Original Article

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Dentistry Section

Antibacterial Effects of Garlic Extracts and Ziziphora Essential Oil on Bacteria Associated with Peri-Implantitis

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ABSTRACT

Introduction: Peri-implantitis is an inflammatory process, characterized by loss of bone around implants and bleeding on probing. Colonization of bacteria in the pocket created around the implant is one of the most important aetiologic factors involved. Although antibiotics are routinely used and are effective agents against this bacterial infection, due to the side effects and drug resistance of these medications, researchers are more interested in using herbal derivatives. There are relatively limited number of studies in this respect.

Aim: To evaluate and compare the antibacterial effects of the extracts of *Allium sativum* and *Ziziphora clinopodioides* essential oil on *S. aureus* and *P. aeruginosa*.

Materials and Methods: In this in vitro study conducted at Tabriz University of Medical Sciences between March 2016 and July 2016, aqueous and methanolic extracts of garlic and ziziphora essential oil were prepared and then their effects on one standard strain of *P. aeruginosa* and two standard strains of *S. aureus* and 18 clinical strains, (nine strains of *P. aeruginosa* and nine strains of *S. aureus*) which had been isolated from wound and blood cultures, were evaluated using the reference broth macro dilution method and disk diffusion technique. Data were evaluated with descriptive statistical techniques and t-test for independent groups, using SPSS 17.

Results: Aqueous and methanolic extracts of garlic did not exhibit inhibitory effects on *S. aureus* and *P. aeruginosa*. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) for ziziphora essential oil against *S. aureus* were 0.25 and 0.5 µg/mL, respectively. The mean diameter of growth inhibition zone for *S. aureus* in the ziziphora essential oil group (0.25µg/mL) and Vancomycin group (10 µg/mL) were 26.09±5.8 and 14.63±1.20 mm respectively (p<0.001). Growth inhibition zone for *P. aeruginosa* were observed in only one (35 mm) and three cases (12.5±3.4 mm) in the ziziphora essential oil group (0.5 µg/mL) and Nalidixic acid group (10 µg/mL) respectively.

Conclusion: The essential oil of ziziphora exhibited a favourable antibacterial effect on *S. aureus*; therefore, its extract can be used as an effective antimicrobial agent alone or in association with other antibiotics in the treatment of infections due to *S. aureus*.

Keywords: Allium sativum, Ziziphora clinopodioides, Minimum inhibitory concentration, Nalidixic acid, Vancomycin

INTRODUCTION

In recent years use of dental implants has increased significantly as an important treatment modality for the reconstruction of the dental system; however, concomitant with this increase in the use of dental implants, the incidence of peri-implantitis has increased, too [1,2]. In this context, the prevalence of peri-implantitis was reported to be around 20-40% [1,3,4].

Peri-implantitis is a multifactorial condition and a local inflammatory reaction, which is associated with the loss of supporting bone in the tissues around the implant [1]; the chief aetiologic agent for this condition is bacterial colonization of the pocket around the implant [4]. Two of these bacterial species are *S. aureus* and *P. aeruginosa* [1,5].

Although antibiotics are routinely used and are effective agents against this bacterial infection, due to the resistance of human pathogenic micro-organisms to these antibacterial agents [6] and the side effects and inefficacy of these medications, researchers are more interested in using herbal derivatives for the treatment of these conditions [7].

In addition, herbal medicines are usually more compatible with the body, and usually have no side effects; therefore, in chronic diseases when such medications are used for a long time, they are more appropriate. In addition, these medications are inexpensive and are readily available [7,8].

Ziziphora is a plant from the *Labitae* or *Lamiaceae* family and has four species in Iran, referred to as *Ziziphora clinopodioides, Ziziphora tenuior, Ziziphora capitata* and *Ziziphora persica* [9,10].

Garlic is a monocotyledones plant with the scientific name *Allium sativum* from the *Alliaceae* family [11,12].

These plants have been used for many centuries as food spices and as medications in traditional medicine for the treatment of different medical conditions [9-12].

Different studies have been carried out on the antibacterial properties of these plants and had conflicted results. In a study, the methanolic extract of ziziphora had a moderate antibacterial effect on *S. aureus* and *P. aeruginosa* [9]. In another study, the ziziphora oil exhibited antibacterial effects only against *S. aureus* at a concentration of 10 µg, with no antibacterial effects against *P. aeruginosa* [10]. But another study reported that the ziziphora oil at the same concentration above had antibacterial effects on both *S. aureus* and *P. aeruginosa* [7].

