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Synergistic antibacterial effect of *Glycyrrhiza glabra* and *Rosmarinus officinalis* against MRSA isolated from Egypt

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ABSTRACT

192 Staphylococcus samples were isolated from patients in Egyptian hospitals and 112 were identified as Staphylococcus aureus using conventional methods based on morphological and biochemical characteristics. Sensitivity of isolates to a range of antibiotics was also tested by disc diffusion method, which revealed that 82% of isolates were MRSA isolates. Methicillin resistance was confirmed using PCR-based molecular approach, 8 isolates were harboured mecA gene and 4 isolates were Borderline S. aureus (BORSA). MICs of different antibiotic classes were determined; results were varied to different antibiotics, where Penicillin and Ampicillin MICs varied from resistant to borderline resistant isolates (512,62µg/ml). Cinnamomum cassia, Syzygium aromaticum, Glycyrrhiza glabra, Rosmarinus officinalis and Salvia officinalis plant extracts were tested to examine their antibacterial activity against MRSA isolates. MIC of tested plant extracts was evaluated by agar dilution method. Diethyl ether extracts of G. glabra, R. officinalis and S. officinalis showed the lowest MICs values (0.05, 0.39, 0.195mg/ml respectively). Investigation of possible synergistic effect upon combination between plant extracts with the lowest MICs values was evaluated by a checkerboard titration assay. Combination between diethyl ether extracts of G. glabra and R. officinalis at conc. 0.0125 and 0.0975 mg/ml respectively showed synergistic effect on MRSA isolates and standard strain. Both extracts shown antibacterial effects on MRSA cells by shrinkage of the protoplasts and disruption of the cytoplasmic membrane evidenced by protein analysis and Microscopic examination of cells pre and post treatment by TEM .These results suggest that these extracts might be used as a promising antibacterial agent.

Keywords: Synergistic effect, Agar well diffusion method, Minimum inhibitory concentration, *Glycyrrhiza glabra, Rosmarinus officinalis.*

INTRODUCTION

Antibacterial drug resistance is a global public health threat, due to the inappropriate use of existing drugs, coinciding with a marked decline in innovative antibacterial drug development. Patients and clinicians are increasingly confronting infections caused by pathogens resistant to all antibacterial drugs in both the inpatient **[1]** and outpatient **[2]** settings.

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a major nosocomial pathogen that possesses substantial threats and economic burdens to patients and hospitals globally. MRSA infection increases the risk of mortality, the length of hospital stay of patients, and extra costs of treatment and a control program for patients and hospital

settings **[3]**. Plant extracts are a valuable source of novel antibacterial compounds to combat pathogenic isolates of methicillin−resistant *Staphylococcus aureus* (MRSA), a global nosocomial infection **[4]**.

C.cassia extract have a better antimicrobial activity at lower effective concentration, for *S. aureus***[5]**. Ethanol extract of *S. aromaticum* buds were found to inhibit the growth of MRSA**[6]**. *G. glabra*, Family *Fabaceae*, also known as liquorice and sweet wood, is native to the Mediterranean and certain areas of Asia. It is a perennial herb which possesses sweet taste. The main taproot, which is harvested for medicinal use, is soft, fibrous, and has a bright yellow interior**[7]**.*G. glabra* showed antibacterial activity against *S. aureus***[8]**. *R. officinalis* family *Lamiaceae*, originally grows in southern Europe. Its herb and oil are commonly used as spice and flavoring agents in food processing for its desirable flavor, high antioxidant activity and lately as antimicrobial agent **[9]**. Rosemary leaves extract can inhibit the growth of bacteria and yeasts. Therefore it can be used as a potential antimicrobial source of natural origin in foods [10].

In our continuous search of the antibacterial activities of medicinal plants, we designed the present work to determine the activity of few medicinally important plants and evaluate their synergistic effect on MRSA

MATERIALS AND METHODS

Test microorganisms

- Prior to obtaining clinical isolates from patients, approval was received from the Ethics Committee of Cairo-University and an informed consent was obtained from all patients.
- Hundred -ninety two clinical *Staphylococcus* isolates were collected from El-Demerdash Hospital and Al Borg Laboratories , from the period of January 2011 to July 2011, from different sites of infection (blood, skin**,** urine , saliva, throat,…etc), male and female patients, and varied range age patients(>5-60 years old).
- *Staphylococcus aureus* isolates were identified biochemically (Catalase test, Coagulase test, DNase test, Mannitol Fermentation, growth on Baird-Parker agar , growth on Vogel Johnson media and Blood agar hemolysis) and microscopically [11]. CHROMagar MRSA medium to identify MRSA isolates.

Susceptibility test and Minimum Inhibitory Concentrations of *Staphylococcus aureus* isolates

Identified *S. aureus* isolates were tested for their resistance to different classes of antibiotics which also used to detect multi-drug resistance among clinical isolates of *S. aureus* [12]. MICs were determined by the agar dilution method in Mueller Hinton agar medium [13].

