

Inhibitory Effect of Mediterranean Sage and Rosemary on Clinical and Community Isolates of Methicillin-Resistant *Staphylococcus aureus*

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Received: April 17, 2014 Revised: May 6, 2014 Accepted: May 9, 2014

Abstract

Plant extracts are traditionally used for treating many infectious and non-infectious diseases. This study aimed at assessing the inhibitory effect of the ethanol extracts of two medicinal plants; Mediterranean Sage (*Salvia libanotica*) and Rosemary (*Rosmarinus officinalis*) on clinical and community strains of methicillin-resistant *Staphylococcus aureus* (MRSA). Ethanol extracts of the two plants were tested for their antibacterial effect against 25 clinical (n=15, 60%) and community (n=10, 40%) strains of MRSA. Rosemary and Mediterranean Sage extracts demonstrated activity against all isolates, 50µl of 100 mg/ml of each plant extract yielded inhibition zone reaching as high as 27 and 30 mm by agar well diffusion method. Effective MICs and MBCs of ethanol extracts of Rosemary and Mediterranean Sage against MRSA were 0.125 to 0.5mg/ml and 0.25 to 1 mg/ml respectively. Mixed ethanol extract of Rosemary and Mediterranean Sage showed antagonistic effect on MRSA strains. These results suggest the potential therapeutic implications of the ethanol extract from Rosemary and Mediterranean sage in the treatment of MRSA infections.

Key words: Mediterranean Sage; Rosemary; MRSA; Jordan.

1. Introduction

Traditionally, the dependence on alternative medicine in developing countries and other parts of the world is clearly obvious, they use preparations of any part of the plants for the purpose of pain relief, infection prevention or even as cosmetics (Ahmad *et al.*, 1998). Studies evaluating medicinal plants as a source of antimicrobials proved that plants' active components might be used as bacterial inhibition agents (Emori and Gaynes, 1993; Cos *et al.*, 2006). Medicinal plants may play a role as a natural source of antimicrobial drugs (Habeeb *et al.*, 2007).

Methicillin-resistant *Staphylococcus aureus* (MRSA) infections of both hospital and community acquired have increased remarkably during the last decade (Chambers, 1997). Hospital acquired MRSA (HA-MRSA) strains are mainly distinguished from community acquired MRSA (CA-MRSA) using molecular techniques. The SCC *mec* in HA-MRSA belongs to type I, II or III and is bigger than that of CA-MRSA, which belongs to SCC *mec* type IV or V. In addition, CA-MRSA strains frequently carry the gene for PVL (Panton Valentine Leukocidin) toxin which is not commonly found in HA-MRSA (Naimi *et*

al., 2003). Antibiotic options for patients with MRSA infection are usually restricted due to the wide range of MRSA antibiotic resistance. This has enhanced researchers to use other natural agents to fight MRSA, especially from medicinal plants (Schito, 2006). In Jordan, data revealed that MRSA infections both hospital and community acquired have been increased in the last few years, with percentage of 62% and 8% respectively (Borg *et al.*, 2007; Aqel *et al.*, 2012).

In a previous study carried out by some authors of this study (Ibrahim *et al.*, 2013), the effect of crude extract of *S. libanotica* and *R. officinalis* against two test strains of *Staphylococcus aureus* ATCC (25923) and an MRSA isolate was clearly identified. The present study aims to further assess the antimicrobial effect of *S. libanotica* and *R. officinalis* against clinical and community isolates of MRSA. Both Mediterranean Sage and Rosemary tested in this study are used traditionally in Jordan for purposes such as the treatment or relief of respiratory and gastrointestinal infections (Obeidat, 2011; Abu-Shanab *et al.*, 2004).

