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Evaluation of Antibacterial Activity of Alcoholic Extract of Rosemary Leaves against Pathogenic Strains

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Abstract

***Background*:** Today, the increased use of antibiotics lead to the incidence of resistant strains. We are faced with lack of new antimicrobial drugs that have fewer side effects than antibiotics. Rosemary is a medicinal plant that has many uses in traditional medicine. In this study, ethanol leaves extract of this plant is tested on various pathogens.

***Materials and Methods*:** In this experimental study, *Rosmarinus officinalis* was used to evaluate the antimicrobial effects on pathogens. Ethanol extract of the leaves of this plant, with concentrations of 400, 200, 100 and 50 mg/l were prepared, and antibacterial activities were evaluated by well diffusion method on strains of *Escherichia coli*, *Pseudomonas aeruginosa*, *Bacillus cereus* and *Staphylococcus aureus*. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) was determined by the microplate method.

***Results*:** In this study, ethanol extract of rosemary leaves at concentrations of 400 mg/ml was active against *Pseudomonas aeruginosa*, *Bacillus cereus*, *Staphylococcus aureus* and *Escherichia coli*. Minimum inhibitory concentration of the extract on the growth of these bacteria from 6.25 mg/ml to 100 mg/ml was change. Also MBC of extract showed range from 12.5 to 200 mg/ml respectively.

***Conclusion*:** Result from these finding suggest that, rosemary extract, on all strains, including *Staphylococcus aureus*, *Escherichia coli*, *Bacillus cereus* and *Pseudomonas aeruginosa*, has an inhibitory effect. Also, the effect of extract, risesed by increasing the concentration.

Introduction

Along with using antibiotics and antimicrobial drugs, now we are seeing the increasing prevalence of antibiotic-resistant strains, especially in infections. The outbreak of these resistant pathogens in patients has made the treatment course more difficult and longer. Although the production of antibiotics increases day by day, but resistance in bacteria has created a major problem worldwide. *Pseudomonas aeruginosa* and *Escherichia coli* are Gram-negative and opportunistic pathogens of Enterobacteriaceae. *Bacillus cereus* and *Staphylococcus aureus* are also of important Gram-positive pathogens in hospitals [1]. Traditionally, plant compounds are used for treatment of hospital infections in advanced countries. Appropriate method in obviating the common problems of antibiotics side effects is using plant drugs with antimicrobial properties.

Plant extracts and essential oil have been studied as important natural antimicrobial agents for years. Rosemary is herbal plant with green, picked and fragrant leaves. Rosemary essential oil is an antimicrobial source and chemical mixture whose antioxidant properties and antimicrobial effects have been proved in various researches. For example, phenolic compound is found in abundant [2, 3]. Essential oil is one of the main compounds of this herb which exists in 1-2%.

It consists of borneole, limonene, camphene, camphor and other compounds like phenolic acid including rosmarinic acid, caffeic acid and chlorogenic acid [3]. Antimicrobial effects of rosemary essential oil and extract on various microorganisms have been studied. Jarrar et al. examined the effect of ethanol extract of rosemary alone and with cefuroxime on methicillin resistant staphylococcus.

They reported that rosemary essential oil with cefuroxime had better synergistic effects on resistant bacteria [4]. In research about *Streptococcus sobrinus*, Tsai et al. stated that methanol extract of rosemary prevented the growth of oral streptococcus well [5].

Vesal-Talab et al. also showed that rosemary essential oil participated with different concentration in antifungal activities. It was found that essential oil had higher antifungal activities [6]. Thus, according to the various antimicrobial potentials, in other studies should be done to determine the range of antimicrobial effects of this medical drug.

In this research, the antimicrobial effect of ethanol extract of rosemary leaves on main hospital pathogens was evaluated through well plate and simultaneously with the method of dilution in micro plate.

Materials and Methods

This experimental study was conducted at Microbiology Research Laboratory, Department of Microbiology; Islamic Azad University of Falavarjan in 2012. The bacterial strains were provided by Microbiological Collection of Tehran Research Center. Herbal samples were collected and identified in cooperation with Herbarium and Herbal Research Center of Isfahan. In each experiment, three iterations were employed to do statistical calculations.

Provision of Bacterial Strains: *Bacillus cereus* (ATCC: 1247), *Staphylococcus aureus* (ATCC: 25923), *Escherichia coli* (ATCC: 25922) and *Pseudomonas aeruginosa* (ATCC: 1430) that were lyophilized, prepared from Microbial Collection of Tehran Research Center and then revived by standard methods.

