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Examining the antibacterial activity of *Artemisia dracunculus* L. extracts using different methods of extraction

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ABSTRACT

The status of medicinal plants in Iranian traditional medicine and rich existence of herbal sources and current problems in curing infections, on the other hand led the scientists to study medicinal plants more accurately. Tarragon (Artemisia dracunculus L.) is a herbaceous plant from Asteraceae family, available in planted and farming forms in Iran. This study aimed to examine the antibacterial activity of Artemisia dracunculus L. in various extraction methods. The Maceration and Soxhlet methods were used to Methanolic extract of Artemisia dracunculus L. and the antibacterial effect of extracts was conducted in various concentrations on *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli* and *Pseudomonas aeruginosa* microorganisms by Disk diffusion, Well Diffusion, Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) methods. The results indicated that both extracts obtained by Maceration and Soxhlet methods had antibacterial effects only on two gram-positive bacteria including Staphylococcus aureus and Bacillus subtilis. Both gram-negative bacteria, Escherichia coli and Pseudomonas aeruginosa, were resistant to the extracts in all concentrations. In the current study, the antibacterial properties of two methanolic extract which obtaiened by maceration and suxhelet methods were examined. According to results, the extract gained by maceration method showed higher antibacterial properties in copmparision with suxhelet method. The maceration obtained extract showed antibacterial properties on gram-positives bacteria in all applied concentration while in suxhelet extract that was only in 200 mg/ml. Gram-negative bacteria had no sensetivity to both extracts in all applied concentrations. The higher sensitivity in gram-positive bacteria to the extracts may result from the fact that gram-negative bacteria contain an outer membrane and a periplasmic space that are not seen in gram-positive bacteria. The outer membrane of gram-negative bacteria is known as a barrier against various antibiotic molecules penetration. On the other hand, this membrane prevents hydrophilic penetration into the bacteria. The periplasmic space contains many enzymes enable it to dissect outer molecules that come from the outer space.

1. Introduction

The spices and essential oils of plants are known as healthy and less-risky materials and have been used in curing of various diseases for centuries (Shan et al., 2007). Being poisonous, negative side effects of synthetic contents, increasing microbial resistance of pathogenic microbes against antibiotics and increasing customers demand for more natural foods

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resulted in fewer trends to use synthetic substances by people and the new areas to investigate their replacements by natural materials will be provided. While natural substances isolated from plants are considered as hopeful resources to replace synthetic materials, aromatic plants contain more effective compounds (Singh et al., 2004). The classic methods for extraction relied on putting plants in suitable solutions; heating and stirring are applied to increase the process. Among the classic methods are Soxhlet, distillation, soaking (Maceration) and Percolation. Soxhlet method is a standard that applied as the main evaluating reference for other methods. This method is used for extracting low or moderately volatile compounds that are resistant to heating (Luque de Castro and Garcia-Ayuso, 1998). The solute selection is significantly important in this method as choosing various solutions results in producing different extracts and various contents (Zarnowski and Suzuki, 2004). The genus Artemisia L. belongs to Asteraceae family contains 35 annual and perennial species spreading in Iran. Estragon (Artemisia dracunculus L.) is planted in farms of Iran (Mozaffaryan, 1998). Artemisia dracunculus L. is an herbaceous, aromatic and perennial plant with direct, branched and rhizomatous stems. The plant height varies from 80 to 150 cm due to climatic conditions. Thin, long and linear leaves are 3-6 cm. The leaf margins are flat, toothless and not downy. The leaf blade is light green and shiny. The flowers are yellow or dark brown and its fruit is Achene (Bown, 1995). This plant is originated from central and south Russia, Siberia, central and north Asia and west America. Today, this plant is cultivated in most parts of the world. Artemisia dracunculus L. vegetate in humid areas and along the rivers (Omidbeygi, 1997; Zaman, 1997; Zargari, 1996). Artemisia dracunculus L. prefers warm and dry climate and complete sunshine. Extremely cold winters and especially heavy soil may be injured the plat root (Yazdani, 2004). In this study, the effects of two extracting method on antibacterial activity of the extract in Artemisia dracunculus L. by two different methods were studied.

