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Chemical composition and antibacterial activity of methanolic extract from *Echinops robustus* on typical food-borne pathogens

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ABSTRACT

This study was conducted to evaluate the chemical composition and antibacterial activity of methanolic extract from *Echinops robustus* on typical food-borne pathogens. Chemical composition of the extract analyzed by gas chromatography-mass spectrometry (GC-MS). The GC-MS analysis of the extract revealed 54 compounds in which Butanal (2.222%), 4-Heptanone (1.717%) and Palmitic acid (4.799%) were the major constituents. The agar disk diffusion method was used to study the antibacterial activity of *Echinops robustus* methanolic extract against 5 bacterial strains. The extract of *Echinops robustus* showed moderate antibacterial activity against two gram-positive and three gram negative microorganisms tested with higher sensitivity for gram positive ones (Clear zone= 10mm for *Bacillus cereus* and 12mm for *Staphylococcus aureus)*. Minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) were quantified by micro-dilution method. The MIC was 25 mg/ml for *Bacillus cereus* and 58 mg/ml for *Escherichia coli* and the MBC values were 400 and >400 mg/ml respectively for the mentioned species. Overall, results presented here suggest that the methanolic extract of *Echinops robustus* possesses antibacterial properties, and is, therefore, a potential source of active ingredients for food and pharmaceutical industry.

Keywords: *Methanolic extract; Echinops robustus; Antibacterial activity; GC/MS.*

1. INTRODUCTION

Spoilage of food systems with microbial infection has been a major concern for decades and also increasing incidence of food-borne diseases makes manufacturers produce safer foods and develop new natural antimicrobial agents. The demand for non-toxic-natural preservatives has been rising with awareness of consumers and reports of the effects of synthetic preservatives [1]. Accordingly, there is a need to introduce alternative antimicrobial agents for food treatment. Using local medicinal herbs for possible antimicrobial applications represents a promise for this need [2]. Plant metabolites including essential oils and extracts has been studied widely for this activity. Essential oils from aromatic plants mostly consist of chemical components such as terpenoids including mono-terpenes, sesquiterpenes and their oxygenated derivatives which easily diffuse into the microbial cell and induce biological reactions [3].

Echinops robustus is a pubescent annual herb widely spreading from the base (Figure1). It is a plant with 40-100cm height and stems are simple or branching from the base covered with white cottony hair with lanceolate leaves. The species is found practically throughout Iran, India, Turkey, Pakistan, Afghanistan and etc. The Plant is bitter which increases the appetite and stimulates liver. It is used in brain diseases, pains in the joints, inflammations and etc. Roots of the plant are used for treating different ailments. The root is used as abortifacient and aphrodisiac, infusion of the root is given in seminal debility, impotence, hysteria, and its decoction is given in dyspepsia, scrofula, syphilis and fevers [4].

Figure 1. *Echinops robustus* herb.

There are few researches on the antimicrobial activity of Echinops genius. Şapci and Vural (2018) studied antimicrobial and antioxidant activity of *Echinops antalyensis* [5]. The research of Gemechu *et al.* (2016) was on the antimicrobial activity of *Echinops kebericho* against human pathogenic bacteria and fungi [6]. As we found no research on chemical composition and antibacterial activity of methanolic extract from *Echinops robustus*, this study was set on typical food-borne pathogens.

2. MATERIALS AND METHODS

2.1. Materials.

Muller Hinton Agar (MHA) and Broth (MHB) were purchased from Merck, Germany. *Echinops robustus* collected from Shamshir-kooh mountain (1480 m height) Baghchegh village in northern Khorasan province (Iran) and extracted with methanol (Merck-Germany) in laboratory condition for 48 hrs.

2.2. Extraction.

Echinops robustus leaves were dried at room temperature and the powdered material was then weighed (300 g), soaked in

1.5 L of methanol (MeOH) for 48 h and filtered through Whatman No1 filter paper. The filtrate obtained was concentrated under reduced pressure (at 68°C) in a rotary evaporator to obtain the crude extracts were kept at 4°C until further uses [7].

2.3. Gas chromatography/mass spectroscopy.

The chemical composition of the extract was analyzed using GC–MS technique. The mass spectrometer was Agilent Technologies 5975 C (Agilent Technologies, Palo Alto, CA, USA) in the electron impact (EI) ionization mode (70ev) and HP- 5MS (bonded and cross-linked 5% phenyl-methylpolysiloxane, 30 mm-0.25 mm, coating thickness 0.25 mm) capillary column (Restek, Bellefonte, PA). Injector and detector temperatures were set at 220°C. The oven temperature was held at 50°C for 30 min, then programmed to 240°C at a rate of 3°C /min. Helium (99.99%) was the carrier gas at a flow rate of 1 ml/min. Diluted samples (1/100 in hexane, v/v) of 1.0 were injected manually [8].

