Original Article

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An Evaluation of the Inhibitory and Synergistic Effects of Alcoholic Extract of Stachys Byzantina on Standard Strains under in vitro Conditions

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ABSTRACT

BACKGROUND AND OBJECTIVE: Plants of mint family with their antimicrobial properties are used to treat infectious diseases. This study aims to analyze the inhibitory effects as well as synergistic and antagonistic effects of alcoholic extract of Stachys byzantina on five standard strains under in vitro conditions.

METHODS: In this experimental study, after gathering, Stachys byzantina was dried in the shade far from direct light. The maceration technique was used to prepare alcoholic extract. The effect of extract was analyzed based on disk diffusion technique using concentrations of 62.5, 125, 250 and 500 mg/ml and about 20 µl of the extract was tested. The microbial strains were purchased in the form of lyophilized strains. In order to determine MIC and MBC, microdilution method was used. Moreover, the synergistic effect of the extract was analyzed with antibiotic.

FINDINGS: In this study, the greatest effect of the extract was observed to be on *Staphylococcus epidermidis* at concentration of 500 mg/ml (23±1.7 mm). However, this effect on *Staphylococcus aureus*, *Streptococcus Group A* and *Pseudomonas aeruginosa* was found to be 18.4±1.8 mm, 14.4±2.4 mm and 11.7±2.4 mm, respectively. In addition, as the concentration of the extract increased, the inhibition zone diameter increased significantly ($p \leq 0.05$). Synergistic activity of the extract was shown with gentamicin, erythromycin and penicillin with *Pseudomonas aeruginosa*. The phytochemical results indicated the presence of alkaloid, carbohydrate, tannin, terpenoid, steroid, saponin and flavonoid.

CONCLUSION: It seems that the extract of Stachys byzantina has inhibitory effect, either alone or in combination with other antimicrobial agents and improves the performance of some of the antibiotics.

KEY WORDS: *Alcoholic extract, Stachys byzantina, Phytochemical compound, Antimicrobial activity, Synergistic effect.*

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Introduction

As the use of antibiotics and antimicrobial drugs increased in recent years, we observe increasing prevalence of pathogenic strains that are resistant to several antibiotics (1). Due to the increased antibiotic resistance, investigations for finding materials with more beneficial effects and less side effects has increased (2). Currently, most of the modern drugs are chemical drugs, whereas about 100% of pharmaceutical products have herbal origin (3). The use of native medicinal plants that are effective in preventing and treating diseases by synthesis of secondary and active ingredients in addition to ecological adaptations has gained an unprecedented status in medical sciences (4). Paying attention to plants with antimicrobial effects can solve to common problems of using antibiotics, particularly the side effects (5). *Stachys byzantine* is one of these medicinal plants with gramineous, stable structure, with plenty of fluff, villous, woolen and long fibers. This plant is the native of Turkey, Armenia and Iran and its dispersion is in different regions of Iran, particularly northern regions, Alborz province and north – west of Iran (6–8).

The Lamiaceae is one of the biggest herbal families with global dispersion. This family with around 200 genera and 2000 to 5000 species is a widespread aromatic plant with medicinal properties. The *Stachys* genus includes about 300 species. *Stachys* was taken from the term Chistets, meaning wound cleanser and healer, indicating wide use of the essences and extracts of this genus as antiseptic and healer of skin diseases (9). Plants of Lamiaceae family with their antimicrobial properties are used in the treatment of infectious diseases, particularly diarrhea, fever, colds, and oral hygiene (10, 11).

In traditional medicine resources of Iran and other countries, several therapeutic effects including hypnotic, sedative, lowering blood pressure, wound healing, stop the bleeding, increase biliary secretion, anti-cough and anti-sore throat, anti-kidney infection and pain reliever effects were attributed to various species of *Stachys* genus (12, 13). In addition, several other studies have mentioned its antioxidant, anti-bacterial, anti-inflammatory and anti-cancer effects, most of which mentioned the quality and quantity of the terpenoid, phenolic and flavonoid ingredients in the essence and extract of various species of *Stachys* genus (14 – 18). Since the vegetation diversity and the quality of active ingredients in the plants of each region are affected by ecological stresses and even the phenology of the species of that region (19, 20), the approach of International Community Health is toward identification of the ecological needs of the species in natural habitats, ethnofarmacology, extraction of the active ingredients and above all, investigating their antioxidant properties in order to revive, cultivate and extract the natural ingredients and ultimately, produce natural and low-risk drugs (21, 22).

Since Gilan Province has diverse habitats of *Stachys Byzantina* because of its special ecologic condition, which are highly important for the traditional medicine of the region, the present study was conducted to analyze the inhibitory and synergistic effects of alcoholic extract of *Stachys byzantine*, collected from Heyran Defile region, on the standard strains under in vitro conditions.

