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Antibacterial properties of essential oils and methanol extracts of *Ziziphora tenuior Lam.* (a native plant) in pre-flowering stage against isolated bacteria from urogenital tract infections

Y. Anzabi [1], A. Khaki [1], A. Rasoli [2], S. Ebrahimpour[3], F. Fallah Rostami[4*]

[1]*Department of Pathobiology, Tabriz Branch, Islamic Azad University, Tabriz, Iran*
[2] *Veterinary College, Tabriz Branch, Islamic Azad University, Tabriz, Iran*.
[3]*Infectious Diseases and Tropical Medicine Research Center, Babol University of Medical Sciences, Babol, Iran.*
[4]*Ministry of Health and Medical Education, Pediatric Neuro Rehabilitation Research Center, University of Social Welfare and Rehabilitation Sciences, Tehran, Iran*

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This study aimed to investigate the antibacterial activity of essential oils and methanol extracts of *Ziziphora tenuior* on some pathogenic bacteria in culture and determination of its minimal inhibition concentration (MIC) and minimal bactericidal concentration (MBC).

The results showed that the MIC for essential oils of *Ziziphora tenuior* was 250 µg/ml for most of the gram-negative bacteria except for *Pseudomonas aeruginosa*. The gram-negative bacteria *Klebsiella pneumoniae* was more sensitive compared to other species of gram-negative bacteria. On the other hand, the MIC of gram-positive bacteria such as *Staphylococcus aureus* was 250 µg/ml, and for other species of *Staphylococcus* it was 500 µg/ml. The results of the MIC determination of the methanol extracts of this plant showed that the combination of these has inhibitory and bactericidal effect on all bacteria under test except for *Pseudomonas aeruginosa*. The MBC of the methanol extract of *Ziziphora tenuior* was 2000 µg/ml for most of the gram-negative bacteria and less for the gram-positive ones. We can conclude that low concentrations of the essential oils of this plant are able to inhibit the growth of the bacteria under study.

Key words: antibacterial effect, *Ziziphora tenuior*, essential oils, methanol extracts, urogenital tract.

INTRODUCTION

Essential oils, plant extracts and their constituents have known anti-bacterial effects [1]. *Ziziphora tenuior* is a genus of Ziziphora and breed of mint and is a one-year herbaceous plant with short stems, 5-15 cm tall and thin, sharp leaves that is scattered in many parts of Iran [2]. This plant grows in wild state in vast areas of Iran like the mountainous regions of Azerbaijan provinces, especially in the mountains of Tabriz [3]. Four species of the plant called *Ziziphora clinopodioides, Ziziphora capitata, Ziziphora persica* and *Ziziphora tenuior* have been identified in Iran. Among the healing properties of this plant sputum collection, carminative and stomach reinforcement can be named. In some areas its powdered leaves mixed with honey are used to treat dysentery [2]. In different areas, the plant powder is used as a garnish on yogurt and dairy products [4]. Also, it is used for treatment of diseases of the stomach and as an antiseptic to relieve colds [5].

Despite the heavy use of plants in the mint family of flavors in Iran, systematic research has not been performed on the antibacterial effects of the *Ziziphora tenuior* extract on pathogenic bacteria. This study aimed to investigate *in vitro* the antibacterial activity of essential oils and methanol extracts of this plant on some pathogenic bacteria isolated from urogenital tract infections.

MATERIALS AND METHODS

Collecting plants used in the study: wild plants of *Ziziphora tenuior* had been collected in the hills around the city of Tabriz in East Azerbaijan province (at a height of 1700-1800 m) during pre-flowering stage and the species of this plant were determined in the Food Hygiene Department of Tabriz Branch, Islamic Azad University (Tabriz - Iran). After collecting the plants, the leaves were dried in appropriate circumstances in shadow and were crushed by mill in order to prepare extracts and essential oils.

Preparation of plant methanol extracts and essential oils: The specialized laboratory at the Veterinary Faculty of Tabriz Branch, Islamic Azad University, used the maceration method to prepare

* To whom all correspondence should be sent:
E-mail: fallahrostami2@gmail.com

methanol extracts of *Ziziphora tenuior*. For this purpose 50 g of each sample was soaked in 80% methanol and 48 h later it was smoothed by filter paper. Extracts were obtained using a rotary machine at 40 to 50°C, concentrated and dried at the same temperature for 2 days and gradually dried [6,7].

For production of essential oils, the water distillation method using a Clevenger apparatus was applied [2,8].