PCR amplification of *mecA* gene

-DNA Extraction

DNA was extracted from 12 *S. aureus* isolates using the boiling method. 2-3single colonies of bacteria were washed twice with 0.5 cc EDTA-Tris buffer. Then 250µl from 1x TE was added and the mixture was incubated at 100ºc for 8 min. then it was harvested by centrifugation at 7500 rpm for 10 min, then centrifugation at 8000 rpm for 5 min. Finally, the supernatant was used for Polymerase Chain Reaction (PCR) assay.

-PCR

Methicillin Resistance gene *mecA* was detected by PCR. Specific primers; primer *mecA F:* 5'AAAATCGATGGTAAAGGTTGGC3' and *mecA R:* 5'AGTTCTGCAGTACCGGATTTGC3' were used to amplify a 533-bp DNA fragment of *mecA* gene.

DNA amplification was carried out in a PCR thermocycler with the following thermal cycling profile: Initial denaturation at 94ºC for 3 min was followed by 30 cycles of amplification with 94ºC for 30 s, annealing at 55ºC for 30s, and extension at 72ºC for 30s (except for the final cycle, which had an extension step of 4 min). The PCR mixture was prepared in a final volume of 25 µl. The amplification mixture consisted of 2.5 µl template DNA, 10 nmole of each primer, 2.5 µl PCR buffer, 2 µl dntp, 0.5 µl mM MgCl2, 15 µl D.W. and 0.5 µl of Taq DNA polymerase [14].

Preparation of extracts

Cinnamon (*Cinnamomum cassia* Family: *Lauraceae*) Bark, Clove (*Syzygium aromaticum* Family: *Myrtaceae*) Buds, Licorice (*Glycyrrhiza glabra* Family: *Fabaceae*) Dried roots, Rosemary (*Rosmarinus officinalis* Family: *Lamiaceae*) Leaf, Sage (*Salvia officinalis* Family: *Lamiaceae*) Leaf were chosen for the study.

The air-dried leaves and plant parts were collected and ground into powder using a sterile pestle and mortar. The grounded plant parts were subjected to extraction process by maceration , 10g of air-dried powder of plant parts was

mixed with 100 ml solvent (ethanol, methanol, diethyl ether, hexane *or* water) at room temperature for 48 hours with occasional stirring [15].

Thereafter, it was filtered through filter paper Wattman filter No. 1.The supernatant was collected and the solvent was evaporated (Polar and non-polar solvents were evaporated at 40-60ºC and water extracts were evaporated at 90-100ºC), in vacuum evaporator to make the final volume about 5ml, transfer to test tube, dissolve in Dimethyl sulfoxid (DMSO) and their antibacterial activity were studied according to The modified agar well diffusion method [16] and MIC of plant extracts was evaluated by agar dilution method in Mueller Hinton agar medium EUCAST [13].

Evaluation of the synergistic effect between plant extracts with lowest MICs
The Effect of combination of plant extracts with lowest MICs on MRSA isolates was evaluated by a checkerboard titration assay in plates [17]. Plant extracts were tested at concentration range (MIC –1/32 MIC) of each plant extract. The Plates of 18ml MHA and 2ml (1ml of each plant extract in combination) were prepared, 2µl of each bacterial strain (104CFU/ml) were inoculated on the Mueller Hinton agar surface. Plates were assessed visually for growth after an 18-24hr incubation period at 37°C.

The Fractional Inhibitory Concentration (FIC) was derived from the lowest Concentration of two plant extracts in combination permitting no visible growth of the test organisms on the MHA plates; FIC indices were calculated using this formula:

FIC index=MIC of X in combination /MIC of X + MIC of Y in combination/ MIC of Y

Synergy (S) = FIC≤ 0.5, Addition (A) = 0.5<FIC<1, Indifference (I) =1<FIC<4 and Antagonism (AN) = FIC≥4.

Studying mode of action by Protein analysis and TEM
-SDS- Polyacrylamide Gel Electrophoresis (SDS-PAGE):
MRSA isolate was harvested after 24 h after exposure to *G. glabra* and *R. officinalis* extracts separately at their MICs, comparing them with cells without treatment as a control. The pelleted cells were resuspended in one volume of sample buffer and incubated for 5 min. at 100 °C, followed by immediate cooling in ice, cell debris removed by centrifugation at maximum speed for 3 min. The supernatant containing total cellular protein was separated on 10% (w/v) SDS-PAGE gels. Discontinuous polyacrylamide gels consist of a resolving or separating (lower) gel and stacking (upper) gel. The stacking gel acts to concentrate large sample volumes, resulting in better band resolution[18].