Methods

Plant collection and extraction: In this experimental study, *Stachys byzantina* leaves were collected from Heyran Defile, and were dried in the shade far from direct light. Then, the dried leaves were powdered using Waring mill. Then, in order to prepare the alcoholic extract, 10 g powder was poured into 50 ml ethanol 96% (Merck, Germany) and was kept at lab temperature for 24 – 72 and was stirred every couple of hours. Then, the supernatant was removed and was concentrated in vacuum using Maceration method (23). In order to standardize the procedure and the reliability, and to compare and assess the antimicrobial effect of the extract extracted by distilled water and ethanol, the dry weight of the extract was determined. First, weight of the tube was measured by a sensitive digital scale (0.0001) and then, 1 ml alcoholic extract was poured into the tube. Then, the content of the tube was dried at room temperature. Finally, the tubes were weighed again and after subtracting the weight of empty tubes, the mean dry weight of ethanoic and aqueous extracts was determined (24).

Preparation of microbial strains: In this research, the tested microbial strains included *Staphylococcus aureus* ATCC 5923, *Staphylococcus epidermidis* ATCC 12228, *Streptococcus group A* ATCC 19615, *Pseudomonas aeruginosa* ATCC 27853 and *Escherichia coli* ATCC 25922, prepared from the center for the collection of industrial and infectious bacteria and fungi in Iranian Research Organization for Science and Technology. The microbial suspension was prepared using 0.5 McFarland standard (25, 26).

Analysis of the antimicrobial effect of the extract: Agar diffusion and disk diffusion methods were used to

analyze the antimicrobial. The bacteria were cultured on Mueller Hinton Agar for 24 hours. A suspension with 0.5 McFarland concentration was prepared in Mueller Hinton Broth (Merck – Germany). Then, 1 ml microbial suspension was cultured on the surface of the plate containing Mueller Hinton Agar and then the blank disks were placed on the surface of the plate infected to the bacteria. 20 µl of different concentrations of the diluted extract was poured on the disks. 24 hours after incubation at 37 ºC, the inhibition zone diameter was measured. Dilution of the extracts was performed using dimethyl sulfoxide (DMSO) 10%. Concentrations of 62.5, 125, 250 and 500 mg/ml extract were used in this study. Chloramphenicol antibiotic was used as the positive control (27).

Based on agar diffusion method, 2 wells with a diameter of 7 mm were created in Mueller Hinton Agar. 0.5 McFarland of the bacterial suspension was cultured on the medium. The control antibiotic was poured in one of the wells and 62.5, 125, 250 and 500 mg/ml extract was poured in another well. Then, the plates were incubated for 24 hours at 37 ºC. The results were recorded based on measuring the diameter of inhibition zone by antibiotic and herbal extract (20, 21, and 28). The Tests were repeated at least 3 times. Microdilution method was used to determine the minimal inhibitory concentration (MIC) and minimum bactericidal concentration (MBC); 0.5 McFarland of the bacterial suspension was prepared in Tryptic Soy Broth (TSB). Dilution series equal to 6.25, 25.5, 50,

100 and 200 mg/ml extract were prepared and 70 µl of them were added to 96 – well microplate that previously contained 70 µl bacterial suspension with 0.5 McFarland opacity. Then, similar tests were performed for positive control (the medium containing bacteria and no extracts) and negative control (the medium without bacteria). Then, the microplates were incubated for 24 hours at 37 ºC. Lowest dilution of the extract, in which no opacity was observed, was reported as the minimal inhibitory concentration. Of all the wells in which the growth of bacteria was completely stopped, plates containing the Muller Hinton Agar culture medium were cultured and were incubated for 24 hours at 37 ºC. Concentrations that were free of bacterial growth, minimum bactericidal concentration was reported (29, 30).

Analysis of the combined effect of herbal extracts and antibiotic disks: To determine the combined effect of herbal extracts and antibiotic disks, disk diffusion method was used. Investigating the synergistic and antagonistic effects of the extract was done using sub-MIC concentrations. The used dilution was equal to 1:2 to 1:4 MIC. The sub-MIC concentration of the extract with 1:2 MIC dilution was added to Mueller Hinton Agar and used as test plate. The bacterial suspension with a concentration of 1.5×10^8 cfu/ml (equal to 0.5 McFarland) was lawn cultured on agar medium containing sub-MIC concentrations by swab and the antibiotic disks were placed on the surface of agar. Then, the inhibition zone diameter of disks was recorded after 24 hours at 37 ºC. To analyze the effect of the extract on the antibiotic, Penicillin (30 µg), gentamicin (10 µg) and erythromycin (15 µg) antibiotic disks (Padtan Teb Co.) were used.

