International Journal of Nature and Life Sciences

IJNLS

https://dergipark.org.tr/tr/pub/ijnls

e-ISSN: 2602-2397 https://doi.org/10.47947/ijnls.1490216

Research Article

Investigating the Antibacterial Effects of *Zataria multiflora* Methanolic Extract on Standard Pathogenic Bacteria in Laboratory Conditions

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Received: May 30, 2024
Accepted: July 23, 2024
Online Published: August 18, 2024

Abstract: Alternative treatments are becoming more and more necessary due to the increasing growth in medication resistance to synthetic antibiotics. The purpose of this study was to examine the antibacterial capabilities of an extract from *Zataria multiflora* (*Z. multiflora*) against a variety of pathogenic bacteria. *Z. multiflora* plants were collected from the natural resources of Isfahan province and verified as *Z. multiflora* by botanists from the Agricultural Jihad Organization. Methanolic extract of *Z. multiflora* was prepared, and its effects were tested at concentrations of 50 mg/ml, 100 mg/ml, 200 mg/ml, and 400 mg/ml using the well diffusion and disk diffusion methods on *Staphylococcus aureus* (*S. aureus*), *Bacillus cereus* (*B. cereus*), *Escherichia coli* (*E. coli*) and *Pseudomonas aeruginosa* (*P. aeruginosa*). Minimum inhibitory concentration of bacterial growth and minimum bacterial pathogen concentration (MIC/MBC) were determined through dilution in tubes. The outcomes showed that both gram-positive and gram-negative bacteria were significantly inhibited by the methanolic extract of *Z. multiflora*. The MBC/MIC analysis revealed that *S. aureus* was the most sensitive, while *E. coli* was the least sensitive to the *Z. multiflora* extract. The study shows the potential of the methanolic extract from *Z. multiflora* against various pathogenic bacteria, including Gram-positive and Gram-negative ones. It suggests it could be a viable alternative to synthetic antibiotics, especially in the face of antibiotic resistance. Further research is needed to understand its mechanisms, optimize extract preparation, and explore its clinical applications.

Keywords: Antibacterial effects; Extract; Medicinal plants; Pathogenic bacteria.

Citation:
Yamchlou, N., Cinisli, K. T., Shadi-Dizajii, A., Ghahremani, Z., Golestani, A., Soleymanpour, K., Hosseini-Karkaj, K., Jafari-Sales, A., Pashazadeh, M. (2024). Investigating the antibacterial effects of *Zataria multiflora* methanolic extract on standard pathogenic bacteria in laboratory conditions. International Journal of Nature and Life Sciences, 8 (2), 102-110.

