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In-Vitro Antibacterial Properties of Sage (Salvia officinalis) Ethanol Extract against Multidrug Resistant Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa and Klebsiella pneumoniae

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Abstract

***Background*:** Due to excessive consumption of synthetic drugs, drug resistance rate of pathogenic bacteria is increasing and the need to find new compounds is necessary. The aim of this study was to investigate the antibacterial effect of ethanol extract of, sage to the four species of common pathogenic bacteria resistant to multiple drugs in vitro such as: *Staphylococcus aureus* (50 strains), *Escherichia coli* (50 strains), *Pseudomonas aeruginosa* (50 strains) and *Klebsiella pneumonia* (50 strains).

***Materials and Methods*:** In this experimental study, antibacterial effect of ethanol extract of sage plants on the development of multi-drug resistant bacteria was performed by well diffusion at concentrations of 50, 400, 100 mg/mLand microdilution method.

***Results*:** Ethanol extracts of sage in well diffusion method showed significant inhibitory effect on the growth of isolated bacteria. The results indicate the inhibitory effects of ethanol extract of sage with MIC (Minimum Inhibitory Concentration)=18.75 mg/mL for *S. aureus*, MIC=26.56 mg/mL for *E. coli*, MIC=33.75 mg/mL for *P. aeruginosa* and with MIC=31.25 mg/mL for *K. pneumoniae*.

***Conclusion*:** In relation with the antibacterial effect of ethanol extracts of Sage on the multi-drug resistant bacteria the use of herbs as an alternative to antibiotics after pharmacological studies, for treatment recommended.

Introduction

Despite the treatment of infectious disease in human history have always considered and in this respect, a lot of efforts to eradicate the factors of this disease has been done but changes in the behavior of microorganisms in various aspects, has led to eradication of many of these microbial agents not associated with success and even with the emergence and spread of new strains of diseases in the range of new features to be added. One important aspect of this is the emergence of resistant strains of bacteria so that in spite of the efforts has been done to produce broad spectrum antimicrobial substances. Today proportional to the progress of science and technology, medicinal plants should be used. Due to bacterial resistance to common antimicrobial drugs and less toxic side effects of natural remedies such as applications of sage extract on the face of bacteria under in vitro condition looks valuable [1-3]. Methicillin-resistant *Staphylococcus aureus* is a major nosocomial and community-acquired infections that today has gained multiple resistances to a wide range of antibiotics, including beta-lactam, the aminoglycosides, the tetracyclines, and macrolides. Therefore today a limited number of anti-staphylococcal antibiotics such as vancomycin, ticoplanin and linozolide are available [4, 5]. *Escherichia coli* and *Klebsiella pneumoniae* are infections clinically important bacteria that especially isolated from patients in hospital. Recently, drug resistance to multiple antibiotics, has considered [6].

Pseudomonas causes most of infections in the hospital. Many of medical instruments are able to transmit this bacterium. *Pseudomonas aeruginosa* is an opportunistic bacterium and is resistant to most antibiotics [7]. Salvia officinalis is a plant of the mint family, and it is a flowering plant, angiosperm, a dicotyledonous, joined petals, order Toby Floral, suborder mangroves, family mint and gender is salvia. Salvia is a perennial plant, evergreen, with woody stems, green leaves and violet blue flowers. The leaves are gray-green in color and in upper surface have wrinkles and in lower surface are almost white with much shorter soft fluff. Some species of the genus Salvia like salvia officinalis has a significant therapeutic effect. It was effective in lowering blood sugar, relaxation and so on. Compounds that have been identified in this plant contain Thujone, 1, 8 cineole, Borneol, Borneol acetate, sesquiterpene, tannins and phenolic acids. Alkan and abd- almageed also showed that, the main components of the essential oil of Salvia officinalis contains alpha - Thujone (24.88%), camphor (16.03%), 1, 8 cineole (9.97%), respectively. Salvia officinalis antimicrobial activity is due to the presence of 1, 8 cineole, alpha - Thujone and camphor [8, 9]. Thus, according to the various antimicrobial activities, in other

studies should be done to determine the range of antimicrobial effect of this herbal drug. In this study, the antibacterial activity of sage ethanol extract was evaluated through well diffusion and microdilution method against multi drug resistant bacteria.

Materials and Methods

This experimental study was conducted at Microbiology Research Laboratory, Department of Microbiology, Islamic Azad University of Falavarjan in 2012.

