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Original Article

Evaluation of the Chemical Compounds and the Antibacterial, Antioxidant and Cytotoxic Activities of *Echinophora Cinerea Boiss* Essential oil

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Abstract

Background and Aim: As a plant of the Apiaceae family, *Echinophora Cinerea Boiss* can grow up to 30 to 100 cm tall. The essential oil of *E. cinerea* contains phenolic compounds. Phenolic Compounds are one of the best sources of natural antioxidants. The present research aimed to evaluate the chemical compounds, and the antibacterial as well as antioxidant and cytotoxic activities of *E. cinerea* essential oil.

Materials and Methods: The hydrodistillation method and gas chromatography coupling with a mass spectrometer (GC/MS) were used to prepare and identify the chemical compounds of the essential oil. The antioxidant capacity of the essential oils was evaluated by DPPH and was compared with standard antioxidants (BHT). The antibacterial test was carried out by Broth Micro Dilution, Minimum Inhibitory Concentration (MIC), and Minimum Bactericidal Concentration (MBC) methods. These oils were evaluated and compared after 24 and 48 h to control the antibiotic.

Results: The main constituents of the essential oil were combined with plant α-phellandrene (32.09), limonene (16.28), *P*-cymene (10.75), α-pinene (9.79), carvacrol (3.79), and β-Myrcene (2.65). IC50 for the essential oil of *E. cinerea* and BHT was 0.74 and 52.72 µg/ml, respectively. Strong antibacterial impacts of the essential oils belonged to *Staphylococcus aureus* (MIC = 0.16 µg/ml and MBC = 0.63 µg/ml. The results of inhibitory effects on tumor cell lung have shown that via increasing the concentration, the inhibitory effects of cell have increased, so that at the highest concentrations, more than 60% of tumor cells were inhibited and the IC50 was 3589.31 µg /ml.

Conclusion: The present study demonstrates that *Echinophora* could have considerable antibacterial, antioxidant and cytotoxicity properties. The essential oil of this plant is effective against important human bacterial pathogens. It is also a determinant of nosocomial infection.

Keywords: *Echinophora Cinerea Boiss*, Antioxidant, Antibacterial, Cytotoxicity

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Introduction

The essential oil of medicinal plants has biological properties. Certain aromatic plants have been used for treating infectious illnesses by phytotherapy. They are of high significance, for they have fewer side effects and low toxicity levels (1). Nowadays, plant materials play pivotal roles in primary health care such as therapeutic remedies in many developing countries (2). Medicinal herbs have long been used in traditional medicine to treat several illnesses owing to their anti-microbial properties (3, 4). Throughout the history, several plant oils have been used for food security and quality improvement (5). *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Salmonella spp*., and *Streptococci* are common foodborne pathogenic bacteria which are frequently isolated from different kinds of foods such as meat, dairy products, seafood and vegetables (6). Food poisoning is caused by the consumption of foods contaminated by Gram-positive and Gram-negative bacteria. Essential oils are complicated blends of volatile secondary metabolites comprised of sesquiterpenes, including carbohydrates, alcohols, ethers, aldehydes, and ketones to which aromatic medicinal herbs owe their fragrant and biological properties (7). *Echinophora* genus is used in customary medicine to strengthen the stomach. This plant with the local name *fyaleh* is used as a seasoning in nutriment, and local people use it in pickle and tomato sauce as a preserver with antimicrobial and antifungal properties (8, 9). Four herbaceous, perennial and aromatic species of *Echinophora* could be found in Iran. Two species of platyloba and cinerea are endemic to Iran, and the other two species, namely sibthorpiana and orientalis, grow in Anatolia, Armenia, Russia, Turkmenistan, Afghanistan, Balkan, Crete, Cyprus, and Syria (10) in addition to Iran. *Echinophora Cinerea Boiss* is a herbaceous, perennial, aromatic plant of Apiacea family whose height is about 30 to 100 cm. It has a glabrous cylindrical stem with needle-shaped leaves alternatively. Moreover, it has small and yellow umbelliferous flowers with inflorescence, and cone-shaped roots. Moreover, its fruits contain small seeds (11). This plant is found in regions above 1500 meters high in Lorestan, particularly in Ashtorankuh, Kuh Kala, Garin Kuh and Sefidkuh where it is abundant (12).

