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CHEMICAL COMPOSITION AND ANTIMICROBIAL ACTIVITY OF THE EXTRACT FROM *ANTHEMIS COELOPODA* BOISS. VAR. *COELOPODA* STEMS AND ROOTS.

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ABSTRACT

The global increasing of bacterial drug resistance as well as the serious side effects of pharmaceutical chemicals, have encouraged researchers to focus on alternative drugs. Plants are able to make compounds with important biological effects such as anti-bacterial activities. Therefore research about anti-bacterial activities of plants as a natural source has been considered to find out a substitution to chemical synthesis. The quality and quantity of hexane extract from *Anthemis coelopoda* Boiss. (Family Compositae) roots and stems were analyzed by GC and GC/MS. The anti-bacterial activities of the chloroform extract of *Anthemis coelopoda* Boiss. roots and stems were evaluated by well diffusion method and disc diffusion method against four bacterial strains including: *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli* and *Salmonella typhi*. Finally minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values by microdilution method were determined. The yields of the roots and stems on dry weight basis were 1%. Twenty-two components in roots and stems (95%) were identified. The major components including: Ascorbic acid 2, 6-dihexadecanoate (32.1%), 1, 2-Benzenedicarboxylic acid, bis (2-methylpropyl) ester (14.8%) and Trioctylamine (6.08%). Maximum inhibition zone (12 mm) of the extract was obtained against *Staphylococcus aureus* and *Bacillus subtilis*, at concentration of 6mg/ml and the best results of MIC and MBC were obtained for *Bacillus subtilis* at the concentration of 0.187 mg/ml and 0.375 mg/ml respectively. This study showed that *Anthemis coelopoda* Boiss. can be considered a natural antibacterial source.

KEYWORDS: *Anthemis coelopoda* Boiss. var. *coelopoda*, Essential oils, Chemical composition, Antimicrobial activity.

INTRODUCTION

Investigation about the therapeutic effects of plants has been a very traditional and ancient idea.[1] Medicinal plants have been the best source of obtaining a diversity of drugs. About 80% of people in developed countries use traditional medicine, compounds derived from medicinal plants. So, research on plants for better understanding their properties, safety and efficiency seems to be important nowadays.[2] Their bioactive substances have a wide range of biological functions, for example antioxidant and antimicrobial activities.[3,4] The antimicrobial and antifungal properties of plants have been recognized for many years, and their rudiment have found applications as naturally occurring antimicrobial and antifungal agents in the field of pharmacology, pharmaceutical botany, phytopathology, medical and clinical microbiology, food preservation and etc. The natural anti-bacterial effects of phytochemical are "biocide". The essential oil rudiments that possess antimicrobial activities have been the subject of many researchers and caused the screening of a wide variety of plant species and have picked structurally unique biologically active compounds. However, less attention has been given to the activities of their original components in the plants tested. For example Antibacterial effects of some species of plants are due to phenols, quinines, flavones, flavonoids, flavonols, tannins, terpenoids, alkaloids substances contained in them, so that if the compound is pure, it has a very high anti-bacterial effect.[1,5 ,6]

"Antibiotic resistance" generally happens because of long-term use of synthetic antibiotics, the original advantage of natural factors is that they do not enhance this resistance and can be used for long-term.[1,5,6] The components of the extract are known to be active against a large variety of microorganisms, including Gram-negative and Gram-positive bacteria. Gram-negative

bacteria were shown to be commonly more resistant than gram-positive bacteria to the antagonistic effects of extract because of the lipopolysaccharide present in the outer membrane.[5]