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1. Introduction

Certainly, the utilization of medicinal herbs has been the most ancient method of disease treatment for humans. Throughout the evolution of civilizations, there has always been a strong bond between humans and plants. Nevertheless, numerous plant species have remained undiscovered, leaving a vast reservoir of untapped and valuable botanical resources. Consequently, plants represent a promising source of potential chemical compounds, with only a fraction of their potential being explored (Jafari-Sales and Hossein-Nezhad, 2020; Jafarı-Sales et al., 2020; Jafarı-Sales and Pashazadeh, 2020; Jafari et al., 2020; Jafari Sales and Shariat, 2020; Mahmoudi et al., 2019). These valuable chemical components hold the potential to serve not only as medicines but also as valuable prototypes for developing pharmaceutical analogs. Moreover, they offer an intriguing tool for comprehending and deciphering biological phenomena (Jafari-Sales et al., 2015; Skaltsa et al., 2003; Skaltsa et al., 1999). Dealing with infectious diseases is a crucial therapeutic obstacle owing to their widespread occurrence. In the 1940s, the discovery of penicillin marked a significant breakthrough, leading to the continuous introduction of new antibiotics for infection treatment. Consequently, synthetic antibiotics became widely utilized in clinical practice for managing various infections. Unfortunately, the overuse of these antimicrobials has resulted in a surge of drug resistance among a majority of bacterial strains against different antibiotics (Weinstein, 2001). This has contributed to the increasing adoption of plants as a safer, cost-effective, and economical alternative to synthetic antibiotics in addressing bacterial infections. Additionally, herbal medicines are more readily accepted and consumed by the population (Mosadegh and Naghibi, 2002; World Health Organization, 1978; 2002). There has been a noticeable increase in worldwide research on different plants and their antibacterial properties in recent years. This growing interest can be attributed to the increased awareness and recognition of the potential therapeutic benefits offered by these plants in combating bacterial infections (Al-Snafi et al., 2021; Cowan, 1999; Jafari-Sales et al., 2021; Mobaiyen et al., 2015; Sales, 2014; Sayyahi et al., 2019; Sayyahi et al., 2021). *Zataria multiflora* (*Z. multiflora*) is known as a plant from the Lamiaceae family, and geographically, its growth occurs only in southwest Asia (central and southern areas of Iran, Pakistan, and Afghanistan) (Golkar et al., 2020). The branches of *Z. multiflora* plant, which are used for some of their medicinal properties, have large amounts of alkaloid, saponin, flavonoid, tannin, and plant disinfectants. Other available bioactive compounds include polymethoxy flavonoids, phenolic acids, and polysaccharides (Izadiyan et al., 2022; Shafaroudi et al., 2022). According to the studies conducted in relation to the chemical compounds of *Z. multiflora* essential oil, it was found that the antimicrobial, antifungal and antioxidant activities of this plant are influenced by high amounts of phenolic compounds such as carvacrol, thymol, linalool, as well as monoterpenes such as p-cymene and γ-terpinen takes place (Golkar et al., 2020; Sadeghi et al., 2015). The leaves have medicinal properties and are utilized for treating coughs. Moreover, the alcoholic extract exhibits antiseptic properties and sparkling effect (Deans et al., 1993; Hornok, 1988). This work aims to evaluate the antibacterial properties of *Z. multiflora* methanolic extract against common pathogenic bacteria, namely *Bacillus cereus* (*B. cereus*), *Escherichia coli* (*E. coli*), *Pseudomonas aeruginosa* (*P. aeruginosa*), and *Staphylococcus aureus* (*S. aureus*).

2. Materials and Methods

2.1. Collection and extraction of vegetarian samples

Plant samples from natural areas around Fuladshahr in Isfahan province (with characteristics of latitude: 32 degrees 29 minutes 26 seconds and longitude: 51 degrees 25 minutes 16 seconds and altitude 1700 meters above sea level) randomly It was collected in the flowering season (August). The samples were collected with sufficient accuracy from a geographical area. Then the collected samples were identified and confirmed by the botanists of the Botany Laboratory and Herbarium of the Ministry of Agriculture Jahad of Isfahan province. Plant samples were dried in a suitable and wide place. After complete drying, the aerial parts (stem and leaves) were separated from the roots and subjected to milling. To maximize the contact surface of the plant material with distilled water, it was finely powdered using an electric mill. For the extraction process, the Soxhlet method was employed. Approximately 300 g of the powdered material was placed in a filter paper soaked with methanol (Merck, Germany) and introduced into the Soxhlet apparatus (Figure1). The apparatus was connected to a flask containing 500 ml of methanol, and the pure extract was obtained through a Rotary (Figure 2). The resulting extract was then diluted using a 5% Dimethyl sulfoxide (DMSO) (Merck, Germany) solvent, resulting in concentrations of 50 mg/ml, 100 mg/ml, 200 mg/ml, and 400 mg/ml. These different concentrations were used for conducting the well

diffusion and disk diffusion tests, as well as for determining the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) following the Clinical and Laboratory Standards Institute (CLSI) protocol (Cockerill, 2013).

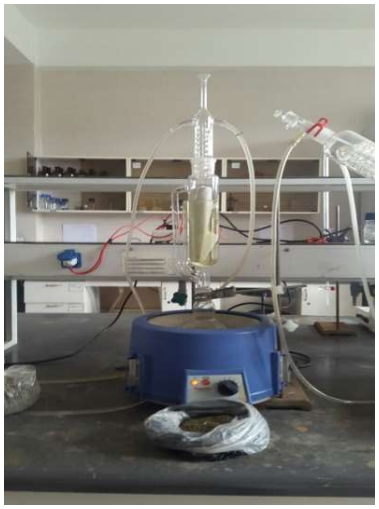

Figure 1. Extraction by using soxhlet.

