIC of 0.75 and 1.56 mg mL^{-1}, respectively. However, the aerial part extract showed a MIC of 3.125 mg mL^{-1} on *S. epidermidis*. The seed and aerial part methanol extracts of *E. altissimum* on *N. meningitides* and *S.pneumonia* exhibited a MIC of only 25 and 12.5 mg mL^{-1}, respectively. Based on the findings, the seed methanol extract demonstrated a better inhibitory effect against tested bacteria than the methanol extract from the aerial and underground parts. The bacteria *E. faecalis, E. coli,* and *A. baumannii* exhibited resistance against *E. altissimum* methanol extract in a serial dilution test.

Table 2. MIC and MBC of *E. altissimum* methanol extracts on infective bacteria (mg mL^{-1}).

Bacteria	Aerial part		Underground part		Seed	
	MIC	MBC	MIC	MBC	MIC	MBC
N.meningitides	25	-	-	-	6.25	25
A. baumannii	-	-	-	-	-	-
K.pneumoniae	12.5	25	25	25	3.125	12.5
P. mirabilis	-	-	12.5	25	25	25
N. gonorrhoeae	-	-	12.5	25	6.25	12.5
E. coli	-	-	-	-	-	-
M. luteus	6.25	12.5	1.56	3.125	0.75	0.75
S. epidermidis	3.125	6.25	6.25	12.5	3.125	6.25
S.pneumonia	-	-	-	-	12.5	-
A. haemolyticum	12.5	25	12.5	-	25	-
S.saprophyticus	-	-	12.5	25	6.25	25
E. faecalis	-	-	-	-	-	-

One of the problems that humans have always faced is the spread of infectious diseases. Antimicrobial resistance and the side effects of synthetic antibiotics have led to research on antimicrobial compounds of plant origin against clinical infections caused by bacteria, fungi, and viruses (Singh et al. 2010(. Due to the presence of alkaline, the family *Boraginaceae* root has antibacterial, antitumor, and anti-inflammatory properties (Chen et al. 2003). A variety of biologically active constituents, including naphthoquinones, flavonoids, terpenoids, and phenols, with antioxidant, anti-inflammatory, antibacterial, and antiviral properties have been isolated from *Echium* species (Hosseini & Abolhassani 2011; Rabbani et al. 2011). Hosseinzadeh et al. (2012) reported the antibacterial properties of cyclohexane, dichloromethane, and methanol extracts of *E. italicum* on *S. epidermidis*, *S. aureus*, *M. luteus,* and *B. cereus* and investigated their MIC. Their results showed the underground part cyclohexane extract exhibited the highest antibacterial activity. Conversely, the seed methanol extract showed a more inhibitory effect in the present study. This difference is probably related to several factors, including species and solvent types, place and time of sample collection, and extraction method. In addition, antimicrobial activity against human pathogenic bacteria and MIC values were reported by Morteza-Semnani and Saeedi (2005).

3.2. Anti-radical activity by DPPH

The antioxidant activity of aerial and underground parts and seed methanol extracts by DPPH is shown in Table 3. Ascorbic acid was used as the standard. Accordingly, the degree of antiradical scavenging increased when the concentrations of methanol extract increased. According to analyzed data, the IC50 values of the underground part and seed extracts exhibited a significant difference compared to the standard. Finally, the aerial part methanol extract of *E. altissimum* exhibited the lowest IC50 of all samples. When free radicals are stored within a cell, oxidative stress is created. The highest contributor to many pathological conditions is oxidative stress (Birben et al. 2012). Asghari et al. (2019) reported the highest DPPH from *E. amoenum* methanol extract was 22.8 µg mL^{-}, which is different from the present study.

Table 3. Antioxidant properties of different organs from *E. altissimum* (IC50: mg mL^{-1}).

	Inhibition percentage of DPPH in different concentrations (mg mL^{-1})					
Organ	0.2	0.4	0.6	0.8	1	IC50
Aerial part	91.72	93.57	94.32	97.12	98.48	0.1054^{c}
Underground part	87.28	90.25	92.57	94.27	97.81	0.1278^{b}
Seed	85.36	88.59	92.89	96.12	98.83	0.1508^{a}
Ascorbic acid	91.3	92.41	96.58	98.47	99.13	0.1091^{c}

3.3. Chemical compositions analysis by GC/MS

The chemical constituents of *E. altissimum* methanol extract is shown in Table 4. Nineteen (82.23%) compounds were measured in *E. altissimum*. The major compounds were rosmarinic acid (28.36%), Myricitrin (12.38%), quercetin (8.21%) and chlorogenic acid (7.29%). The major constituents of *E. italicum* extract included rosmarinic acid, chlorogenic acid, phydroxybenzoic acid, quercetin, caempherol and rutin (Bo$\check{c}$ković et al 2017). Similarly, rosmarinic acid was found to be a major compound in the present study. The dominant chemicals of *E. italicum* essential oil from north of Iran were hexadecanol (27.1 %) and pulegone (8.8 %) (Morteza-Semnani et al. 2009). The dominant chemical compositions of *E. italicum* essential oil included hexadecaol (27.1%) and polygon (8.8%) (Morteza-Semnani & Saeedi 2005). Their study also reported that the dominant compounds of *E. amoenum* essential oil from Mazandaran province, Iran, were thymol (19.5%) and carvacrol (%7.5%). Conversely, hexadecanol was not identified in our research. Another study reported the strong antioxidant, antidiabetic, antithrombotic, antiinflammatory, and anticarcinogenic activities of rosmarinic acid and rutin (Petersen & Simmonds 2003).