The aerial parts of the plant are used for different purposes. *Echinophora* with the local name *fyaleh*, for instance, has been used for flavoring in food industry (12). This plant is considered as a stomach tonic, diuretic and anti-cancer herb (13). Antifungal effects of yeast extract on the *Trichophyton rubrum*, *Microsporum gypsum*, *Trichophyton mentagrophytes*, *Epidermophyton floccosum*, *Microsporum canis* and *Candida Albicans* have been proved (14-17). The use of steam distillation method indicated that the extracted essential oil contained %34.43 *P*-Cymene, %21.18 α-phellandrene, 3.31% α-pinene and the use of HD-SME (Headspace solvent micro-extraction) method revealed that there were % 40.64 α-phellandrene, %17.28 z-β-Ocymen, %1.18 *P*-Cymene, % 5.18 α-pinene (13). Moreover, the investigation of the volatile compounds of the aerial part of *E. cinerea* by Sajjadi *et al*. revealed that there were 27 compounds, most notably % 40.6 α-phellandrene, %16.5 α-pinene, %9.8 β- phellandrene, % 7.5 *P*- Cymene, %5/4 linalool, and % 4.8 citronellol (18). In another study that examined the essential oil of *Echinophora cinerea* flowering branch in Fars Province, 19 compounds, including % 61.4 α-phellandrene, %10.7 β- phellandrene, %9.6 α-pinene and %6.1 *P*-Cymen were identified (19). The nocardial effect of this plant on human pathogens such as Nocardia, Asteroides and Brazilian has been substantially reported (20). The present study evaluated the chemical constituents, and the anti-bacterial, anti-oxidant as well as cytotoxicity properties of the essential oil *E. Cinerea*.

Materials and Methods

Plant Collection and Identification

The aerial parts of plants were gathered in June 2018 from South Khorramabad, Lorestan Province, and then were transferred to the laboratory for analysis.

Extraction of the Essential oils

Steam distillation method was used to extract the essential oils in the laboratory, and the essential oils were processed by Clevenger apparatus (BP British pharmacopeia, U.K.). The aerial parts (100 g) including

stems and leaves were completely dried at ambient temperature in the shade and then were milled. Duration of essential oil extraction was 4 h. After extraction, the product was dehydrated using sodium sulfate and was kept in the dark in bottles at 4 °C until being used (21).

GC Instrument

Gas chromatography (Beifen 3420 capillary model) and chromatograph column specifications BP-5 (5% phenyl: 95% polydimethyl siloxane) with fused silica capillary column (30m x 0.25 mm Internal diameter, 0.25. m film thickness) were used to identify the chemical components of the essential oils. The temperature for the column was programmed at 50 °C. After five minutes, it was stopped at that temperature, and then it was increased to 280 °C by the rate of 6 °C/min. The equipped detector was FID, and helium gas with % 99/999 purity was used as the carrier gas.

GC-MS Instrument

Gas chromatography coupled with mass spectrometry (GC-MS) was used for further exploration. The isolation and measurement of the samples were carried out using TRACE GC (GC/MS) owned by Thermo Quest-Finnigan companies coupled with mass spectrometry TRACE MS. To isolate the compound, Fused Silica DBX-type of 95% (polydimethylsiloxane) that was 60 meters long with the inner dimensions of 250 mm and the thickness of 25 was used. At the column temperature of 60°C to 250 °C, the rate of 5 °C/min increased, and then reached the speed of 30°C min to 280°C. Subsequently, the temperature was kept for 2 min. The injection site temperature and the detector temperature were set at 250 and 260°C respectively. Helium gas with a speed of 1/1 with %99/999 purity was used as the carrier gas. Spectrometer condition was exactly in accordance with GC. Only the ionization energy of 70 electron volts was used. Moreover, normal hydrocarbons (C8-C20) was used for the retention of indices to identify the range of injection under the injected sample.

