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Comparative Evaluation of the Antibacterial Activities of Essential Oils of *Iris pseudacorus* and *Urtica dioica* Native North of Iran

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In this study, the effects Antibacterial activity of *Urtica dioica* and *Iris pseudacorus* essential oils, native plant northern of Iran. were investigated for some selected bacteria. The influence of essential oils was tested by the using of disk diffusion and micro-broth dilution methods against standard strains of the picked out bacteria. Gas Chromatography /Mass Spectroscopy (GC/MS) analysis, bioactivity determination, Minimum inhibitory concentrations (MIC) and minimum bactericidal concentration (MBC) of essential oils were utilized for this goal. This study showed that, Inhibition zone diameter varied from 11 to 19 mm and 9 to 17mm for *Urtica dioica* and *Iris pseudacorus* respectively. In contrast, this figure fluctuated from 19 to 28 mm and 7 to 17 mm for *Gentamicin* and *Ampicillin* separately. By the application of micro-broth dilution technique, MICs for 1% essential oils were 1.8 -7.5 μg/mL and 3.75 -15 μg/mL for, Urtica dioica and Iris pseudacorus against gram-positive and gram-negative bacteria individually. Furthermore, the MBCs of herbal essences were 1.8-15 μg/mL for, *Urtica dioica* and 15-30 μg/mL for *Iris*. The application of essential oils for the bio-control of diseases, as a novel emerging alternative to antimicrobial treatments, lead to safer and more environmental management for infective diseases.

Key word: Antimicrobial effects, Essentials oil, *Urtica dioica*, *Iris pseudacorus*.

The four full seasons and the various climates in our country, Iran, have certainly contributed to the variety of flora in Iran, some of which demonstrate wonderful therapeutic effects. This is of particular interest when one considers such problems as antibiotic resistance and other side effects of synthetic drugs which have caused global interest in the growth of new disciplines such as pharmacognosy. Nowadays, medicinal plants have many applications in people's lives. They can be used in the pharmaceutical compounds, cosmetic, sanitary and nutritional industries[1]. In this century, medicinal plants and their derivatives consist of 20% and 80% of the prescriptions in developed and developing countries respectively[2].Based on the evidence of traditional physic, particularly Iranian's medicine, it is obvious that scientists can achieve more effective drugs by medicinal plants[3-5]. Recent experiences have indicated that chemical drugs have many undesirable effects although they have sufficient proficiency. On the other hand, natural products which are accompanied with other materials have always had biological balance so they aren't accumulated in the body. Consequently,

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fewer side effects are made by this type of medicine[6].The resistant properties of essences have been known from ancient eras[7] and todays, medicinal plants are very valuable in the industry and scientific researches because of their antimicrobial and antioxidant activities[8]. Over the past 20 years, there has been a lot of interest in the investigation of natural materials as sources of new antibacterial agents and different extracts from medicinal plants have been tested. Many reports show the effectiveness of traditional herbs against microorganisms, as a result, plants are one of the bedrocks for modern medicine to attain new principles[9]. *Urtica dioica* which is a member of *Urticaceae* class, its Latin name is *Nettle,* has many important functions in traditional treatment because it has a lot of curable effects. There are many reports which show this plant is very effective in the treatment of blood pressure, diabetes, and Prostate Hyperplasia, Rheumatoid arthritis and Allergic rhinitis[10]. Antimicrobial activities of alcoholic and aqueous extracts of the separate parts of *Urtica* were investigated on the *Staphylococcus aureus*, *Escherichia coli*, *Bacillus subtilis*, *Pseudomonas aeruginosa* and *Candida albicans* in the Islamic Azad University Science- Research Tehran. Its summary illustrated that alcoholic extract of *Urtica* seed had the greatest influence on the gram positive bacteria; leaves extract had the maximum effect on the gram negative bacteria, its blossom oil had the highest impact on the antifungal attribute and aqueous essence had positive effect on the all bacteria except Pseudomonas[11].