-TEM
It was carried out using JEOL (jem 1230 EM) Transmission Electron microscopy, in National Research Center. MRSA incula were prepared from 12 h age cultures and standardized to 108 CFU/ml. The stock extracts were at concentration 0.05mg/ml for *G. glabra* extract and 0.39mg/ml for *R. officinalis* extract. One milliliter of 10^8 CFU/ml of MRSA isolate suspensions were incubated with 1 ml of each plant extracts solution for 24h at 37°C. After incubation, the cells were centrifuged at 6000 rpm for 15 min and washed twice with 0.01 M potassium phosphate buffer (pH 7.0). The samples obtained in the form of pellets after centrifugation, which were fixed with 2% glutaraldehyde for 2 h at 4º C. The pellets thus obtained were dehydrated in a gradient ethanol (10-100%). Up to 40% ethanol centrifugation carried out after which the cells were transferred onto the slide and treated up to 100% ethanol followed by drying the slides in desiccators[19].

Statistical analysis
Data of antibacterial activity of the most potent plant were conducted in triplicates for consistency of results and statistical purpose. Statistical analysis was performed using the statistical package SPSS v 20.0 (SPSS for Windows; SPSS Inc., Chicago, IL). Mean comparison was made through one sample t-test. P values less than 0.05 (95% confidence level) are reported as statistically significant, for determination of the suitable solvents of the most potent plants.

RESULTS

Phenotypic and biochemical characteristics of *S.aureus* isolates
Clinical isolates in this study were identified based on their morphological and biochemical characteristics. Examination of isolates grown on nutrient agar plates revealed the characteristic appearance of staphylococcal colonies.

Bacterial colonies were golden yellow, circular, smooth and glistening. Analysis of Gram-stained cultures of all 192 isolates showed that they were Gram-positive cocci arranged in bunches a characteristic morphology of staphylococci. 112 isolates were able to ferment Mannitol when grown on Mannitol salts agar (MSA) medium and were positive to Catalase test, Coagulase test and DNase test, also growth on Baird-Parker agar shows black in colour with clear zones produced around them , while growth on Vogel Johnson media identified it by its ability to reduce Tellurite (producing grey black colonies) and ferment Mannitol (given yellow color around the colonies) and Blood agar hemolysis result in clearing of the red from the surrounding medium around the colony of *Staphylococcus aureus* produces alpha toxin which typically causes wide zones of beta (complete) hemolysis.

Methicillin resistance among isolates

Methicillin Resistant *Staphylococcus aureus* (MRSA) isolate were identified by susceptibility to Methicillin antibiotic disc and by using CHROMagar MRSA medium that produce mauve-colored colonies resulting from hydrolysis of the chromogenic substrate.

Multi-drug resistance among *S. aureus* isolates

The susceptibility Percentages to different antibiotic classes (23 antibiotics) of identified 112 *S.aureus* isolates were described in Table 1 and Figure 1, we can deduce that the highest resistance percentages were achieved by Oxacillin, Ampicillin, Methicillin and Vancomycin with resistance percentage 83.2, 82.3,82 and 80.5% respectively. From 112 *S.aureus* isolates about 82% of the isolates were identified as MRSA and for easy going we randomly choose 12 MRSA isolate from them and renumbered them again to complete our studies on.

Table 1: Susceptibility Profile 112 *S. aureus* isolates

Antibiotics	Resistance %	Intermediate %	Sensitive %
Gentamycin (CN)	67.25	12.39	20.36
Neomycin(N)	69.03	22.12	8.85
Amikacin(AK)	61.06	18.58	20.35
Cephalaxein (CL)	76.99	8.85	14.16
Cefuroxime (CXM)	76.99	10.62	12.39
Cefotaxime (CTX)	68.14	20.35	11.5
Cefepime (FEP)	75.22	14.16	10.62
Ipenenem (IPM)	55.75	5.3	38.94
Naldixic acid(NA)	69.03	16.8	14.16
Ciprofloxacin(CIP)	64.6	15.04	20.35
Norfloxacin(NOR)	64.6	15.04	20.35
Levofloxacin(LEV)	66.37	15.04	18.6
Azithromycin(AZM)	75.22	10.62	14.16
Erythromycin (E)	76.99	9.73	13.27
Ampicillin(AMP)	82.3	7.08	10.62
Oxacillin(OX)	83.2	7.96	8.85
Methicillin(MET)	82	9	9
Sulphonamides&Tri-methoprim (SXT)	59.3	16.81	23.9
Doxycycline(DO)	54.87	19.47	25.66
Chloramphenicol (C)	62.83	9.7	27.4
Vancomycin (VA)	80.5	11.5	9.7
Clindamycin (DA)	49.6	23	11.5
Nitrofurantoin (F)	57.5	15.9	26.5