Antioxidant Assay

To review the antioxidant properties, free radical 2, 2-diphenyl-1, 1-picrylhydrazyl was determined. This substance is a lipophilic free radical with the maximum absorption at 517 nm. During the reaction, DPPH reacts with antioxidant substances. Subsequently, the reduction of its amount with decreasing wavelength is directly related to the free radicals responsible for the coloration of the solution and the alteration from dark purple to yellow (22). Absorption of the solution at 515-517nm wave shows the number of free radicals in the medium. The kinetics of this reaction follows the predicted steps.

Rapid response, slow response, and the reaction of free radicals with phenols occur rapidly within 5 min, between 5 to 30 min, and over 30 min respectively. Radical reactions with some other species occur at lower speeds. After a few hours, by decreasing the absorption mode, equilibrium could be observed (23). To accomplish the performance, 0.3 ml of different concentrations of oil was poured into the test tubes, and then 2.7 ml of methanol solution DPPH (6×10^{-5}M) was added. The prepared solution was mixed continuously for 60 min in darkness. Subsequently, using UV-Vis (Jenway Model 6715) spectrophotometer, absorbance of the solution was read at 517 nm. Using Eq.1, DPPH free RSA radical neutralizing power was analyze 24) according to the following formula:

(Eq.1)

$$RSA(\%) = 100 \times \left(\frac{A_{blank} - A_{sample}}{A_{blank}} \right)$$

Antibacterial Activity Assay

Standard bacteria were prepared from microbiology laboratory (Bu Ali Hospital, Tehran) and stored in the freezer. Clones of each strain were cultured in medium trials. The strains of *Staphylococcus aureus* are ATCC: 10690, *Pseudomonas aeruginosa* ATCC: 27853, *Listeria monocytogenes* ATCC: 13932, *Escherichia coli* ATCC: 11775, *Salmonella enterica* ATCC: 1709, *Salmonella typhi* PTCC: 1609, *Salmonella paratyphi* PTCC: 1230, *Staphylococcus epidermidis* PTCC: 1435, *Streptococcus agalactiae* PTCC: 1768, *Enterococcus faecalis* ATCC: 29219 and *C. albicans PTCC:* 5072. The minimum inhibitory concentration (MIC) was determined in sterile 96-well plates using the microdilution method. Initially, 100 µl of Mueller-Hinton Broth medium (Merck, Germany) was added to 96-well microplates. Subsequently, 50 µl oil was poured into the first well of rows, and then from the second house to the third and so on until the ninth house was diluted. In another raw, 50 µl of antibiotics of gentamicin, vancomycin, and amphotericin B with optimal sensitivity was poured into the test bacteria.

Finally, 50 µl of diluted microbial suspension was added to all wells in equal of McFarland half-pipe. After 24 h of incubation at 37°C, the plate was checked.

To determine the minimum bactericidal concentration (MBC), all the wells without opacity were separately cultured on the Muller Milton agar. After 24 hours, the lowest concentration of the essential oil at which bacteria had not grown was considered. A comparison was made between the results pertaining to the antibiotics and the standard table of the National Committee for Clinical Laboratory Standards (NCCLS). Vancomycin 128µg/ml, and Gentamicin 128µg/ml were used as positive control (based on the type of bacteria).

The Cytotoxicity Assay of A549 Cell Line

We obtained the cell line of human lung carcinomatous tissue (NCBI code No.: C137; ATCC No.: CCL-185) from the Pasteur Institute, Tehran, Iran. A549 cells were grown in RPMI 1640 added with 10% FBS, 1% (w/v) glutamine, 100U/ml penicillin and 100 µg/ml streptomycin. Subsequently, they were cultured in a humid atmosphere at 37°C in 5% CO_2. The antitumor agent decreased the MTT [3-(4,5-dimethylthiazol-2-yl)- 2,5-diphenyltetrazolium bromide] by mitochondrial dehydrogenase to the formazan blue artefact, which is a reflection of the normal mitochondrial efficiency and cell viability (25). In short, the cells (5×10^4) were seeded in wells comprising 100 µl of the RPMI medium supplemented with 10% FBS in a 96-well plate. Twenty-four hours after the accomplishment of adhesion, distinct concentrations of the essential oils were added to triplicate wells over the range of 500 to 31 µg/ml. After 2 days, 10 µl of MTT (5 mg/ml stock solution) was added and the plates were incubated in duration of 4 hours. The medium was discarded, and the formazan blue formed in the cells was dissolved with 100 µl of dimethylsulphoxide (DMSO). Quantification of formazan was achieved using an ELISA microplate reader (SLT, Austria) at 490 nm. The calculation of cell viability curves was carried out with regard to the control cells. The antitumor assay was expressed as the concentration of drug inhibiting cell growth by 50% (IC50). All the tests and investigations were conducted in triplicate, and the mean values were recorded.

