IJASR International Journal of Academic Scientific Research
ISSN: 2272-6446 Volume 3, Issue 1 (February – March 2015), PP 34-40
www.ijasrjournal.org

Antibacterial activity of four herbal extracts against methicillin resistant bacteria isolates collected from Almadinah hospitals, Saudi Arabia.

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ABSTRACT

The purpose of this study was to explore antimicrobial effect of four plant extracts Mentha cervina , Mentha longifolia,Ocimum basilicum and Origanum vulgare against multi-drug resistance (MDR) Staphylococcus aureus isolates collected from Al madinah Almounawarah hospitals , Saudi Arabia. MRSA is resistant to not only methicillin and other β- lactam antibacterial agents but also other antibacterial agents; therefore new agents are needed to treat MRSA. Antibacterial activity of the medicinal plant extract was determined using well diffusion assays and paper disc diffusion method. The medicinal plant extract exhibited antibacterial activity against multi-drug resistance (MRSA) Staphylococcus aureus. The synergetic effect was clearly observed with Mentha longifolia were combined with Ocimmum basilicum , and followed by Mentha longifolia + Origanum vulgare, Mentha longifolia + Mentha cervina and Ocimmum basilicum + Mentha cervina then , Origanum vulgare + Ocimmum basilicum and Origanum vulgare + Mentha cervina and showed the strongest antimicrobial activity. Our results confirmed that ethanolic extracts, could be a possible source to obtain new and effective herbal medicines to treat infections caused by multi-drug resistant strains of microorganisms from community as well as hospital settings. In addition ethanolic extracts of our medicinal plants may have the potential to restore the effectiveness of β-lactam Antibacterial agents against MRSA.

Keywords: *Herbal extract, antibacterial activity,MIC,FIC,MRSA.*

INTRODUCTION

The antibacterial agents constitute the principal basis for human infection therapy, as their application might lead to the effective eradication of many serious infectious diseases. Unfortunately, the extensive and massive use of these antibacterial drugs has resulted in the emergence of multi antibiotics resistant bacterial pathogens [1].

Antibacterial drugs have been extensively used for more than fifty years and this over use has resulted in the acquisition of specific bacterial resistance for most of the used antibiotics as consequences of arising new resistant antibiotics bacterial genes. Many bacterial pathogens exhibited antibiotic resistance characters including *Staphylococcus aureus* which is responsible for many illnesses including suppuration abscess formation many biogenic infections and might lead to fatal human septicemia. Accordingly, antibiotic resistant *Staphylococcus aureus* constitute an arising challenge to human health, as it is a wide spread infection between hospitalized patents.

Staphylococcus aureus resistance is not only for methicillin and other B-lactam antibacterial drugs, but it might extend to other antibacterial drugs which again might result in severe consequences and high medication cost ending with high mortality rate between patients [2]. Antibiotic resistance are also extended to many other bacterial pathogens , accordingly , it became very urgent to operate more advanced research for new effective antibacterial agents to overcome such emerging resistance [3]. Recently, herbal medicine attracted the attention of many researchers all over the world and various plants have been investigated for their antibiotic

effect. It is well known that the traditional herbal medicine have been used for centuries as crude materials, and recently extracted with fractionation [4].

MATERIAL AND METHODS

Specimens:

In our present study, 80 bacterial isolates were collected from Al Madinah hospitals, Saudi Arabia. Out of these, 30 samples were positive for *Staphylococcus aureus*. Only 10 MRSA isolates were considered and included in our present study.