2. Materials and Methods

2.1. Providing the plant and extracting the essence

Artemisia dracunculus L. was collected from the Ahmadi,s market garden of Tonekabon, Iran in May 2014. Then the samples were identified and shade-dried with dry air and were kept in an appropriate place for the examinations. The Maceration and Soxhlet methods were used for extracting.

2.2. Extracting by Maceration method

In order to extraction 30g dried samples was weighted and powdered by electrical grinder. The powder was transferred to a beaker and 300 mL of methanol solution was added. The samples were set on a shaker in 45°C for 48 hours. Then the solution were purified by filter paper and transferred to dissecting tray to evaporation solvent.

2.3. Extracting by Soxhlet method

Plant powder in an amount of 30g was weighted carefully, placed in filter paper and put in Soxhlet set and 300ml methanol (98%) was poured in Round-bottom flask and Soxhlet was conducted for 3 hours. Then, the flask containing the solution was transferred to dissecting tray to evaporation of solvent. The extracts were transferred to 4°C refrigerator to further application.

2.4. Examining the antibacterial activity of extracts

Disk diffusion, well Diffusion, Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) tests were used to investigate the antibacterial activity of the extracts. Two gram–positive bacteria including *Staphylococcus aureus* (PTCC=1113) and *Bacillus subtilis* (PTCC= 1156), and two gram-negative bacteria, *Escherichia coli* (PTCC= 1399) and *Pseudomonas aeruginosa* (PTCC= 1599) were provided from the center of industrial bacteria and fungi.

2.5. Providing microbial suspension

Each microorganism was transferred to Mueller-Hinton agar culture was incubated in oven 37°C for 24 hours. Then, 5-10 distinct colonies from the purified culture were solved in 3-4 ml sterile distilled water to obtain 0.5

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McFarland. The number of microorganisms was about 1.5×10^8 CFU/ml in above conditions.

2.6. Disk diffusion method

In Disk diffusion method, the blank sterile disks with 6mm diameter were put in sterile glass plates; and 50µL of the extracts (25-50-100-200 mg/mL) was poured on the sampler. A suspension equivalent to 0.5 McFarland was provided by prepared microorganism after 24 hour of cultivation; the steady culture was administered for the bacteria through using swap on Mueller-Hinton agar culture level. The disks containing the extracts were put on agar level; and the plates were incubated in 37°C for 24 hours. The sensitivity or resistance of the microorganisms was determined by measuring the diameter of non growth halo around each disk.

2.7. *Well Diffusion method*

In this method, 10 µl of microbial suspension was added to the culture medium and the method was conducted after culturing on the plate. Then, 100 µL of diluted extract by DMSO (25-50-100-200 mg/mL), with different amounts, was poured in each well; and after incubation the microorganisms growth was reported by millimeter. All measurements were done in three repetitions.

2.8. Minimum Inhibitory Concentration (MIC)

Eleven tubes containing 1mL Mueller-Hinton agar culture were used to determine Minimum Inhibitory Concentration (MIC). The extract was poured in the first tube (in 1 to 5 ratio with watery DMSO) and mixed. Then, 1ml was taken from the mixture of first tube, containing 2ml extract and Mueller-Hinton agar culture, and added to second tube; after that 1ml was taken from this tube and added to the next one. It was repeated to the tenth tube. In order to have similar mass in the tubes, 1ml was taken from the tenth tube and poured away (the resulted concentrations from the first tube to the tenth are as follows , from left to right: 105-5×102-25×103-125×102-62.5×10^2-31.25×10^2-15.625×10^2-781.25-390.625-195.3125). The eleventh tube doesn't contain any extract and is considered as negative control. Then, 10µL microbial suspension which is equal to Mc .Farland 0.5, was added to the 1st – 11th tubes and were placed in an incubator for 24 hours and in 37°C. The results were reported by observing darkness or no darkness.

