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Synergistic Effects of Plant Extracts and Antibiotics on *Staphylococcus aureus* Strains Isolated from Clinical Specimens

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Abstract: This study has been done to evaluate the interaction between water extracts of *Psidium guajava*, *Rosmarinus officinalis*, *Salvia fruticosa*, *Majorana syriaca*, *Ocimum basilucum, Syzygium aromaticum*, *Laurus nobilis* and *Rosa damascena* alone and then synergy testing of these extracts with known antimicrobial agents of different mechanisms (protein synthesis inhibition: oxytetracycline HCl and gentamicin sulfate; cell wall synthesis inhibition: penicillin G and cephalexin; folic acid synthesis inhibition: Sulfadimethoxine as sodium; and nucleic acid synthesis inhibition: enrofloxacin) using both well-diffusion and microdilution method. This study was conducted against five *S. aureus* isolates; one is Methicillin-resistant *Staphylococcus aureus* (MRSA) and 4 Methicillin-sensitive *Staphylococcus aureus* (MSSA). The results of the conducted experiments using well-diffusion method demonstrate that these plants showed *in vitro* interactions between antimicrobial agents and plant extracts were additive against the five strains of *S. aureus*, while using microdilution method showed synergistic effects between combination of antibiotics and plant extracts with significant reduction in the MICs of the test antibiotics against these strains of *S. aureus*. This change in MIC was noticed in all plant extracts against test antibiotics including these plants showed weak antibacterial activity by well diffusion method. Also our results showed that synergism effect between antimicrobial agent and plant extract was occurred in both sensitive and resistant strains but the magnitude of minimum fold inhibition in resistant strains especially MRSA strain was higher than the sensitive strains.

Key words: Plant extracts · Synergistic effects · Antimicrobial agents · Microdilution method · Well diffusion method

INTRODUCTION

The wide use of antibiotics in the treatment of bacterial infections has led to the emergence and spread of resistant strains. Methicillin-resistant *Staphylococcus aureus* (MRSA) is a major cause of nosocomial infections. MRSA infections are very difficult to cure because MRSA strains are resistance against almost all clinically available antibiotics. For most MRSA strains, glycopeptide-type drugs such as vancomycin are the only effective antimicrobial agents [1]. However, vancomycin-resistant *S. aureus* (VRSA) has been reported [2-4]. Thus, it is extremely important to find new antimicrobial agents or new ways that are effective for the treatment of infectious diseases caused by drug-resistant bacteria including MRSA [5].

Few studies have found that the efficacy of antimicrobial agents can be improved by combining them with crude plant extracts against different pathogens including *S. aureus*, *P. aeruginosa*, *E. coli*, Extended Spectrum β-lactamases-producing multidrug-resistant *E. coli* and vancomycin-resistant enterococci (*Enterococus fecalis*) [6-18]. Some Palestinian plants exhibit significant potency against human bacterial [19-21]. But plant extracts as antimicrobials are rarely used as systemic antibiotics at present, this may be due to their low level of activity, especially against Gram-negative bacteria. Here we are trying to investigate an alternative approach to the treatment of bacterial infections by able to change the phenotype of a resistant pathogen to certain antibiotics to more susceptible pathogen to that antibiotics. This alternative approach has been evaluated

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using *in vitro* interaction between water plant extracts of the following plants: *Psidium guajava*, *Rosmarinus officinalis*, *Salvia fruticosa*, *Majorana syriaca*, *Ocimum basilucum*, *Syzygium aromaticum*, *Laurus nobilis*, *Rosa damascena* and certain known antimicrobial drugs such as oxytetracycline HCl, gentamicin sulphate, penicillin G, cephalexin and enrofloxacin using well diffusion method and minimum inhibitory concentration (MIC) by microdilution method against 4 Methicillin-sensitive *Staphylococcus aureus* (MSSA) and one strain MRSA.