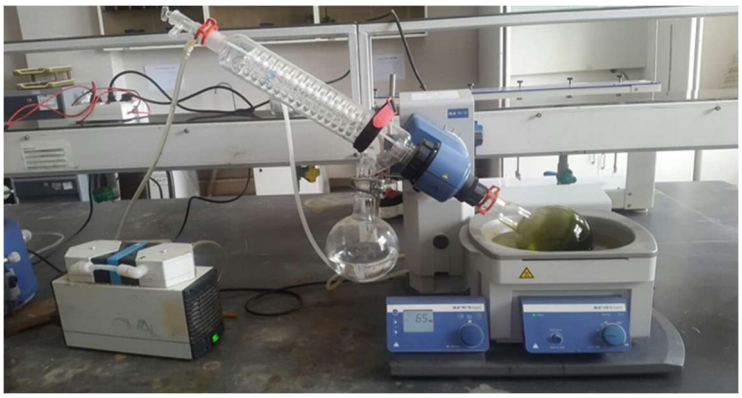

Figure 2. Purified extract using a rotary.

2.2. Determination of antibacterial effect of extract

Strains of *S. aureus* (American Type Culture Collection-ATCC: 25923), *B. cereus* (Persian Type Culture Collection-PTCC: 1052), *E. coli* (ATCC: 25922), and *P. aeruginosa* (ATCC: 27853) are prepared as a lyophilized of Pasteur Institute of Iran. To commence the testing process, susceptible bacteria were adjusted to the standard 0.5 McFarland turbidity and subsequently spread onto the surface of Mueller-Hinton Agar (MHA) (Merck, Germany) culture medium using a sterile swab. Following this, wells with a diameter of 5 mm and spaced 2 cm apart were introduced on the medium, and specific dilutions of the aforementioned extract were placed into the wells. The extent of the inhibition zone's diameter served as an indicator of the extract's concentration. The diameter of the inhibition zone was measured and compared to the designated standard to establish the antibacterial efficacy of the test extract. It was discovered that there was a linear relationship between the halo size and the logarithm of the extract's concentration (Neef et al., 1995). The disk diffusion method shares similarities with the well diffusion method, but instead of creating wells on the agar surface, disks (blank disks manufactured in Padtan-Teb co, Iran) soaked with various concentrations of methanolic extract are utilized. The procedure involves the following steps: Initially, sterile disks are soaked in methanolic extract concentrations of 50 mg/ml, 100 mg/ml, 200 mg/ml, and 400 mg/ml, and then left to dry. Next, sterilized swabs are immersed in a microbial suspension and allowed to dry. The swab is then pressed against the side of the tube to remove excess solution and streaked over the surface of the agar plate. Streptomycin antibiotics (Padtan-Teb, Iran) were used as a positive control, and DMSO was used as a negative control. After incubating the plates

at 37 °C for 16-18 hours, the diameter of the inhibition zones is measured in millimeters using a caliper (Shariff, 2001). The dilution tube method was used to conduct the MIC and MBC testing. The methanolic extract was diluted to concentrations of 0.78, 1.56, 3.125, 6.25, 12.5, 25, 50, 100, and 200 mg/ml in Mueller-Hinton Broth (MHB) (Merck, Germany) culture medium in order to calculate the MIC. The produced microbial suspension was then added to each dilution in a volume of 1 ml. Negative control tubes with the culture media free of bacteria and positive control tubes with the culture medium alone (without extracts) were made. All tubes were then placed in an incubator at 37 °C for 24 hours. Following the incubation period, the tubes were checked for the presence or absence of bacterial growth. The MIC is the lowest extract dilution at which no bacterial growth was detected. Samples from every tube in which no bacterial growth was seen were cultivated on MHA plates in order to calculate the MBC. The injected plates underwent an additional 24-hour incubation period at 37 °C. The MBC was defined as the extract concentration in the tube containing no bacterial growth on the agar plate (Alizadeh et al., 2012). Software from SPSS (version 26, SPSS Inc., Chicago, IL, USA) was used to examine the experimental results. The one-way analysis of variance (ANOVA) and the least significant difference (LSD) test were used to group and compare the means. A significance level of $p < 0.01$ was deemed statistically significant in this investigation.