Provision of plant samples: The fresh leaves of *Rosmarinus officinalis* were collected and dried in a place not exposed to sun light. Then the dried leaves were crushed to powder. Fifty gram of leaves were weighted and poured into a sterilized erlenmeyer flask. In the next step, 250 milliliter ethanol (98%) was added so that the herb's compounds were dissolved. Erlenmeyer flask containing alcohol and herb powder was placed into shaker for 48 hours until the solvent may exert their effect at the temperature of 40ºC. Next, rotary was employed to eliminate the solvent. Finally, the Rosemary extracts were preserved in sterilized dishes at refrigerator and to prevent from the light effects, they were wrapped with aluminum covers. Extracts with concentrations of 50, 100, 200 and 400 were provided and solved in 5% dimethylsulfoxide (DMSO). Then they were used in well diffusion and MIC tests. To prepare a microbial suspension, several colonies from 24 h newly cultured were transferred into Mueller Hinton Broth medium. The turbidity was 0.5 McFarland standards (1.5×10^8 bacteria per milliliter). This suspension was then 0.01 diluted to achieve the turbidity of 1.5×10^6, because the turbidity of suspension has considerable influence on accuracy of results. In this research, the antibacterial effect of ethanol extracts from Rosemary was examined through well plate and micro plate methods. In well plate method, suspension with turbidity of 1.5×10^6 was steadily cultured in the surface of Mueller Hinton Agar plate. Then some 6 mm diameter wells were made in appropriate intervals and 100 microliters extract was poured into each. DMSO was used as negative control and antibiotic chloramphenicol as positive control. Next, the cultured media were heated in 37ºC incubator for 24 hours. Ultimately, plates were evaluated in terms of inhibition zone. Inhibition zone diameter was measured by millimeter [7, 8].

Determination of MIC: The dilution in microplate method was used to determine Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC) of different leaves concentration extracts of rosmarinus officinallis. The strains of mentioned pathogenic bacteria were put under a 24 h culture process in Mueller Hinton Broth medium at the temperature of 37ºC. Dilution serials of 6.25, 25.2, 50, 100, 200 and 400 mg/ml were provided from the extract and 70 µl of them were added into 96 cells micro plates containing 70 µl bacteria suspension with turbidity of half McFarland. Then the similar tests were done for positive control (medium with bacteria and without extract) and negative control (medium without bacteria). Afterwards, micro plates were heated in a 37ºC incubator. The minimum dilution with no seen turbidity was reported as MIC. All tests were repeated three times and the average was presented [9-12].

Determination of MBC: Considering MIC results, the Minimum Bactericidal Concentration was also specified. From all cells in which the growth of bacteria was stopped, plates containing Mueller Hinton Agar were cultured and heated at 37ºC for 24 hours. Concentrations without bacteria growth were reported as MBC. All the tests were repeated three times and the results were averaged. Data were analyzed by statistical SPSS-14 Software. To study the significant difference, variance analysis and χ^2 tests were used and their difference was calculated in a significant level ($p\leq0.001$) [13, 14].

Results

The results of antibacterial activity of *Rosmarinus officinalis* leaves extracts were shown in table 1. All extracts showed dose dependent activity which increases with increase in concentration (Table 1). Antibiotic chloramphenicol was positive control and DMSO was the negative control. As the table shows, ethanol extract has prevented from the growth of bacteria such as *Pseudomonas aeruginosa*, *Escherichia coli*, *Bacillus cereus* and *Staphylococcus aureus*. Also, by increasing the concentration of methanol extract, the inhibition zone increased ($p\leq0.001$).

Additionally, the results revealed that contrary to the similar studies, rosemary extract had effect on Pseudomonas aeruginosa. Moreover, the concentration of 400 mg/ml has more effect on *Pseudomonas aeruginosa* and *Staphylococcus aureus*. The values of MIC and MBC of ethanol extract of Rosemary leaves has been presented in table 2 against the referred bacteria.

