

Full Length Research Paper

Effect of tyrosol on *Staphylococcus aureus* antimicrobial susceptibility, biofilm formation and virulence factors

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Staphylococcus aureus is considered the most common causative agent of community and hospital-acquired infections. The isolates with increased virulence and antimicrobial resistance, especially methicillin resistant *S. aureus* (MRSA), represent a challenge in hospitals and health care facilities worldwide. The effect of tyrosol, a phenolic quorum sensing compound of *Candida albicans*, was studied on the sensitivity of clinical MRSA isolates to antibiotics. Besides having antibacterial activity, subinhibitory concentrations (3.5 - 14.3 mM) of tyrosol increased the susceptibility to antimicrobial. It gave either synergistic or additive effect when combined with gentamicin, amikacin and ciprofloxacin. Also, the effect of such concentrations on virulence factors production was investigated. Biofilm formed was significantly decreased in most of the tested isolates ($P \leq 0.0001$). In addition, it significantly decreased the production of protease and lipase enzymes. Overall, these results represent a promising method for inhibiting *S. aureus* either by reducing its resistance to antibiotics or decreasing the production of virulence factors.

Key words: *Staphylococcus aureus*, tyrosol, antibiotic sensitivity, virulence factors.

INTRODUCTION

Staphylococcus aureus is a commensal organism that can asymptotically colonize healthy individuals. Also, it is one of the important pathogens of both hospital and community acquired infections (Gordon and Lowy, 2008; Plata et al., 2009). This pathogen can cause a wide range of infections which may be superficial, systemic or toxin mediated (Kluytmans et al., 1997; Schito, 2006).

Shortly after the first introduction of methicillin in 1960,

methicillin resistant *S. aureus* isolates were reported. MRSA is characterized by the presence of *mecA* gene that results in decreased affinity for β -lactam antibiotics as it encodes penicillin binding proteins 2A (PBP2A) (Gordon and Lowy, 2008). MRSA is considered a major problem worldwide as it results in worse consequences such as prolonged time of hospitalization, increased cost of treatment and mortality (Onelum et al., 2015). The

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increasing prevalence of MRSA requires the development of new treatment strategies for serious infections which became an urgent matter (Shah, 2005).

S. aureus possesses an arsenal of virulence determinants that promote tissue colonization, invasion, avoiding the immune systems and dissemination of the disease (Bien et al., 2011). These determinants include adherence factors (adhesins) and secreted products (exoproteins). Adhesins are involved in attachment and initiation of colonization. The organism can reside in biofilm in prosthetic materials causing its persistence and escaping the host defense and antimicrobials (Donlan and Costerton, 2002). Exoproteins such as exotoxins and enzymes (lipases, proteases, hyaluronidase, hemolysins and nucleases) are produced during infection to help in invasion and destruction of tissues (Dinges et al., 2000).

Tyrosol (2-(4-Hydroxyphenyl) ethanol) is a known phenolic compound which is a quorum-sensing molecule of *Candida albicans*. Similar to farnesol, tyrosol is released into the growth medium and accelerates the formation of germ tubes (Alem et al., 2006). Therefore, it appears that tyrosol and farnesol control positively and negatively the morphogenesis in *C. albicans*.

Moreover, it has antioxidant properties and scavenging effects on reactive oxygen and nitrogen species that are implicated in human pathologies such as cardiovascular and thrombotic diseases. It is found in food sources such as olive oil, olive leaves and green tea (Miro-Casas et al., 2003; Romero and Brenes, 2012) and terrestrial fungi. Tyrosol also showed antimicrobial activity (Alem et al., 2006; Guimarães et al., 2009).

Therefore, this study aims to evaluate the effect of tyrosol on the antimicrobial sensitivity of clinical MRSA isolates, in addition to its effect on biofilm and production of some virulence factors.

MATERIALS AND METHODS

Bacterial isolates and reagents

In this study, clinical isolates of *S. aureus* (MRSA) were isolated from Mansoura University Hospitals. MRSA was identified according to sensitivity to cefoxitin disc (CLSI, 2014) and presence of *mecA* gene by PCR (Kondo et al., 2007). They were stored at -70°C. Tyrosol and p-nitrophenylpalmitate were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Mueller-Hinton broth (CAMHB, Sigma-Aldrich), LB broth and egg-yolk tellurite emulsion were obtained from Oxoid. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was obtained from SERVA Electrophoresis GmbH (Germany).