3. Results

Through the implementation of both the well diffusion and disk diffusion methods, the investigation into the impact of *Z. multiflora* extract on the tested bacteria revealed a significant inhibitory effect. As the concentration of the methanolic extracts increased, the inhibitory effect became more pronounced, as evidenced by the enlargement of the non-growth zones. Moreover, this study demonstrated that the inhibitory effect of *Z. multiflora* extract was more pronounced on gram-positive bacteria com methanolic pared to gram-negative bacteria. Detailed results, illustrating the effects of various concentrations of methanolic extract using the well diffusion and disk diffusion methods, can be found in Tables 1 and 2. Notably, these two methods exhibited variations in the diameter of the non-growth zones observed in the bacteria.

Table 1. The diameter of the bacterial inhibitory zone measured in millimeters at various methanolic extract concentrations using Agar well diffusion.

Extract concentration / Bacteria strain	50 mg/ml	100mg/ml	200mg/ml	400mg/ml	Negative control	Positive control
S. aureus	7	9.70	12.20	14	-	17
B. cereus	6.20	8.50	10.15	12	-	16.70
P. aeruginosa	--	--	8	10.60	-	13.80
E. coli	--	--	7.70	9	-	14

Table 2. The diameter of the bacteria's no-growth zone measured in millimeters at various methanolic extract concentrations using the disc diffusion method.

Extract concentration / Bacteria strain	50 mg/ml	100mg/ml	200mg/ml	400mg/ml	Negative control	Positive control
S. aureus	8	10	12	14.20	-	17.26
B. cereus	7.20	9	11.15	13	-	16.17
P. aeruginosa	--	8.10	10	11.10	-	14.65
E. coli	--	--	9.40	10.54	-	14.73

The results of the MBC/MIC test showed that the herb extract was most susceptible to *S. aureus* bacteria and least susceptible to *E. coli*. Table 3 contains the comprehensive findings of the MBC/MIC test conducted by the tube method on methanolic extracts against the chosen bacteria.

Table 3. MBC / MIC mg / ml test for bacteria in millimeters at different concentrations.

Extract concentration Bacteria strain	MIC mg/ml	MBC mg/ml
S. aureus	12.5	25
B. cereus	12.5	50
P. aeruginosa	25	50
E. coli	50	100