Tested bacteria: The examined bacteria in this study included 9 species of bacteria isolated from UTI of patients referred to a number of clinics of Tabriz City in 2014 (Table 1).

Table 1. The isolated bacteria tested in this study

	Name of isolated bacteria
1	*Staphylococcus aureus*
2	*Staphylococcus epidermidis*
3	*Staphylococcus saprophyticus*
4	*Escherichia coli*
5	*Klebsiella pneumonia*
6	*Proteus vulgaris*
7	*Enterobacter aerogenes*
8	*Citrobacter frundii*
9	*Pseudomonas aeruginosa*

Preparation of bacterial suspensions: for preparation of such suspension a 24 h old culture of each bacterium is needed. Hence, 24 h before the test the stored cultures were inoculated into BHI agar medium and incubated for 24 h at 37°C. The colonized medium was washed with normal saline solution, the bacterial suspensions were diluted with normal saline and their turbidity was set equivalent to that of a standard tube 0.5 McFarland. The test suspension contained 1.5×10^8 CFU/ml [9,10].

Antibacterial effects of methanol extracts and essential oils of Ziziphora tenuior

Agar Disk Diffusion: To evaluate the antibacterial effects of essential oils and methanol extracts of the mentioned plant the disk diffusion in agar method was used. It should be explained that the disks containing extracts were prepared from sterile blank disks manufactured by Padtan Teb Company (Tehran-Iran). Thus the blank disks were placed in tubes containing essential oils and methanol extracts of *Ziziphora tenuior* for 30 to 50 min and following the complete absorption by the disk, the disks were placed at 44-45°C until completely dry and ready [11]. Then 100 ml of the prepared suspensions of all isolated bacteria were cultured separately on the surface of Mueller-Hinton agar medium. Using sterile forceps, the disks impregnated with essential oils and methanol extracts of *Ziziphora tenuior* were placed at a certain distance from each other and from the edge of the plate in the medium and were fixed with little pressure on the medium. Then the plates were incubated for 24 h at 37°C and the antibacterial activity was recorded by measuring the diameter of the inhibition zone around the disks. To make sure, the experiment was repeated three times for each isolated bacterium. The mean inhibition zone diameter in the three replicates was registered as the final diameter [12,13]. The standard antibiotic ampicillin (10 µg/disk) was used as positive control.

Minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC): For this purpose, dilution methods were used**.** In order to determine MIC for essential oils and methanol extracts of *Ziziphora tenuior*; a series of 10 test tubes was used, 8 tubes for testing the different dilutions of essential oils and methanol extracts and two tubes as test controls. Each of these compounds was tested separately with different dilutions from tube number one, containing 5.62 µg/ml up to tube number eight with concentration of 8 mg/ml of the plant extract and essential oil in BHI broth medium plus 1 ml of the tested bacterial suspension which contains 1.5×10^8 CFU/ml bacteria. Also, a tube containing 9 ml BHI broth medium plus 1 ml of essential oil and extract as antibacterial compounds control and a tube containing 9 ml the medium as well as 1 ml of bacterial suspension were prepared as isolated bacteria control. All test tubes were incubated at 37°C for 24 h. After the incubation period the tubes' inoculated turbidity due to bacterial growth was studied. This method was repeated 3 times for each isolated bacterium. The extracts and essential oils dilution containing the lowest concentration and the maximum inhibition of bacterial growth was considered as the MIC of the compounds separately (essential oils or methanol extracts). Also, the lowest concentration of the methanol extracts and essential oils where no bacterial growth was observed, cultured by the pure plate method, was considered as the MBC of the essential oils and methanol extracts.

For this purpose, 1 ml of each concentration was mixed with 20 ml of BHI agar at 45°C in Petri dishes and after closing the agar and incubation for 24 h, the incubated plates were controlled for the presence of microbial growth. Dilution plates containing the lowest concentration of the extract and no colonies of bacteria were found and were selected as MBC of the corresponding extract [14,15].

Table 2. Mean of inhibition zone (mm) ±SD of tested antibacterial compounds (essential oils and methanol extracts of *Ziziphora tenuior* and ampicillin as the standard antibiotic).