Organism Identification:

API Staph-Ident strip system. The API Staph Ident. Strips consist of a series of 10 microcupules containing dehydrated substrates, nutrient media, or both. The alkaline phosphatase (Phs), β-glucosidase (Gls), and, β-glucuronidase (Glc) microcupules contain chromogenic substrates and positive reactions are noted by the liberation of p-nitrophenol from them.The urease (Ure) and arginine utilization (Arg) microcupules contain phenol red as an indicator to detect alkaline end products. Indicators for the detection of acid production including mannose (Mne), mannitol (Man), trehalose (Tre), and salicin (Sal) microcupules contain cresol red were practically used. The, 3-galactosidase (Ngp) microcupule contains 2-naphthol-13-D-galactopyranoside and with the addition of a reagent composed of for STAPH-IDENT composed of (0.35% fast blue BB salt in 2-methoxyethanol) for the detection of the free β-naphthol. Test organisms were removed from an overnight culture inoculated on Trypticase soy agar plates with the addition of 5% sheep blood, using a sterile cotton swab. The inoculated swab was agitated in 2 ml of sterile 0.85% saline so that the final turbidity was approximately that of a no. 3 McFarland (BaSO4) turbidity standard. Each microcupule on the strip was filled with three drops (approximately 80, µl) of the above-described bacterial suspension. After inoculation, strips (placed into plastic trays with lids) were incubated for 5 h at 35°C in air and then read. Two drops of STAPH-IDENT reagent were added to the Ngp microcupule, and color development was recorded after 30 s. Positive reactions were converted to a four-digit profile to confirm the organism identification at the species level following the manufacturer instructions. The following 10 biochemical tests are divided into the four groups arranged in order on the strip: Phs, Ure, and Gls; Mne, Man, and Tre; Sal, GIc, and Arg; and Ngp. A value of one, two, and four is assigned to the first, second, and third positive biochemical test, respectively, in each group. A value of zero is assigned to a negative result. Each resulting profile number represents a unique combination of positive reactions [5].

Plant extracts preparation:

Plants: *Mentha Cervina,Mentha longifolia* **,***Origanum vulgare* and *Ocimum basilicum* were collected from Al Madinah Almounawarah Saudi Arabia. The collected plants were subjected to scientific identification using the scientific identification manuals used with the kind help of our botany specialized colleges in the biology dept. faculty of science . Plants was rinsed twice with tab water then, leaves were separated and washed again using sterilized water for the elimination of soil and dust particles and massive surface contaminants , then left in shade for drying . Finally, plant material was pulverized using domestic blender to powder form. Powdered material (100g) was mixed with one liter of 70% methanol and kept 7 days, in closed lab glass Bottles at room temperature. After 7 days, mixture was filtered through whatman filter paper. The filtered extract was subjected to rotary evaporator. The water bath temperature was adjusted to 45 C^0 .The semisolid extract produced was kept in a laminar flow cabinet for 3 days for methanol evaporation .The stock solutions were prepared from the dried herbal extracts using 50% ethanol to achieve a concentration of 100 mg/ml for each of the tested plant extracts [6].

Antibacterial assay:

The tested of *Staphylococcus aureus* isolates were processed for susceptibility tests using the method of Bauer-Kirby [7]. Sterile paper discs (5mm) were loaded with 100 µl of 100 mg / ml of each of the tested ethanolic plant extracts and their combinations **.**The concentrations of the tested plant extract combinations were prepared in the ratio of 1:1 v/v. Dilutions of bacterial suspensions were prepared using McFarland standard tubes [$1x10^{8}$ CFU/ ml] .Sterilized plates containing Muller Hinton Agar were loaded with the bacterial suspension and the discs were loaded with various tested plant extracts, separately and in combinations. The extract loaded discs aseptically placed on the inoculated agar plate's surface. All plates were incubated at 37^{0}C for 24 hrs then the diameter of the formed inhibition zones around each disc was recorded and compared according to clinical laboratory standard institute [8]. The experiment was carried out in triplicates using controls

Minimum inhibitory concentrations assay:

The MIC was determined by micro-broth dilution methods [10]. The tested extracts were serially diluted in Mueller-Hinton broth (Oxoid) medium. Duplicate tubes of each dilution (0.391, 0.780, 1.563, 3.125, 6.25, 12.5, 25.0, 50.0,75.0,and 100 mg/ml) were inoculated with 5 x 10^{5} cells (cfu) of the tested *Staphylococcus aureus* strain then cultures were incubated at 37^{0}C for 18 h. MICs were considered as the least concentration of each extract with no visible bacterial growth in terms of turbidity. The plant extract combinations were prepared in the ratio of 1:1(v/v). The FICs index of plant extract combinations was calculated as the sum of each component FIC in a combination and interpreted as either synergistic (≤0.5), additive (0.5–1.0), indifferent (1–4.0) or antagonistic (≥4.0) [16]

Statistical Analysis

All the experimental results were expressed as mean ± standard deviation then statistically analyzed using the Microsoft Excel Software 2010 program [9].