2.4. Organisms and inoculation conditions.

Aqueous extract of *Echinops echinatus* was tested against five bacterial strains: tow gram positives including *Staphylococcus aureus* (PTCC 1431) and *Bacillus cereus* (PTCC 1015); and three gram negatives including *Escherichia coli* (PTCC 1399), *Salmonella entrica* (PTCC 1709) and *Pseudomonas aeroginosa* (PTCC 1430) obtained from Persian Type Culture Collection (PTCC, Iran). The bacteria strains were first grown on MHA at 37°C for 24 hrs prior to seeding on to the nutrient agar. Finally, suspensions were adjusted to 0.5 Mc-Farland standard turbidity. Bacterial suspensions were standardized to concentrations of 1.5×10^8 CFU/ml [9].

2.5. Antimicrobial assay.

The methanolic extract of *Echinops robustus* was tested using agar disc diffusion technique for determination of the growth inhibition zones and also broth macro-dilution method was used to determine the MIC and MBC [10].

2.6. Disk diffusion test.

The antimicrobial activity test was done on methanolic extract of *Echinops robustus* leaves using disk diffusion method against the mentioned bacterial strains. Measured amounts of the test samples were dissolved in dimethyl sulfoxide (DMSO) to give solutions of known concentration (100, 200, 300 and 400 mg/ml). Then, sterile filter paper discs (6 mm diameters) were placed on plates containing MHA seeded with the test organisms. Plates were kept at 4°C for 15 min to allow maximum diffusion and then inverted and incubated at 37°C for 24 hours. In the case of antimicrobial property, inhibition of microbial growth appears by clear and distinct areas defined as zone of inhibition. Gentamycin and DMSO were considered respectively as positive and negative controls [11].

2.7. Minimum Inhibitory Concentration (MIC) test.

The MIC test was studied using micro-dilution method. The 96-well plates were prepared by dispensing into each well 95µL of MHB and 5µL of the inoculum. One-hundred microliters of the extract (concentration of 400 mg/ml) were added into the first well, followed by two-fold dilution until the 9th well. The wells of column 10 were filled with 195µL of MHB (negative control). The wells of the last column were used as a positive control which contained 195µL of MHB and 5µL of the inoculum. The plates were screened visually after incubation at 37°C for 24 h for broth turbidity. The minimum bactericidal concentration (MBC) is the lowest concentration of the essential oil that can kill 99.9% of the bacterial population after incubation for 18–24 h at 37°C. It was calculated by inoculating the content of the well indicating the MIC and the wells that precede it in an agar plate [12].

3. RESULTS

The composition of the extract from *Echinops robustus* is shown in Table 1 which indicates 54 different components in the composition. Based on the results, Butanal (2.222%), 4-Heptanone (1.717%) and Palmitic acid (4.799%) were the major constituents.

According to Table 2, the antimicrobial activity by disk diffusion technique showed the clear zone of the extract (at 400 mg/ml) on *S.aureus, Bacillus cereus*, *S.entrica, P.aeroginosa* and DMSO (control) respectively at 10, 12, 8, 8, 8 and 0mm. This herbal extract had weak antimicrobial activity at concentration lower than 400 mg/ml. Also the MIC and MBC were respectively were in the ranges of 8.3-58.3 mg/mL for MIC and 125- >400 mg/mL for MBC at 400 mg/ml extract concentration. According to Table 3, the methanolic extract of *Echinops robustus* had the lowest MBC (125 mg/ml) on *S. aureus*, as the only none spore-former gram (+) strain. Data shows that the extract had a more bacteriostatic effect on gram (+) bacteria than the gram (-) ones. Gemechu *et al.* (2016) showed that among the tested microorganisms, *S. aureus*, was the most sensitive microbe to alcohol based extracts from *Echinops kebericho* which is in agreement with our findings [6]. Bin et al. (2017) showed that the essential oil from *Echinops ritro* exerted potent inhibitory effects against *E. coli*, *S. aureus*, and *Salmonella* Enteritidis with MIC values of 2.5, 0.15, and 0.6 mg/ ml; also, the MBC values were 7.5, 1.0, and 3.0 mg/ml, indicating that the essential oil possessed remarkable antibacterial and bactericidal properties [13].

Plant extracts and essential oils can control microbial growth which depends on the chemical composition which is dependent on the method of extraction and solvent type. Şapci and Vural (2018) indicated that the antimicrobial and antifungal activity of *Echinops emiliae* extract could be change with the type of used extracts [5]. Besides, they are used in other industries including traditional medicine, dietary supplements, recombinant protein manufacturing and functional foods [14]. Flavonoids are the main group of compounds with antimicrobial and antioxidant activity. The antimicrobial activity is due to their permeability into the cell and cellular secretory processes [15]. Some studies emphasize that gram-negative bacteria are more resistant to herbal essence and extracts while others claim in versus. Our study indicates that gram (-) bacteria were more resistant than positive ones regarding *Echinops echinatus.* The higher resistance of gram-negative bacteria to antimicrobial agents is reported before and is attributed to the lipopolysaccharides existed in their outer membranes which make them resistant to antibiotics, detergent and hydrophilic dyes [1]. This lipopolysaccharide layer, blocks the penetration of hydrophobic components of oils and so the gram (-) bacteria are found to be more resistant to the essential oils effects [16].