Iris pseudacorus which is classified in the *Iridacea*e family, named Yellow flag because of yellow flowers, is usually seen around the paddy. Its rhizome is very useful in the cure of respiratory problems (mucolytic) and kidney diseases (diuretic)[12].

Another study presented that there were 31 chemical compounds such as *Aristolone, Bogurjunene, Cuparene, Camphor, G elemene, t cadinol, a cadinol* and *a muurolenein* in *Iris*. This research also demonstrated that *Iris* essence had antibacterial and antifungal activity. For example its antibacterial activity against *Bacillus subtilis*, *Escherichia coli*, *Staphylococcus aureus*, *Clostridium sporogenes*, *Clostridium perfringens*, *Salmonella typhi* and *Yersinia enterocolitica* was proved[13].Daniel and his colleagues did a research, 2006, about the antimicrobial activity of *Iris* rhizome essential oils in Italy. This experiment represented that there were different amount of MIC for various bacteria such as *aureus* (512mg/ml), *faecalis* (125 mg/ml), *pseudomonas* (31.25 mg/ml), *Escherichia coli* (7.8 mg/ml) and *cereus* (15.62 mg/ml)[14].

Both of these plants are well known in the traditional medicine and their history is very long. In this work, antibacterial activity of *Urtica* leaves and *Iris* rhizomes against 5 different gram-positive and gram-negative bacteria was studied and their components were analyzed.

MATERIALS AND METHODS

Plant material and extraction procedure

Urtica dioica and *Iris pseudacorus* were collected from *Lothian's* farmlands, a city located in the north of Iran, and then the leaves of *Urtica* and the rhizomes of *Iris* were separated and dried in the suitable conditions. After milling, essences were prepared by Clevenger (Hydro distillation method) for 4 hours. Extracts were placed in bottle plastic and then stored at -19°C to prevent from oxidation, polymerization and destruction. Because of high hydrophobicity, volatilization and the low amount of dissolving, DMSO, as an emulsifier, was added to prohibit of the changes in the essence activity.10 µl of each essence was placed in 2 tubes, subsequently 200µl of DMSO was added to each tube and they are shaken for good mixing. DMSO was selected for this research because it doesn't have any effects on chemical oils. For this goal, 3 dilutions were prepared (1%, 0.1%, and 0.01%) and the best result was allocated to 1%. Consequently, the concentration of each tube was 1%.

Bacteria used in this study includes: *E. coli* ATCC1533, *E. faecalis* PTCC1239, *B. cereus* PTCC1565, native strain of *P. aeruginosa*, *S.aureus* and *K. pneumonia*.5 lyophilized standard bacterial strains, including gram-positive and gram-negative bacteria. In order to determine the antimicrobial activity of disk-diffusion method was used; 120µL of each bacterial strain with turbidity 0.5 McFarland ca. 108 colony forming units (CFU) ml31), was inoculated on Mueller-Hinton agar using a sterile L-hockey stick. 10 µL of each diluted essence was placed on the blank disks and they were put in the plate which its surface was covered by paradigm.

Commercial antibiotic disks were placed in another plate to compare zone diameter made by antibiotic and essence. Next, the plates were laid in the bacteria incubated at 37°C for 24 h. Microbial growth was determined by inhibition zone diameter and for each bacterial strain, pure solvent instead of essence was used for control samples. This experiment was repeated three times and the average number was calculated and shown in Table1 and Figure1.Commercial antibiotic disks were *gentamicin* (G) and *ampicillin* (A). Antibacterial effects of this experiment were repeated for three times and results were investigated by ANOVA method and all data were in the range of 5% (meaningful range).