Identifying the nature of organic compounds: To identify the organic compounds in the sample of dried powder, maceration method was used and the organic compounds in the herbal sample were analyzed using the available standard methods (31 – 34).

Statistical analysis: The inhibition zone diameter and the antimicrobial effect of the extract were analyzed using SPSS ver.14, one-way ANOVA and supplementary tests (Tukey HSD) and $p<0.05$ was considered significant.

Results

Results of this study demonstrated that the antimicrobial effect of the alcoholic extract of *Stachys byzantine* is dose-dependent and as the concentration increases, the inhibition zone diameter increases significantly ($p\leq0.05$). This study showed that the type of bacteria and the concentration of extract affects the inhibition zone diameter; the inhibition zone diameter at 500 mg/ml alcoholic extract was 23.2±1.7, 18.4±1.8, 14.4±2.4 and 11.7±2.4 for *Staphylococcus epidermidis*, *Staphylococcus aureus*, *Streptococcus group A* and *Pseudomonas aeruginosa*, respectively. Lowest level of susceptibility was observed in regard with *Escherichia coli* (5±2.1) (Table 1). Moreover, as the concentration of the extract increased, its antibacterial activity against the bacteria of the test increased. There was a significant difference between various concentrations in *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Streptococcus group A* compared with negative control ($p<0.05$). The inhibition zone diameter was not significantly different regarding *Pseudomonas aeruginosa* and the antibacterial effects of difference concentrations of the extract was almost similar on this bacterium (Table 2).

Table 1. The mean inhibition zone diameter of the selected bacteria in the presence of alcoholic extract

Extract concentration Microorganism	62.5 mg/ml	125 mg/ml	250 mg/ml	500 mg/ml	p-Value
Staphylococcus aureus	11±0.9 [a]	12.2±1.7 [b]	14.5±2.3 [c]	18.4±1.8 [d]	0.045
Staphylococcus epidermidis	18±2.1 [a]	20.2±1.7 [bc]	21.5±1.7 [c]	23.2±1.7 [d]	0.025
Streptococcus group A	7.5±1.9 [a]	10.2±2.7 [b]	12.5±2.9 [c]	14.4±2.4 [d]	0.015
Pseudomonas aeruginosa	8.2±0.4 [a]	9±1.8 [ab]	10.5±2.9 [bc]	11.7±2.4 [d]	0.052
Escherichia coli	2±0.5 [a]	2.8±0.5 [ab]	3.2±0.9 [b]	5±2.1 [c]	0.96

Dissimilar letters indicate a significant difference ($p \leq 0.05$).

Table 2. The results of minimal inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) in different concentrations of *Stachys Byzantina* extract using Microdilution broth method based on mg/ml

Microorganism	MIC (mg/ml)	MBC (mg/ml)
Staphylococcus aureus	12.5	25
Staphylococcus epidermidis	50	100
Streptococcus group A	50	100
Pseudomonas aeruginosa	50	100
Escherichia coli	100	100

Results indicate that the highest inhibitory and antibacterial effect of the alcoholic extract of this plant was on *Staphylococcus aureus*. The extract of *Stachys Byzantina* increased the antibiotic effect of gentamicin, erythromycin and penicillin against *Pseudomonas aeruginosa*. In addition, the use of this extract along with gentamicin, erythromycin and penicillin antibiotics increased the antagonistic properties of the selected bacteria. The interaction between the extract and gentamicin antibiotic revealed no effect on the growth of *Staphylococcus aureus*. In addition, combined use of penicillin and the extract had antagonistic effects against *Staphylococcus epidermidis*.

Synergistic effect of extract on penicillin and erythromycin against *Streptococcus group A* and *Pseudomonas aeruginosa* was shown. However, it had no effect on *Escherichia coli* regarding the antibiotic performance (Table 3). The results of phytochemical experiments are illustrated in Table 4.

Table 3. Evaluating the synergistic and antagonistic effects of alcoholic extract of *Stachys byzantina* along with gentamicin, erythromycin and penicillin antibiotics on selected bacteria based on mm

Antibiotics Microorganism	Erythromycin A(AE)	Gentamicin A(AE)	Penicillin A(AE)
Staphylococcus aureus	15.21	15.13	15.15
Staphylococcus epidermidis	19.25	19.20	19.12
Streptococcus group A	12.20	12.16	12.20
Pseudomonas aeruginosa	11.22	11.35	11.21
Escherichia coli	0	0	0

A: The inhibition zone diameter for the target antibiotic. AE: The inhibition zone diameter for the antibiotic and the extract

Table 4. Phytochemical composition of alcoholic extract of *Stachys byzantine*

Organic composition	
Alkaloids	+
Amino acids	-
Carbohydrates	+
Flavonoids	+
Glycosides	+
Tannins	+
Phenol	-
Terpenoids	+
Steroids	+
Saponin	+
Volatile oils	-

(+) Present, (-) absent

Discussion

Results of this study demonstrated that the alcoholic extract of this plant at 500 mg/ml concentration has considerable antibacterial effects on *Staphylococcus epidermidis*, however, it does not have antibacterial effects at lower concentrations. The presence or absence of a significant difference in the inhibition zone diameter at various concentrations can be attributed to the level of active ingredients in the extracts and we may conclude that the inhibition zone diameter increases with increased concentration.