Name of isolated bacteria	Essential oils of *Ziziphora tenuior* (10µl/disk)	Methanol extracts of *Ziziphora tenuior* (10µl/disk)	Antibiotic Ampicillin (10µg/disk)
Staphylococcus aureus	22±0.1	19±0.1	13± 0.1
Staphylococcus epidermidis	20±0.1	19.5±0.2	14±0.1
Staphylococcus saprophyticus	21±0.1	19±0.1	14±0.1
Escherichia coli	21±0.1	20±0.1	13±0.1
Klebsiella pneumonia	29±0.1	26±0.1	19±0.1
Proteus vulgaris	19±0.1	17±0.1	9±0.1
Enterobacter aerogenes	19±0.1	17.5±0.2	11±0.1
Citrobacter frundii	20±0.1	19±0.1	4±0.1
Pseudomonas aeruginosa	0	0	0

Note: Significant difference: P<0.05, Subset for alfa=0.05

Table 3. Results of minimal inhibition concentration (MIC) with different concentrations of the *Ziziphora tenuior* essential oils

Name of isolated bacteria	Essential oils concentration (µg/ml)							
	62.5	125	250	500	1000	2000	4000	8000
Staphylococcus aureus	+	+	+	-	-	-	-	-
Staphylococcus epidermidis	+	+	+	+	-	-	-	-
Staphylococcus saprophyticus	+	+	+	+	-	-	-	-
Escherichia coli	+	+	-	-	-	-	-	-
Klebsiella pneumonia	+	-	-	-	-	-	-	-
Proteus vulgaris	+	+	-	-	-	-	-	-
Enterobacter aerogenes	+	+	-	-	-	-	-	-
Citrobacter frundii	+	+	-	-	-	-	-	-
Pseudomonas aeruginosa	+	+	+	+	+	+	+	+

Note: The symbol (+) indicates the growth of bacteria and the sign (-) indicates the absence of bacterial growth.

Table 4. Results of minimal inhibition concentration (MIC) with different concentrations of the *Ziziphora tenuior* methanol extracts.

Name of isolated bacteria	Methanol extract concentration (µg/ml)							
	62.5	125	250	500	1000	2000	4000	8000
Staphylococcus aureus	+	+	+	-	-	-	-	-
Staphylococcus epidermidis	+	+	+	+	+	-	-	-
Staphylococcus saprophyticus	+	+	+	+	-	-	-	-
Escherichia coli	+	+	+	+	+	-	-	-
Klebsiella pneumonia	+	+	-	-	-	-	-	-
Proteus vulgaris	+	+	+	-	-	-	-	-
Enterobacter aerogenes	+	+	+	+	+	-	-	-
Citrobacter frundii	+	+	+	+	-	-	-	-
Pseudomonas aeruginosa	+	+	+	+	+	+	+	+

Note: The symbol (+) indicates the growth of bacteria and the sign (-) indicates the absence of bacterial growth.

Table 5. Results of the minimal bactericidal concentration (MBC) with different concentrations of the *Ziziphora tenuior* essential oils

Name of isolated bacteria	Essential oils concentration (µg/ml)							
	62.5	125	250	500	1000	2000	4000	8000
Staphylococcus aureus	+	+	+	-	-	-	-	-
Staphylococcus epidermidis	+	+	+	+	+	+	-	-
Staphylococcus saprophyticus	+	+	+	-	-	-	-	-
Escherichia coli	+	+	-	-	-	-	-	-
Klebsiella pneumonia	+	+	-	-	-	-	-	-
Proteus vulgaris	+	+	-	-	-	-	-	-
Enterobacter aerogenes	+	+	-	-	-	-	-	-
Citrobacter frundii	+	+	-	-	-	-	-	-
Pseudomonas aeruginosa	+	+	+	+	+	+	+	+

Note: The symbol (+) indicates the growth of bacteria and the sign (-) indicates the absence of bacterial growth.

Table 6. Results of the minimal bactericidal concentration (MBC) with different concentrations of the *Ziziphora tenuior* methanol extracts

Name of isolated bacteria	Methanol extract concentration (μg/ml)							
	62.5	125	250	500	1000	2000	4000	8000
Staphylococcus epidermidis	+	+	+	+	-	-	-	-
Staphylococcus saprophyticus	+	+	+	+	-	-	-	-
Escherichia coli	+	+	+	+	+	-	-	-
Klebsiella pneumonia	+	+	+	+	-	-	-	-
Proteus vulgaris	+	+	+	+	+	-	-	-
Enterobacter aerogenes	+	+	+	+	+	-	-	-
Citrobacter frundii	+	+	+	+	+	-	-	-
Pseudomonas aeruginosa	+	+	+	+	+	+	+	+

Note: The symbol (+) indicates the growth of bacteria and the sign (-) indicates the absence of bacterial growth.

The Tukey test was used for the comparison of samples. Also, in order to determine which samples have significant mean differences, ANOVA with equal frequency was used.
It is crucial that the statistical methods used in the comparison between MBC and MIC of plant extracts and essential oils were descriptive statistics.