RESULTS

Antibiotic susceptibility test:

The tested 10 *Staphylococcus aureus* isolates had shown to be multidrug resistant. The multidrug resistance ranged from 5 to 10 antibiotics (Table 1). Two *S.aureus* strains were resistant to all the tested 10 antibiotics , 2 were resistant to 8 antibiotics , 2 were resistant to 7 antibiotics , 3 isolates showed resistance to 6 antibiotics and only one strain was resistant to 5 antibiotics (Table 1) .

Table 1.Antibiotic resistance profile of *Staphylococcus aureus*.

Isolate no.	*Isolates*	*Class*	*Antibiotic Resistance Pattern*	*No. of resistant A.b.*	*No. of sensitive A.b.*	*Type of resistence*
1	Staph. 1	MRSA	G-Of-Cf-R-Va-E-C-P-M-Ox	10	0	Multi drug resistant
2	Staph. 2	MRSA	Of-Cf-R- C-P-M-Ox	7	3	Multi drug resistant
3	Staph. 3	MRSA	Of-Cf- Va-E- P-M-Ox	7	3	Multi drug resistant
4	Staph. 4	MRSA	R-Va-E-P-M-Ox	6	4	Multi drug resistant
5	Staph. 5	MRSA	G-Of-Cf-R-Va-E-C-P-M-Ox	10	0	Multi drug resistant
6	Staph..6	MRSA	G-Va-P-M-Ox	5	5	Multi drug resistant
7	Staph. 7	MRSA	Of-Va-C-P-M-Ox	6	4	Multi drug resistant
8	Staph. 8	MRSA	Of-Cf-Va-P-M-Ox	6	4	Multi drug resistant
9	Staph. 9	MRSA	G-R-Va-E-C-P-M-Ox	8	2	Multi drug resistant
10	Staph. 10	MRSA	G-Of-Cf-R-Va-E-C-P-M-Ox	10	0	Multi drug resistant

Staph. *Staphylococcus aureus*, MRSA: Methicillin Resistant *Staphylococcus aureus*, Ab*:* Antibiotic,. M: Methicillin, G: Gentamicin, Of: Ofloxacin, Cf: Ciprofloxacin, R: Rifampicin, Ox: Oxacillin Va: Vancomicin, E: Erythromycin,C: Chloramphenicol, P: PenicillinG.

The susceptibility pattern of the herbal ethanolic extract showed that the inhibition zone diameter ranged from 14 to 18, 5 mm. The highest effect was exerted by *Origanum vulgare* (18.5 ± 0.521) the least effect was recorded by *Ocimum basilicum* (14 ± 0.133), and both of *Mentha longifolia* and *Mentha cervina* have moderate effect (16.5 ± 0.415 & 15± 0.231) mm respectively (Table 2).

Table 2. Susceptibility pattern (inhibition zone diameter) of crude ethanolic herbal extracts against MDR *Staphylococcus aureus*.

Extracts	Zone Diameter (mm)
	Mean SD
Mentha longifolia	16.5 ± 0.415
Origanum vulgare	18.5 ± 0.521
Ocimum basilicum	14.00 ± 0.133
Mentha cervina	15.00 ± 0.231

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Herbal extracts combinations proved being more effective against the resistant *Staphylococcus aureus strains* . The susceptibility patterns of these extracts in terms of the inhibition zone diameter was higher than that of the individual plants extracts . These resulted in inhibition zones diameter ranged from 18.67 mm to 27.67 mm with the highest effect for (*Mentha longifolia* + *Ocimum basilicum)* , *(Ocimum basilicum* + *Mentha cervina*) and *(Mentha longifolia* + *Origanum vulgare***)** combinations, with 27.67 ± 0.577 , 27.33 ± 0.577 ,and 26 ± 0.816 respectively . The herbal combinations (*Origanum vulgare* + *Ocimum basilicum) and (Origanum vulgare* + *Mentha cervina*) had a moderate effect , 21 ± 1.1101 and 18.67 ± 1.528 respectively (Table 3).

