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Evaluation of Antibacterial Activity of Ethanolic Extract of *Malva neglecta* and *Althaea officinalis L.* on Antibiotic-Resistant Strains of *Staphylococcus aureus*

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ABSTRACT

With increasing awareness of the dangerous effects of synthetic antibiotics, the demand for natural herbal drugs and cheap without side effects has increased. The present study aims to investigate the antibacterial effect of ethanolic extract of *Malva neglecta* and *Althaea officinalis L.* on antibiotic-resistant strains of *Staphylococcus aureus*. After collecting herbs and approval of its scientific name by botanists of Iranian Agriculture Organization, the extract of *Malva neglecta* and *Althaea officinalis L* were prepared by vacuum distillation procedure. Then minimum inhibitory concentration and minimum bactericidal concentration (MBC) of the extracts were determined on *S. aureus* strains isolated from patients with broth micro dilution and agar dilution method. Results showed that MIC/MBC of ethanolic extracts of *Malva neglecta* and and *Althaea officinalis L.* against *S. aureus* were 6.5/13 $mgml^{-1}$ and 3.2/6.5 $mgml^{-1}$, respectively. These findings suggest that *Malva neglecta* and *Althaea officinalis L.* can be considered as a group of herbs with anti-bacterial properties. Thus, it can be used widely in traditional medicine in this regard.

Key words: Antibacterial effect, Malva neglecta extract, Althaea officinalis extract, Staphylococcus aureus

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1. INTRODUCTION

The genus *Staphylococcus* is easily grown in many culture environments and is metabolically active. *S. aureus* species has become one of the most important health problems in the world due to its potential pathogenesis of disease and increasing resistance against antimicrobial drugs. *S. aureus* is resistant to *penicillin* because of producing beta-lactamase enzymes. *Methicillin* tolerance in *S. aureus* suggests the resistance to all *penicillins* including *penicillinase* and *cephalosporins* (1, 2). Despite their antibiotic treatment, the infections of these bacteria are with considerable complications. Thus, preventing the infections of this bacterium and finding the causes of its diffusion in hospitals are of prominent importance. *S. aureus* causes a wide range of diseases including *endocarditis*, *osteomyelitis*, *pneumonia*, and toxic shock syndrome. According to the above mentioned points and increasing resistance of *Staphylococcus* sp. to other antibiotics such as erythromycin and tetracycline and the emergence of vancomycin intermediate *Staphylococcus aureus* (VISA) and vancomycin resistant *Staphylococcus aureus* (VRSA) strains, continuous efforts were made to find new antimicrobial drugs (2). Without a doubt, the oldest human approach to the treatment of diseases has been the use of medicinal plants. There has always been a close connection between human being and plants during the development of all human civilizations; however, most plant species have not been recognized yet and a long time is left to discover the new and valuable plant resources. In this way, the plants can be seen as a source of potentially useful biochemicals, only a part of which has been exploited (3-5). One of the most important health challenges is to cope with infectious diseases because of their high prevalence and spread. After the identification of penicillinin in1940s and expanding its use in treatment, new antibiotics were everyday presented for the treatment of infections. The result of this expansion was the clinical

use of natural and synthetic antibiotics in the clinical treatment of the infection. Overuse of antimicrobial drugs lead to increased drug resistance of bacterial species against different antibiotics (6). This issue has been one reason for the growing use of plants as natural, safe, accessible, and affordable materials compared to synthetic antibiotics to treat bacterial infections. Moreover, these herbal medicines are more acceptable for the people to use (7-9). These are the reasons for increasing the new wave of international studies, and the introduction of anti-bacterial effects of different plants in recent years (10). In this study, two species of medicinal plants, namely *Malva neglecta* and *Althaea officinalis L.*, have been considered. *Malva neglecta* is a genus of perennial plants in the family *Malvaceae* with maximum 50 cm height (Figure 1).

Figure 1. ***Malva neglecta***

Its leaves are rounded with five pink or white petals. The chemical components of the *Malva neglecta* include vitamins A, B, and C, tannins, plenty of enameled materials, sugar, calcium oxalate, resinous substances, pectic, and a pigment called *Malvine*. *Malva neglecta* family has approximately 120 genus and 1500 species. *Malva neglecta* is used as medicinal plant in the treatment of different diseases such as common cold and to relieve cough in different parts of Iran. Its leaves and flowers are the most important used parts. Cure properties of this plant caused it to be used as a valuable and useful medicine to relieve the bruise, inflammation, mosquito bite and etc. It is consumed for the treatment of urinary tract or digestive system diseases as well (11-14). *Althaea officinalis L.* is a medicinal plant which in traditional medicine was used as an anti-cough, mucous thinner medicine for the respiratory tract (15), a perennial herb whose stem is erect, with a height of 0.55 to 1.5 meter with rounded leaves (Figure 2).