RESULTS AND DISCUSSION

RESULTS

The results show that the essential oils and methanol extracts of *Ziziphora tenuior* have no effect on *Pseudomonas aeroginosa* at the tested concentrations. At a 5% level of confidence there is no significant difference between the antibacterial effects of essential oils and methanol extracts on *E. coli*, *Citrobacter frundii* and *Enterobacter aerogenes*, but there is a significant difference between other bacteria. Also the strongest antibacterial effect was on *Klebsiella pneumoniae*. Compared with the positive control (ampicilin), in most of the cases except *Pseudomonas aeruginosa*, the essential oils and methanol extracts showed higher antibacterial activity and this function is more pronounced for the essential oils. The results also showed that the minimal inhibition concentration (MIC) for the essential oils of *Ziziphora tenuior* was ≤125 μg/ml for most of the gram-negative bacteria except for *Pseudomonas aeruginosa* (Tables 2,3). The minimal bactericidal concentration (MBC) of essential oils for the above mentioned bacteria was equal to their MIC (the essential oils did not affect *Pseudomonas aeruginosa*). On the other hand, the minimal inhibition concentration of gram-positive bacteria such as *Staphylococcus aureus* was 250 μg/ml, even though for other species of *Staphylococcus* it was 500 μg/ml (Table 3).

In general, these results indicated that among the gram-positive bacteria, *Staphylococcus aureus* has the highest sensitivity against the used concentrations of the *Ziziphora tenuior* essential oils in our study. The 6 gram-negative isolated bacteria were more sensitive to essential oils. *Klebsiella pneumoniae* was similar to the other species of gram-negative bacteria.

On the other hand, the results of the MIC determination of methanol extracts of *Ziziphora tenuior* showed that the extract has inhibitory and bactericidal effect on all tested bacteria except *Pseudomonas aeruginosa* (Table 3). The MBC of these extracts for most isolates were equivalent with their MIC (Tables 4,5,6).

The results of this study showed that, in general, low concentrations of the essential oils of *Ziziphora tenuior* compared to its methanol extracts, are able of inhibiting the growth of the studied bacteria.

DISCUSSION

Through thousands of years, the inhibitory effect of spices, herbal extracts and essential oils are known; but in recent years the effect of aromatic extracts, essential oils and herbal ingredients of these oils on pathogenic bacteria and microorganisms causing food spoilage is of great interest and represents the efforts of researchers to use natural preservatives derived from plants sources, instead of chemical preservatives [16,17]. The effect of these substances was studied on important food-borne isolates like *E. coli*, [18], *Salmonella enteritidis* [19-21], *Bacillus cereus* [22,23], *Staphylococcus aureus* [19,20], *Listeria monocytogenes* [24].

Analysis of essential oils from different plants showed the presence of different combinations. The original composition of the essential oils of mint family's plants is thymol and carvacrol. The strong anti-microbial effect of carvacrol has been established by researchers [25, 26]. Ozturk and Ercisli showed that the essence of Kakoty is

formed of 31.86% poligon, 12.21% senion, 10.48% limonen, 9.13% menthol, 6.88% beta-pinene, 6.73% menton, 3.5% peperitnon, 4.18% peperiton [27]. The main component of the essential oils of some mint family's plants including Kakoty, is poligon. Poligon has antibacterial and antifungal properties and is particularly effective for the different isolates of *Salmonella* [28]. According to this study, essential oils of *Ziziphora tenuior* showed a stronger antibacterial impact compared to its methanol extract and this antibacterial activity is probably associated with poligon which is an essential component of Kakoty essence. Results of Salehi *et al*. study [8] on the antimicrobial effect of Kakoty's extract showed that Kakoty extract can inhibit the growth of gram-negative bacteria *Klebsiella pneumoniae* and *Escherichia coli*. Besides lack of antibacterial activity against *Pseudomonas aeruginosa* in a methanol extract of *Ziziphora tenuior* (Kakoty) the results from the studies [8,29] are consistent with the results of the present study. Results of the study of Salehi et al. [8] also suggest that the extract can inhibit the growth of *Staphylococcus epidermidis* and *Bacillus subtilis*. Studies of Ercili and Ozturk [27,30] also showed that mountains' Kakoty extract and persica Kakoty are capable to prevent growing a wide range of gram-positive and gram-negative pathogenic bacteria. In this study, the essential oils of *Ziziphora tenuior* have an inhibitory and bactericidal effect on most of the gram-negative bacteria but has no effect on *Pseudomonas aeruginosa*, which is in agreement with the results of Sharopov et al., [3] in which the experiment was done on the Kakoty. Their results also showed that the essence of Kakoty can prevent the growth of gram-negative bacteria; *Escherichia coli* and *Enterobacter aerogenes*, but had no effect on *Pseudomonas aeruginosa*. Also, the above results are the same as results of Salehi et al. [8] on the antibacterial effect of mountains' Kakoty essence. Their study showed that essential oils of mountains' Kakoty can prevent the growth of gram-negative bacteria, *Klebsiella pneumoniae* and *Escherichia coli* and mountains' Kakoty essence is not active against *Pseudomonas aeruginosa*. The results of this study showed that the essential oils of *Ziziphora tenuior* have good anti-bacterial effect on the tested gram-negative bacteria. Based on Sharopov et al. [3] the anti-bacterial effect of Kakoty essence native for Turkey has been observed on gram-positive bacteria, *Staphylococcus aureus and Bacillus subtilis*. The results of Salehi, et al. [8] showed that mountains' Kakoty essence can prevent the growth of gram-positive bacteria, *Bacillus subtilis* and *Staphylococcus aureus.*