Table 1. Antibacterial activity of Rosmarinus officinalis leaves extracts against tested bacteria measured in millimeter

Bacteria	50	100	200	400	Negative	Positive
S. aureus	18	18	20	22	-	23
Bacillus cereus	-	-	13	13	-	14
Escherichia coli	-	-	13	13	-	14
P. aeruginosa	12	16	16	19	-	21

Table 2. MIC and MBC of leaves extracts against tested bacteria (mg/ml)

Bacteria	MIC	MBC
Staphylococcus aureus	6.25	12.5
Bacillus cereus	50	100
Escherichia coli	100	200
Pseudomonas aeruginosa	100	200

The results determined that in tested bacteria, there was a significant difference ($p \leq 0.001$) in terms of sensitivity to ethanol extract. In other words, the most sensitivity was observed in Staphylococcus aureus and the least was seen in Bacillus cereus and Escherichia coli. So, as it shown in this table, MIC is between 6.25 to 100 mg/ml and MBC is between 12.5 to 200 mg/ml ($p \leq 0.001$).

Discussion

The results indicated that ethanol extract of Rosemary with concentration about 200 mg/ml has prevented from the growth of Gram-negative bacteria and with concentration between 20 to 100 mg/ml from Gram-positive bacteria. Thus, the research represents the antibacterial effects of this medical herb on Gram-negative and Gram-positive pathogenic bacteria. Growth inhibitory effect of the extract starts from the concentration about 20 mg/ml and by gradual increase of concentration, inhibition zone increases as well. Inhibition zone rosemary ethanolic extract on *Staphylococcus aureus* was between 18 to 22 mm and obtained MIC was about 6.25 mg/ml. It was observed in *Pseudomonas aeruginosa* between 12 to 19 mm. concerning the method of extraction and preventing from using high temperature to decrease the rate of destruction of effective herbal compound, there is a partial difference between these results and the similar studies.

Jarrar et al. examined the effect of ethanol extract alone and with cefuroxime on staphylococcus resistant to methicillin. The achieved MIC was about 0.3 to 3.1 mg/ml which had a significant difference with current studies. The difference may be due to using antibiotic with this herbal extract. The research also determined that using rosemary extract along with cefuroxime would have synergic effects on all resistant bacteria. In a research, Tsai et al. looked at effects of ethanol extract of rosemary and calculated MIC at 1.42 mg/ml. In this research, the extract, prevents well from the growth of oral streptococcus. Having studied the effects of rosemary and clove buds essential oil and extract on Botrytis cinerea, Vesal-Talab et al. reported that in general, ethanol extract of both had no antifungal effect. By fumigation method, clove buds essential oil with concentration of about 600 to 650 ppm had antifungal activity. Rosemary essential oil had such activities in concentration of about 600 to 650 ppm. The results showed that antifungal activities were based on concentration, product application method and the type of plant. Also, the essential oil had higher effect than extract [4-6].

In another studies, Aziza Kamal showed the effects of rosemary essential oil on Gram-positive *Staphylococcus aureus* and *Bacillus cereus* and reported that the effect is higher on Gram-positive bacteria than on *Pseudomonas aeruginosa* and *Escherichia coli*. He stated that according GC-MS, isocarnosol is the biggest compound in rosemary [15-17]. In study of Rozman et al., the effect of rosemary extract on different types of listeria was studied and MIC was specified between 625 to 5000 mg/ml. This research found out that the resistance of listeria depends on rosemary extract, chosen extract; various types of listeria and various concentration of extract [17]. In a research over antimicrobial effects of rosemary essential oil, Fu et al. indicated that the extent of inhibition zone in *Staphylococcus aureus* was 18 mm and MIC was 0.125, which shows the resemblance of these results with current studies [18]. By methods of disk diffusion and dilution in tube, Soltan-Dallal et al. [19], evaluated the antimicrobial effects of rosemary essential oil on *Staphylococcus aureus* resistant to methicillin. The diameter inhibition zone was observed about 20 mm. MIC was 1.40 mg/ml and MBC was 2.8. By comparing these results, we can say that the effect of essential oil is higher than extract and these results are more consistent with Soltan-Dalal's results. Today, one of the main treatment problems is development of antibiotic resistance, which has attracted special attention. Since antibacterial effects of rosemary essential oil and extract on various types of bacteria have been proved, they are recommended in treatment of infection-induced pathogen bacteria. It is suggested that further research on medicinal plants, would be the promising alternatives for chemical medicines which have unwanted side effects despite of effectiveness.

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Authors' Contributions

All authors had equal role in design, work, statistical analysis and manuscript writing.

Conflict of Interest

The authors declare no conflict of interest.

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