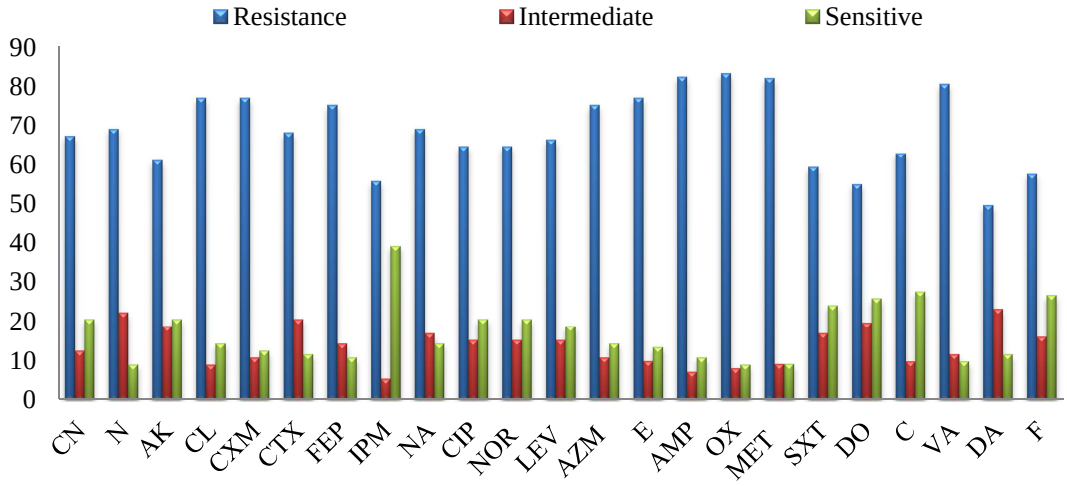

Figure 1: Susceptibility Percentages of 112 *S.aureus* to antibiotic classes

MIC of different antibiotic classes against *S. aureus* isolates

Results in Figure 2, clarify variable range of MICs towards different antibiotic classes and variability of MRSA isolates, where Penicillin and Ampicillin MICs varied from resistant to borderline resistant isolates (512,62µg/ml).

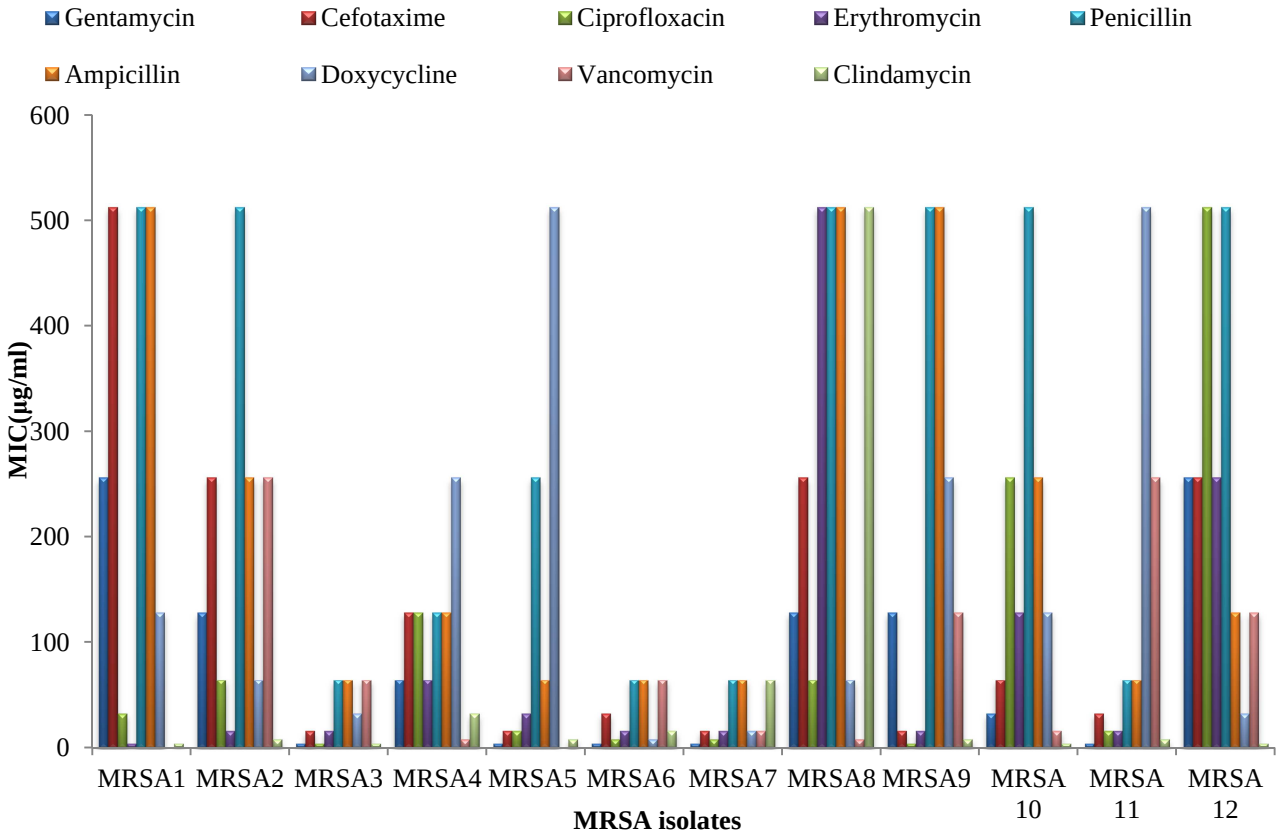

Figure 2: MICs of different antibiotic classes to MRSA isolates

Molecular confirmation of MRSA isolates

To confirm methicillin resistance in MRSA isolates on the molecular level, we tested the presence of *mecA* gene in isolates. Results in