Determination of MIC

The minimal inhibitory concentrations (MICs) of tyrosol, gentamicin, amikacin and ciprofloxacin against *S. aureus* isolates were determined by the broth microdilution method according to CLSI (2014). Briefly, two fold serial dilutions of each tested drug in 96 well microtitreplate were inoculated by cultures of tested isolates. Positive and negative controls were included in each plate. The microtitre plate was incubated at 37°C, the endpoint was read

spectrophotometrically using microplate reader (BioTek ELx800) after 24 h and the MIC was determined.

Determination of the combined effect of tyrosol and antibiotics

The combined effect for Tyrosol/gentamicin, tyrosol/amikacin and tyrosol/ciprofloxacin was measured by Checkerboard microdilution method (Orhan et al., 2005). The concentration of each drug ranged from 2-fold to 1/32 MIC. One hundred microliter of each antibiotic was combined with 100 µl of tyrosol in 96 well microtitre plates. The plates were inoculated with 20 µl of an overnight LB culture giving a final concentration of 5.0×10^5 CFU/ml per well. Plates were incubated for 24 h at 37°C. To evaluate the interaction, the fractional inhibitory concentration index (FICI) was calculated for each combination according to White et al. (1996) and Abdelmegeed and Shaaban (2013).

Measuring the effect of tyrosol on metabolic activity

To evaluate the effect of tyrosol on vitality of *S. aureus* isolates, MTT reduction assay was conducted on both tyrosol treated and non-treated cells at a defined time point (Montoro et al., 2005). First, a suspension of an overnight *S. aureus* culture (100 µl) was dispensed into selected wells of a 96-well plate containing tyrosol (3.5-228.8 mM) and incubated for 24 h at 37°C. Next, 10 µl of MTT solution [5 g/l in phosphate buffered saline (PBS) (10 mM Na₂PO₄, 1.8 mM KH₂PO₄, 137 mM NaCl, 2.7 mM KCl, pH 7.0), filter sterilize by bacterial filter (0.22 µm)] was added to each well and the plate was re-incubated for 4 h. Aspiration of the well solution was performed followed by addition of 50 µL of dimethylsulfoxide (DMSO) solution to the wells. The plate was shaken for 5 min. A change in color from yellow to violet was measured with a microtitre plate reader (BioTek ELx800) at 540 nm. All assays were carried out in triplicate.

Biofilm formation

Biofilm was developed in a 96 well microtiter plate (Nunc, New York, NY, USA). *S. aureus* cultures (100 µl) were incubated for 24 h at 37°C in the presence of different concentrations of tyrosol (3.5-14.3 mM) and the procedure was completed according to Eid et al. (2012) and El-Mowafy et al. (2014).

Effect of tyrosol on *S. aureus* virulence factors

The effect of tyrosol on the production of total protease, lipase and lecithinase enzymes by tested isolates was done with formerly prepared culture supernatant. Total protease activity was measured using the modified skim milk assay as previously described (El-Mowafy et al., 2014). The lipase assay was carried out using p-nitrophenyl palmitate as a substrate as previously described (EL-Sokkary et al., 2011).

The lecithinase activity was measured by mixing 100 µl of the filtrate with or without tyrosol and 100 µl of the egg-yolk suspension (egg-yolk tellurite emulsion 20 ml/L; NaCl, 5 g/L). The pH was adjusted to 7.8 in individual wells of polystyrene 96-well plates then the plates were incubated at 37°C for 24 h and absorbance was measured at 600 nm using microplate reader. All experiments were carried out in triplicates.

