



Synergistic effect between some antimicrobial agents and rosemary (*rosmarinus officinalis*) toward *staphylococcus aureus* – *in-vitro*

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ABSTRACT

The present study was carried out to evaluate the antimicrobial activity of ethanol plant extract of rosemary (*Rosmarinus officinalis*) and / with five antimicrobial agents of different mechanisms (oxytetracycline Hcl, amoxicillin, cefquinome, sulphaquinoxaline and danofloxacin) against field strain of *S. aureus*. By using agar well diffusion method, the mean zone of inhibition (mm) of ethanol extract of Rosemary were 21.67±0.33, 20.33±0.33, 19.67±0.17, 18.83±0.17, 18.17±0.17, 16.33±0.33 and 11.5±0.29 mm at different concentrations. While the mean zone of inhibition (mm) of different antimicrobial agents at different concentrations for amoxacillin was 24±0.29, 20.67±0.33, 17.67±0.33 and 14.17±0.17mm and for cefquinome was 20.83±0.17, 18±0.29 and 13.5±0.29 mm. no inhibition zones were detected with danofloxacin, sulphaquinoxalline and oxytetracycline Hcl. By macrodilution method, the MIC of rosemary was 0.048mg/ml and the MICs for the aforementioned antimicrobial agents were 0.015, 0.25, 1.0, 8.0 and 16 µg/ml respectively. The synergistic effects were recorded by FIC index between 0.006 and 0.00038 mg/ml of rosemary with 0.125 and 4.0 µg/ml of cefquinome and sulphaquinoxalline respectively.

Keywords: Rosemary, Antimicrobial agents, MICs, *S.aureus*

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1. INTRODUCTION

The incorrect and overuse of existing antimicrobials is becoming a formidable threat in the fight against disease due to the emergence of multi-drug resistant strains. Thus, alternatives to the standard treatments with single agents become necessary. In clinical practice, combination antibiotic therapy is being used in an attempt to broaden the bacterial spectrum and to avoid the emergence of resistant strains (Lambert, 2000 and van Vuuren et al., 2009). Furthermore, to avoid the undesirable toxic effects of antimicrobial therapy, the combination with herbal products may be an innovative alternative to prescriptive treatment protocols (Harris, 2002). Higher plants produce hundreds to thousands of diverse chemical compounds with different

biological activities. Accordingly, secondary products have both a defensive role against herbivory, pathogen attack, and inter-plant competition. They also have an attractant role toward beneficial organisms such as pollinators or symbionts (Briskin, 2000 and Mhanna and Adwan, 2008). Combinations of antimicrobials that demonstrate an in vitro synergism against infecting strains are more likely to result in successful therapeutic protocols. Thus, evidence of in vitro synergism could be useful in selecting most favorable combinations of antimicrobials for the practical therapy of serious bacterial infections (Hooton et al., 1984 and Aiyegoro and Okoh, 2009). Drug synergism between known antimicrobial agents and bioactive plant extracts is a

novel concept and has been recently reported (Nascimento *et al.*, 2000, Aqil *et al.*, 2005, Betoni *et al.*, 2006 and Mhanna and Adwan, 2008). The current study was planned to assess the antimicrobial activity of ethanol extract of rosemary and some antimicrobials agents such as oxytetracycline HCl; amoxicillin; cephquinome; sulphaquinoxaline sodium and danofloxacin. Both agar gel diffusion method against *S.aureus* strain and estimation of MICs by using macrodilution method were applied to evaluate *in-vitro* interaction between the used plant extract and antimicrobial agents.

2. MATERIAL AND METHODS

2.1. Bacterial strain

The examined strain of coagulase positive *S.aureus* from rabbits was provided from Microbiology Dep., Faculty of Veterinary Medicine, Benha University. The bacterial suspension was adjusted to be the proper density 1.5×10^6 bacteria / ml. (Golshani and Sharifzadeh, 2013).