Figure **3** showed that 532-bp fragment of *mecA* was amplified from the DNA of all MRSA isolates, among the 12 selected *S. aureus* isolates that demonstrates resistance to methicillin by the disc diffusion test, only 8 isolate(1,2,4,5,8,9,10 and 12) harboured *mecA* gene and 4 isolates(3,6,7,11) were Borderline *S. aureus* (BORSA) isolates**.**

Figure 3: Electrophoretic profile of PCR products of MRSA isolates, Lane M: marker 50bp ladder, Lane N: negative control, Lane 1, 2,4,5,8,9,10 and 12: positive *mecA* gene, Lane 3, 6, 7 and 11: *mecA* gene was not detected

Antibacterial activity and Minimum inhibitory concentration (MIC)

C. cassia, S. aromaticum, G. glabra, R. officinalis and *S. officinalis* showed marked inhibitory effect against almost all studied MRSA isolates. The results given in

Figure 4 indicates that the antibacterial activity of methanol extract of *C. cassia* and *S. aromaticum*, and diethyl ether extract of *G. glabra*, *R. officinalis* and *S. officinalis* were the most potent extracts against MRSA isolates.

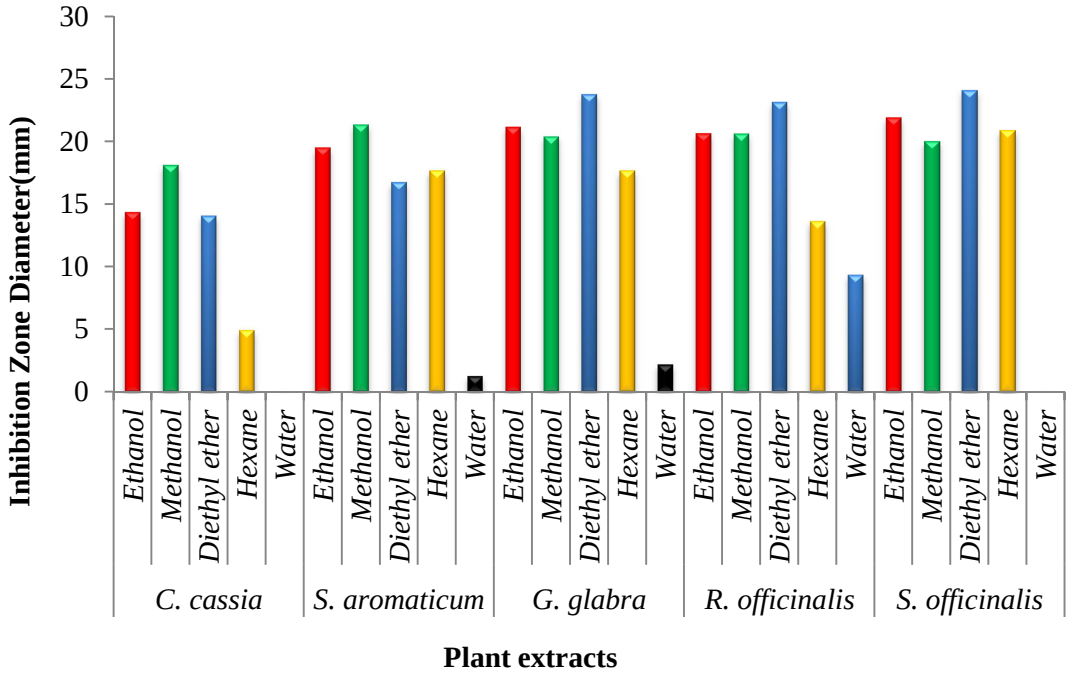

Figure 4: Mean inhibition zone diameter of plant extracts

MIC of methanol extract of *C. cassia* and *S. aromaticum*, and diethyl ether extract of *G. glabra*, *R. officinalis and S. officinalis* was recorded in Table 2, clarify that plant extracts with the lowest MIC values were *G. glabra*=0.05mg/ml, *R. officinalis*=0.39mg/ml and *S. officinalis*=0.195mg/ml.

Table 2: Minimum Inhibitory Concentration of plant extracts on MRSA

Plant/extract	MIC(mg/ml)
C. cassia/methanol extract	1.56
S. aromaticum /methanol extract	0.78
G. glabra/diethyl ether extract	0.05
R. officinalis/diethyl ether extract	0.39
S. officinalis /diethyl ether extract	0.195

Synergistic effect between plant extracts

Upon combination between plant extracts with lowest MICs values we find that, combination between *G. glabra* and *R. officinalis* diethyl ether extract was the only one which shows a case of synergy. There is a reduction in MIC of *G. glabra* and *R. officinalis* diethyl ether extract to 0.0125mg/ml and 0.097mg/ml respectively (Table 3). So the efficacy of each extract can be improved by combination together.