Determination of MIC

MIC is the lowest concentration of an essence that is needed to hinder the growth of bacteria [15] so different concentrations of essence must be provided and the maximum concentration was 30µL and other solutions were diluted to ½:30, 15, 7.5, 3.75 and 1.8 in this work. Micro-dilution broth method was used for the determination of MIC. Micro-plates with 96 wells, 8 rows with 12 wells, 250 µL volumes, were used for this goal. Essence concentration was 30 µL in the first well so 7.5 µL of essence was mixed with 222.5 µL of DMSO and 100µL of this solution was poured in the first well and then 100 µL of Trypticase Soy Broth ,TSB,(containing bacterium) was added. After several pipe ting for better mixing, 100 µL of this solution, the first well, was transferred to the second well and at this stage 100 µL of microbial broth was added and this method continued to the fifth well. The sixth well of each row was considered as the negative control and only 100µL of DMSO was added. The seventh and eighth wells were positive controls. It means that they contained 100 µL of *ampicillin* and *Gentamycin* individually. In the last step, micro-plate surface was covered by par film, incubated at 37°C for 24 hours, and it was inspected by turbidimetry. The first well which was completely transparent and had no bacterial growth was considered for MIC. The results are shown in Table2 and Figure2.

Determination of MBC

MBC is the lowest concentration of essence at which 99.99% of bacteria are killed[16]. For measurement of this figure, 10 µL of the content of without turbidity wells was cultivated on the Mueller-Hinton plate. After 24 hours (37°C), the numbers of colonies were counted and the first well which had equal or less than 3 colonies was regarded as MBC. The results are shown in Table2 and Figure2.

Gas Chromatography /Mass Spectroscopy (GC/MS)

The constituents of the volatile oil were identified by gas chromatography (model Hewlett-Packard-GC6890) which was combined with mass spectrometry(Mass 5973N).The column was HP-5MS(30 mm×0.25 mm internal diameter, 0.25 µm film thickness) and Helium (2 ml/min) was used as a carrier gas. Certain amount of essence was dissolved in diethyl ether and 1 µL of this phase was injected to the GC-MS.Samples were injected while the injection temperature was 250 °C. The temperature of capillary column was 60 °C for 3 minutes and then programmed to 250 °C at 8°C / min and held for 10 min.The identification of the compounds was performed by comparing their retention indices and mass spectra with Wiley275 library installed on the instrument and NIST Mass Spectral.The relative proportions of the essential oil constituents were expressed as percentages obtained by peak area normalization and integration, all relative response factors being taken as one. Chromatogram and the percentage of each constituent are shown in the Figure 3, 4.

The effect of pH and temperature

5 ml of each essence (100 mg/ml) was placed in the tube. Firstly, it was conserved in the refrigerator (4°C) and then maintained between 60-100 °C for 30 minutes in the Benmari. After this step, all experiments were done for antibacterial activity. The influence of pH on the methanolic extracts was inquired in a wide range.3 different pH, 2.5-5-10, were regulated by HCL and NaOH (1N) and the tubes were kept for 30 min. To conclude, pH and temperature had no effect on the antibacterial activity so pH 7 was selected for the experiment. The antimicrobial activity of essences depends on the hydrophilic and lipophilic property.

RESULTS AND DISCUSSION

The Inhibition zone diameter of selected bacteria compared with *Urtica dioica* leaves essence illustrated that this essence had the

highest activity against *K. pneumonia* and *B. cereus.* In addition, average activity was related to *S. aureus* and *P. aeruginosa*. Whereas, the maximum diameter of inhibition growth for *Iris* was only related to *B.cereus* and slightly on the *S. aureus* and *P aeruginosa.* Furthermore, negligible effects were observed for *K. pneumonia*, *E. faecalis* and *E. coli*. It can be deduced that the effects of *Urtica dioica* oils was better than *Ampicillin* except for *E. faecalis* and *E. coli*. Conversely, *Ampicillin* had more effects than *Urtica dioica* and *Iris pseudacorus* for these bacteria. It is interesting that antibacterial influence of *Gentamicin* was more desirable than *Urtica dioica* oil in all conditions (Table 1).