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2. Materials and Methods

2.1. Plant Samples and Manipulation

Two medicinal plants, *Salvia libanotica* (Mediterranean Sage) and *Rosmarinus officinalis* (Rosemary) were tested in this study (Table 1). The experimental part of the study was done in the department of microbiology and immunology, faculty of medicine, Mu'tah University, from 1 May 2013 to 15 September 2013. Plants were purchased locally from the markets. Leaves were collected to be air dried for 14 days and then powdered using mortar and pestle.

Table 1. Profile of the two medicinal plants used

Botanical Name	Family	Local Name	Plant part used
Rosmarinus officinalis	Lamiaceae	Hasa-alban	leaves
Salvia libanotica	Lamiaceae	Miramiyah	leaves

2.2. Ethanol Extracts Preparation

One hundred grams of each powder were extracted by cold maceration with 80% ethanol for 48 hrs at room temperature. Filtration by Whatman filters paper no. 2; evaporation and concentration of the extract under low pressure were applied consequently for each plant extract (Ahmad *et al.*, 1998). Powder samples were stored at 4°C. Primary active components and essential oils extraction were performed according to Böszörményi (2009). Briefly, oil extraction was done under water steam distillation for 3 hrs of 30 gm of the plants powder.

2.3. Test Organisms

A total of 25 non-repeat MRSA strains, 15 (60%) strains from different patients admitted to Al-Karak hospital, Jordan, and other 10 (40%) MRSA nasal swabs strains obtained from healthy individuals in the year 2013 were studied. The clinical strains were isolated from respiratory samples (n = 3; 20%), wound swabs (n = 3; 20%), urine (n = 4; 26.6%), pus (n = 1; 6.7%), catheter tip (n = 1; 6.7%), blood (n = 3; 20%). Isolates were identified morphologically and biochemically by standard microbiological procedures using Gram stain, catalase test, coagulase test and an API system (bioMérieux, France). Cefoxitin (30 µg) discs (Oxoid, Basingstoke, UK) were used for methicillin resistance determination. Susceptibility tests were applied according to guidelines of Clinical Standards Laboratory Institute (CLSI, 2012). Detection of *MecA* gene (encoding high-level resistance to methicillin) and 16S rRNA gene (internal control) were performed with DNA extraction, primers and amplification conditions according to Petinaki (2001). *Staphylococcus aureus* ATCC 29213 and 2 methicillin-susceptible *Staphylococcus aureus* (MSSA) strains were used as control strains during susceptibility testing and PCR procedures. Bacterial samples were preserved in transport media at 4°C and subcultured overnight before use. Consent was obtained from all participants after explaining the purpose of the study. The ethics and

scientific committee of the faculty of medicine at Mu'tah University approved the study (approval no. 22/2/12).

2.4. Agar Well Diffusion Method

Extracts inhibition effects were assessed by agar well diffusion method according to the National Committee for Clinical Laboratory Standards (NCCLS, 2000a). About 20 ml of Mueller Hinton agar (Oxoid, Hampshire, England) was poured into Petri dishes. After solidification, inoculum of 0.5 McFarland of each test strain was seeded on the media. Allowing inoculum to dry; 5 mm size wells was made with sterile borer. About 50 µl of 100 mg/ml of each extract was introduced into the well and plates were incubated at 37°C for 24 hrs. All samples were tested in duplicates. Other 2-32 mg/ml dilutions were prepared to determine the concentration effect (CE) on bacterial inhibition. Water, vancomycin (30 µg) and oxacillin (1 µg) disks were used as negative and positive controls (Abu-Shanab *et al.*, 2004). Antimicrobial effect was determined by measuring the diameter of zone of inhibition around the holes and disks.

2.5. Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC) by Broth Dilution

MIC and MBC were detected by using different extract concentrations from 0.0625 mg/ml to 32 mg/ml. In a test tube equal volume (0.5 ml) of both plant extracts and nutrient broth were mixed together. Standard microbial inoculums were added to each tube (0.1 ml of $1-2 \times 10^8$ cfu/ml). Tubes were aerobically incubated at 37°C for 24 hrs according to NCCLS (2000b). Vancomycin and oxacillin dilution tubes were prepared and used as positive and negative controls in all tests.