The genus *Anthemis* L. (tribe Anthemideae Cass.), is the second largest in the Compositae family' [7] and includes 130 species widespread in the Mediterranean, South West Asia and South Africa.[8] There are 39 species in Iran, among which 15 are endemic.[9] From the Roman times up to now, *Anthemis* species have been commonly used as folk remedies, insecticides and dyes.[10] The genus *Anthemis* is widely used in pharmaceutics, cosmetics and food craft. The flowers of the genus *Anthemis* have well-documented use as germicide and healing herbs, the main components being natural flavonoids and essential oils.[11] The essential oil from *A. nobilis* flowers is commonly used for pharmaceuticals, food additives, as well as an important source in aromatic and cosmetic manufacturing.[12,13,15]

All of the extracts of A.tinctoria prevent the growth of most of the tested bacteria.[14] The total extract of *A. cotula* flowers exhibit antibacterial activity against both Gram-negative and Gram-positive bacteria.[2] Methanol extract of *A. gayana* flowers and leaves has a high antibacterial effect.[17]

This study investigates the quality and quantity of roots and stems extracts of *Anthemis coelopoda* Boiss. by GC and GC/MS and the antibacterial effect of this plant by agar diffusion and determination of MIC and MBC by micro dilution method.

MATERIALS AND METHODS

Materials

Solvents including chloroform ($CHCl_3$), n-hexane (C_6H_{14}) and dimethyl sulfoxide (DMSO) were purchased from Merck.

Cultures including Mueller-Hinton broth (MHB) and Mueller-Hinton agar (MHA) were from Merck.

Blank, penicillin, gentamicin and vancomycin control disks from mast antibiotic discs.

Collection of plant

Anthemis coelopoda Boiss. were collected from around the area of Bojnourd (North Khorasan Province, Iran) in the June of 2015. The voucher specimen was incorporated at the herbarium of Islamic Azad University of Pharmaceutical Sciences.

Preparation of chloroform and hexane extracts

The root and stem were dried under the shade and ground into fine powder using electric blender, then, 100 g of powder were extracted with 1500 ml chloroform, by maceration extraction during 72h and three stages. The residue was dried and then evaporated by rotary. The dried extract was stored at 4°C until used.

Some of the extract in a separate bowl with 50 ml n-hexane were mixed and separated.

GC-MS Analysis

The Gas chromatography-Mass spectrometry (GC-MS) analysis of hexane extract of *A.coelopoda* has been done by using a GC-MS (Model; QP 2010-plus series, Shimadzu, Japan) equipped with 5ms column of 30m length, 0.25mm diameter and 0.25µm film thickness. The column oven temperature was programmed from 60°C to 240°C for 6°C min^{-1} and 240°C to 270˚Cfor 5 °C min^{-1}. Ionization of the sample components was done in electron impact mode (EI, 70 eV). The temperature of the injector was fixed to 250°C and one of the detector to 300°C. Helium (99.9995% purity) was the carrier gas stabilized with a flow rate of 1.5 ml min^{-1}. The mass range from 35-600 m/z was scanned at a rate of 0.3 scans/s. 1.0 µL of the hexane extract of *A.coelopoda* was injected with a Hamilton syringe to the GC-MS manually for total ion chromatographic analysis in split injection technique. Total running time of GC-MS is 47min. The relative percentage of the each compound from extract was expressed as percentage with peak area normalization.

Identification of Components

Identification of bioactive compounds in the hexane extract components was carried out by comparison of their relative retention (RT) times with those of reliable samples or by comparison of their relative retention index (RRI) to series of n-alkanes. Computer implementation of commercial databases (Wiley GC/MS Library, Adams Library, Mass Finder 3 Library).[18,19]

Bacterial strains and reagents

Four representative strains used for antibacterial susceptibility testing were obtained from faculty of pharmacy, Azad University of Tehran. These included: Gram-positive: *Staphylococcus aureus* (ATCC: 25923), *Bacillus subtilis* (ATCC: 1720) and Gram negative: *Escherichia coli* (ATCC: 1399), *Salmonella typhi* (ATCC: 1639). The cultures of bacteria were maintained in their suitable agar slants at 4°C throughout the study and used as stock cultures.