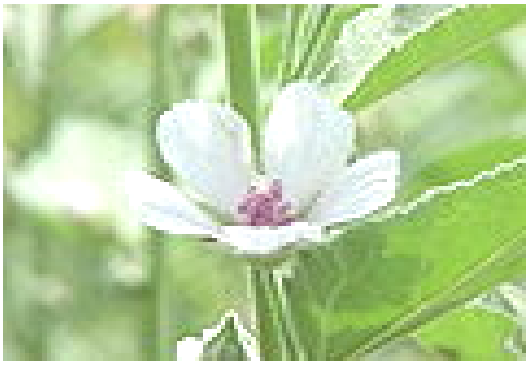

Figure 2. ***Althaea officinalis L***

All parts of the plant are soft and velvety with white or pink flowers beside the leaves. It is reported that this plant is useful in removing dryness and irritation of the throat and chest due to the presence of polysaccharide (16). Traditionally, its flowers and root are used as an antiseptic for disinfection of skin wounds (17). The present study aims to investigate the antibacterial effects of the ethanolic extract of *Malva neglecta* and *Althaea officinalis L.* on antibiotic-resistant strains of *S. aureus*.

2. MATERIALS AND METHODS

2.1. Preparation of the bacterial strain and its antibiotic resistance

In this study, 30 samples were selected from clinical samples (urine) of Razi Hospital in Marand county of Tabriz in Iran, and were cultured on mediums of mannitol salt agar and blood agar. Pure isolates obtained from the synthetic media were identified in terms of genus using catalase tests and other biochemical tests, and finally *S. aureus* species were identified with tube and lamivudine coagulase test and examining agglutination. Then, the purified isolate ware antibiogramed by Kirby-Bauer method on Mueller-Hinton agar medium, and their sensitivity to antibiotics was examined. The antibiotics used in the present study are *trimethoprim/sulfamethoxazole* (SXT 10+10 µg), *Ampicillin* (AM 10 µg), *Ceftazidime* (SAZ 30µg), *Cefoxitin* (CF 5 µg), *Amikacin* (AN 30 µg), *Penicillin* (P 10 µg), *Ceftriaxone* (CRO 30 µg), *Erythromycin* (E 15 µg), *Tetracycline* (TE 30 µg), made in PadTan Teb company (Tehran, Iran). After 24 h incubation at temperature 37℃, the diameter of the inhibition zone is measured and sensitivity and resistance of the isolates were determined and the results were compared with the standard table.

2.2. Sampling and preparation of plant extraction

Plant samples were collected in two stages at the end of May and early June from natural areas around Marand town in East-Azerbaijan province of Iran, Eishabad village. Once the samples were collected and transported, they were cleaned and dried in the large space and proper conditions away from direct sunlight. After complete drying of the samples, the stems and leaves shoots were separated from the roots and prepared to grind. After grinding, 100 g of the herbal powder was mixed with the

ratio of 1: 8 with distilled water and 80% ethanol. The mixtures are kept for 48 hat lab temperature and were mixed by a glass rod each hour. The mentioned mixtures are filtered by four-layer sterile gas and funnel. To separate the impurities in the extract, it is centrifuged at 2500 rpm for 20 min at temperature 4℃. Then, the filtered extract was transferred to a distilled system in vacuum to remove the solvents and finally to obtain a strong extract. The extract is divided by sterile microbial filters, 0.45 micron, and in 1.5 ml micro tubes all at sterile conditions and was preserved at temperature -80 ℃ (18, 19).

2.3. Determining the antibacterial effect of the extracts by agar well diffusion and determining MIC and MBC values of the extracts by macrodilution method