Results and Discussion

Essential Oil Extraction and Identification of the Compounds

As an aromatic, herbaceous plant from the Apiaceae family, *E. cinerea* grows primarily in the Mediterranean regions, and its names are Khusharuz, Tigh turagh, and Keshandar (18). It is also known as *fyaleh*, which is used as spice (12). The *Echinophora* essential oil is a colorless or yellowish liquid containing %32.16 *α*-phellandrene, %16.28 limonene, %10.75 *P*-Cymene, %9.79 *α*-pinene, %3.79 carvacrol, and %2.65 *β*-Myrcene). In the present study, the efficiency of *E. cinerea* essential oil was 1.39±0.13 %. Its constituent compounds were identified after examining the GC-MS. The results pertaining to the essential oil have been presented in Table 1 and Figure 1. The main compounds identified were %32.9 *α*-phellandrene, %16.28 limonene, %10.75 *P*- Cymene, %9.79 *α*- pinene, %3.79 carvacrol, and %2.65 *β*-Myrcene. The results of the present research confirm the findings of previous studies (36). Moreover, the phytochemical analysis is partly in agreement with previous studies. According to the present research, *α*- phellandrene is one of the major components of *E. cinerea* essential oil but its value varies in distinct reports. This variety might be related to the harvest time, habitats and climatic conditions.

Antioxidant Property

The results have shown IC_{50} 0.74 and BHT 52.72 µg/ml for the essential oil of *E. cinerea* (Table 2).

Anti-Bacterial Properties (Assay)

The antibacterial properties of sterols, alkaloids, triterpenes, polyphenols, flavonoids and saponins have been reported (27, 28). Even though the mechanisms related to the antimicrobial activities of essential oils have not been thoroughly identified, several types of action including, for instance, degradation of the bacterial cell wall, alteration of the proteins of the cytoplasmic membrane, alteration of membrane permeability, inactivation of extracellular enzymes, decrease of intracellular ATP, leakage of cellular contents, coagulation of cytoplasm, and interruption of electron flow and active conveyance have been proposed (29-31). Certain studies have indicated the exact mechanism of some oil components. Moreover,

Table 1: Chemical Compound *Echinophora Cinerea Boiss essential Oil*.

No	RI	Compound name	Area%
1	856	trimethyl cyclopentadiene	0.7
2	870	m-xylene	0.06
3	938	α-thujene	0.99
4	939	α-pinene	9.79
5	955	Camphene	0.15
6	977	Sabinene	0.94
7	984	β-pinene	0.8
8	990	β-Myrcene	2.65
9	994	dehydro-1,8-cineole	0.04
10	1016	α-phellandrene	32.09
11	1017	(MS)	0.68
12	1021	α-Terpinene	0.18
13	1029	P-cymene	10.75
14	1037	limonene	16.28
15	1061	γ-Terpinene	0.73
16	1092	α-Terpinolene	0.41
17	1094	fenchone	0.27
18	1099	linalool	0.91
19	1101	6-camphenone	0.25
20	1130	p-menth-2-en-1-ol	0.23
21	1146	1-terpineol	0.13
22	1163	(MS)	0.8
23	1174	safranal	0.41
24	1176	(MS)	0.53
25	1184	4-terpineol	0.23
26	1188	(MS)	0.1
27	1192	cryptone	0.09
28	1197	(MS)	0.26
29	1227	A-phellandrene epoxide	0.92
30	1230	(MS)	0.47
31	1251	(MS)	0.49
32	1253	linalyl acetate	0.19
33	1301	carvacrol	3.79
34	1333	(MS)	0.25
35	1351	α-Terpinyl acetate	0.39
36	1402	cis- jasmone	0.13
37	1437	trans caryophyllene	0.05