Antibacterial susceptibility testing

Disc diffusion method

Antimicrobial tests were carried out by the disc diffusion method explained by Murray and his co-worker in 1999.[20] The dried chloroform extract was dissolved in Dimethyl sulfoxide 20% (DMSO) to a final concentrations of 6, 3, 1.5, 0.75, 0.3, 0.09, 0.04 mg/ml.

The concentration of suspension of tested bacteria was adjusted to 10^8 CFU/ml prepared 0.5 McFarland turbidity standards. Each Microorganisms suspension was spread over the surface of MHA. Blank discs in different concentrations of extract were dipped for several hours and then were placed over the culture medium containing bacterial suspension. Gentamicin, penicillin and

vancomycin were used as positive controls and chloroform and DMSO 20% were used as negative controls.

Well diffusion method

Antimicrobial tests were carried out by the well diffusion method directed by Official Circular 198.[21] In well diffusion method 60 µl of Serial dilutions of the extract (6, 3, 1.5, 0.75, 0.3, 0.09, 0.04 mg/ml) were load in the wells (diameter of 6 mm) embedded in the culture plate containing bacterial strain (bacterial strains cultured in MHA with bacterial suspension).

The inoculated plates were incubated for 18-24 h at 37°C. The diameters of inhibition zones were used as a measure of antibacterial activity and each assay was repeated three times.

Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The MIC of the chloroform extract against the microorganisms under investigation was assessed according to the broth microdilution method, as previously reported, [18] with minor modifications. Briefly, 12mg of the chloroform extract was dissolved in DMSO 20% and serial diluted with the medium to the desired concentrations. MIC tests were carried out in microplate wells. Each of well filled with 100 µl Mueller-Hinton broth. 100 µl of the soluble extract was added to the first test well and mixed. Then, 100µl from the first well added to the second well, mixed and so the series was continued. Microorganisms were cultured overnight at 37˚C in MHA. 100µl of suspension of tested bacteria (10^8 CFU/ml) was added to each well. Positive growth control including 100 µl Mueller-Hinton broth and 100 µl and 100µl of suspension of tested bacteria (10^8 CFU/ml). Negative growth control including 100µl 100 µl Mueller-Hinton broth and series dilution extract. Microplates were incubated for 24 h at 37°C.

Minimum inhibitory concentration (MIC) was defined as a lowest concentration of the plant extract which resulted in inhibition of visual growth with comparison to positive and negative control .MIC determination was repeated three times for each microorganism.

Also the MBC of the chloroformic extract against the microorganisms was measured as explained.[22]

Minimal bactericidal concentrations (MBC) were determined by subculturing 100 µl of the culture from each negative well and from positive growth control over MHA. The plates were incubated for 24 h at 37˚C and MBC was defined as the lowest concentration of the plant extract producing no growth and no colony.

RESULTS AND DISCUSSION

Chemical Composition from the Extract of *Anthemis coelopoda* BOISS. Stems and Roots.

The yields of the roots and stems on dry weight basis were 1%. Twenty-tow components in root and stem (95%) were identified (**Table 1**). The major components includes: Ascorbic acid 2, 6-dihexadecanoate (32.1%), 1,2Benzenedicarboxylic acid, bis (2-methylpropyl) ester (14.8%), Trioctylamine (6.08%). Based on studies on the flower heads essential oil of *Anthemis tinctoria* L. by capillary GC/MS, 48 components of the oil were identified, that major constituents were 1,8-cineole (7.9%), β-pinene (7,3%), decanoic acid (5.4%) and α-pinene (4.4%).[23] The studies undertaken on *Anthemis melampodina* showed that the main components of the oils were Santolinatriene (27.33%), Pinene (6.44%) and Sabinene (6.09%).[24] In *Anthemis xylopoda* the major components were Borneol (31.80%), Carvacrol (12.67%), 1,8-cineole (5.45%) and 2,5,5-trimethyl-3,6-heptadien-2-ol (5.10%) for flowers and Borneol (30.15%), 1,8-cineole (16.74%), α,β-Thujone (12.08%), 2,5,5-trimethyl-3,6-heptadien-2-ol (8.50%) and Carvacrol (5.21) for leaves reported.[11] The main component of the *Anthemis talyshensis* A. aerial parts oil were Berneol (18.2%), α-eudesmol (13.3%), Hexadecanoicacid (9.5%), γ-eudesmol(8.6%) and Elemol(7.6%).[25] In *A.coelopoda* Boiss. flower oil, the major constituents were cis-chrysanthenyl acetate (27.3%), hexyl-butanoate (16.0%), and Myrcene (7.0%), while the leaf oil contained Isobornyl-formate (30.6%), Trans-ethyl chrysanthemumate (15%) and p-mentha-1,5-diene-8-ol (13.7.4%).[26] The oil of the *Anthemis mazandranica* aerial parts contained 19 compounds that main component was Eugenol (35.5%) and Oxygenated monoterpenes (59.11%) were the most abundant group of compounds.[27]