Regarding the inhibitory effects of the extract on the growth of microorganisms, this research is consistent with the report by Skaltsa et al., however, regarding the fact that the ethanolic extract had no effect on *Pseudomonas aeruginosa* and *Escherichia coli*, the two studies were not consistent (13). This may be related to higher level of active ingredient in the ethanolic extracts. Overall, gram positive bacteria are more sensitive to herbal extracts than gram negative bacteria (35). This may be attributed to the intrinsic tolerance of gram negative bacteria as well as the nature of plants and their compositions. Different studies have demonstrated that the cell wall of the gram positive bacteria are more sensitive to many antibiotics, antimicrobial chemicals (36) and even many herbal medicines (37), compared with gram negative bacteria. Considering the differences in the antimicrobial effects of theses microorganisms, one can conclude that the difference in the studied strains is very important. A study by Dulger et al. demonstrated that the methanolic extract of various species of *Stachys* had significant effects on several bacteria including *Escherichia coli, Staphylococcus aureus, Klebsiella pneumoniae, Pseudomonas aeruginosa, Proteus vulgaris, Bacillus cereus, Mycobacterium smegmatis* and *Listeria monocytogenes* (38).

Moreover, in a study about the antibacterial effects of the essence of *S.chrysantha* and *S.cadina*, it was shown that the studies plants had suitable antibacterial effects on gram positive bacteria *Golden Staphylococcus* and *Streptococcus Epidermis* and gram negative bacteria *Escherichia coli, Klebsiella pneumoniae* and *Pseudomonas aeruginosa*. Studies conducted on various species of *Stachys* genus indicate its antibacterial effects (13). MIC results demonstrated that the ethanolic extract of this plant for *Staphylococcus aureus, Staphylococcus epidermidis, Streptococcus group A* and *Pseudomonas aeruginosa* was between 12.5 to 100 mg/ml; therefore, we may conclude that highest resistance belonged to the gram negative bacteria *Escherichia coli*. In this study, the antibacterial effects of some common antibiotics were testes on the standard bacterial strains. Results demonstrated that the alcoholic extract of *Stachys byzantine* only has antagonistic effects regarding the antibiotic function of penicillin against *Staphylococcus epidermidis* and has no effect regarding the function of gentamicin against *Staphylococcus aureus*, however, increases the susceptibility of *Pseudomonas aeruginosa* to penicillin, gentamicin and erythromycin. The extract of the plant had synergistic effect regarding the penicillin and erythromycin function against *Streptococcus group A* and *Pseudomonas aeruginosa* and had no effect on the function of other antibiotics in these two strains.

Since bacterial resistance is increasing and resistance is easily transferred from resistant bacteria to susceptible bacteria in various ways and causes resistance to the commonly consumed antibiotics, *S.byzanthina* extract, similar to other derivatives of medicinal plants with antibacterial properties, can be used as an alternative or complementary composition for curing bacterial infections (39).

Considering the problems caused by antibiotic resistance in the treatment of infections, it seems necessary to find a solution for this problem. On the other hand, food and supplements that are used by different people and patients may have strengthening or inhibitory effects on the function of antibiotics and the active ingredients of the medicinal plants may help to return the susceptibility of the bacteria that lost their susceptibility to the antibiotics in the current conditions (40). The results of phytochemical experiments demonstrated that the ethanolic extract of this plant contain Alkaloids, carbohydrates, tannins, terpenoid, steroids, saponins and flavonoids. Researches in recent years have indicated that flavonoids have inhibitory effects on a wide range of microorganisms; this property may be due to binding to extracellular proteins, binding to the cell wall of the bacteria or disruption of the membrane of bacteria (41).

Results of this study demonstrated that the extract of *Stachys Byzantina* has antibacterial effects and this study suggests the possibility of using this plant as an antibacterial material, particularly in cases of drug resistance. Moreover, this extract has synergistic effects with several antibiotics including penicillin and erythromycin and can improve the effect of these drugs, which provides the possibility of being used as

complementary drug. Furthermore, we need to conduct further researches under in vivo conditions to assess the effective concentration and the active ingredients of this extract on target bacteria and clinical strains and the side effects at these concentration, so that we can introduce this extract as a new antibacterial drug to the world after taking the supplementary steps.

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