Table 3: Combination between *G. glabra* and *R. officinalis* diethyl ether extract

		***R. officinalis* mg/ml**						
		0.39	**0.195**	**0.0975**	**0.048**	**0.024**	**0.0125**	**0.006**
***G. glabra* mg/ml**	**0.05**	NG	NG	NG	G	G	G	G
	0.025	NG	NG	NG	G	G	G	G
	0.0125	NG	NG	NG	G	G	G	G
	0.0625	NG	NG	G	G	G	G	G
	0.003	NG	NG	G	G	G	G	G
	0.0015	NG	NG	G	G	G	G	G
	0.0008	NG	NG	G	G	G	G	G

Studying mode of action of plant extracts by:

-Protein analysis

From

Figure 5 we can deduce that *G. glabra* extract induce a kind of over expression of certain proteins which may have an inhibitory effect on cell growth and its virulence effect, as revealed from the broad bands in lane 3 in comparison with those bands in lane 4 of untreated cells, in contrary with the effect of *R. officinalis* extract there is a kind of down expression of major soluble proteins which, may be the reason of its antibacterial activity, as revealed from the fused faint wide band in SDS-page electrophoresis that is loaded with the same amount of *G. glabra* extract and untreated cells.

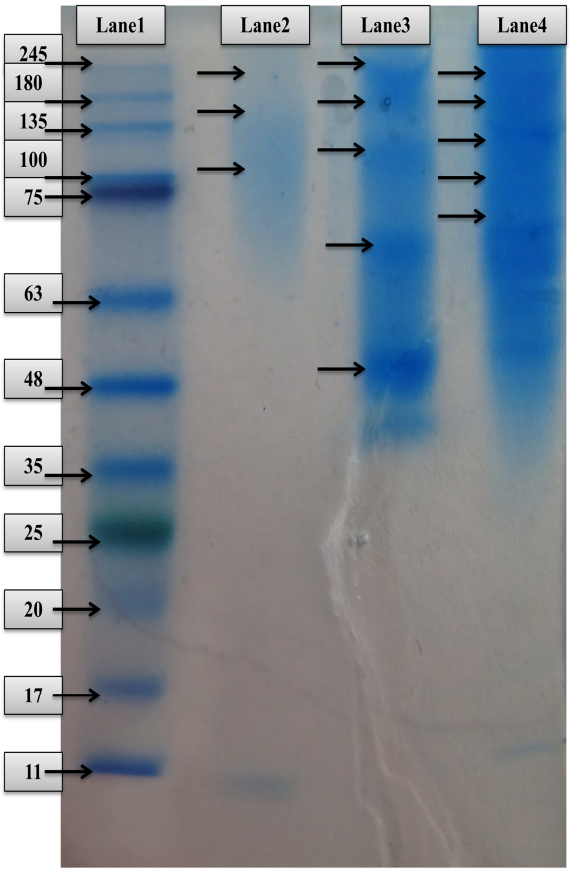

Figure 5: SDS-page Photograph: Lane 1: represent protein marker, Lane 2: represent MRSA cells treated with R. officinalis extract, Lane 3: represent MRSA cells treated with G. glabra extract and Lane 4 represent untreated MRSA cells, black arrows points to bands in each lane.

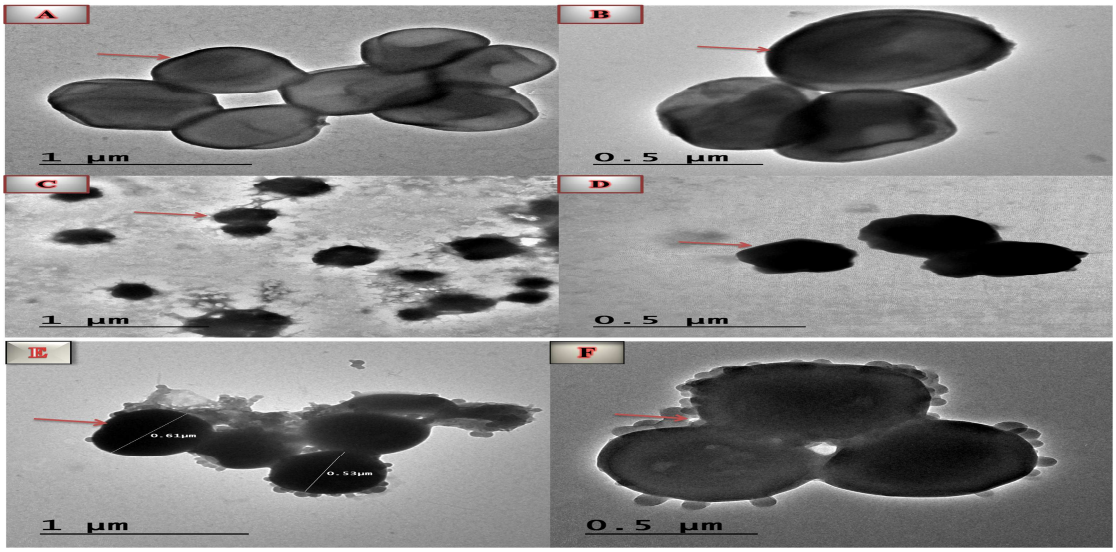

Figure 6: TEM Photograph of MRSA isolate treated with R. officinalis and G. glabra diethyl ether extracts showing the cytological changes in cells: (A) & (B) control cells, (C) & (D) cells treated with *R. officinalis* extract, (E) & (F) cells treated with *G. glabra* extract.