Table4: The chemical compositions of *E. altissimum* methanol extract by GCMS.

Z. altissimum	Compound content (%)
Rosmarinic acid	28.36
Myricitrin	12.38
Naringenin	4.27
Hydrocaffeic acid	2.08
Deoxyshikonin	1.22
Chlorogenic acid	7.29
Shikonin	5.33
Caempherol	2.59
3,3-dimethylacrylshikonin	1.02
Luteolin	0.88
Quercetin	8.21
Delphinidin	0.33
Peonidin	1.28
Acetylshikonin	0.66
Tiglylshikonin	1.88
Rabdosiin	0.7
Apigenin	1.12
β-sitosterol	0.52
Pulegone	2.11
Total	82.23

In addition, the anxiolytic and antioxidant activity of chlorogenic acid has been reported (Bouayed et al. 2007). According to Ghassemi et al.پ(2003) results, chemical compositions of α cadinene (24.2%), n-hexadecane (8.7%), n-pentadecane (5.6%), and viridiflorol (4.9%) were reported from *E. amoenum* (Qazvin province, Iran). Some factors, including light, temperature, water, time, and growing place, affect the quality and quantity of chemical compositions (Samy & Gopalakrishnakone 2008).

3.4. Determination of total phenolic and flavonoid contents

The total flavonoid and phenolic contents from methanol extracts of the aerial, underground, and seed parts of *E. altissimum* are represented in Table 5. Additionally, the phenolic content of the seed and aerial and underground parts was measured as 154.27, 170.23, and 246.12 (mgGA/DWg), and the flavonoid content was calculated as 3.28, 4.87, and 6.29 (mgQ/DWg), respectively. Eventually, the highest total phenolic and flavonoid contents were observed in the seed methanol extract of *E. altissimum*. Based on the findings, the different samples of *E. altissimum* methanol extract showed a significant difference in phenolic and flavonoid contents.

Table 5. Total phenolic and flavonoid contents of different organs from *E. altissimum*

Organ	Aerial part	Underground part	Seed
Phenol (mgGA/DWg)	154.27^{c}	170.23^{b}	246.12^{a}
Flavonoid (mgQ/DWg)	3.28^{c}	4.87^{b}	6.29^{a}

The secondary metabolites, including saponins and tannins, have been reported from the aerial parts of *E. italicum* (Fazly Bazzaz et al. 1997). Phytochemical analysis of *E. italicum* showed a condensed tannin and antioxidant content of 21.49 (mgGA/DWg) and 112.92 (µgAA/g), respectively (Romeiras et al. 2011). Their results also showed the total phenolic and flavonoid content were1540 (mgGA/DWg) and 4.54 (mgQ/DWg), respectively, from the petal aqueous extract of *E. amoenum* (Pilerood & Prakash 2014). The phenolic content of that study was higher compared to the present research, and conversely, the flavonoid content of the methanol extract from the seeds and underground part of *E. altissimum* in the present study is higher than the mentioned studyپs results. These differences are probably related to the difference in species and type of extract. The highest phenolic content of 119.5 (mgGA/DWg) and highest flavonoid content of 62.17 (mgQ/DWg) were reported from *E. amoenum* seeds (Abbaszadeh et al. 2013). Similarly, contents in the present study, the seed methanol extract of *E. altissimum* showed the highest phenolic and flavonoid. Hashemi et al. (2019) exhibited phenol and flavonoid contents, as well as antioxidant activities, from *E. amoenum* methanol extract ranged from 54.16-688.97 (mgGA/DWg), 13.38-146.60 (mgQ/DWg) and 9.5-1472.4 (µg mL^{-}), respectively. Contrary to our study, phenolic contents of 109.15±0.51 and 105.22±0.07 (mgGA/g) and flavonoid contents of 25.16±0.19 and 25.03±0.29 (mgRU/g) were previously reported from ethanol and chloroform extracts of *E. italicum* (Boϛković et al. 2017). This difference is related to the type of solvent, the sample collection place, and the extraction method.

4. Conclusion

The seed extract exhibited the highest effect on tested pathogenic bacteria compared to the other samples. The chemical compositions were determined by GC/MS. In addition, the secondary metabolites with antibacterial activity, including phenol and flavonoid, were measured. The highest compound was determined as rosmarinic acid. Based on the findings, *Echium altissimum* can be introduced as a medicinal plant for antibiotics production and antimicrobial drug synthesis in medical and microbiology science.

Conflict of Interests

There is no conflict of interest.

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Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

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