Statistical analysis

Statistical analysis was done using GraphPad Prism. One way

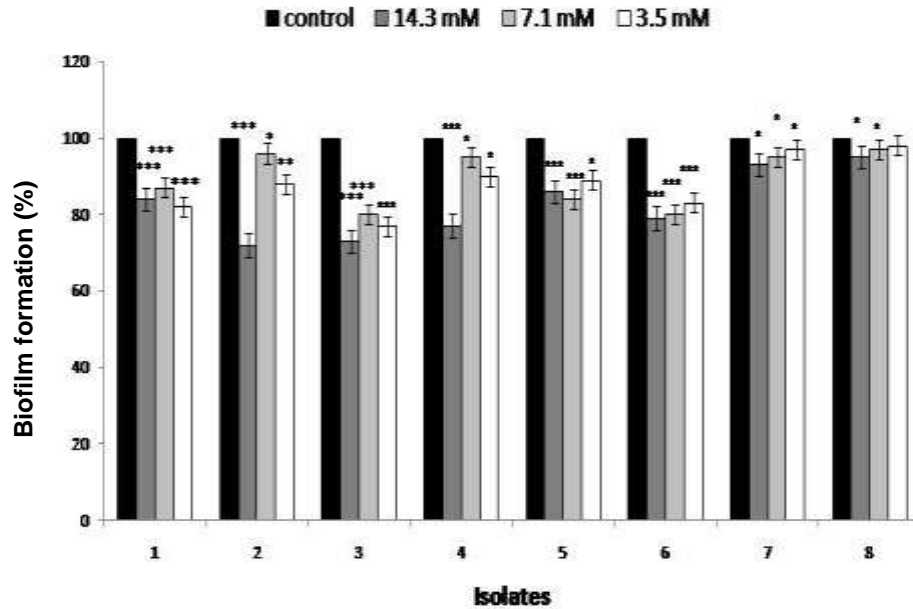


Figure 1. The effect of subinhibitory concentrations (3.5-14.3 mM) of tyrosol on biofilm formation. (***: $P \leq 0.0001$, **: $P \leq 0.001$, *: $P \leq 0.05$).

ANOVA followed by Turkey posttest were performed to compare the differences between the control and treated isolates. A P value ≤ 0.05 was considered statistically significant.

RESULTS

MIC determination

The MIC of tyrosol, gentamicin, amikacin and ciprofloxacin was determined by microbroth dilution method. The MIC of tyrosol was found to be 114.4 mM for all the tested isolates. The MIC for gentamicin, amikacin and ciprofloxacin were 62.5-8000, 17.5-70 and 125-500 $\mu\text{g/ml}$, respectively.

The combined effect of tyrosol and antibiotics

The effect of tyrosol/gentamicin combination was found to be synergistic in 66% of tested isolates ($\text{FICI} = 0.28-0.5$). Besides that, tyrosol/amikacin and tyrosol/ciprofloxacin combinations exhibited synergistic effect in two isolates ($\text{FICI} = 0.37$). For the other isolates, these three combinations showed an additive effect ($\text{FICI} = 0.51-0.62$).

Effect of tyrosol on the metabolic activity

MTT, an oxidation-reduction indicator was added to suspensions in the presence of tyrosol. The vitality assay indicated that incubation of cells with 114.4 and 228.8

mM of tyrosol (1x and 2x MIC) resulted in no change in color from yellow to violet, showing that these concentrations prevented the oxidation-reduction reactions in the suspensions. Tyrosol (28.6- 57.2 mM) partially inhibited oxidation-reduction reactions. Lower concentrations (3.5 - 14.3 mM) had no effect on the viability of tested isolates. Therefore, these subinhibitory concentrations were used for studying the effect of tyrosol on the production of virulence factors.

Biofilm formation

The effect of 3.5 - 14.3 mM of tyrosol on biofilm formation was estimated. All the tested concentrations reduced the formed biofilm of the isolates significantly except for one isolate in which (3.5 mM) tyrosol reduced the formed biofilm in non-significant manner (Figure 1). The maximum decrease (30%) in biofilm formation was attained using 3.5 mM of tyrosol among isolates 2, 3 and 4.