-TEM

The morphology of MRSA treated cells was examined using Transmission Electron Microscope (TEM). MRSA cells were grown in incubator shaker at 37 °C for 24 h. in the presence of 0.05 mg/ml of *G. glabra* extract and 0.39 mg/ml of *R. officinalis* separately. As illustrated in

Figure 6 untreated cells of MRSA (control) typically had a structured nucleus and a cytoplasm with several elements of endomembrane system enveloped by a regular, intact cell wall plasma membrane lying closely to the cell wall. After being exposed to either *G. glabra* or *R. officinalis* extract, cells appeared vertically more oblong accompanied by the shrinkage of protoplast and disruption of cytoplasmatic membrane. In addition, the cytoplasmic volume is contemplated decreasing following the cell membrane invagination causing notable structural disorganization within the cell cytoplasm.

DISCUSSION

High prevalence of multidrug resistance indicates serious need for antibiotics surveillance program. Karthy*et al.* [20]stated that antibiotic resistance is the ability of a microorganism to withstand the effects of an antibiotic. The extensive use of antibiotics over the last 50 years has led to the emergence of bacterial resistance and to the dissemination of resistance genes among pathogenic microorganisms. The aim of the present work was mainly designed to investigate antibacterial activity of few medicinally important plants and evaluate their synergistic effect on MRSA.

Data in the present study also revealed that most of *Staphylococcus aureus* isolates were resistant to multiple antibiotics. These high proportions of multi-drug resistant isolates could be attributed in part to the antibiotic misuse as exposure to antimicrobial agents is considered a risk factor for the acquisition and transmission of MRSA [21].

Our data was alarming and revealed even higher proportions of MRSA isolates among identified *S. aureus* isolates. Susceptibility tests revealed that out of 112 *S.aureus* isolates, 82% of them were confirmed to be MRSA. In recent years, MRSA has become widespread world-wide. Also in a recent study, Bassyouni*et al.* [22] reported the detection of 53% of MRSA among the studied *S. aureus* clinical isolates . In the United States, the proportion of MRSA isolates increased from 2.4% in 1970s to 55% in 1990s [23] [24]. Similar increase in the resistance rate and detection of new MRSA strains were also reported in Europe [25] [26] and in other parts of the world [23]. Other studies conducted in Turkey reported higher proportions of MRSA in nosocomial *S. aureus* strains isolated from blood stream infections [27]. The prevalence of MRSA in Egypt in the years 2003, 2004, and 2005 was studied. Percentages of MRSA isolates were 33%, 50%, and 63% of the studied invasive *S. aureus* isolates respectively [28]. The percentage of MRSA isolates in our study is much higher and indicates the rapid increase in the prevalence of MRSA over years. These results are also in agreement with the increased rate of drug resistance reported all over the world [27] [23] [29].

In this study we observed isolates that were methicillin-resistant by disk diffusion method ,also *mecA* gene was not detected by PCR method ,as appeared in MRSA isolates 3,6,7 and 11.Probably this resistance is phenotype, associated with modifications in native PBPs, beta-lactamase hyperproduction, or possibly a methicillinase [14]. Strains of *S. aureus* for which Oxacillin MICs are 1-2 μg/ml have been called acquired resistant, borderline, or partial borderline susceptible[30]. Resistance to methicillin primarily derives from acquisition of the *mecA* gene, not native in this species, which codes a modified penicillin-binding protein (PBP2a) with low affinity for β-lactams [31].

The ethno-botanical approach assumes that the traditional uses of plants can offer strong clues to the biological activity of plants and their usage in medical treatment. Our data also showed that some antimicrobial substances could only be extracted by organic solvents, where most of the antibacterial principles were either polar or non-polar and were extracted through the organic solvent medium as shown by Britto [32]. Similar results were shown by Krishna*et al.* [33] and Singh and Singh [34] .Also it was reported that organic solvents are clearly better solvents of antimicrobial agents[35].The polarity of antibacterial compounds make them more readily extracted by organic solvents, and using organic solvents does not negatively affect their bioactivity against bacterial species.

In our study, methanol extract of *C.cassia* has a mean inhibition zone diameter ~18.13mm .It was reported byShan*et al.* [36] that methanol and ethanol extracts of Cinnamomum bark were most effective against *B. cereus*, *E. coli* and *S. aureus*. These results confirmed the observation of Fan and Chen [37], Yuste and Fung [38]. San*et al.* [39] also reported that cinnamon showed potent and maximum activity among some plants extracts against *Staphylococcus aureus*.