In this method, a bacterial suspension by the tested species equivalent to a standard turbidity 0.5 McFarland standard was provided at first and each of the samples was diffused for Mueller–Hinton agar culture media by sterile swap. On the above culture media, some wells with the diameter 5 mm was immediately created and a 90 µl aliquot of various dilutions are inoculated inside the well and were kept for 24 h in incubator at temperature 37℃ and finally the bacteria non-growth zones were measured (measuring the diameter of inhibition zone by caliper). To determine MIC and MBC of the extracts, macrodilution method was applied. To do this, the concentrations 0.8 to 418 $mg.ml^{-1}$ were provided at first in Mueller–Hinton broth. Then, by adding 1 ml microbial suspension equivalent to standard turbidity 0.5 McFarland standard, the final concentrations of the extract is regulated as 0.4 to 209 $mg.ml^{-1}$. The tubes were incubated at temperature 37 ℃ for 24 h. The turbidity of the tubes was investigated daily and is subcultured on Mueller–Hinton media. This was done in triplicates. Beside test tubes, to determine MIC positive control including bacteria is done in the media without extract to compare the turbidity of the test tubes. The first tube of the low concentration of extract without turbidity of bacterial growth was computed as MIC concentration and the first tube of low concentrations of the extract in which added bacteria was removed was computed as the MBC concentration (18-21). The results of the experiments are analyzed by SPSS (version 19) software and the samples were plotted by Excel software. ANOVA one-way test (one-way variance analysis) and LSD were used for classification and comparison of means.

3. RESULTS AND DISCUSSION

3.1. Bacterial response to antibiotics

The results of the biochemical analysis of the isolated bacteria showed that the isolates purified catalase, coagulase and mannitol were positive. Antibacterial activity of multiple antibiotics against the isolates obtained in *in vitro* was evaluated. The results of this experiment showed that each bacterial isolate had a similar behavior in presence of the antibiotic; however, some differences were seen among the isolates in terms of antibiotic sensitivity or resistance. Differences were also observed in the inhibition mechanism of antibiotics during which penicillin and erythromycin antibiotics demonstrated the lowest antibiotic effect (Diagram 1).

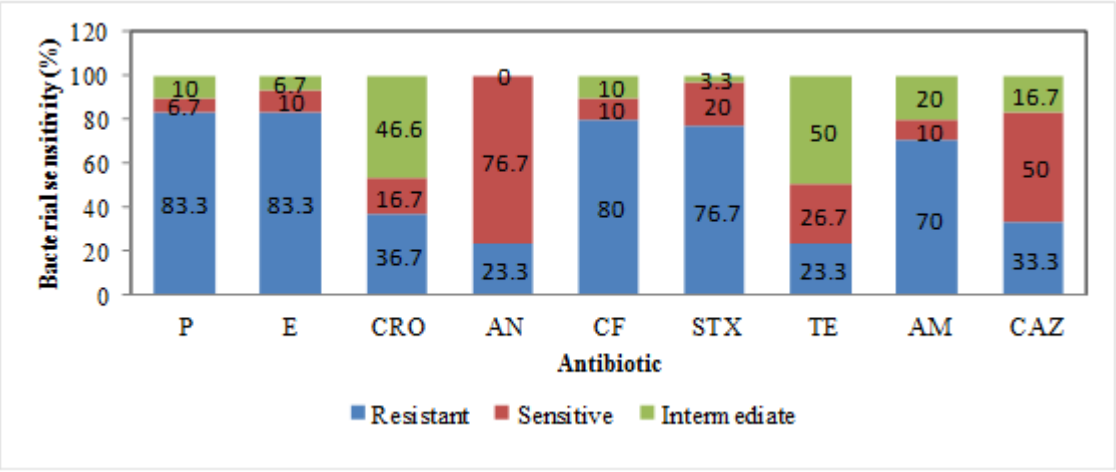

Diagram 1. Sensitivity model of isolates of *Staphylococcus aureus* against antibiotic (%)

The highest resistance rates were observed with penicillin (83.3%), erythromycin (83.3%), cefoxitin (80%), and 50% isolate against ceftazidime were sensitive . 11 isolates were resistant to most antibiotics.

3.2. Antibacterial properties of the extracts

Geographical and climatic diversity has caused the existence of a rich and varied source of plant species in Iran. Some of these plants have medicinal properties such as antibacterial ones**.** Findings of the present study about antibacterial effects of plant extracts showed that ethanolic extract of *Malva neglecta* and *Althaea officinalis L.* had antibacterial effect on the studied bacteria, with the highest effect for *Althaea officinalis L.* extracts. The findings related to antibacterial effects of *Malva neglecta* and *Althaea officinalis L.* were shown in Table 1 and the results of MIC and MBC by macrodilution of *S. aureus* isolates of patients were shown in Table 2.