Table.3 Susceptibility pattern(inhibition zone diameter) of medicinal plants ethanolic extracts combinations on MDR *Staphylococcus aureus.*

Extracts combinations	Zone Diameter (mm)
	Mean SD
Mentha longifolia + *Origanum vulgare*	26 ± 0.816
Mentha longifolia + *Ocimum basilicum*	27.67 ± 0.577
Mentha longifolia + *Mentha cervina*	24.33 ± 1.155
Origanum vulgare + *Ocimum basilicum*	21 ± 1.110
Origanum vulgare + *Mentha cervina*	18.67 ± 1.528
Ocimum basilicum + *Mentha cervina*	27.33 ± 0.577

The minimum inhibitory concentration (MIC) values of the tested ethanolic plant extracts ranged between 12.50 to 50.00 (mg/ml) .The most potent inhibition was recorded to *Origanum vulgare* with 12.50 (mg/ml) ,while the least potent inhibition was attributed to *Ocimum basilicum* and *Mentha cervina with* 50.00 (mg/ml) each (Table 4). The minimum inhibitory concentration (MIC) values of the tested plant extracts combinations were in the range of 6.25 To 50.00 (mg/ml) . The combination (*Mentha longifolia* + *Origanum vulgare*) showed the highest inhibition potency with 6.25 (mg/ml) while the least potent inhibition was recorded by the combination (*Mentha longifolia* + *Mentha cervina*) (Table 5) .

Table 4. Minimum inhibitory concentration (mg/ml) values of individual plant extracts

Plant extract	MIC(mg/ml)
Mentha longifolia	25.00
Origanum vulgare	12.50
Ocimum basilicum	50.00
Mentha cervina	50.00

MIC:Minimum inhibitory concentration

Table 5. Minimuml inhibitory concentration (mg/mL) values of plant extracts combinations and their FIC indices .

Extracts combinations	MIC mg/ml	FIC index	
Mentha longifolia + *Origanum vulgare*	6.25	0.75	additive
Mentha longifolia +*Ocimum basilicum*	25.00	1.50	indifferent
Mentha longifolia + *Mentha cervina*	50.00	3.00	indifferent
Origanum vulgare +*Ocimum basilicum*	25.00	2.50	indifferent
Origanum vulgare + *Mentha cervina*	25.00	2.50	indifferent
Ocimum basilicum +*Mentha cervina*	25.00	1.00	indifferent

DISCUSSION

As consequences of the massive antibiotic use in the last fifty years, the acquired bacterial antibiotic resistance and the arising of resistance genes had become evident. Accordingly, many pathogenic microbes including *Staphylococcus aureus* constitute a real threat to human health. These antibiotic resistant pathogens are responsible for a variety of infections which might end with fatal septicemia of infected humans. The increasing incidence, of *S. aureus* antibiotic resistance particularly in hospitals towards methicillin and wide range of other antimicrobial agents, resulted in announced therapy difficulty [5]. Methicilline resistant *Staphylococcus aureus* [MRSA] constitute a human health problem causing a real life threat to hospitalized patients. Hospital acquired *Staphylococcus aureus* infection still difficult to treat with known antibacterial agents Consequently, several attempts were exerted to control this phenomenon spread through the search for the discovery of alternative effective herbal therapy for treating the infection with MRSA .[10].