MATERIALS AND METHODS

Plant Material and Extract Preparation: The plant materials used in this study consisted of *Psidium guajava* (leaf), *Rosmarinus officinalis* (leaf), *Salvia fruticosa* (leaf), *Majorana syriaca* (leaf), *Ocimum basilucum* (leaf), *Rosa damascena* (flower) which are growing in Palestine and *Syzygium aromaticum* (dried flowerbud), *Laurus nobilis* (leaf) were collected from Palestinian markets. These plants were identified by Dr. Firas. D. Sawalha, Department of Plant Production, Faculty of Agriculture, An-Najah National University, Nablus, Palestine. Water extract was prepared as describe previously [21]. Approximately of 30 g of dried plant materials were separately powdered, extracted by adding 150-200 ml boiled distilled water and then kept for 1 h. The extracts were filtered through Whatman No. 2 filter paper under vacuum. Extracts were concentrated to dryness at 37°C. Then, 100 mg of the dry residue was dissolved in 1 ml of sterile distilled water.

Antimicrobial Agents and Antibiotics: Five antimicrobial agents were evaluated for synergism assays with different plant extracts. These agents included oxytetracycline HCl (10%), enrofloxacin (10%), getamicin Sulphate (50%), cephalexin (0.15%) and penicillin G (penicillin G procaine 900000 and penicillin G sodium 300,000 I.U). All these antimicrobial agents were produced by Jerusalem Pharmaceutical CO. Balsam branch except penicillin G was produced by Birzeit-Palestine Pharamaceutical CO and were diluted to a final concentration 100 μg ml^{-1} except penicillin G to 100 I.U ml^{-1} for well diffusion method and 200 μg ml^{-1} and 200 I.U ml^{-1} for MIC test.

Bacterial Strains: Five *S. aureus* isolates (one MRSA and 4 MSSA) used in this study were recovered from urine sample, Thabet Thabet's Hospital, Toulkarm; semen sample, Al-Zaka Hospital, Toulkarm; diabetic foot wound, Al-Makased Hospital, Jerusalem; chest wound, Al-Makased Hospital, Jerusalem and leg wound, Al-Makased Hospital, Jerusalem. All isolates were identified in the microbiology laboratories of An-Najah National University, Palestine, by Gram stain, culture properties on nutrient agar and mannitol salt agar, catalase test, detection of hemolysis on 5% blood agar and by tube coagulase reaction. *S. aureus* strains were tested for methicillin resistance using the disk diffusion method [22]. Oxacillin (1 μg) and methicillin (5 μg) disks (Oxoid) were used. Zones of inhibition were determined in accordance with procedures of the National Committee for Clinical Laboratory Standard [23], *S. aureus* isolates considered susceptible to methicillin and oxacillin if inhibition zones are =14 and =13 mm, respectively. A reference strain [Bacillus subtilis ATCC6633] was also tested.

Antimicrobial Activity Tests

Determination of the Combined Activity Using Well-diffusion Method: Antibacterial activity was measured using a well diffusion method according to the National Committee for Clinical Laboratory Standard [24]. Briefly, Petri plates containing approximately 25-30 ml of Mueller Hinton agar medium were inoculated using a cotton swab with a 4-6 h old culture of the bacterial strains. Wells (6 mm diameter) were punched in the agar and filled with 30 μl of plant extracts or antibiotics and in case of synergism effect 30 μl of each has been added into well. Replicate of each plate has been done. The plates were incubated at 37°C for 18-24 h. The antibacterial activity was assessed by measuring the inhibition zone diameter (mm) around the well. The average of three replicates for each extract, antibiotic and combination has been calculated. Synergism effect was considered when combinations exhibited with enlargement of combined inhibition zone size by ≥5 mm [9].

Determination of MIC by Microdilution Method: MIC of antibiotic was determined by the microdilution method as described by [25]. The antibiotic was serial diluted in Mueller Hinton broth. Plant extracts solution were separately added into wells in a final concentration 1.5 mg ml^{-1}, then bacterial inoculum size of 104 CFU ml^{-1} was added to each well. Controls without plant extracts, without bacterial inoculum or with plant extracts only were also included in the experiment. Each plant extract was run in duplicate. The test plates were incubated at 37°C

for 18 h. The MIC was taken as the minimum concentration of the dilutions that inhibited the growth of the test microorganism.