In this research, In Vitro effects of *Urtica dioica* and *Iris pseudacorus* essence on the selected pathogenic bacteria for the determination of the early antimicrobial activity due to disk diffusion in agar compared with control was done. Micro-broth dilution method with micro-plate, when there is no turbidity, was applied to identify MIC and culture from without turbidity wells on the agar environment was considered to determine MBC. It is obvious that the average activity of essence can be result of the reaction of its components because resultant of this reaction is positive or sometimes is negative. Definitely, different effectiveness of two essences in MIC and MBC can be result of ecological, geographical, climatic factors and the age of plant on the mixing of various population of one or combined sort. By regarding the different percentages of volatile ingredients in each essence, this type of researches can lead to recognition of the variety of theses essences(Table 2).

It is necessary to do more research to formulate these drugs in comparison with common antibiotics although MIC essences were significant in used concentration. While antimicrobial activities of medical plants have been shown in these types of articles but the main goal is to achieve more knowledge about effective matter and their applications for treatment of diseases. Sufficient scientific evidences of the pharmacologic effects of the plants on bacterial and fungal human

Table 1. Bioactivity antimicrobial activity of Urtica dioica and Iris pseudacorus essential oils

Essence and Antibiotic IZD	Ampicillin (A) Millimeter(1)	Gentamicin(G) Millimeter	Urtica leaves essence Millimeter	Iris rhizome essence Millimeter
B. cereus	14	28	19	17
S. aureus	16	23	18	14
K. pneumonia	12	24	20	10
P. aeruginosa	7	20	17	12
E. faecalis	17	19	14	9
E. coli	13	23	11	10

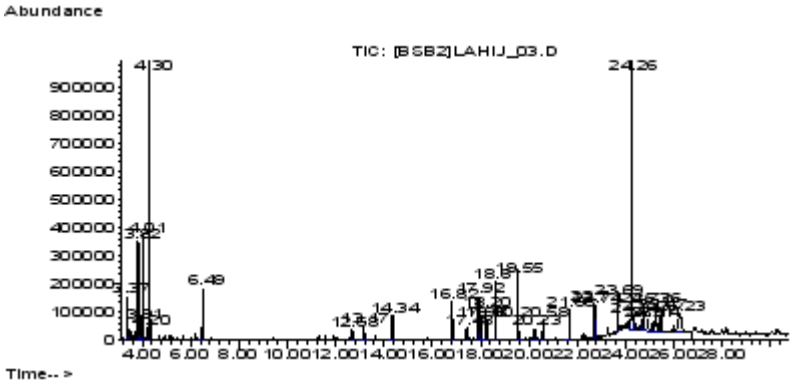
*Inhibition Zone of Diameter

Table 2. Evaluation of antimicrobial activity of *Urtica dioica* and *Iris pseudacorus* essential oils to determine the MIC and MBC

Essence and Antibiotic Microorganism IZD	Ampicillin (A) (mm)	Gentamicin (G) (mm)	Urtica leaves essence		*Iris* rhizome essence	
			MIC	MBC	MIC	MBC
B. cereus	14	28	1.8	1.8	3.75	15
S. aureus	16	23	3.75	3.75	7.5	>15
P. aeruginosa	7	20	3.75	3.75	7.5	>15
K. pneumonia	12	24	3.75	7.5	7.5	30
E. faeacalis	17	19	7.5	15	15	>30
E. coli	13	23	7.5	15	15	>30

