*Journal of Medicinal and chemical Sciences*

*Journal homepage: www.jmchemsci.com*

Original Research Article

# Antimicrobial effects of *Rosmarinus officinalis* methanolic extract on *Staphylococcus aureus, Bacillus cereus, Escherichia coli and Pseudomonas aeruginosa* in laboratory conditions

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**ARTICLE INFORMATION**

Received: 01 June 2019
Received in revised: 22 June 2019
Accepted: 10 July 2019
Available online: 01 April 2020

DOI: 10.26655/jmchemsci.2020.2.2

**KEYWORDS**

Antimicrobial effects
Extract
Rosmarinus officinalis

**ABSTRACT**

Treatment of bacterial infections with chemicals has led to drug resistance. Therefore, research to replace herbal treatments with less side effects is of a great importance. Therefore, this study aims at investigating the antimicrobial effects of methanolic extract of *Rosmarinus officinalis* on some gram positive and gram negative bacteria. In this research study, after collecting the plant and confirming its scientific name, *R. officinalis* extract was prepared using Soxhlet extractor method at the concentrations of 20-400 mg/mL and the antimicrobial effects of the extract using agar well diffusion and determination of the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) from dilution method against standard bacteria of *Staphylococcus aureus, Bacillus cereus, Escherichia coli* and *Pseudomonas aeruginosa.* Methanolic extract of *R. officinalis* plant has an inhibitory effect on *P. aeruginosa, E. coli* and *S. aureus* so that it has the highest sensitivity to methanolic extracts of *R. officinalis* in *P. aeruginosa* with a 19.8 mm the zone of growth inhibition and the lowest sensitivity to *S. aureus* with the zone of growth inhibition 14.4 mm. The results of this study showed that *R. officinalis* extract has a significant effect on tested bacteria, and further research is required to identify, quantify, and purify its effective compounds.



**Journal of Medicinal and Chemical Sciences:** http://www.jmchemsci.com/

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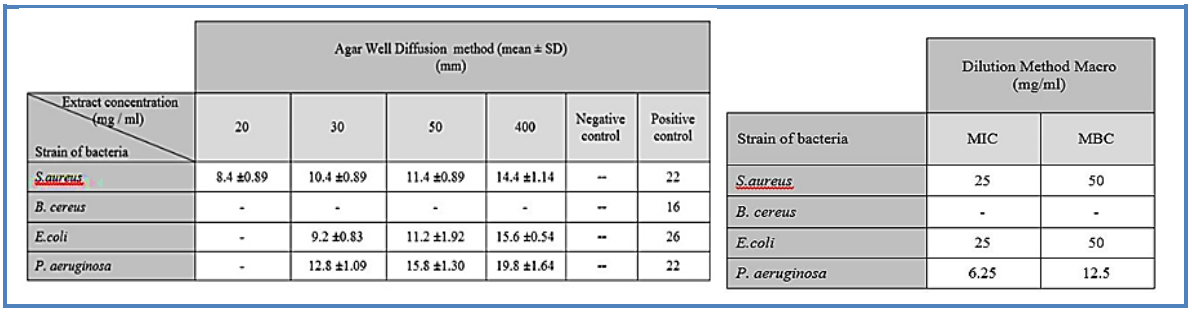
## Graphical Abstract



| Extract concentration (mg / ml) / Strain of bacteria | Agar Well Diffusion method (mean ± SD) (mm) | | | | | |
|---|---|---|---|---|---|---|
| | 20 | 30 | 50 | 400 | Negative control | Positive control |
| *S.aureus* | 8.4 ±0.89 | 10.4 ±0.89 | 11.4 ±0.89 | 14.4 ±1.14 | -- | 22 |
| *B. cereus* | - | - | - | - | -- | 16 |
| *E.coli* | - | 9.2 ±0.83 | 11.2 ±1.92 | 15.6 ±0.54 | -- | 26 |
| *P. aeruginosa* | - | 12.8 ±1.09 | 15.8 ±1.30 | 19.8 ±1.64 | -- | 22 |

| Strain of bacteria | Dilution Method Macro (mg/ml) | |
|---|---|---|
| | MIC | MBC |
| *S.aureus* | 25 | 50 |
| *B. cereus* | - | - |
| *E.coli* | 25 | 50 |
| *P. aeruginosa* | 6.25 | 12.5 |