RESULTS

The results of the conducted experiments using well-diffusion method demonstrate that these plants contain bioactive compounds some of them has a weak effect such as *Ocimum basilucum* and *L. nobilis*. *In vitro* interactions between antimicrobial agents and plant extracts using the previous method were additive against the five strains of *S. aureus*, while using microdilution method showed synergistic effects between combination of antibiotics and plant extracts with significant reduction in the MICs of the test antibiotics against 5 strains of *S. aureus*. This change in MIC was noticed in all plant extracts against test antibiotics including these

Table 1: Minimum inhibitory concentration of antibiotics alone and in combination with aqueous plant extracts against 5 clinical isolates (4 MSSA and 1 MRSA) of *S. aureus* using microdilution method

	MIC (mg l^{-1})			
Antibiotic a/Plant extrat	Four strains of MSSA	One strain of MRSA	Minimum fold inhibition for MSSA strains	Minimum fold inhibition for MRSA strain
CN	1.563-3.125	25-50		
P. guajava + CN	0.0244-0.0488	0.195	32	128
R. officinalis + CN	0.0244	0.39	64	64
S. fruticosa + CN	0.0122-0.0244	0.195	64	128
M. syriaca + CN	0.0244-0.0488	0.78	32	32
O. basilicum + CN	0.0122-0.0244	0.39	32	64
S. aromaticum + CN	0.0122-0.0244	<0.0488	64	>512
L. nobilis + CN	0.0488-0.0977	0.39	16	64
R. damascena + CN	0.0122-0.0244	<0.0488	64	>512
ENR	0.195-0.39	0.39		
P. guajava + ENR	<6.1X10-3	<6.1X10-3	>32	>64
R. officinalis + ENR	<6.1X10-3	<6.1X10-3	>32	>64
S. fruticosa + ENR	<6.1X10-3	<6.1X10-3	>32	>64
M. syriaca + ENR	<6.1X10-3	<6.1X10-3	>32	>64
O. basilicum + ENR	<6.1X10-3	<6.1X10-3	>32	>64
S. aromaticum + ENR	<6.1X10-3	<6.1X10-3	>32	>64
L. nobilis + ENR	<6.1X10-3	<6.1X10-3	>32	>64
R. damascena + ENR	<6.1X10-3	<6.1X10-3	>32	>64
OT	0.78-6.25	25-50		
P. guajava + OT	<0.0244	0.0977-0.195	=32	128
R. officinalis + OT	<0.0244-0.0488	0.39	=16	64
S. fruticosa + OT	<0.0244	0.195	>32	128
M. syriaca + OT	0.0488	0.195-0.39	16	64
O. basilicum + OT	0.0488	0.39	16	64
S. aromaticum + OT	<0.0244	<0.0488	>32	>512
L. nobilis + OT	0.0488	0.78	16	32
R. damascena + OT	<0.0244	<0.0488	>32	>512
CL	0.195-0.39	50		
P. guajava + CL	<6.1X10-3	0.0488	>32	1024
R. officinalis + CL	<6.1X10-3	0.0977	>32	512
S. fruticosa + CL	<6.1X10-3	0.0977	>32	512
M. syriaca + CL	<6.1X10-3	0.39	>32	128
O. basilicum + CL	<6.1X10-3	0.39	>32	128
S. aromaticum + CL	<6.1X10-3	<0.0488	>32	>1024
L. nobilis + CL	<6.1X10-3	0.39-0.78	>32	64
R. damascena + CL	<6.1X10-3	<0.0488	>32	>1024
P	>100	>100		
P. guajava + P	1.563-3.125	0.0977	>32	1024
R. officinalis + P	0.78-1.563	0.195	>64	512
S. fruticosa + P	0.39-1.563	0.0488	>64	2048
M. syriaca + P	0.78-1.563	0.39	>64	256
O. basilicum + P	1.563-3.125	0.39	>32	256
S. aromaticum + P	0.78	<0.0488	>128	>2048
L. nobilis + P	1.563-3.125	0.39	>32	256
R. damascena + P	0.78	<0.0488	>128	>2048

a P, Penicillin G; CN, Gentamicin sulphate; CL, Cephalexin; SDM, sulfadimethoxine as sodium; ENR, Enrofloxacin; OT, Oxytetracycline Hcl

plants showed weak antibacterial activity by well diffusion method. Also our results showed that synergism effect between antimicrobial agent and plant extract was occurred in both sensitive and resistant strains but the magnitude of minimum fold inhibition in resistant strains especially MRSA strain was higher than the sensitive strains. Minimum fold inhibition with drug-plant extract combinations against these strains is presented in Table 1.