Table: 1 Phytoconstituents identified in the hexane extract of *A.coelopoda* Boiss. by GC-MS

NO	Name of the compound	Retention Time	Peak area%
1	Octanal	5.888	2.25
2	Dodecane	10.419	0.90
3	Tridecane	12.784	1.19
4	Tetradecane	15.063	0.72
5	Heneicosane	19.303	4.19
6	Oleic acid	22.525	2.41
7	3,7,11,15-Tetramethyl-2-hexedecen-1-ol (phytol)	23.850	2.08
8	2-Pentadecanone ,6,10,14-trimethyl	24.005	0.53
9	1,2-Benzenedicarboxylic acid, bis (2-methylpropyl) ester	24.530	14.85
10	Ascorbic acid 2,6-dihexadecanoate	26.085	32.12

11	Eicosane	26.605	1.25
12	D -Nerolidol	27.065	0.57
13	Ethyl iso-allocholate	27.400	0.33
14	D-Norandrostan-16-ol,acetate	28.640	0.64
15	Thunbergol	29.470	3.67
16	Octadecyl chloride	29.775	1.71
17	Trioctylamine	31.650	6.08
18	Bumetrizole	36.635	0.86
19	1,6,10,14,22-Tetracosahexaen-3-ol,2,6,10,15,19,23-hexamethyl	42.175	4.42
20	7,8-Epoxylanostan-11-ol,3-acetoxy	43.425	5.92
21	Lanosta-8,24-dien-3-one	43.889	1.99
22	Lupeol	44.996	1.58
	Total		95

The anti-microbial activities of chloroform extract of *Anthemis coelopoda* Boiss.

The anti-microbial activities were examined against four Gram-positive and Gram-negative bacteria via two method, disc diffusion and well diffusion, and its potency was qualitatively assessed by inhibition zone diameter. (**Table 2**, **Table3**).

Table: 2 The antibacterial activity of *Anthemis coelopoda* Boiss. $CHCl_3$ extract against bacterial strains tested based on agar disc diffusion method (n=3)

	Zone of Inhibition in mm			
Concentration	***S.aureus***	***B.subtilis***	***E.coli***	***S.typhi***
6mg/ml	12 ± 1.00	12± 0.00	11 ± 1.00	11.6 ± 0.57
3mg/ml	11± 1.00	11± 0.00	10.3 ± 0.57	11± 1.00
1.5mg/ml	11 ± 1.00	11± 0.00	10± 0.00	10.6 ± 0.57
0.75mg/ml	10 ±0.00	10.3± 0.57	9.6± 0.57	9.6 ± 0.57
0.3mg/ml	9.3 ± 0.57	10.3±0.57	9.3± 0.57	9.3 ±0.57
0.18mg/ml	9 ± 0.00	10±0.00	9± 0.00	9 ± 0.00
0.09mg/ml	9 ± 0.00	9±0.00	8.3 ± 0.57	8.5 ± 0.57
0.04mg/ml	9 ± 0.00	8.6 ±0.57	8.3 ± 0.57	-