2.9. Minimum Bactericidal Concentration (MBC)

This method was done after MIC method; while the tubes were cultured in darkness on Mueller-Hinton agar culture and incubated in 37°C to determine Minimum Bactericidal Concentration (MBC). The colonies were counted by serial dilution. The first tube with decreased bacterial numbers, more than one milliliter, was chosen as Minimum Bactericidal Concentration.

2.10. Statistical analysis

ANOVA and T-test were used for statistical analysis and comparing the means in independent communities. Describing the data and drawing the diagrams was done by Excel software.

3. Results

In this study to investigate the antibacterial activity of two extracts by Maceration and Soxhlet methods. By the Disk diffusion and Well Diffusion, Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) methods were applied. The results of data variance indicated that the effect of extracting method, density and their interaction on the diameter of non growth halo around in disk diffusion method was significant in 1% level. Comparing the means indicated that ethanol extract in Maceration method contains more antibacterial activity than the extracts in Soxhlet method. According to Table 1, both extracts contain inhibitory effects on gram-positive bacteria; but the gram negative bacteria are not sensitive to extracts with different concentration. In Maceration method, all concentration indicated antibacterial activity on gram-positive bacteria. While in Soxhlet

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method, the antibacterial activity was observed only in 200 mg/mL density. The diameter of halo was enhanced by increasing the extracts density.

Investigating the results of variance analysis in well diffusion test indicated that there is a significant difference between extracting method and density on antibacterial properties of extracts significantly (P≤0.01). In this test, the extracted methanolextract by Maceration method indicated antibacterial activity in 200 mg/mL density on *Staphylococcus aureus* and *Bacillus subtilis*. The extracted essence by Maceration method contains lethal effect only on *Staphylococcus aureus* in all concertration (25-50-100-200 mg/mL), while it had antibacterial effects on Bacillus subtilis only in 200 mg/mL (Table 2).

The results of MIC and MBC tests indicated that both extracts obtained by Maceration and Soxhlet methods had antibacterial effects on gram- positive bacteria, *Staphylococcus aureus* and *Bacillus subtilis*. The results of MIC and MBC are given in Table 3 (concentrations are reported in µg/mL). The results of this study indicated that both extracts had no effects on gram negative bacteria.

4. Discussion

Disk diffusion, Well Diffusion methods MIC and MBC were used for studying the antibacterial activity of Artemisia dracunculus L. extract gained by Maceration and Soxhlet methods. The results of variance analysis indicated that there is statistically significant difference between extracting method and density on diameter of non growth haloat 1% discrimination among the bacteria, *Staphylococcus aureus* was the most sensitive sample to both extracts in comparison with two gram-negative bacteria, *Escherichia coli* and *Pseudomonas aeruginosa*. The higher sensitivity in gram-positive bacteria to the extracts may result from the fact that these bacteria contain one-layer cell wall, while that is multi layer in gram-negative bacteria. In the other words, gram-negative bacteria contain an outer membrane and a periplasmic space that are not seen in gram-positive bacteria. Thr outer membrane of gram-negative bacteria is known as a barrier against various antibiotic molecules penetration. On the other hand, this membrane prevents hydrophilic penetration into the bacteria. The periplasmic space contains many enzymes enable it to dissect outer molecules that come from the outer space (Elgayyar, 2001). Nakhaee Moghadam et al. investigated the aqueous and methanolic extract of *Cuminum cyminum* L and *Artemisia dracunculus* on *Helicobacter pylori* in laboratory condition. Due to the results of methanolic extract in both *Cuminum cyminum* L. and *Artemisia dracunculus* L. methanolic had more anti-Helicobacter *pylori* activity than aqueous extract (P< 0.001). Minimum Inhibitory *Concentration* (MIC) of *Cuminum cyminum* L. and *Artemisia dracunculus* L. methanol extracts was 691 µg/mL. Minimum Bactericidal Concentration (MBC) of methanol extract in *Artemisia dracunculus* L. is higher than its MIC and *Cuminum cyminum* L. methaniol extract MBC is equal to its MIC. The methanol extracts of both plants kept their antibacterial activity after heating by autoclave for 20 minutes. Some parts of *Artemisia dracunculus* L. antibacterial effects may be related to saponine and tannin compounds. According to Zargari (2007) and Mirheidari (2003) the various compounds which can be found in this plant including, Artemidin, acids like Butyric acid, Hydroxybenzoic acid, Rosmarinic acid, Salicylic acid, Artemisia ketone, Camphene, Carbohydrate and Monosaccharide like Fructose, Phenol phytoesters and peroxidase. Tannins are phenolic polymer that can be connect to prpteins, their antimicrobial activity may be due to inactivation of enzymes covering transporter proteins or other ones (Cowan, 1999).