Effect of tyrosol on virulence factors

Sub-inhibitory concentrations (3.5 - 14.3 mM) of tyrosol were found to significantly decrease protease and lipase enzyme production in MRSA isolates 3, 5 and 7. Tyrosol (14.3 mM) significantly decreased protease and lipase release with $P < 0.05$ by 35-40% (Figure 2) and 25-40% (Figure 3), respectively. On the other hand, lecithinase enzyme production was increased significantly in all the

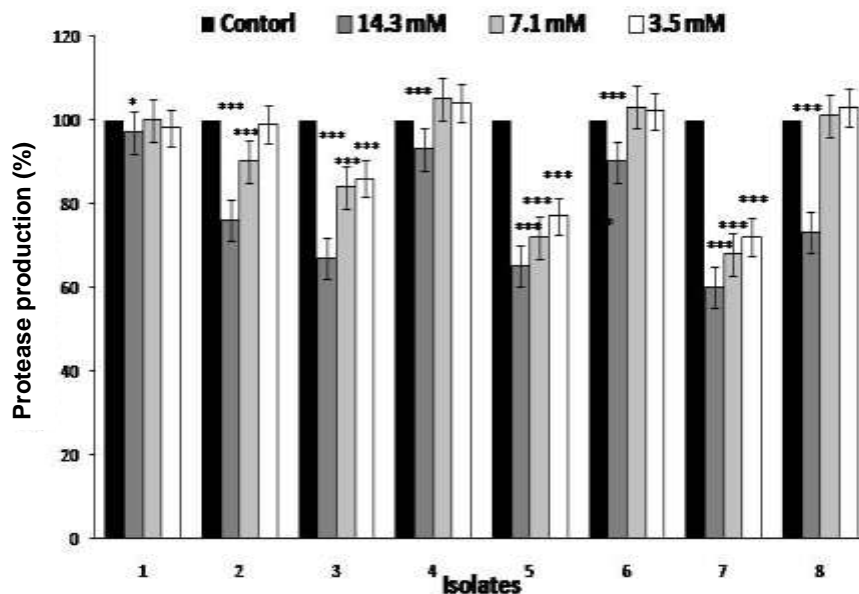


Figure 2. The effect of subinhibitory concentrations (3.5-14.3 mM) of tyrosol on protease enzyme production. (***, P≤0.0001, **, P≤0.001, *, P≤0.05)

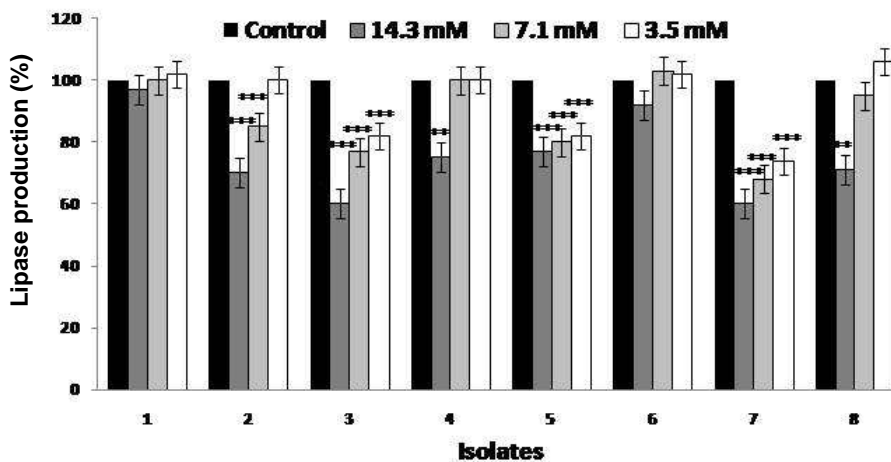


Figure 3. The effect of subinhibitory concentrations (3.5-14.3 mM) of tyrosol on lipase enzyme production. (***, P≤0.0001, **, P≤0.001, *, P≤0.05).

tested isolates except for one isolate in which 3.5 mM of tyrosol led to non-significant increase (Figure 4).

DISCUSSION

There is mounting indication in the literature for the prominence of polymicrobial infections in which organisms interact with each other synergistically or inhibiting the effect of their pathogenesis and patient health (Harriott and Noverr, 2009). *C. albicans* cohabits with various

human microbiota in multiple sites which give rise to mixed species biofilm (Shirliff et al., 2009; Peleg et al., 2010; Ovchinnikova et al., 2012). In poly-microbial blood stream infections, *C. albicans* was co-isolated with *S. aureus* in 11% of cases (Klotz et al., 2007).