Table: 3 The antibacterial activity of *Anthemis coelopoda* Boiss. $CHCl_3$ extract against to bacterial strains tested based on agar well diffusion method (n=3)

Concentration	Zone of Inhibition in mm			
	S.aureus	***B.subtilis***	***E.coli***	***S.typhi***
6mg/ml	11.3 ± 0.57	12± 0.00	11 ± 1.00	11± 0.00
3mg/ml	10.3 ± 0.57	11.6± 0.57	10.6 ± 0.57	10.6 ± 0.57
1.5mg/ml	9.6 ± 0.57	11.6± 0.57	10.3 ± 0.57	10.3 ± 0.57
0.75mg/ml	8.6 ± 0.57	10.6± 0.57	10± 0.00	10± 0.00
0.3mg/ml	-	10.3±0.57	9.6± 0.57	9.3 ±0.57
0.18mg/ml	-	9.6±0.57	9.3 ± 0.57	9 ± 1.00
0.09mg/ml	-	9.3±0.57	9.3 ± 0.57	9 ± 1.00
0.04mg/ml	-	8.6±0.57	8.6 ± 0.57	8.6±0.57

The *Anthemis coelopoda* Boiss. roots and stems extract showed anti-microbial activity but microbial susceptibility demonstrated different behaviors in closer observation. Generally, the Gram-positive bacteria are more susceptible than Gram-negative bacteria due to the differences in their cell wall structure. Due to having outer membrane as a barrier to many environmental substances, Gram–negative bacteria are resistant against antibiotics.[28,29] In our studies the Gram-positive bacteria i.e., *Staphylococcus aureus* and *Bacillus subtilis*, are more susceptible than Gram-negative bacteria i.e., *Escherichia coli* and *Salmonella typhi*. In the disc diffusion method maximum inhibition zone (12 mm) of extract was obtained against *Staphylococcus aureus* and *Bacillus subtilis*, at concentration of 6mg/ml. In the well diffusion method it happened with 12 mm against *Bacillus subtilis* and 11.3mm against *Staphylococcus aureus* at 6mg/ml. However, in the disc diffusion method no effect was observed at concentration of 0.09mg/ml extract on *Salmonella typhi* but in the well diffusion method, at concentration of 0.09mg/ml extract on *Staphylococcus aureus*.

Results demonstrated that there is direct relationship between dilution and inhibition zone. So the more dilution increased, the more zoon diameter decreased.

In two figures below, the average zone of inhibition of antibacterial activities of chloroformic extract against bacterial strains by two method, disc diffusion and well diffusion, are compared. (**Figure1, Figure2**).

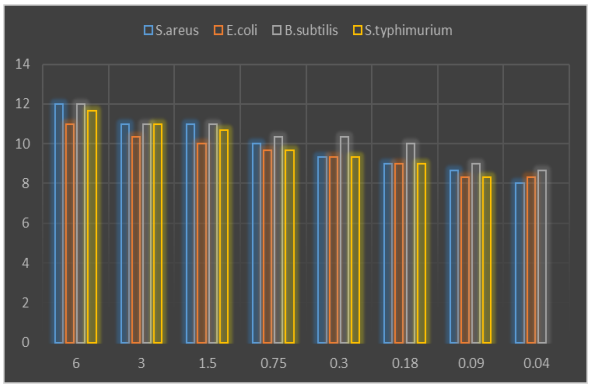

Figure1: The antibacterial activity of *Anthemis coelopoda* Boiss. $CHCl_3$ extract against bacterial strains tested based on agar disc diffusion method.