Also, most studies suggest that the susceptibility of gram-negative bacteria against antibacterial compounds is less than that of gram-positive ones which may be due to the presence of an outer membrane in the structure of their cell walls. Gram-positive bacteria have a large amount of mucopeptide compositions in their cell wall while gram-negative bacteria have only a thin layer of mucopeptide and much of their cell wall's structure is made of lipoprotein and lipo polysaccharide (LPS) and it seems that for this reason they are more resistant to anti-bacterial substances. These results are consistent with the results obtained in this study [18].

CONCLUSIONS

In this study it was found that the essential oils and methanol extracts of *Ziziphora tenuior* (Kakoty) have anti-bacterial effects on the tested bacteria except *Pseudomonas aeruginosa*, therefore, it seems that the above mentioned compounds can be used as antibacterial agents against a broad spectrum of bacteria causing urogenital tract infections. It is suggested that in this connection supplementary study should be done on animal models.

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АНТИБАКТЕРИАЛНИ СВОЙСТВА НА ЕТЕРИЧНИ МАСЛА И МЕТАНОЛОВИ ЕКСТРАКТИ ОТ *Ziziphoratenuior lam.* (ЕСТЕСТВЕН ПРОДУКТ) ПРЕДИ ЦЪФТЕЖА СПРЯМО БАКТЕРИИ, ИЗОЛИРАНИ ПРИ ИНФЕКЦИИ ОТ УРИНАЛНИЯ ТРАКТ

И. Анзаби[1], А. Кхаки [1], А. Расоли[2], С. Ебрахимпур[3], Ф. Фалах Ростами[4*]

[1]Департамент по патобиология, Ислямски университет „Азад", Клон Табриз, Иран
[2] Колеж по ветеринарство, Ислямски университет „Азад", Клон Табриз, Иран.
[3]Изследователски център по инфекциозни и тропически болести, Медицински университет в Бабол, Иран
[4]Министерство на здравеопазване и медицинско образование, Изследователски център по психиатрия и поведенчески науки, Медицински университет „Мазандаран", Сари, Иран

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(Резюме)

Изследването има за цел да изучи антрибактериалната активност на етерични масла и метанолови екстракти от растението *Ziziphoratenuior* върху някои патогенни бактериии определянето минималните концентрации на инхибиране (MIC) и минималните бактерицидни концентрации (MBC).

Резултатите показват, че за маслата MIC е 250 µg/ml спрямо повечето Грам-отрицателни бактерии с изключение на *Pseudomonas aeruginosa*. Грам-отрицателните бактерии *Klebsiella pneumoniae* е по-чувствителна спрямо други видове грам-отрицателни бактерии. От друга страна MIC за Грам-положителните бактерии като *Staphylococcus aureus* е 250 µg/ml, а задруги видове от рода *Staphylococcus* тя е 500 µg/ml. Резултатите от определянето на MIC за метаноловите екстракти от същото растение показват, че комбинацията от тях има инхибиторен и бактерициден ефект за всички изследвани бактерии освен *Pseudomonas aeruginosa*. MBC на метаноловите екстракти е 2000 µg/ml за повечето от Грам-отрицателните и по-малко за Грам-положителните. Може да се заключи, ниските концентрации на етеричните масла от това растение са в състояние да инхибират растежа на изследваните бактерии.