## Introduction

One of the most important achievements for treatment of most diseases is the use of medicinal herbs in such a way that in the development of all civilizations there has always been a close connection between man and plant [1-5]. Although most plant species are known to date, there is still a long way to discover new and valuable herbal resources [6, 7]. Medicinal plants are one of the important sources of antimicrobial agents in different countries. About 60-90% of the population uses plant drugs in developing countries [8, 9]**.** So, plants can be considered as the source of potential chemical substances, only part of which has been exploited. These potentially useful chemicals can be used not only as a drug but also as an unrivaled model as the starting point for making pharmaceutical analogues, and as an interesting tool for understanding better biological phenomena [10-13]. *Rosmarinus officinalis* belongs to the Lamiacea family and is widely known as a medicinal herb among many countries. This plant has anti-bacterial effects, antifungal, anti-oxidant and native to the Mediterranean and Asia [14-17]. Antimicrobial properties of the *R. officinalis* derived from phenolic compounds: carnosol, rosmarinic acid, caffeic acid, flavonoids including diosmin, luteolin, and mono terpenes, such as camphor, cineol and borneol [18]. *R. officinalis* is resistant to dehydrated stress and can continue to grow under drought conditions [19]. Various studies have reported the effects of *R. officinalis* plants on food preservation from oxidation and microbial contamination[20-22]. In traditional medicine, this plant is used for anti-asthma effects, food digestion, sedative, headache, circulatory disorders, that increased visual acuity, anti-rheumatism and memory stimulus [23-24]. Campo *et al.* [14] showed that the minimum inhibitory concentration of the methanolic extract of *R. officinalis* for different bacteria was different and started from 0.06% for *B. cereus* and reaches 0.1% for *L. mesenteroides*. Therefore, the aim of this study was to evaluate the antimicrobial properties of methanolic extract of *R. officinalis* plants on some of the gram-positive and gram-negative bacteria**.**

## Methods and Materials

In this work, the plant samples were collected from the natural arenas of the greenhouses of Marand city. The specimens were dried in a large, well-groomed space, and prepared for grinding. Extraction was performed using the Soxhlet extractor method[25]. So that 60 gr dried powdered leaves with 300 mL of methanolic as a solvent for 8 h were placed in a Soxhlet Extractor. This solvent was evaporated slowly at 40 °C using a rotary evaporator and concentrated extract was obtained**.** The extracts were concentrated with