2.2. Plant sample

2.2.1. Rosmary

Rosemary (*Rosmarinus officinalis*) is a woody, perennial herb with fragrant evergreen needle-like leaves. It is a member of the mint Family: Lamiaceae, Genus: Rosemarinus. The leaves are 2-4 cm long and 2-5 mm broad, green above, and white below with dense short woolly hairs. The flowers are variable in color, being white, pink, purple, or blue (Fritzweiss and Fintelmann, 2000 and Mhanna and Adwan, 2008).

2.2.2. Plant Extract preparation

Ten grams of dried ground plant material was added to 500 ml conical flasks plus 150 ml of solvent (ethanol 99.9%). Ratio (1 : 15) of the plant material and solvent with slow mixing, the extract solution was filtered the solvent was removed using rotary evaporator at 40 °C to obtain the crude extract and then it was kept in sterile bottles

at 4°C until use, the resulted deposit was dissolved in distilled water to prepare the different concentrations. (Parish and Davidson, 1993).

2.3. Media used

Mullar Hinton agar and broth (Himedia) were used for evaluation of antibacterial activity by agar well diffusion method (NCCLS, 1993) and determination of MICs by macrodilution method (Hindler, 1995) respectively.

2.4. Antimicrobial agents

Different antimicrobial agents as amoxicillin, sulphaquinoxaline and oxytetracycline HCl (El-Nasr Pharmaceutical Chemical Co.), cefquinome (Internet International GmbH) and danofloxacin (Adwia).

2.5. FIC index calculation

Antimicrobial agents were used at concentration (1/2 MIC) in combination of rosemary. Type of interaction between plant extract of rosemary and antibiotics was determined through calculation of FIC Index. The FIC (Fractional Inhibitory Concentration) value for each agent was calculated using the formula (El-Kalek and Mohamed 2012). FIC (antibiotic) = MIC of antibiotic in combination / MIC of antibiotic alone. FIC (extract) = MIC of extract in combination / MIC of extract alone. FIC Index = FIC (antibiotic) + FIC (extract). Synergistic: if the FIC indices were <1. Additive: if the FIC indices were = 1. Indifferent: if the FIC indices were between 1 and 2. Antagonistic: if the FIC indices were >2

3. RESULTS

3.1. Antibacterial activity of ethanol extract of rosemary

Agar well diffusion method was used to determine the mean inhibition zones of Rosemary which were 21.67 ± 0.33 , 20.33 ± 0.33 , 19.67 ± 0.17 , 18.83 ± 0.17 , 18.17 ± 0.17 , 16.33 ± 0.33 , 11.5 ± 0.29 and zero mm at different concentrations

(100,50, 25,12.5, 6.25, 3.125, 1.56 and 0.78) against field isolate of *S.aureus* respectively (Table, 1).

3.2. *Determination of antibacterial activity of antimicrobial agents*

Mean zones of inhibition of five Antimicrobial agents (amoxicillin, cefquinome, danofloxacin, sulphaquinoxaline and oxytetracycline) were determined against field isolate of *S.aureus* using agar well diffusion method. amoxicillin and cefquinome showed high inhibitory effect but danofloxacin, sulphaquinoxaline and oxytetracycline HCL showed no inhibitory effect (Table 2).

3.3. *Determination of MIC of rosemary ethanol extract*

The result of MIC of rosemary ethanol extract was 0.048 mg/ml by using macrodilution method.

3.4. *Determination of MICs of antimicrobial agents*

The MICs of antibacterial agents were 0.015, 0.25, 1.0, 8.0 and 16.0µg/ml for the different antimicrobial agents (amoxicillin, cefquinome, danofloxacin, sulphquinoxaline and oxytetracycline respectively).

Table (1): Inhibition zones (mm) of rosemary ethanol extract.

rosemary concentrations(mg/ml)	Zones of inhibition(mm)			Mean ± *SE
	Experiment 1	Experiment 2	Experiment 3	
100	22	21	22	21.67±0.33
50	20	20	21	20.33±0.33
25	19.5	19.5	20	19.67±0.17
12.5	19	18.5	19	18.83±0.17
6.25	18.5	18	18	18.17±0.17
3.125	16	17	16	16.33±0.33
1.56	12	11.5	11	11.5 ±0.29
0.78	*-	*-	*-	*-

*- = no inhibition zone.