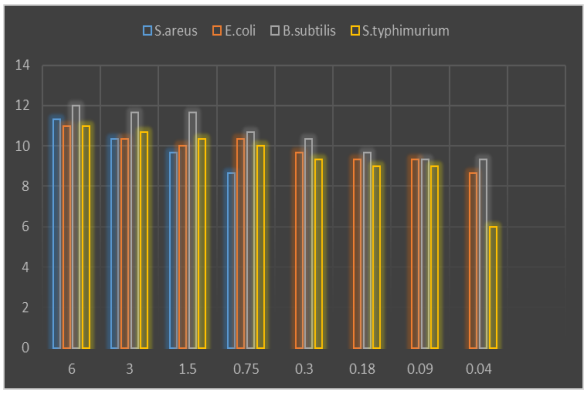

Figure2: The antibacterial activity of *Anthemis coelopoda* Boiss. $CHCl_3$ extract against bacterial strains tested based on agar well diffusion method.

As the negative control Chloroform and DMSO 20% showed no antibacterial activity. The anti-microbial activities of positive control including Gentamicin, Penicillin and Vancomycin against microorganisms by disc diffusion method were also examined and their potencies were qualitatively assessed by inhibition zone diameter. (Table 4) The result showed that penicillin has good anti-bacterial activity against Gram-positive bacteria i.e., *Staphylococcus aureus* and *Bacillus subtilis*, and no activity against Gram-negative bacteria i.e., *Escherichia coli* and *Salmonella typhi*. The highest anti-bacterial activity of Vancomycin was observed against *Bacillus subtilis* and the lowest against *Escherichia coli*. Gentamicin exhibited high activity against all bacteria tested.

The anti-bacterial activity of *Anthemis coelopoda* Boiss. against all of the tested bacteria was observed less effective than antibacterial activity of suggested antibiotics. However, was more effective than penicillin in Gram-negative bacteria.

Table 4: The anti-bacterial activity of antibiotics against bacterial strains tested based on agar disc diffusion method.

Organisms	Zone of Inhibition in mm		
	Penicillin	Vancomycin	Gentamicin
S.aureus	40	20	20
B.subtilis	40	22	31
E.coli	-	8	25
S.typhi	-	20	20

The MIC and MBC of *Anthemis coelopoda* Boiss. chloroformic extract was determined against bacterial strains by microdilution method. Minimum concentration having no opacity, is reported as MIC; and minimum concentration no growth in subculturing of negative well over MHA, is reported as MBC. (**Table 3, Figure 3**) The result showed strong antibacterial activity against *Bacillus subtilis* with MIC of 0.187 mg/ml and MBC of 0.375mg/ml and weak antibacterial activity against Salmonella *typhi* with MIC of 1.5 mg/ml and MBC of 1.5mg/ml.

Table 3: The MIC and MBC of *Anthemis coelopoda* Boiss. chloroformic extract against the microorganism tested in microdilution assay.

Organisms	Zone of Inhibition in mm	
	MIC mg/ml	MBC mg/ml
S.areus	0.375	0.375

B.subtilis	0.187	0.375
E.coli	0.75	0.75
S.typhi	1.5	1.5

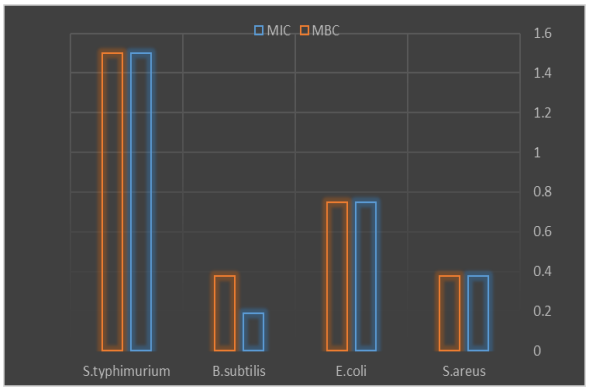

Figure3: The MIC and MBC of *Anthemis coelopoda* Boiss. chloroformic extract against the microorganism tested in microdilution assay. (MIC and MBC mg/ml)