5% DMSO solvent, concentrations of 20-400 mg/mL were prepared for use in minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), and agar well diffusion. The microorganisms used in this study were *Staphylococcus aureus* ATCC 25923, *Bacillus cereus* ATCC 1052, *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 (the microbial collections of the University of Tehran). To achieve a concentration of $1.5 \times 10^6$ CFU / mL, a microbial suspension with a McFarland turbidity Standard 0.5 was diluted to 0.01. In order to investigate the antimicrobial activity of methanolic extract of 4 concentrations of 20, 30, 50, and 400 mg/mL of methanolic extract of the plant in DMSO 5% solvent was prepared. In this study, the antimicrobial activity of the methanolic extract was investigated using agar well diffusion and dilution test. In agar well diffusion method, 500 mL of microbial suspensions of $1.5 \times 10^6$ CFU/mL was transferred onto agar medium and cultured in a 3-way sterile swab. Then, pits of diameter 6 mm and 2.5 cm apart were created at the agar surface. Subsequently, 100 µL of concentrations of 20, 30, 50 and 400 mg/mL were injected from methanolic extract into each well. The negative control was obtained using a solution that was used to dissolve the extracts (5% DMSO) and also used as a positive control for chloramphenicol antibiotics. Then the plates were incubated for 24 h at 37 °C and after a certain time (24 h), in terms of forming or not forming a non-growth zone in millimeters, was measured. The minimum inhibitory concentration and minimum bactericidal concentration of methanolic extract were determined using the dilution method. In this method, to determine the MIC, methanolic extracts from dilutions of 25.6, 12.5, 25, 50, 100, and 200 mg/mL were obtained in Mueller Hinton Broth medium. Then, to each dilution, 1 mL of active bacterial suspension was added. Besides the tubes, positive control (The culture medium containing bacteria, without extracts) and negative control (non-bacterial culture) were used. Finally, the tubes were incubated for 24 h at 37 °C. After incubation, the tubes were examined for turbidity induced by the inoculated bacterial growth and the last dilution in which no turbidity was observed (no growth) as MIC was considered. Subsequently, all tubes in which no bacterial growth was observed were sampled and determined by cultivating the minimum concentration of MBC in the plate. To reduce the error of the test, each of the above experiments was repeated five times. SPSS software version 18 was used to analyze the data. To study the significant difference was found between the results of ANOVA and chi-square and the difference between the groups was significant at the significance level of $p < 0.05$.

## Results and Discussion

The results of this study indicated that the methanolic extract of *R. officinalis* plant has antimicrobial activity against the standard bacteria. Also, the concentration of extract increased significantly ($P < 0.05$), which indicated that the anti-bacterial effect of the extract It is concentration dependent. Comparison of gram positive and gram negative bacteria in different concentrations of methanolic extract of *R. officinalis* showed that the antibacterial effect of this plant on gram negative bacteria is higher than gram positive bacteria. The methanolic extracts of *R. officinalis* plant had the highest effect on *P.aeruginosa* with diameter of the no-growth zone, 19.8±1.64 mm. Also, this extract did not affect *B. cereus* bacteria. These results indicate that there is a significant difference in the sensitivity of the extract of *R. officinalis* plants among the tested bacteria ($p < 0.05$). In other words, there is the

highest sensitivity to methanolic extract of *R. officinalis* plants in *P. aeruginosa* and the least susceptibility to *S. aureus*. The MIC and MBC values are presented in Table 2. The minimum inhibitory concentration of the bacteria is between 6.25 and 25 mg/mL. The Minimum bactericidal concentration was between 12.5 and 50 mg/mL.

**Table 1.** Average the diameter of inhibition zone

| | Agar Well Diffusion method (mean ± SD) (mm) | | | | | |
|---|---|---|---|---|---|---|
| **Extract concentration (mg / mL) Strain of bacteria** | 20 | 30 | 50 | 400 | Negative control | Positive control |
| ***S.aureus*** | 0.89 ±8.4 | 0.89 ±10.4 | 0.89 ±11.4 | 1.14 ±14.4 | -- | 22 |
| ***B. cereus*** | - | - | - | - | -- | 16 |
| ***E.coli*** | - | 0.83 ±9.2 | 1.92 ±11.2 | 0.54 ±15.6 | -- | 26 |
| ***P. aeruginosa*** | - | 1.09 ±12.8 | 1.30 ±15.8 | 1.64 ±19.8 | -- | 22 |

**Table 2.** Minimum inhibitory concentration and Minimum Bactericidal Concentration of methanolic extract of *R. officinalis* plants on tested bacteria

| | Macro Dilution Method (mg/mL) | |
|---|---|---|
| **Strain of bacteria** | MBC | MIC |
| ***S.aureus*** | 50 | 25 |
| ***B. cereus*** | - | - |
| ***E.coli*** | 50 | 25 |
| ***P. aeruginosa*** | 12.5 | 6.25 |