4. Discussion

In response to the rising bacterial resistance to certain antibiotics, there has been a concerted endeavor to explore and harness plant compounds for treating various ailments. Plants have long been integral to human health and well-being, with medicinal plants offering valuable properties such as anti-bacterial, anti-parasitic, anti-fungal, and antioxidant effects. Studies conducted by Atapour et al. and Ghannadi et al., showed that the methanolic extract of *Z. multiflora* plant has the most inhibitory effect on *Helicobacter pylori* (*H. pylori*) bacteria (Atapour et al., 2009; Ghannadi et al., 2004). Studies conducted by Fazeli et al., and Omidpanah et al., indicate that the alcoholic extract of the *Z. multiflora* plant can inhibit the growth of *E.coli* (Fazeli et al., 2007; Omidpanah et al., 2016). In research conducted by Goudarzi et al., it was observed that *Z. multiflora* extract exhibited a promising antibacterial effect against *E. coli* at a concentration of 0.78 mg/ml (Goudarzi et al., 2006). In the study, *Z. multiflora* demonstrated the highest inhibitory effect on *S. aureus* and the lowest effect on *E. coli* (Foladvand et al., 1970). Moreover, Sharififar et al. demonstrated the efficacy of *Z. multiflora* extract against standard strains of *E. coli* and *S. aureus* (Sharififar et al., 2007). Azizkhani et al., showed in their research that the growth of *S. aureus* bacteria decreases with increasing concentration of *Z. multiflora* (Azizkhani et al., 2012). Motevasel et al., Ojagh et al., Owlia et al., and Sabzikar et al. proved that the most inhibitory effect of *Z. multiflora* extract was on the inhibition of *S. aureus* (Motevasel et al., 2014; Ojagh et al., 2016; Owlia et al., 2006; Sabzikar et al., 2020). Studies by Sheikholeslami et al., and Yadegar et al. about the *Z. multiflora* plant showed that the hydroethanolic extract of this plant has a significant inhibitory effect on Methicillin-resistant *S. aureus* bacteria (MRSA) (Sheikholeslami et al., 2016; Yadegar et al., 2010). Fallah et al., Hatami Pirghibi et al., Karbasizade et al., and Meskini et al., by investigating the antibacterial properties of *Z. multiflora* extract, showed that the ethanolic and methanolic extracts of this plant have a great inhibitory effect on *P. aeruginosa* bacteria (Fallah et al., 2013; Hatami Pirghibi et al., 2021; Karbasizade et al., 2017; Meskini et al., 2019). Javan's study found a synergistic effect of *Trachyspermum ammi* L. and *Z. multiflora* on *S. aureus* and *B. cereus*, while an increasing effect was observed on *E. coli*, *Salmonella typhimurium* (*S. typhimurium*), and *Listeria monocytogenes* (*L. monocytogenes*)(Jebelli Javan, 2016). Zare Bidaki et al. reported that among the studied bacteria, *B. cereus* showed the highest susceptibility to the essential oil of *Zataria multiflora*, while *P. aeruginosa* exhibited the least susceptibility (Zare Bidaki et al., 2015). Safari et al., found that the alcoholic and hydroalcoholic extracts of *Z. multiflora* have a significant inhibitory effect on *Streptococcus iniae* (*S. iniae*) (Safari et al., 2015). During their study in 2019, Rezvannejad et al. showed that the ethanolic and methanolic extract of *Z. multiflora* plant has a significant effect on the bacterium *Peanibacillus alevi* (*P. alevi*) (Rezvannejad et al., 2019). Dadashi et al., and Taherpour et al., showed the highest inhibitory effect of *Z. multiflora* extract against *Klebsiella pneumonia* (*K. pneumonia*) and *Vibrio cholera* (*V. cholera*) bacteria respectively during their research (Dadashi et al., 2016; Taherpour et al., 2015). Pirbalouti et al.'s research on the antibacterial properties of *Z. multiflora* showed that the aqueous extract of this plant has a good inhibitory effect on *Vibrio harveyi* (*V. harveyi*) and *Vibrio parahaemolyticus* (*V. parahaemolyticus*) bacteria (Pirbalouti et al., 2011). The study of Davazdahemami and Behbahani, regarding the inhibitory effect of *Z. multiflora* plant showed that the methanolic extract of this plant has a significant antibacterial effect on *Bacillus subtilis* (*B. subtilis*) and *Sterptococcus pyogenes* (*S. pyogenes*) bacteria (Davazdahemami and Behbahani, 2022). According to the research conducted by some researchers on the antibacterial effects of *Z. multiflora* essential oil, it was found that this plant has an inhibitory effect on *E. coli*, *S. aureus*, *P. aeruginosa* and *B. cereus* bacteria (Andacheh et al., 2020; Pourhosseini et al., 2020; Ramezanpour et al., 2016).

5. Conclusions

The experimental data presented in this study have demonstrated the potent antibacterial properties of the extract derived from the medicinal plant. The extract was found to be effective against a range of clinically-relevant bacterial strains, including *S. aureus*, *B. cereus*, *P. aeruginosa*, and *E. coli*. These promising results suggest that this plant extract holds significant potential as a natural source for the development of novel herbal antimicrobial agents. To further substantiate these findings and explore the practical applications, additional research is warranted. Preclinical studies involving laboratory animal models would be valuable in evaluating the *in vivo* efficacy and safety profile of the plant extract. Given the rising concerns over antibiotic resistance and the growing interest in natural, plant-based therapeutics, the antibacterial potency demonstrated by this extract represents an important step forward. With appropriate further investigations, this discovery could potentially pave the way for the introduction of effective, plant-derived antimicrobial agents to combat the threat of resistant bacterial infections.

Conflicts of Interests
Authors declare that there is no conflict of interests

Financial Disclosure
Author declare no financial support.

Statement contribution of the authors
Idea: AJS, MP, Design: KTC, ASD, Supervision: AJS, MP, Equipment: ZG, AG, Data collection and processing: KTC, ASD, AJS, Analysis and commentation: NY, KS, KH, Literature review: KTC, ASD, Writing: NY, ZG, AG, KS, KH, Review: AJS, MH

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