In a study, the aqueous and methanolic extracts of garlic had no effects on *S. aureus* and *P. aeruginosa* at a concentration of 2 mg/disk [13]. However, in another study, the aqueous and methanolic

extracts of garlic with MIC of 0.5-1 mg/mL exhibited antibacterial activity against both *S. aureus* and *P. aeruginosa* [14]. While in another study, the MIC for *S. aureus* was >50 mg/mL, with >125 mg/mL for *P. aeruginosa* [15].

Considering what was discussed above and given the conflicted results about antibacterial activity of ziziphora and garlic, which have usually had shortcomings, and relatively limited number of studies in this respect, especially about antibacterial activity of ziziphora, the present study was undertaken to evaluate and compare the antibacterial effects of the extracts of *Allium sativum* and *Ziziphora clinopodioides* essential oil on *S. aureus* and *P. aeruginosa*.

MATERIALS AND METHODS

In this in vitro study conducted at Tabriz University of Medical Sciences between March 2016 and July 2016, a standard strain of *P. aeruginosa* (PAOI) and two standard strains of *S. aureus* (ATCC822 and ATCC25923) were procured from Pasteur Institute of Iran. Eighteen clinical strains, nine strains of *P. aeruginosa* and nine strains of *S. aureus*, were procured too. These clinical strains had been isolated from wound and blood cultures of patients and stored at Imam Reza Hospital at Tabriz, Iran. Detailed informed consent form was obtained from the patients. All the ethical and the humanity considerations were observed and performed according to the Helsinki Declaration of 1975, as revised in 2008. This study was approved by the Ethics Committee of Tabriz University of Medical Sciences in Iran.

Garlic and ziziphora were procured at sufficient amounts from pharmaceutical companies and confirmed by the Herbarium of the Pharmaceutical Faculty of Tabriz University of Medical Sciences. Then the aqueous and methanolic extracts of garlic and ziziphora essential oil [7-9] were prepared and their effects on these bacterial species were evaluated. The standard Vancomycin antibiotic (10 µg/disk) for *S. aureus* and Nalidixic acid (10 µg/disk) for *P. aeruginosa* were used for all the strains separately as positive controls.

Preparation of the aqueous extract of garlic: After peeling the garlics (500 g), they were stored in a freezer for 24 hours and after creating cracks in each, they were placed at ambient temperature for one hour. Then a mixer was used to produce a garlic mixture with the use of sterile distilled water (1 mL of distilled water for each gram of garlic); then the garlic mixture was filtered through sterile gauze pieces and whatman paper filters. The resultant mixture was centrifuged in a refrigerator containing centrifuge machine at 500 rpm for 30 minutes in order to remove any impurities such as cellulose and cellular debris from the extract; the final result was a clear and yellow coloured solution. The 1/2, 1/4, 1/8 and 1/32 dilutions of garlic extract were prepared (220, 110, 55 and 27.5 mg/mL concentrations, respectively) and sterilized with 0.22 µm porous filters and stored in a refrigerator for subsequent uses [11,14,15].

Preparation of the methanolic extract of garlic: In order to prepare the methanolic extract of garlic at a concentration of 10 mg/mL, the peeled garlics were milled in association with methanol. The resultant mixture was immersed in 100 mL of 70% methanol and covered to prevent the evaporation of its volatile constituents. This raw extract was preserved in a revolving vibrator at 240-340 rpm for two days, followed by centrifugation at 8000 g for 10 minutes. Then the supernatant was collected and stored at -20°C so that it could be used for the evaluation of its antibacterial activity in culture media [11,14,15].

Preparation of ziziphora essential oil: In order to prepare the essential oil of ziziphora at concentrations of 2, 1, 0.5 and 0.25 µg/mL, a Clevenger apparatus and distillation technique with water were used. To this end, the dry plant of ziziphora was powdered thoroughly and boiled with distilled water in a flask for four hours. Then the power supply of the apparatus was cut off. Finally, half an hour after turning off the machine, its outlet was opened slowly so that the liquid could flow out. Due to the adhesion of the ziziphora extract to the glass walls of the machine, a solvent, called petroleum ether was used, which easily dissolves the extract in itself and then is sublimated leaving the extract behind [8-10].