38	1444	γ-Elemene	0.11
39	1487	γ-Curcumene	0.15
40	1498	Germacrene D	0.05
41	1548	Kessane	0.54
42	1577	Germacrene B	0.21
43	1604	caryophyllene oxide	0.1
44	1618	carotol	0.15
45	1683	dodecalactone	0.05
46	1814	hexadecanal	0.08
47	1838	neophytadiene	0.06
48	1959	Palmitic acid	1.76
49	2032	(MS)	0.21
50	2243	(MS)	6.28
51	2300	(MS)	2.1
Total identified compounds			87.81%
Total unidentified compounds			12.17
Total compounds			99.98

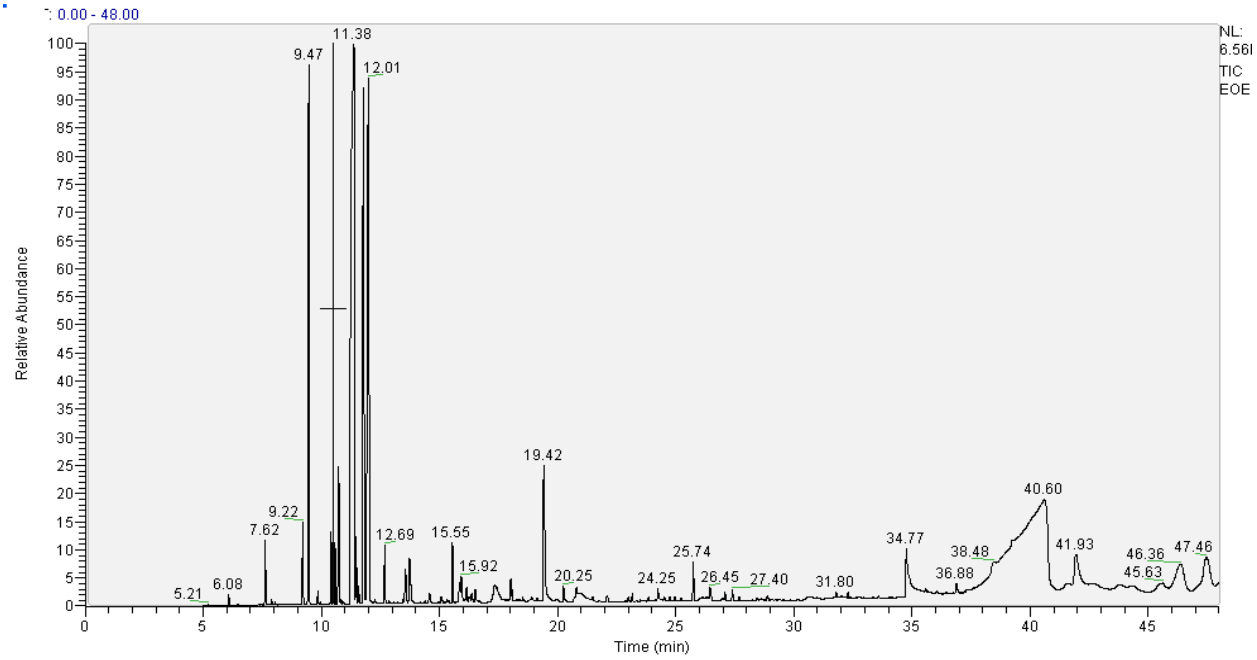

Figure 1. Chromatogram (MS) of the essential oil of *E. cinerea*.

p-Cymene amasses in great quantities and acts by expanding the membrane phospholipids via extending spaces through which ion leakage is likely to take place (30, 31). In this study, the third composition contained the most essential components of *E. cinerea* of the p-Cymene (10.75%). Differences in the chemical

Table 2: The results pertaining to the antioxidant capacity of the essential oil of *E. cinerea* (DPPH method).