DISCUSSION

Combined antibiotic therapy has been shown to delay the emergency of bacteria resistance and may also produce desirable synergistic effects in the treatment of bacteria infection. Drug synergism between known antibiotics and bioactive plant extracts is a novel concept and could be beneficial (synergistic or additive interaction) or deleterious (antagonistic or toxic outcome). Despite the abundant literature about the antimicrobial properties of plant extracts, none of the plant derived chemicals have successfully been used for clinical use as antibiotics [26].

In our experiments, despite that some plant extracts showed weak antimicrobial effect using well diffusion method, the interactions between antibiotics and plant extracts were mainly additive against the five strains of MSSA and MRSA. This could be attributed to the inability of higher concentrations of plant extracts to diffuse through the nutrient agar medium. This impairment in drug diffusion is a major limitation in the evaluation of the antimicrobial effects of plant extracts using the agar diffusion method [11].

In this study, synergism effect resulting from the combination of antimicrobial agents with crude plant extracts was verified for all plants. Our results were consistent with previous *in vitro* studies which reported synergistic effects with significant reduction in the MICs of the antibiotics due to combination of different antimicrobial agents with different crude plant extracts against *S. aureus* strains [10-11, 13-15, 18] and stand out as veritable sources of potential resistance modifying agents [27-29].

In these experiments, the change in MIC was noticed in all plant extracts against test antibiotics including these plants showed weak antibacterial activity. These results were in agreement with a previous report who mentioned a synergetic effect even these extracts did not show any activity by themselves [17]. In addition, results showed that synergism effect was occurred in both sensitive and resistant strains but the magnitude of minimum fold inhibition in sensitive is less than resistant strains. These results were consistent with that which showed that synergistic interactions occurred in both resistant and sensitive *S. aureus* [15-16].

All plant extracts showed a decrease in MIC to test antimicrobial agents and this could be referred to that these crude extracts have many different phytochemicals [30], which might inhibit bacteria by different mechanisms. This double attack of both agents on different target sites of the bacteria could theoretically lead to either an additive or a synergistic effect [11]. Screening for such activities in crude extracts is the first step in identifying leads for isolation of such compounds and some plants have provided good indications of these potentials for use in combination with antimicrobial therapy. Further separation and purification of the crude extracts might show an increase in bioactivity than the crude extracts. This may be due to numerous compounds within the crude extracts may have interfered with the actions of one another. Once they were separated by various purification methods however, the inhibiting effect of one on the other had reduced significantly [6].

Here we recommended the evaluation of the exact drug-plant ratio at which the interaction in maximal between the plant extract and antimicrobial drug. A wider study with increase in the number of drugs, increase number of clinical isolates especially MRSA and the identification of the effective compounds in the crude extract are also necessary in order to establish the mode of action against the *S. aureus* isolates and the mechanism of synergy, which is fundamental to development of pharmacological agents to treat diseases by *S. aureus* using medicinal plants.

In conclusion, the results of this study were encouraging, although clinical controlled studies are needed to define the real efficacy and possible toxic effects *in vivo*. This study probably suggests the possibility of concurrent use of these antimicrobial drugs and extracts in combination in treating infections caused by *S. aureus* strains or at least the concomitant administration of these plants and antimicrobial drugs may not impair the antimicrobial activity of these antibiotics. However, it is hard to predict synergistic effects *in vivo* on the basis of the presented *in vitro* evidence alone because it is difficult to estimate the *in vivo* concentration of active ingredients, especially the bioavailable concentration of free (active) ingredients,

after plants have been ingested. Therefore, our results revealed the importance of plant extracts when associated with antibiotics to control bacteria.

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