Test tube with no visible growth (no turbidity) was considered as the lowest extract concentration that inhibits bacterial growth (MIC), whereas, tube with no visible growth that yielding no colonies when subcultured on extract or drug free nutrient agar was considered as MBC tube (Weckesser *et al.*, 2007).

3. Results

3.1. Primary Assessment of Antimicrobial Activity

Qualitatively and quantitatively antibacterial activities of *S. libanotica* and *R. officinalis* against MRSA were in vitro assessed (Table 2). Fifty micro-liters of 100 mg/ml of *R. officinalis* and *S. libanotica* showed the greater effect with MIC and MBC values range of 0.125-0.25 mg/ml and 0.25-0.5 mg/ml and 0.25-0.5 mg/ml and 0.5-1 mg/ml of each plant extract respectively (Table 3).

Our results indicate that ethanol extract mixture of both *S. libanotica* and *R. officinalis* will diminish their potent antibacterial effect against MRSA with MIC and MBC increasing up to 4 to 8 mg/ml and 8 to 16 mg/ml, respectively (Table 3). 16S rRNA gene was positive for all staphylococcal strains. PCR product of *MecA* gene was detected in all MRSA strains but not MSSA strains.

Table 2. Antibacterial activity of the ethanol crude plant extract on *S. aureus* strains

Plant used	MRSA ^a (n=10)	MRSA ^b (n=15)	^c <i>S. aureus</i> and MSSA
<i>Rosmarinus officinalis</i>	28 [#]	26	28
<i>Salvia libanotica</i>	23	22	26
Vancomycin 30µg	21	21	22
Oxacillin 1µg	NA	NA	16
Negative control (Water)	NA	NA	NA

[#]Inhibition zone average in mm; ^c*S. aureus* = standard strain used ATCC 29213.

^a Community MRSA isolates; ^b Clinical MRSA isolates; ⁿ = number

Vancomycin, Oxacillin = standard antibacterial drug used as positive control of MRSA tested. NA=No activity

Table 3. MIC and MBC (mg/ml) of ethanol extracts of Rosemary and Mediterranean Sage and their combination on MRSA.

Plant used	MRSA ^a (n=10)		MRSA ^b (n=15)	
	MIC	MBC	MIC	MBC
<i>Rosmarinus officinalis</i>	0.125-0.25	0.25-0.5	0.25-0.5	0.5-1
<i>Salvia libanotica</i>	0.25-0.5	0.5-1	0.25-0.5	0.5-1
<i>R. officinalis/ S. libanotica</i>	4 – 8	8 – 16	4 – 8	8 – 16
Vancomycin	0.25-0.5	0.5-1	0.5-1	1-2
Oxacillin	NA	NA	NA	NA

Vancomycin, Oxacillin = standard antibacterial drug used as positive and negative controls. ^a Community MRSA isolates; ^b Clinical MRSA isolates; ⁿ = number

of MRSA tested. NA=No activity

Table 4. The concentration effect of the ethanol extracts of Rosemary and Mediterranean Sage on *S. aureus* and MRSA strains

Plant extract	Conc. (mg/ml)	Diameter (mean ± SD) of inhibition zone (mm) including well diameter of 6 mm		
		<i>Staphylococcus aureus</i> ATCC 29213	Community MRSA (n=10)	Clinical MRSA (n=15)
Rosemary	2	10.66 ± 1.15	8.75 ± 1.06	8.0 ± 1.04
	4	11.83 ± 0.28	10.5 ± 0.7	9.5 ± 0.1
	8	15.33 ± 1.52	14.75 ± 1.76	13.05 ± 1.5
	16	16.66 ± 0.57	17.00 ± 2.82	16.00 ± 2.5
	32	20.00 ± 0	19.00 ± 1.41	18.00 ± 1.4
Sage	2	8.16 ± 1.04	8.00 ± 0	7.8 ± 0.2
	4	10.16 ± 0.28	9.25 ± 1.06	9.1 ± 1.0
	8	16.33 ± 1.52	13.00 ± 1.41	12.00 ± 1.3
	16	17.66 ± 2.02	16.25 ± 0.35	15.5 ± 0.75
	32	19.00 ± 1.73	17.5 ± 0.7	16.2 ± 0.5