Syzygium aromaticum (commonly known as clove) is among the *Myrtaceae* family which demonstrated significant antimicrobial activities against wide range of microorganisms. Results showed mean inhibition zone diameter ~21.3mm,, which lines with Pandey and Singh [40] who found that ethanol and methanol extracts of *S. aromaticum* showed good inhibitory activity in comparison of selected antibiotic (tetracycline) against *Ps. aeruginosa*, *E. coli* and *S. aureus*. Concerning the potent effect of *S. aromaticum* plant, the results were in good accordance with those reported by many investigators [41] ,[42] ,[43] ,[44] and 45].
The wide range of therapeutic properties of the root of *Glycyrrhiza* is well known [46].In our data it is recorded that *G. glabra* diethyl ether extract showed the most potent activity over its other extracts with mean inhibition zone diameter ~23.72mm. *G. glabra* extract has shown magnificent antibacterial effect and is well known for its expectorant and demulcent activity [47]. Our results correspond with Mowrey [48] who stated that the alcoholic extracts of *Glycyrrhiza* have displayed antimicrobial activity. He*et al.* [49] indicated the antibacterial activity due to pterocarpenes ,glycyrrhizol A and glycyrrhizol B and Tsukiyama*et al.* [46] reported the presence of Licochalcone A.
Rosemary (*Rosmarinus officinalis* L.) is commonly used as spice and flavoring agents in food processing for its desirable flavor. Our experimental results recorded that *R. officinalis* diethyl ether extract mean inhibition zone diameter was ~ 23.103mm, which is explained by the presence of phenolic compounds in *R. officinalis* ,with its high antimicrobial activity against both Gram-positive and Gram-negative bacteria [50]. [50].

Our results revealed the high antibacterial activity of *S. officinalis* diethyl ether extract mean inhibition zone diameter ~ 24.051mm. These findings agree with that reported by Gutierrez*et al.* [51] who confirmed that *S. officinalis* have antibacterial activity against food borne bacteria. The same results obtained byHoriuchi*et al.* [52] who confirmed the antimicrobial activity of *S. officinalis* against *Streptococcus pneumoniae* and methicillin-resistant *Staphylococcus aureus* (M RSA).

Our experimental data indicated a synergistic interaction between *G. glabra* and *R. officinalis* extracts at 0.0125mg/ml and 0.097mg/ml respectively. Combinations of two or more compounds are generally superior to the use of a single compound, especially for the treatment of serious infections caused by antibiotic resistant bacteria. it was stated inhibition of ß-lactamase by natural plants is remarkable, although they were not directly tested against this specific enzyme isolated from MRSA[53].

G. glabra extract induce a kind of over expression of certain proteins which may have an inhibitory effect on cell growth and its virulence effect, as revealed from the broad bands in lane 3 in comparison with those bands in lane 4 of untreated cells, in contrary with the effect of *R. officinalis* extract there is a kind of down expression of major soluble proteins which, may be the reason of its antibacterial activity, as revealed from the fused faint wide band in SDS-page electrophoresis , that is loaded with the same amount of *G.glabra* extract and untreated cells. Release of intracellular proteins is hallmark for the membrane damage and/or loss of membrane integrity [54]. On the other hand, it was suggested that the disappearance of protein bands might point to a specific mechanism, ultimately leading to bacterial death [55].

Transmission electron microscopy (TEM) is a microscopic technique commonly used for the analysis of materials on the nanoscale. Because it uses electrons, which have a shorter wavelength than light, it is capable of achieving resolution a thousand times better than can be achieved with a light microscope [56].The morphology of MRSA treated cells was examined using TEM. It was found that the untreated (control) of MRSA cells typically had a structured nucleus and a cytoplasm. After being exposed to either *G. glabra* or *R. officinalis* extract, cells appeared vertically more oblong accompanied by the shrinkage of protoplast and disruption of cytoplasmatic membrane. In addition, the cytoplasmic volume is contemplated decreasing following the cell membrane invagination causing notable structural disorganization within the cell cytoplasm. Some authors have suggested that the damage to the cell wall and cytoplasmic membrane was the loss of structural integrity and the ability of the membrane to act as a permeability barrier [57]. The distortion of the cell physical structure would cause the expansion and destabilization of the membrane and would increase membrane fluidity, which in turn would increase passive permeability [58] and manifest itself as a leakage of various vital intracellular constituents, such as ions, protein, nucleic acids, and amino acids [58]. In another study, it was found that the antibacterial substances can easily destroy the bacterial cell wall and cytoplasmic membrane and result in a leakage of the cytoplasm and its coagulation [59].

CONCLUSION

These data led us to approach the conclusion that, the plants extract mixture of *G. glabra* and *R. officinalis* extracts may interfere with bacterial protein synthesis, and cell wall formation and can be used as a promising antibacterial agent

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