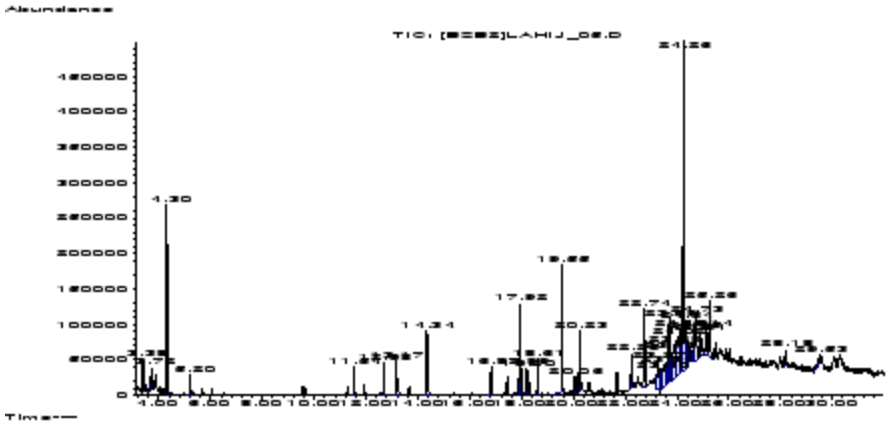
* Inhibition Zone Diameter

diseases have not been reported until this time. Furthermore synergistic influences of effective matter must be studied relative to total oils. The impact of seasonable change parameter on the quality and quantity results must be considered too. Some of them may change to other materials so their concentrations will increase or decrease. It is interesting point about GC analysis that the highest and lowest percentages were *DEHP* (41.7%), *Gamma-Dodecalactone* (1.11%) and *DEHP* (17.5 %), *Trans-Linalool oxide* (0.53%) for *Urtica* and *Iris* respectively. As a result, probably, antimicrobial activities aren't only related to these components and other elements such as weather and region can affect these results. According to obtained results in this study, these essences (1%) compared with positive control had significant inhibition growth ($p< 0.05$). To conclude, these oils can be apparently used as preservative and anti-bacterial factors at least for these studied bacteria .GC showed that there were Di-(2-ethylhexyl) Phthalate, Palmitic Acid, Gamma-Dodecalactone, Methyl Oleate and Stearic acid in both of *Urtica* and *Iris*. As mentioned above, the maximum volume was allocated to Di-(2-ethylhexyl) Phthalate for both of them. After that, the highest percentages were devoted to Eucalyptol (11.4%) and Palmitic Acid (6.75%) for *Urtica* and *Iris* individually (Figure 1 , 2). Other studies indicated that *Urtica* didn't have any effect on the *Bacillus subtilis*, *Bacillus cereus* and *Bacillus pumilus* but it affected on the *Pseudomonas aeruginosa* and *pseudomonas fluorescence*. In other words, Inhibition zone diameter was 10 mm for both of them but MIC was 20 and 25 for *aeruginosa* and *fluorescence* correspondingly[11].



No	R_t	Name of Compound	%
1	3.82	Propylene Glycol	2.25
2	4.01	Diethylene Glycol = DEG = Digol	2.21
3	4.30	1,8-Cineole = Eucalyptol	11.40
4	6.49	Ethyl Benzoate	1.96
5	14.35	Gamma-Dodecalactone = 4-octylbutane-4-olide	1.11
6	16.82	Di iso-Butyl Phthalate	2.01
7	17.94	Palmitic Acid	4.30
8	18.20	Dibenzosuberone	1.47
9	18.30	Ethyl Palmitate	1.39
10	18.61	4-methyl-2,6-di-t-butyl Phenol = BHT	1.66
11	19.56	Methyl Oleate	2.04
12	20.22	Stearic acid	1.31
13	20.59	Ethyl Stearate	2.19
14	21.70	Trico sane	1.29
15	24.25	Di-(2-ethylhexyl) Phthalate = DEHP = DOP	41.07

Fig. 1. Chromatogram and Component of *Urtica leaves*



No	R_t	Name of Compound	%
1	4.30	1,8-Cineole = Eucalyptol	4.13
2	5.20	Guaiacol = Guaipol = 2-methoxyphenol	1.36
3	9.66	Capric Acid = Decanoic Acid	1.70
4	12.68	Dicyclohexyl Methanone = dicyclohexyl Ketone	1.87
5	13.17	Diethyl Phthalate	2.13
6	14.35	Gamma-Dodecalactone = 4-octylbutane-4-olide	3.76
7	17.48	Methyl Palmitate	1.49
8	17.94	Palmitic Acid	6.75
9	19.56	Methyl Oleate	6.07
10	20.24	Stearic Acid	6.30
11	22.74	Dioctyl Adipate = DOA	2.83
12	24.25	Di-(2-ethylhexyl) Phthalate = DEHP = DOP	17.52
13	25.25	Triphenyl Phosphinsulphid (Ph3P=S)	1.79

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