3.2. Determination of Concentration Effect (CE)

Results of CE for the bacterial samples are listed in Table 4. Ethanol extracts of both *S. libanotica* and *R.*

officinalis revealed inhibitory action on *Staphylococcus aureus* ATCC 29213 and MRSA in all added doses. No significant difference in bacterial inhibition zone for both extracts was noticed for all isolates sources at any specified concentration. Nevertheless, increasing concentrations resulted in greater inhibition zone for *S. libanotica* and *R. officinalis* against all isolates.

4. Discussion

World Health Organization encouraged health systems in different countries since 1980s to interact with herbal medicine for identifying and assessing means that build up bases for new and safe herbal agents which can be used for treatment of infectious and noninfectious diseases (WHO, 1978). The development of new antibacterial drugs for the treatment of MRSA infections is of increasing interest (Schito, 2006). Herbal medicine has long been used in Jordan for the treatment of various ailments (Obeidat, 2011; Ibrahim *et al.*, 2010). The incidence of MRSA infections in Jordan was obvious over the last few years (Aqel *et al.*, 2012). MRSA detection rate has been increasing especially in the hospitals. The resistance has been emerging not only to methicillin but also to other many antibiotics, including vancomycin leading to further restriction on available antibiotic options (Bakri *et al.*, 2007; Mohammad, 2010).

Experiments of microbial growth inhibition by agar well diffusion and broth dilution methods revealed that two plants (*S. libanotica* and *R. officinalis*) were active against MRSA strains. Similar antimicrobial results were obtained by other researchers (Abu-Shanab *et al.*, 2006; Obeidat, 2011).

In this study, volatile oils and other triterpenoids, the active components determined primarily from *S. libanotica* and *R. officinalis* showed antibacterial activity against MRSA, *S. aureus* and other tested isolates, this is in agreement with previous Jordanian study by Al-Bakri *et al.* (2010). A study by Nascimento *et al.* (2000) showed that the active chemical constituents obtained from *S. libanotica* and *R. officinalis* were mainly flavonoids, phenolic acids, rosmarinic, caffeic, chlorogenic acids, carnosol, diterpenes, camphor, thuyone and cineole; all these compounds and oils have remarkable antimicrobial activity. In the current study, the MIC and MBC results revealed that low extract concentration had a bacteriostatic action, whereas bactericidal action was detected at higher concentrations, this might be due to increased intracellular uptake of the extract and more cellular damage. The antagonistic effect of the combination of ethanol extracts of *S. libanotica* and *R. officinalis* was the major finding of our study; this finding is in agreement with other previous study by Abu-Shanab *et al.* (2006). Therefore, the combination of both previous extracts which are traditionally used in Jordan against number of diseases obviously has no efficient antibacterial effect.

5. Conclusion

Our previous study by Ibrahim *et al.* (2013) showed the inhibitory action of crud extract of *S. libanotica* and *R. officinalis* against two test strains of *S. aureus*; therefore,

and depending on the previous results, we tested the antibacterial effect of *S. libanotica* and *R. officinalis* against clinical and community strains of MRSA as a second phase. The results presented here indicate that *R. officinalis* and *S. libanotica* extract preparations can be used as antistaphylococcal agent, for both MSSA and MRSA infections. *In vivo* assessment of the dose, toxicity, tolerance and clearance of the active elements of the herbal plants need more investigations.

Acknowledgement

We would like to thank Mr. Shadi Jaber and Mr. Madhar Abumurad for their technical help.

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