Table 1. Antibacterial effects of *Malva neglecta* and *Althaea officinalis L.* on antibiotic-resistant *Staphylococcus aureus* (mean diameter of non-growth haloes ± SD in mm)

Plant type	Concentration (mg.ml^{-1})							
	1.6	3.2	6.5	13	26.1	52.2	104.5	209
Malva neglecta	0	0	7.3±0.16	10.5±0.16	12±0.81	15.6±0.18	17.5±0.16	19.66±0.47
Althaea officinalis L.	0	8.3±0.5	10.66±0.47	12.16±0.18	14.66±0.47	16±0.66	18.5±0.16	20.5±0.26

Table 2. MIC and MBC determination by macrodilution of *Malva neglecta* and *Althaea officinalis L* on antibiotic-resistant strains of *Staphylococcus aureus*

Plant	Ethanol extract concentrations (mgml^{-1})	
	MIC	MBC
Malva neglecta	6.5	13
Althaea officinalis L.	3.2	6.5

Here, the level $p<0.05$ is significant. The misuse and overuse of antibiotics leads to antibiotic resistance among bacteria and consequent treatment complications and increased healthcare costs. The resistance of *S. aureus* to antimicrobial agents can be encoded by both chromosomes and plasmids (22). Regarding the increase of bacterial resistance to certain types of antibiotics, attempts have been made to use compounds from plants and to explore their applications in the treatment of various diseases. Plants have played a very important role in maintaining the health and in improving the quality of human life from thousands of years ago. Medicinal herbs have beneficial properties including the antibacterial, anti-parasitic, antifungal, and antioxidant (23). In this study, it was found that ethanolic extracts of *Althaea officinalis L.* had more antibacterial activity on tested bacteria compared to those of *Malva neglecta*. According to the MIC and MBC values, it can be said that *Althaea officinalis L*. and *Malva neglecta* extracts had good antibacterial activity even at low concentrations; therefore, it seems that the ethanolic extract of *Malva neglecta* and *Althaea officinalis L* are able to inhibit the growth of the studied bacterium *in vitro*. Rosario and Beatriz (2003) (24) studied the antibacterial activity of a species called *Malva lavatera*. It was found that ethanol extract of this plant had a weak antibacterial effect on *S. aureus*, *Pseudomonas aeruginosa*, *Bacillus subtilis* and *Escherichia coli*. In our investigation, the water extract of *Malva neglecta* had more antibacterial effect than ethanolic extracts that is in agreement with the above mentioned finding (24). In a study by Shahidi (2004) done on 10 different species of bacteria, it was shown that the methanol extract of *Malva sylvestris* had antibacterial effect on *Bacillus pumilus.* Agar-well diffusion method was used in this study and the diameters of non-growth haloes of 10-14 mm were measured (25). Dulgar and Gonuz (2004) in Turkey studied the ethanolic extract of 16 plants including *Malva rotundifolio* and examined their antibacterial effect on some of gram-positive and gram-negative bacteria using agar-well diffusion method. Their results revealed that the extract of *Malva rotundifolio* had strong inhibiting effect on *S. aureus*, *P. aeruginosa*, *B. subtilis*, *K. pneumoniae* and *E. coli* (26). Dost Mohamadi *et al* (2012) showed that water and ethanolic extracts of *Malva neglecta* with nanosilver particles had inhibitory effect on *S. aureus* and *Salmonella typhimurium* which were consistent with our findings (27). Eshraghi *et al* (2009) showed the antibacterial effect of *Malva neglecta* on *Nocardia strain* (17). Zareii *et al* (2014) determined the inhibitory effect of *Alceadigitata L.* on gram-positive and gram-negative bacteria, especially on *S. aureus* (28). The antibacterial property of hexane extracts of flower and root of *Althaea officinalis* collected from northwest of Iran (Khalkhal city) were found on some gram-positive and gram-negative bacteria (*E. coli*, *P. aeruginosa*, *K. pneumoniae*, *B. subtilis*, *Enterococcus faecalis*, *S. aureus* and *Staphylococcus epidermidis*) as well as on three fungal species (*Aspergillus niger*, *Candida albicans* and *Saccharomyces cerevisiae*) by Valiei *et al* (2011) (29). In another study on root, flower and leaves of *Althaea officinalis* in Pakistan, the antibacterial effect of this plant on gram-negative *E. coli*, *P. aeruginosa* and gram-positive *S. aureus* were shown, and it was found out that the highest antibacterial effect was on *S. aureus*. The gram-positive bacteria had the greatest sensitivity to the effect of plant extracts compared to the gram-negative bacteria (30).

4. CONCLUSION

In total, results in laboratory conditions showed that the extract of *Malva neglecta* and *Althaea officinalis* L. had antibacterial effect on antibiotic-resistant strains of *S. aureus*. Further investigations on laboratory animals, the extract can be used as a better alternative for producing new medicinal plants against this bacterium with minimal side effects.

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AUTHORS CONTRIBUTION

This work was carried out in collaboration among all authors.

CONFLICT OF INTEREST

The authors declared no potential conflicts of interests with respect to the authorship and/or publication of this article.

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