Preparation of 0.5 McFarland suspension: In order to prepare the microbial suspension, a 24 hour culture of each bacterial species is required. Therefore, 24 hours before the test, a reserve culture (the strains recultured on nutrient agar) was inoculated into the steep nutrient agar culture medium and incubated at 37°C for 24 hours. Then the surface colonies of the culture medium were irrigated with normal saline solution and the microbial suspension was diluted with normal saline solution to achieve a suspension absorption rate equal to 0.5 McFarland solution absorption rate at 530 nm wavelength; in other words, the resultant suspension contained 1.5×10^8 CFU/mL.

Determination of antibacterial effects of ziziphora and garlic by determining of MIC and MBC and Disk diffusion methods: Antibacterial activity testing for determination of MIC and MBC for each bacterial strains was performed by the reference broth macro dilution method (CLSI, document M27-A2) [16].

Additional antibacterial test was performed only for ziziphora essential oil. For this purpose the technique of diffusion in agar was used with the use of disk. After immersion of a sterile swab in the microbial suspension, the swab was pressed against the tube wall to remove excess solution, followed by dragging it on all the surfaces of the Petri disk containing Mueller-Hinton agar culture. In the next stage a sterile tweezer was used to place disks impregnated with the ziziphora essential oil (with MIC of each bacterial strains) on the surface of agar plates with mild pressure. The petridishes were incubated at 37°C for 16-18 hours.

Then the diameters of growth inhibition zones were measured in mm using an accurate ruler. In addition, the Vancomycin standard antibiotic was used to inhibit *S. aureus* and Nalidixic acid was used to inhibit *P. aeruginosa* (10 µg/disk) as positive controls. All the tests were repeated three times. Data were analyzed with descriptive statistics (means ± standard deviations) and t-test for independent groups, using SPSS 17. Statistical significance was set at $P<0.05$.

RESULTS

None of the concentrations (from 1 to 1/32) prepared from the aqueous and methanolic extracts of garlic exhibited inhibitory effects on *S. aureus* and *P. aeruginosa*.

MIC and MBC for ziziphora essential oil against *S. aureus* were 0.25 and 0.5 µg/mL, respectively. Ziziphora essential oil with MIC of 0.25 µg/mL, in only one case exhibited inhibitory effects on *P. aeruginosa* [Table/Fig-1].

Based on the results of disk diffusion method, the ziziphora essential oil (with MIC for each bacterial strains) inhibited *S. aureus* bacterial species, with a mean diameter of growth inhibition zone of 26.09±5.83 mm. In the Vancomycin group (control group),

		SS[a] 1	SS 2	CS[b] 1	CS 2	CS 3	CS 4	CS 5	CS 6	CS 7	CS 8	CS 9
S. aureus	$MIC_{(\mu g/mL)}$	0.01	0.12	0.25	0.25	0.25	0.12	0.25	0.25	0.06	0.25	0.12
	$MBC_{(\mu g/mL)}$	0.25	0.25	0.5	0.5	0.5	-	-	0.5	-	0.5	0.5
P. aeruginosa	$MIC_{(\mu g/mL)}$	-	Not tested	-	-	0.25	-	-	-	-	-	-
	$MBC_{(\mu g/mL)}$	-	Not tested	-	-	-	-	-	-	-	-	-

[Table/Fig-1]: Antibacterial activity testing of ziziphora essential oil for determination of MIC and MBC for each bacterial strains.
[a] Standard Strain, [b] Clinical Strain

the mean diameter of growth inhibition zone for *S. aureus* was 14.63±1.20 mm. The t-test for independent groups showed that this difference was significant statistically (p<0.001). In relation to the effect of ziziphora essential oil on *P. aeruginosa*, in only one case the diameter of growth inhibition zone was 35 mm and in the other cases no growth inhibition zone was observed. In the control group of this bacterial species (Nalidixic acid), resulted in three cases of growth inhibition zones, with a mean size of 12.5±3.4 mm. In the remaining cases (seven cases), there was no growth inhibition zone [Table/Fig-2,3].

Mean Difference	-11.46
Standard Error Difference	1.795
95% CI	-15.204 to -7.716
Test statistic t	-6.386
DF	20
Significance level	p < 0.001

[Table/Fig-2]: Results of t-test for equality of mean diameter of growth inhibition zone between ziziphora essential oil and vancomycin.