***E. cinerea* essential oil**	**concentration**	**RSA (%)**	**IC_{50}**
Essential oil (µg/ml)	2	86.30±0.01	0.74
	1	65.01±0.03	
	0.5	46.10±0.09	
	0.25	44.34±0.05	
	0.125	23.30±0.08	
	0.0625	17.49±0.12	
BHT(µg/ml)	100	24.34±1.05	52.72
	80	28.46±1.55	
	60	47.50±2.05	
	40	52.05±1.97	
	20	74.21±1.87	
	10	77.46±1.89	

Table 3: The mean values of the MBC and MIC concentrations (µg/ml) of the essential oil of *E. cinerea*.

Microorganisms	***E. cinerea essential oil***		**Antibiotics**	
	MIC	**MBC**	**MIC**	**MBC**
			Vancomycin	
L. monocytogenes ATCC 13932	> 660	> 660	1	64
S. aureus ATCC 10690	> 660	> 660	16	64
S. agalactiae PTCC 1768	165	330	2	64
E. faecalis ATCC 29219	165	640	32	32
S. *epidermidis ATCC 1435*	165	165	2	16
			Gentamycin	
P. aeruginosa ATCC 27853	> 660	> 660	0.5	64
S. typhi PTCC 1609	20.62	330	0.5	16
S. para*typhi* PTCC 1230	20.62	330	0.5	1
E.coli ATCC 11775	41.25	660	16	0
S. enterica ATCC 1709	41.25	330	0.5	0.5
			Amphotericin B	
C. albicans PTCC 5072	10.31	0	8.33	0

composition of the essential oils in the findings and available reports could be due to differences in the harvest season, climate settings, geographic area of growing, portions of plants, extraction methods, and

Table 4: The cytotoxicity effect of *Echinophora cinerea* essential oil on A549 cell line.

***Echinophora cinerea* Essential oil**	**Concentration (µg / ml)**	**RSA (%)**	**IC_{50}**
	5000	61±0.07	3589.31
	2500	45±0.04	
	1250	30±0.14	
	625	24±0.03	
	312	24±0.07	
	156	19±0.03	
	78	18±0.05	
	39	17±0.05	

time (32). According to the results of other studies, *Echinophora* genus is the source of phenolic compounds and flavonoids which have durable antioxidant activities (33-35). Antibacterial effects of essential oil could be attributed to p-cymene, linalool, α-pinene, carvacrol, and terpinene (36). In another research, the antibacterial activity of the essential oil was attributed to ocimene, α-pinene, myrcene, and α-phellandrene (26). It is noteworthy that the essential oil of *E. cinerea* was more effective on gram-negative bacteria in the present research (Table 3).

The Cytotoxicity Property of the A549 Cell Line

The investigation of the inhibitory effects of *Echinophora cinerea* essential oil on tumor cell lung indicated that the inhibitory effects of cells have increased via increasing the concentration, so that at the highest concentrations, more than 60% of tumor cells, were inhibited and the IC50 was 3589.31 µg /ml (Table 4). Generally, the decrease in tumor growth can be due to the decrease in cell proliferation or an increase in cell death during necrosis or apoptosis, or a combination of both of them (37). The decrease in IC50 indicates strong essential oil activity (38). In a similar study, examining the anti-tumor effects of *Echinophora platyloba* methanolic extract, Zare Shahneh *et al*. showed that apoptosis is the main mechanism in the death of fibrosarcoma cells (WEHI-164) in mice (37). In another study, Entezari *et al*. examined the antimutagenicity and anticancer activities of *E. platyloba*. The results of their research showed that the methanolic extract could prevent reversible mutations in the Ames test. Moreover, the results confirmed a concentration-dependent inhibitory effect on human leukemia cancer cells (NB4) (39). Their findings were consistent with our study.

Conclusion

The present study demonstrates that *Echinophora* could have considerable antibacterial, antioxidant and cytotoxicity properties. The essential oil of this plant is effective against important human bacterial pathogens. It is also a determinant of nosocomial infection.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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