*SE = Standard Error.

Table (2): Inhibition zones (mm) of Amoxicillin and Cefquinome.

Conc. µg/ml	Zones of inhibition (mm)								Mean ± SD
	Amoxacillin				Cefquinome				
	Exp. 1	Exp. 2	Exp. 3	Mean± SD	Exp. 1	Exp. 2	Exp. 3		
8.0	24	23.5	24.5	24 ±0.29	21	20.5	21	20.83±0.17	
4.0	21	20	21	20.67±0.33	17.5	18	18.5	18 ±0.29	
2.0	17	18	18	17.67±0.33	13.5	14	13	13.5 ±0.29	
1.0	14	14.5	14	14.17±0.17	*-	*-	*-	*-	
0.5	*-	*-	*-	*-	*-	*-	*-	*-	
0.25	*-	*-	*-	*-	*-	*-	*-	*-	
0.125	*-	*-	*-	*-	*-	*-	*-	*-	
0.0625	*-	*-	*-	*-	*-	*-	*-	*-	

*- = no inhibition zone.

*SE = Standard Error.

Table (3): Types of interactions between rosemary and different antimicrobial agents

Antimicrobial agent	FIC of rosemary	FIC index	Type of Interaction
amoxicillin	2.0	2.5	Antagonism
cefquinome	0.125	0.625	Synergism
danofloxacin	2.0	2.5	Antagonism
sulphaquinoxaline	0.008	0.508	Synergism
oxytetracycline	2.0	2.5	Antagonism

3.5. Determination of synergistic effect between rosemary and antimicrobial agents.

The synergistic interaction was determined between rosemary and cefquinome and between rosemary and sulphquinoxaline, but rosemary showed antagonistic interaction with amoxicillin, danofloxacin and oxytetracycline (Table, 3).

4. DISCUSSION

The current results indicated that ethanol extract of rosemary leaves with concentrations between 1.56 - 100 mg/ml has the ability to inhibit the growth of *S.aureus* on Muller Hinton agar with inhibition zones from 11.5±0.29 – 21.67±0.33 mm. These results were in accordance with those recorded by Rasheed *et al.*, (2010), Al Laham and Al Fadel (2013) and Golshani and Sharifzadeh (2013). The MIC for rosemary in this study was reported as 0.048 mg/ml while Rasheed *et al.*, (2010) and Golshani and Sharifzadeh (2013) reported MIC for rosemary as 0.5 mg/ml and 6.25 mg/ml respectively. The wide difference between the aforementioned results might be attributed to the method of extraction and the use of relatively higher temperature, which decrease the rate of destruction of effective herbal compound. The present study revealed that amoxicillin had inhibitory effect on Muller Hinton agar medium against *S.aureus* at concentrations 1.0–8.0µg/ml with inhibition zone between 14.17±0.17- 24±0.29 mm. Other concentrations which ranged between of 0.0625 - 0.5 µg/ml showed no inhibitory effect on *S.aureus*. MIC of amoxicillin