		SS[a] 1	SS 2	CS[b] 1	CS 2	CS 3	CS 4	CS 5	CS 6	CS 7	CS 8	CS 9	Total
S. aureus	$MIC_{(\mu g/mL)}$	0.01	0.12	0.25	0.25	0.25	0.12	0.25	0.25	0.06	0.25	0.12	0.25
	Diameter of Growth Inhibition $zone_{(mm)}$	12	30	25	30	30	23	24	26	35	27	25	26.09±5.83
	$Vancomycin_{(mm)}$	14	14	14	15	12	15	15	16	14	16	16	14.63±1.20
P. aeruginosa	$MIC_{(\mu g/mL)}$	-	Not tested	-	-	0.25	-	-	-	-	-	-	-
	Diameter of Growth Inhibition $zone_{(mm)}$	0	Not tested	0	0	35	0	0	0	0	0	0	-
	$Nalidixic\ acid_{(mm)}$	10	Not tested	0	9	15	0	0	0	0	0	0	-

[Table/Fig-3]: Results of disk diffusion method for antibacterial activity testing of ziziphora essential oil for each bacterial strains.
[a] Standard Strain, [b] Clinical Strain

DISCUSSION

Antibiotics are invaluable medications for the treatment of many human diseases; however, excessive use of these medications results in microbial resistance. Therefore, researchers have prioritized research on different parts of plants with medicinal uses in order to discover new drugs with herbal origins [6,12]. In addition, variations in geographic and climatic conditions in Iran have made Iran a country with a rich and diverse source of plant species [8-10]. In the present study, two plants indigenous to East Azerbaijan Province in Iran, i.e., garlic and ziziphora, were evaluated in relation to their antibacterial activity against *S. aureus* and *P. aeruginosa*. The results showed that the ziziphora essential oil had antibacterial activity against *S. aureus* with MIC and MBC of 0.25 and 0.5 µg/mL. In addition, the ziziphora extract exhibited better antibacterial activity against *S. aureus* compared to Vancomycin; however, *P. aeruginosa* was resistant to this extract. In a study by Shahla SN et al., ziziphora essential oil had no effects on *P. aeruginosa*, even at a concentration of 4 µg/m; however, it exhibited antibacterial activity against *S. aureus*. The MIC and MBC were 0.5 µg/mL in that study [8].

Salehi P et al., showed that the methanolic extract of ziziphora had a moderate antibacterial activity against *S. aureus* and *P. aeruginosa* [9]. In a study by Sonboli A et al., ziziphora oil, at a concentration of 10 µg, exhibited antibacterial activity only against *S. aureus*, with no antibacterial activity against *P. aeruginosa* [10]. However, in a study by Ozturk S et al., ziziphora essential oil exhibited antibacterial activity against both *S. aureus* and *P. aeruginosa* at the same concentration [7]. In addition, in the present study, the aqueous and methanolic extracts of garlic had no antibacterial activity against *S. aureus* and *P. aeruginosa*. In a study by Bakht J et al., the aqueous and methanolic extracts of garlic exhibited no antibacterial activity against *S. aureus* and *P. aeruginosa* at a concentration of 2 mg/disk [13], while in a study by Gull J et al., the aqueous and methanolic extracts of garlic exhibited antibacterial activity against both *S. aureus* and *P. aeruginosa* at an MIC of 0.5-1 mg/mL [14]. In addition, in a study by Abubakar E-mM et al., aqueous and ethanolic extracts of garlic exhibited antibacterial activity against *S. aureus* and *P. aeruginosa*, with MICs of 50 mg/mL for *S. aureus* and 125 mg/mL for *P. aeruginosa*. In addition, the aqueous extract had a stronger antibacterial activity [15]. Deresse D, too, reported that garlic had a stronger concentration dependent antibacterial effect on *S. aureus*, i.e., the antibacterial activity increased with an increase in concentration [17].

LIMITATION

The garlic and ziziphora used in the present study were grown in East Azerbaijan Province region and given diversities in the species of plants in different regions, it is suggested that plants from other regions, should also be used in similar studies. In addition, it is possible that the behaviors of extracts are different in vivo and in vitro; as a result, the antibacterial activities of these plants should be evaluated in vivo, too. The differences in the results might be attributed to differences in oil extraction methods, in the microbial strains, in microbial tests and in the quality and biologic conditions of the plants in different regions. In this context, it appears it is necessary to carry out further studies with different concentrations of plant extracts and with the use of different microbial tests and oil extraction techniques.

CONCLUSION

The results of the present study showed that the essential oil of ziziphora had favourable antibacterial activity against *S. aureus*, with no such effect on *P. aeruginosa*. So Ziziphora can be used in some liquids, such as buttermilk as antimicrobial flavours. Also, it can be used in manufacturing of mouthwashes and toothpastes. In addition, the aqueous and methanolic extracts of garlic had no antibacterial effects on *S. aureus* and *P. aeruginosa*.

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