Conclusion

In the current study, the antibacterial properties of two methanolic extract which obtaiened by maceration and suxhelet methods were examined. According to results, the extract gained by maceration method showed higher antibacterial properties in copmparision with suxhelet method. The maceration obtained extract showed antibacterial properties on gram-positives bacteria in all applied concentration while in suxhelet extract that was only in 200 mg/ml. Gram-negative bacteria had no sensetivity to both extracts in all applied concentrations. The higher sensitivity in gram-positive bacteria to the extracts may result from

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the fact that gram-negative bacteria contain an outer membrane and a periplasmic space that are not seen in gram-positive bacteria. The outer membrane of gram-negative bacteria is known as a barrier against various antibiotic molecules penetration. On the other hand, this membrane prevents hydrophilic penetration into the bacteria. The periplasmic space contains many enzymes enable it to dissect outer molecules that come from the outer space.

Table 1. comparing the means of Maceration and Soxhlet extracts on diameter of non-growth halo (mm) in disk diffusion method

microorganisms	Extraction method (Disc)							
	Maceration method				Soxhlet method			
	25	50	100	200	25	50	100	200
Staphylococcus aureus	11.5 ± 0.5	12	13.6 ± 0.5	14.6 ± 0.5	-	-	-	9.3 ± 0.3
Bacillus subtilis	11	11.8 ± 0.1	12.5 ± 0.2	11 ± 1	-	-	-	9
Escherichia coli	-	-	-	-	-	-	-	-
Pseudomonas aeruginosa	-	-	-	-	-	-	-	-

Standard deviation ± mean

Table 2. Comparing the means of Maceration and Soxhlet extracts on the diameter of non-growth halo (mm) in well diffusion method

microorganisms	Extraction method (hole)							
	Maceration method				Soxhlet method			
	25	50	100	200	25	50	100	200
Staphylococcus aureus	-	-	-	12 ± 0.5	7.3 ± 0.3	10.3 ± 0.3	10.5 ± 0.5	11.3 ± 0.3
Bacillus subtilis	-	-	-	7.6 ± 0.3	-	-	-	-
Escherichia coli	-	-	-	-	-	-	-	-
Pseudomonas aeruginosa	-	-	-	-	-	-	-	-

Standard deviation ± mean

Table 3: Results of MIC and MBC tests from extracts by Maceration and Soxhlet methods

microorganisms	**Extraction method**			
	Maceration method		Soxhlet method	
	MIC	MBC	MIC	MBC
Staphylococcus aureus	62.5×10^2	125×10^2	125×10^2	25×10^3
Bacillus subtilis	125×10^2	25×10^3	125×10^2	25×10^3
Escherichia coli	-	-	-	-
Pseudomonas aeruginosa	-	-	-	-

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