Tested Strains: This clinical study was done on 200 samples including 50 samples of MDR (Multi Drug Resistant) *S. aureus*, 50 samples of MDR *E. coli*, 50 samples of MDR *P. aeruginosa*, 50 samples of MDR *Klebsiella pneumoniae* isolated from human infections, including urinary tract infections, respiratory infections, ear infections, skin infections, wounds, abscesses, and sputum and the isolate was achieved from three hospitals in Isfahan (al-Zahra, Shariati and Gharazi). After collecting samples for diagnosis of bacterial genera and species, biochemical tests were used.

Antibiotics susceptibility test: The test was done by using the agar disk diffusion method or the Kirby-Bauer method as recommended by the National Committee for Clinical Laboratory Standard (NCCLS) on bacterial strains of *S. aureus*, *E. coli*, *P. aeruginosa* and *K. pneumoniae* were resistant to more standard drugs. The antibiotic discs used in this test are: erythromycin, ampicillin, amoxicillin, ampicillin, amikacin, imipenem, penicillin, piperacillin, tobramycin, tazocin, tetracycline, gentamicin, cefataxime, cephalexin, ceftazidime, cefixime, ciprofloxacin, carbenicillin, clindamycin, co-amoxiclav, cloxacillin, co-trimoxazole, nitrofurantoin, vancomycin. To perform this test, the accurate identification of species of bacteria, *S. aureus*, *P. aeruginosa*, *K. pneumoniae*, *E. coli*, colonies of strains tested in Mueller Hinton broth (MHB) and then suspended in standard 0.5 McFarland was conducted to compare. If turbidity ensures it matches perfectly sterile cotton swab using four different bacteria in the culture medium Mueller Hinton agar (MHA) has cultivated and antibiotic discs used in sterile conditions with respect to the distance of two centimeters away from each other placed on medium Mueller Hinton agar plates. After incubation for 18 to 24 h at 37ºC, the results obtained with the standard provided by the National Committee for Clinical Laboratory Standards were compared to susceptible and resistant [10].

Preparation of ethanol extract of sage plant: Sage plant (leaves and stems) from the agriculture and natural resources research center of Isfahan was prepared and dried powder was then carried by the electric crusher. Sage ethanolic extract of the plant was carried out by solvent ethanol using soxhlet apparatus. At first, 50 g of plant powder was weighed by the scales and were placed in the middle of the filter paper and then was placed in a soxhlet tank, then 250 mL of ethanol was poured into the soxhlet flask and extraction was done for 8 hours. During this time the extract obtained was poured in sterilized glass plates were placed at room temperature to dry. The extract can be stored until use in the refrigerator at 4ºC [11].

Antibacterial effect of ethanol extract of Salvia by well diffusion method: To evaluate the antimicrobial activity of ethanol extract of salvia leaf and stem concentrations of 50, 400, 100 mg/mL of ethanol extract was prepared by solution of DMSO 10%. In order to perform well diffusion method, 24 hour culture of *S. aureus*, *E. coli*, *P. aeruginosa* and *K. pneumoniae* with turbidity equivalent to a standard 0.5 McFarland were prepared in MHB medium. The turbidity of the suspension for greater precision in absorption spectrophotometer 0.8 is set. Then suspension obtained cultivated in 4 directions by using sterile swabs on MHA medium. After 0.5 hours a sterile Pasteur pipette using to created Wells with a diameter of 6 mm with respect to the distance 2.5 cm apart on the culture. Hundred µL of different concentrations of ethanol extract prepared from each well were added separately. DMSO 10% of the solution as negative control and 30 µg vancomycin disk antibiotics for *S. aureus* and 10 µg gentamicin disks antibiotics for bacteria, *E. coli*, *P. aeruginosa* and *K. pneumoniae* were used as positive controls. After 24 h incubation at 37ºC, the diameter of inhibition zone around each well was determined using a ruler. In order to confirm the results, experiments were repeated at 3 times [12].

Antibacterial effect of ethanol extract of Salvia by micro dilution method: Dilutions of 400, 200, 100, 50, 25, 12.5 mg/L of ethanol extract of Salvia were prepared in sterile tubes. Then 100 µL of each dilution by using a sampler to the higher concentrations less were added to 1 to 6 sterile U-shaped, Round-bottom 96-well micro plate then 100 microliter of 24 h culture of bacteria *S. aureus*, *E. coli*, *P. aeruginosa* and *K. pneumoniae* with a turbidity equivalent to a standard 0.5 McFarland were separately added to the wells containing 100 µL of the extract. In well No. 7, 100 µL of bacterial suspension with a turbidity equivalent of the bacteria standard 0.5 McFarland Separately with 100 µL MHB medium as a positive control and well No. 8, 200 µL of sterile MHB medium was added as a negative control. Optical density of wells in micro plate ELISA reader at a wavelength of 630 nm was read. Then Micro plate was incubated for 24 h at 37ºC. After 24 h of re-absorption of light at a wavelength of 630 nm was determined by ELISA reader. Compared before and after incubation the optical density of each well and also check the wells, turbidity the lowest concentration of the test substance in the wells was related to no turbidity was observed in the concentration as the minimum inhibitory concentration (MIC) is considered. In order to determine the minimum bactericidal concentration (MBC) of the tested extracts, from the MIC concentration Wells and three wells of further concentration which no detectable turbidity by sterile cotton swab was cultured on MHA medium and was incubated for 24 h at 37ºC. After 24 hours of incubation, the plates were examined for bacteria growth. Concentration of tested extract on solid medium that no