In the present study, the analysis of the growth inhibition activity by the disk diffusion method showed that the tested medicinal plants (*Mentha longifolia*, *Origanum vulgare*, *Ocimmum basilicum* and *Mentha cervina)* commonly used as a traditional medicine were active against the hospital isolated MRSA strains.Our results came parallel and complementary to the previous herbal antibacterial studies in relation to types of antimicrobial herbal extracts. Ten *Staphylococcus aureus* isolates recovered from hospitalized patients proved being resistant to several different anti bacterial drugs (Table1)**.** The ten isolates showed a multidrug resistant profile and three isolates were resistant to the 10 tested antibiotics, confirming their role as a real threat to patients health which might end with mortality. Also, mostly the wound infections isolated strains had multiple antibacterial drug resistance as expected from the previously wide and massive use of antibacterial agents.

Medicinal plants became targeted for the production of new effective antibacterial drugs production for overcoming the extensive occurrence of multi antibacterial drug resistance. In our current study four herbal extracts were applied and proved being efficient as antibacterial agents including *Mentha longifolia*, *Origanum vulgare*, *Ocimmum basilicum* and *Mentha cervina* . It has been postulated previously that these herbal extracts might work through inhibition of different cellular biotic functions including ion leakage from the cells and disrupting the plasma membrane permeability [11].

Herbal extracts and remedies have been extensively used by humans all over the world since ancient times for the cure of various human illnesses. Our current results revealing the active and efficient antibiotic role of the tested herbal extracts (Table 2) came in parallel with these previous reports and confirmed the great value of using selected herbal extracts as efficient alternatives for the known synthetic antibacterial drugs using in vitro bioassays [12].

In addition, our results confirmed the use of such herbal extracts in combinations exerted a remarkable antibacterial capacity towards resistant bacterial strains of *Staphylococcus aureus*, and these came in agreement with these findings reported the gram positive bacteria sensitivity more than that of the gram negative bacteria against the tested essential oils of the Lamiacae family [13]. Moreover, other reports confirmed that plant extracts were more efficient as antibacterial agents more than the standard drugs streptomycin and penicillin [14]. Also, our results came in agreement with the finding of Habiba [15] reporting the announced

antibacterial activity of *M.pulegium* essential oil against gram positive bacteria more efficiently than gram negative bacteria .

In addition the antibacterial activity of *M.spicata* and *M.pulegium* was confirmed and been used in treating illness resulting from microbial infection [15]. The MIC, of all the extracts exhibiting antimicrobial activity were found to be in the range of 12.5 to 50 mg/ml; which is equal to one fourth or half of the concentration used in agar plates experiments . However, these values were recorded with crude herbal extracts. The determination and the isolation of the active compound might be more effective and a much lower MIC could be achieved for the purified extracts. The results of screening showed that the tested ethanolic herbal combination of *Mentha longifolia* + *Ocimum basilicum* , *Mentha longifolia* + *Mentha cervina, Origanum vulgare* + *Ocimum basilicum, Origanum vulgare* + *Mentha cervina and Ocimum basilicum* + *Mentha cervina* were discouraging as these exerted no difference than their individual components . However, the combination of *Mentha longifolia* + *Origanum vulgare* gave a promising results of the lowest MIC,also its fraction index showed additive effect to the combination of the two extract components . This gives a promising effect especially through the effective components analysis which might lead to the discovery of new natural antibacterial pharmaceuticals for therapeutic needs.

CONCLUSIONS

Our present study confirmed that the tested ethanolic plant extracts either individually or in combinations of (*Mentha longifolia , Origanum vulgare*), (*Mentha longifolia , Ocimmum basilicum*) , (*Mentha longifolia , Mentha cervina*), (*Origanum vulgare , Ocimmum basilicum*) , (*Origanum vulgare , Mentha cervina*) and (*Ocimmum basilicum , Mentha cervina*) constitute a promisng effective material for a new and effective therapy for those infections not responding to other synthetic antibacterial drugs . These tested herbal remedies might constitute a good aid for the effective eradication of multi-drug resistant bacterial strains commonly recovered from hospitalized patients.

Acknowledgment: The authors are deeply indebted for all the hospitals staff for their kind cooperation in supplying us with the studied bacterial isolates.

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