Due to the increased resistance of bacteria to some types of antibiotics, efforts have been made to achieve and use of plant compounds and their application in the treatment of various diseases. Plants have played a major role in maintaining health and improving the quality of life of humans thousands of years ago. Medicinal plants have beneficial properties, including anti-bacterial, anti-parasitic, anti-fungal and anti-oxidant properties [26]. The results of this study showed that the diameter of inhibition zone of *R. officinalis* methanol extract on *P. aeruginosa* was 12.8 to 19.8 mg/mL, *E. coli* 9.2 to 15.6 mg/mL and *S. aureus* 8.4 to 14.4 mg /mL. In a study by Golshani and Dawoodi on the antimicrobial effects of methanolic extract of *R. officinalis* leaves in 2013, the highest levels of diameter of inhibition zone of the extract on the bacteria were *P.aeruginosa* (18 mg/mL), *S. aureus* (15 mg/mL) and *E.coli* (14 mg/mL) which is consistent with the results of the findings [27]. Gislene *et al*. [28], with the study of the antimicrobial effects of *R. officinalis* essential oils on different bacteria, showed that the diameter of the inhibition zone of this essential oil on *S. aureus* was 18 mm. Fu *et al*. [23], in a study titled "Antimicrobial effects of *R. officinalis* essential oil," showed that the diameter of inhibition zone of the essential oil on the *S. aureus* bacterium is 18 mm**.** Other

studies have shown the effects of *R. officinalis* essential oils on gram-positive bacteria of *S. aureus* and *B. cereus***.** [29-30] Ahmady-asbchin and Mostafapour, in 2018, studied the essential oil *of R. officinalis* plant, which has an antibacterial effect on *E. coli, S. aureus, S. epidermidis, E. faecalis* and *P. mirabilis*, which this property varies depending on the dilutions of essential oils and bacterial species. Which had the highest effect on *P. mirabilis* and had the least effect on *E. faecalis*. [31] Ahmady-asbchin et al., in another study titled antimicrobial effects of Rosemary extract on some gram-negative and gram-positive bacteria showed that the extract had different effects at different dilutions, so that in dilutions 1, 1.2 and 1.4 P. mirabilis and *E. faecalis* were the most susceptible and most resistant bacteria, respectively [32]. Soltan Dallal *et al*. [33], reviewed the antimicrobial effects of *R. officinalis* essential oil with disc diffusion and dilution methods on methicillin-resistant *S.aureus*, Showed that the diameter of the inhibition zone was 20 mm and MIC/MBC were 40.1 and 81.2 mg/mL, respectively**.** By comparing these results, we can say that the effect of Rosemary essential oil is much higher than that of extract. Mashreghi and Momtazi by examining the antimicrobial effects of *R. officinalis* alcohol extract on *E. coli* O157, showed that this extract does not have much effect in the early stages of bacterial growth and its effects are more pronounced when bacteria grow and propagate [34]. There are some differences in the amount of antimicrobial effects observed in this study and similar studies due to differences in plant growth locations. Differences in antimicrobial effects indicate differences in the active ingredients of the plant.

## Conclusion

In general, the results of the experiments showed that the extract from *R. officinalis* herb has antimicrobial activity against *P. aeruginosa, E. coli and S. aureus*. Although clinical trials on patients after the use of *R. officinalis* extract are recommended for confirmation of these data, so that it can be placed in the category of herbs available to patients**.**

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**How to cite this manuscript:** Abolfazl Jafari-Sales*, Parisa Hossein-Nezhad. Antimicrobial effects of rosmarinus officinalis methanolic extract on staphylococcus aureus, bacillus cereus, escherichia coli and pseudomonas aeruginosa in laboratory conditions. *Journal of Medicinal and Chemical Sciences*, 2020, 3(2), 103-108. DOI: 10.26655/jmchemsci.2020.2.2