growth of tested bacteria was observed, as MBC was considered. In order to confirm the results, experiments were repeated at 3times [13].

Statistical Analysis: For statistical analysis of the data were performed using statistical software SPSS-17 and Kruskal-Wallis test and their difference was calculated in a significant level ($p \leq 0.001$).

Results

Antibiogram test results were shown on table 1, 2, 3 and 4. According to studies carried out in this study (Table 5) the ethanol extract of the leaves and stems of Salvia at concentrations of 50, 100, 400 mg/mL had anti-bacterial effects against bacterial strains of multidrug-resistant *S. aureus*, *E. coli*, *P. aeruginosa* and *K. pneumoniae*. In this study, ethanol extract of Salvia on multidrug-resistant Gram-positive *Staphylococcus aureus* is the most effective. 3 bacterium *Escherichia coli*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* have shown sensitivity to ethanol extract of the leaves and stems of sage. All extracts showed dose dependent activity which increases with increase in concentration. The values of MIC and MBC of ethanol extract of sage have been presented in table 6 against the referred bacteria. As the table shows, ethanol extract has prevented from the growth of bacteria such as *S. aureus*, *E. coli*, *P. aeroginosa* and *K. pneumonia*.

Table 1. Percentage of antibiotic sensitivity and resistance of S.aureus

Antibiotic	Sensitive	Resistant
Erythromycin	12	88
Amoxicillin	64	36
Penicillin	24	76
Cefotaxime	54	46
Gentamicin	64	36
Clindamycin	34	66
Vancomycin	64	36
Tetracycline	16	84
Cloxacillin	20	80
Cefalexin	54	46
Ciprofloxacin	72	28
Imipenem	66	34

Table 2. Percentage of antibiotic sensitivity and resistance of E.coli

Antibiotic	Sensitive	Resistant
Erythromycin	4	96
Tobramycin	58	42
Co-Amoxiclav	36	64
Cefotaxime	56	44
Amoxicillin	36	64
Gentamicin	30	70
Co-Trimoxazole	12	88
Vancomycin	5	95
Cefixime	48	52
Cloxacillin	21	79
Cefalexin	52	48
Ciprofloxacin	46	54
Ampicillin	8	92

Table 3. Percentage of antibiotic sensitivity and resistance of K. pneumoniae

Antibiotic	Sensitive	Resistant
Erythromycin	8	92
Tobramycin	40	60
Co-Amoxiclav	20	80
Cefotaxime	16	84
Amoxicillin	12	88
Gentamicin	48	52
Co-Trimoxazole	40	60
Vancomycin	6	94
Cefixime	12	88
Cloxacillin	3	97
Cefalexin	16	84
Ciprofloxacin	40	60
Ampicillin	12	88

Table 4. Percentage of antibiotic sensitivity and resistance of P. aeroginosa

Antibiotic	Sensitive	Resistant
Tobramycin	36	64
Ampicillin	26	74
Gentamicin	54	46
Co-Trimoxazole	38	62
Imipenem	16	84
Ceftazidime	40	60
Nitrofurantoin	30	70
Amikacin	34	66
Cefotaxime	38	62
Carbenicillin	24	76
Tazocin	50	50
Piperacillin	36	64
Cefalexin	42	58
Ciprofloxacin	22	78

Table 5. Antibacterial activity of Salvia officinalis ethanol extract against tested bacteria measured in millimeter

Bacteria	50 mg/mL	100 mg/mL	400 mg/mL	Control-	Control+
S. aureus	9.0133	11.7467	13.4000	-	11.7200
E. coli	4.8533	6.6133	9.3200	-	5.5200
P.aeruginosa	3.8667	7.0267	9.1467	-	8.6267
K.pneumoniae	2.0800	3.4933	6.1333	-	9.7200

Table 6. MIC and MBC of sage ethanol extracts against tested bacteria (mg/mL)

Bacteria	Number	MIC	MBC
S. aureus	50	18.75	37.5
E. coli	50	26.56	53.125
P. aeruginosa	50	33.75	67.5
K. pneumoniae	50	31.25	51.25

Also, by increasing the concentration of ethanol extract, the inhibition zone increased ($p \leq 0.001$). The results determined that in tested bacteria, there was a significant difference ($p \leq 0.001$) in terms of sensitivity to ethanol extract. In other words, the most sensitivity was observed in *S. aureus* and the least was seen in *K. pneumoniae*.