The comparison of antibacterial activity between *A.coelopoda* and the other species of the *Anthemis* genus, the studies on the antibacterial activity of the methanolic, dichloromethane, hexane, ethyl acetate, 1-butanol and hot water extract fractions of aerial parts of *Anthemis tinctoria* against *Staphylococcus aureus*, *Enterococcus faecalis*, *Escherichia coli* and *Pseudomonas aeruginosa*, as compared to the fractions, higher activity was observed in dichloromethane fraction at 5mg/disk against *Staphylococcus aureus* with inhibition zone of 16mm but none of the fractions, showed activity against *Escherichia coli.*[30] While chloroform extract of *A. coelopoda* have anti-bacterial activity against *Escherichia coli*. (In concentration of 6mg/ml with inhibition zoon of 11 mm).

The anti-bacterial activity of the ethyl acetate, acetone, chloroform and ethanol from *Anthemis coelopoda Var.bourgaei* and *Anthemis tinctoria Var. pallida* against ten gram-positive and Gram-negative bacteria were investigated; maximum anti-bacterial activity of *Anthemis coelopoda Var.bourgaei* was reported in ethyl acetate fraction against *Pseudomonas aeruginosa* in dilution of 133.3mg/ml with inhibition zoon of 17mm. It should be mentioned that chloroform and ethanol extracts showed no anti-bacterial activity against bacterial strain tested.[15]

Meanwhile maximum anti-bacterial activity of *Anthemis tinctoria Var. pallida* was reported in ethyl acetate fraction against *Proteus mirabilis* in dilution of 133.3mg/ml with inhibition zoon of 12mm. Chloroform, ethanol and acetone extracts fractions showed no anti-bacterial activity against bacterial strain tested.[15]

The anti-microbial activity of the ethanol, acetone, ethyl acetate and chloroform extracts of *Anthemis aciphylla* Boiss. *var.aciphylla* were tested in vitro against 26 bacterial and 3 yeast species. The results showed the highest antimicrobial activities were obtained with ethyl acetate extract against *Xanthomonas campestris*, in dilution of 13.3 mg/ml and inhibition zoon of 38mm, In addition maximum inhibitory effect of chloroform extract was observed against *Bacillus cereus* in concentration of 13.3mg/ml and inhibition zoon of 18mm. Most bacterial strains studied including: *klebsiella pneumoniae, Pseudomonas gladioli pv. agricola, Staphylococcus aureus, Pseudomonas lachrymans, Aeromonas hydrophila, Yersinia enterocolitica, Salmonella typhimurium, Pseudomonas syringae, Proteus vulgaris* and *Candida albicans* did not show sensitivity to chloroform fraction.[31]

The antibacterial activity of the extract of *Anthemis gayana* leaf and flower were evaluated against *Pseudomonas aeruginosa* (clinical), *Pseudomonas aeruginosa* (standard), *Staphylococcus aureus* (standard), *Staphylococcus aureus* (clinical). The result showed that Maximum inhibition zone (25±0.45 mm) of leaf extract is observed against *Pseudomonas aeruginosa* (clinical); in dilution of 500 mg/ml. The minimum inhibition zone (0 mm) of leaf and flower extract was observed against *Staphylococcus aureus* (standard), in 62.5 mg/ml dilution.[17]

CONCLUSION

This study shows an overview analysis the quality and quantity of hexane extract of *Anthemis coelopoda* Boiss. *var. coeiopoda* stem and root growing in Bojnord, performed by GC/MS. The main components of this extract include: Ascorbic acid 2,6-dihexadecanoate (32.1%), 1,2-Benzenedicarboxylic acid, bis (2-methylpropyl) ester (14.8%), Trioctylamine (6.08%). GC-MS analysis of hexane extract of *A.coelopoda* showed a spectrum of compounds having a variety of bioactivities.

Based on these results the chloroform extract of *Anthemis coelopoda* Boiss. showed moderate anti-microbial activity against the tested Gram-positive and Gram-negative bacteria.

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