Table 1. Chemical composition determined by GC–MS analysis of the extract from *Echinops robustus*

Number	Name	RT (min)	% of Total
1	Pyridine	3.297	**0.13**
2	1-Fluoropropane	7.942	**0.108**
3	Methyl thiocyanate	8.094	**0.125**
4	Vinyl Ether Divinyl oxide	8.816	**0.104**
5	2-Pentenal	9.067	**0.13**
6	Thiophene	12.238	**0.159**
7	Gentamicin a	12.349	**0.16**
8	Butanal	19.938	**2.222**
9	Thiophene Thiole	21.5	**0.301**
10	Thiophene, 2,3-dihydro-	22.048	**0.517**
11	1,2-Benzenediol Pyrocatechol	22.52	**0.224**
12	Decanoic acid (CAS) Capric acid	22.893	**0.18**
13	4-vinylphenol p-vinylphenol	23.569	**0.375**
14	2-Pyrrolidinone	23.715	**0.267**
15	4-Heptanone	25.055	**1.717**
16	Diisopropyl sulfide	26.996	**0.383**
17	8-Hydroxylinalool	27.276	**0.3**
18	Phenol-2-carboxylic acid Salonil	27.626	**0.416**
19	1,8-Cineole	29.392	**0,092**
20	3-Piperidinol	30.126	**0,086**
21	Hexanedioic acid (CAS) Adipic acid	30.785	**0.765**
22	Vanillin 4-Formyl-2-methoxyphenol	31.42	**0.087**
23	Pyrogallol	31.583	**0.143**
24	Octanal	32.801	**0.11**
25	3,4,5-trimethyl-Phenol	33.314	**0.957**
26	cis-Z-.alpha.-Bisabolene epoxide	34.48	**0.194**
27	3-Nonanone	35.086	**0.229**
28	Paraben	36.829	**0.485**
29	Vanillic acid	38.135	**0.439**
30	Lauric acid	38.461	**0.401**
31	3-methyl-Butanal	41.434	**0.883**
32	Cyclohexanol	43.211	**0.244**
33	Coniferyl alcohol Coniferol	44.698	**0.32**
34	Myristic acid	45.817	**0.794**
35	Phenanthrene	46.096	**0.228**
36	Anthracene	46.487	**0.213**
37	Octadecane	47.309	**0.586**
38	Carbazole	48.113	**0.093**
39	Pentadecanoic acid	49.221	**0.2**
40	Myo-Inositol	50.491	**0.271**
41	Thianthrene	50.818	**0.404**
42	4-Methyl-8-hydroxyquinoline	51.855	**1.113**
43	Palmitic acid	52.718	**4.799**
44	Linoleic acid	57.847	**2.651**
45	Oleic Acid	58.138	**0.755**
46	Stearic acid	58.768	**0.992**
47	Lauric acid	59.479	**0.186**
48	Benzen ethanol	60.592	**0.592**
49	Capric acid	64.404	**0.289**
50	Caryophyllene oxide	65.156	**0.083**
51	Alpha -Terpineol	66.258	**0.235**
52	Eugenol	69.423	**0.378**
53	Linolic acid	72.529	**0.403**
54	Squalene	76.452	**0.371**

Table 2. Results of disc-diffusion test and inhibition zones (mm) for methanolic extract of *Echinops robustus.*

Microorganism	Concentration of methanolic extract (mg ml^{-1})				Positive control	Negative control
	50	**100**	**200**	**400**	**Gentamicin**	**DMSO**
Bacillus cereus	0	8	9	10	29	0
Staphylococcus aureus	0	5	10	12	26	0
Salmonella enterica	0	2	7	8	25	0
Escherichia coli	0	3	6	8	25	0
Pseudomonas aeroginosa	0	3	6	8	22	0

Table 3. MIC and MBC for methanolic extract of *Echinops robustus* (mg ml^{-1}).

Microorganism	*B. cereus*	*S. aureus*	*S. enterica*	*E. coli*	*Pseudomonas aeroginosa*
MIC	**20.8±5.2**	**8.3±3.6**	**25±6.6**	**58.3±10.2**	**25±4.8**
MBC	**400±55**	**125±28**	>400	>400	>400

Data are the mean values for triplicate (X±SD)

4. CONCLUSIONS

This study characterized the chemical composition and antibacterial properties of *Echinops robustus* methanolic extract in northern Khorasan province (Iran). Results showed that the extract was characterized by 54 different components which had antimicrobial activity against pathogens tested in the current study. The results emphasize that more attention is needed on this native herb and the possible applications in food systems.

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