Discussion

The result indicated that ethanol extract of sage with concentration of 50, 400,100 mg/mL has prevented from the growth of both Gram negative and Gram positive bacteria. Thus, the research represents the antibacterial effects on this medicinal herb on Gram negative and

Gram positive multidrug resistant bacteria. By gradual increase of concentration, inhibition zone increases as well. The most sensitivity was observed in *S. aureus* and the least was seen in *K. pneumoniae*. The result indicate the inhibitory effects of ethanol extract of Sage with MIC (Minimum Inhibitory Concentration) = 18.75 mg/mL for S. aureus, MIC=26.56 mg/mL for *E. coli*, MIC=33.75 mg/ml for *P. aeruginosa* and with MIC=31.25 mg/mL for *K. pneumoniae*. Concerning the method of extraction and preventing from using high temperature to avoid herbal compound destruction, there is a partial difference between these results and the similar studies. Rasooli et al. revealed that the sage essential oil had antibacterial activity against *S. aureus* and *E. coli* and MIC=11 mm for *S. aureus* and MIC=23mm for *E. coli*. This study proved our results are right which showed the essential oil had higher effect than ethanol extract [14].

Velickovic et al. reported in 2003. Sage ethanol extract possesses antibacterial activity against standard strains of Gram positive bacteria (*S. aureus* ATCC6538, *B. subtilis* ATCC6633) and Gram negative bacteria (*E. coli* ATCC25922, *P. aeroginosa* ATCC9027 and *S. enteritidis* ATCC13076) and the result showed the inhibitory effect of sage ethanol extract with MIC= 10 mg/mL for *S. aureus*, MIC= 6 mg/mL for *B. subtilis*, MIC= 60 mg/mL for *E. coli*, MIC= 60 mg/mL for *P. aeroginosa* and MIC= 50 mg/mL for *S. enteritidis* which had a significant differences with current studies. Lai and et al. reported in 2004. Sage ethanolic extract against the bacteria *Bacillus cereus*, *Staphylococcus aureus* and *Vibrio parahaemolyticus* is effective and in present study the sage ethanol extract has antibacterial activity against multidrug resistant *S. aureus*, *E. coli*, *P. aeroginosa* and *K. pneumonia* [15]. Culafic et al. reprted, Sage essential oil and its fractions showed a significant antibacterial effect against *S. aureus* and *B. subtilis*. The minimum inhibitory concentrations were 1.25-2.5 µL/mL for *S. aureus* and 0.15-2.5 µL/mL for *B. subtilis* [16]. Present study proved antibacterial effect of sage ethanol extract against selected multidrug resistant bacteria. Also, the essential oil had higher effect than ethanol extract.

In study of Khalil et al. the sage essential oil was effective against both Gram positive (*S. aureus* and Streptococcus group D) and Gram negative bacteria (*E. coli*, *S. typhi* and *P. aeruginosa*) and the antibacterial effect against Gram positive bacteria was more than Gram negative bacteria which had similar result with this study. By comparing these results, we found that the effect of essential oil is higher than ethanol extract. Application of plant extracts with known antimicrobial properties can also be of great importance in the treatment of disease. In recent years, studies in different countries to prove the effectiveness of plants have been conducted. Many plants are used for antimicrobial therapy because the compounds are synthesized in the secondary metabolism of plants. Many studies have shown that extracts of plants belonging to the Lamiaceae such as sage has biological activity against bacteria and yeasts. The ethanol extract of Salvia officinalis can prevent multi-drug resistant bacteria *S. aureus*, *E. coli*, *P. aeruginosa* and *K. pneumoniae*. Since in many countries growing resistance to antibiotics is seen. These results, especially the effect of high concentrations of ethanol extract on the bacteria can be important. However, the clinical application of this plant are needed more and larger studies and if successful and standardization of results. These plants can use as an alternative instead of inert and ineffective antimicrobial drugs currently used.

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Authors' Contributions

All authors had equal role in design, work, statistical analysis and manuscript writing.

Conflict of Interest

The aut5hors declare no conflict of interest.

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