using macrodilution method was 0.015 µg/ml. These results were in accordance with Idamokoro *et al.* (2013) who stated that amoxicillin (0.01 µg/ml) against *S.aureus* showed inhibition zone of 23 ±1.5mm using agar well diffusion method and MIC of 6.25x10⁻⁴ mg/ml using the broth microdilution method. Similar findings were recorded by Palaksha *et al.*, (2013) who claimed that the mean inhibitory zone was 14.5±0.2887 mg/ml and Nnamani *et al.*, (2012) who added that MIC of amoxicillin against *S.aureus* was 0.025±0.002 mg/ml. On the other hand Ghannadi *et al.*, (2012) stated that amoxicillin (25 µg/disc) against *S.aureus* using disc diffusion method achieved inhibition zone 39.5±0.1 mm. Nazima *et al.*, (2010) recorded no inhibition against *S. aureus* using disc diffusion method for amoxicillin. The results indicated that cefquinome had inhibitory effect on Muller Hinton agar medium against *S.aureus* at concentrations 2.0-8.0 µg/ml with inhibition zone between 13.5±0.29–20.83±0.17 mm. This was in agreement with Al-Khafaji (2004) who recorded the inhibitory zone as 19.2 mm. concentrations of 0.0625 – 1.0 µg/ml showed no inhibitory effect on *S.aureus*. Minimum Inhibitory Concentration (MIC) of cefquinome using macrodilution method was 0.25 µg/ml. Quan-chao *et al.*, (2009) stated that MIC₉₀ of cefquinome on *S. aureus* using the microdilution method was 0.125 µg/ml. The results indicated that danofloxacin, sulphaquinoxaline and oxytetracycline had no inhibitory effect on Muller Hinton agar medium against *S.aureus* at concentrations 0.0625 – 8.0µg/ml. MIC of danofloxacin, sulphaquinoxaline and oxytetracycline

using macrodilution method were 1.0, 8.0 and 16.0 µg/ml respectively. Mhanna and Adwan (2008) who studied the antibacterial activity of antibiotics at concentration 100 µg/ml against 4 strains of *S.aureus* using agar well diffusion method and minimum inhibitory concentration using micro dilution method. The inhibition zones were 16.6-28.3, 27-30 and 8.3-9.3mm for oxytetracycline HCl, enrofloxacin and sulphadimethoxine sodium respectively. MICs were 0.78-6.25, 0.39-0.195 and >100 mg/ml respectively. Moreover, Adeshina *et al.*, (2011) studied the antibiotic sensitivity pattern of *S.aureus* to ofloxacin (30 µg) and tetracycline (30 µg). They stated that *S.aureus* was sensitive to ofloxacin and resistant to tetracycline. The present results indicated that cefquinome and sulphaquinoxaline showed synergism with rosemary ethanol extract against *S.aureus*. But showed antagonism with amoxicillin, danofloxacin and oxytetracycline. These results were in agreement with Mhanna and Adwan (2008) who evaluated the interaction between water extract of rosemary and known antimicrobial agents of different mechanisms (oxytetracycline HCl, gentamicin sulfate, penicillin G, cephalixin; sulfadimethoxine sodium and enrofloxacin) using microdilution method against five *S.aureus* isolates resembled synergistic effects. On the other, hand van Vuuren *et al.*, (2009) reported that interaction of the essential oils (*Mentha piperita* and *Rosmarinus officinalis*) with ciprofloxacin against *S.aureus* indicated mainly antagonistic profiles. The current results were in accordance with Jarrar *et al.*, (2010) who recorded that there was synergistic effect between ethanol extract of rosemary and cefuroxime against methicillin-resistant *Staphylococcus aureus* and Toroglu (2011) who recorded antagonism for the combination of Rosemary essential oil and gentamycin antibiotic discs (10 µg), but synergism with cephalothin. Shaaban *et al.*, (2013) stated that a synergistic effect was seen in *S.aureus* by using disc diffusion method

when combination of rosemary essential oil and gentamycin antibiotic discs (10µg) were applied. While there was no effect of rosemary with erythromycin (15µg). An increase in antibacterial activity of rosemary ethanol extract when combined with gentamycin or tetracycline was reported by Thorria (2013). In contrast a decrease in its antibacterial activity when combined with ampicillin but no difference with amoxicillin against *S.aureus*. There was an increase in inhibition zone in its combination with cephalothin by using disc diffusion method.

5. CONCLUSION

Ethanol plant extract of rosemary has antimicrobial activity against local strain of *S. aureus* and has a synergistic effect with cefquinome and sulphquinoxaline. These results signify that rosemary extract potentiates the antimicrobial action of antibiotics, suggesting a possible utilization of this herb in combination therapy against staphylococcal infections *in vivo*.

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