Besides that, *C. albicans* and *S. aureus* can form complex polymicrobial biofilms on various mucosal surfaces in which *S. aureus* is associated with hyphal cells (Peters et al., 2010). The effect of *C. albicans* itself on *S. aureus* has been extensively investigated previously (Harriott and Noverr, 2009; Ovchinnikova et

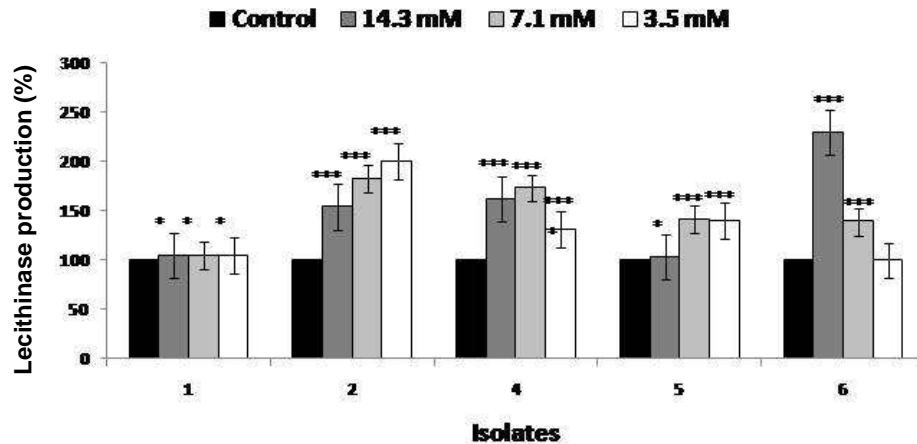


Figure 4. The effect of subinhibitory concentrations (3.5-14.3 mM) of tyrosol on lecithinase enzyme production. (***: $P \leq 0.0001$, **: $P \leq 0.001$, *: $P \leq 0.05$).

al., 2012; Lin et al., 2013; Schlecht et al., 2015). The effect of farnesol, *C. albicans* quorum sensing compound, was studied on *S. aureus* in various reports (Unnanuntana et al., 2009; Kaneko et al., 2011; Cerca et al., 2013). Till now, no available investigation was done to evaluate the effect of the other *C. albicans* quorum sensing compound (tyrosol) on the sensitivity of *S. aureus* to antibiotics and virulence production.

In this study, the authors were interested in determining how tyrosol might affect the antibiotic sensitivity, biofilm and some virulence factors production (protease, lipase and lecithinase) by Egyptian clinical isolates of *S. aureus*.

Tyrosol exhibits antibacterial effect against *S. aureus*. In contrast to these results, Papadopoulou et al. (2005) showed that tyrosol is least likely to exhibit any antimicrobial activity on *S. aureus*, *E. coli* and *C. albicans*. Being a phenolic compound may account for this antibacterial activity by having membrane-active properties which induce progressive leakage of intracellular constituents, including K^+ (Lambert and Hammond, 1973). Aside from being a quorum sensing compound, tyrosol is one of the simple phenols found in olive oil providing benefits to health. Medina et al. (2006) reported that hydroxytyrosol and tyrosol are olive oil phenolic compounds having bactericidal activity against the tested foodborne pathogens including *S. aureus*.

The ability of tyrosol to sensitize the tested isolates to antibiotics (gentamicin, amikacin and ciprofloxacin) was assessed. It enhanced the activity of the three antibiotics against *S. aureus* isolates. About 66.7% of tested isolates exhibited sensitivity towards the tyrosol/gentamicin combination. For amikacin and ciprofloxacin, tyrosol exhibited a synergistic effect in two out of eight isolates and showed additive effect in the other isolates. The synergistic effect shown by tyrosol/ antibiotic combinations may be explained by the possible membrane permeabilizing action of tyrosol which increases the

sensitivity of *S. aureus* to antibiotics. A similar finding was reported by Jabra-Rizk et al. (2006) who showed that farnesol increases the sensitivity of tested mecithillin sensitive *S. aureus* isolates to gentamicin and this increase was not shown in MRSA isolates. Unnanuntana et al. (2009) have demonstrated that farnesol/gentamicin combination has no effect on the sensitivity of *S. aureus* to gentamicin. Concerning farnesol, it has variable activities against different organisms in combination with different antimicrobials. For example, a study performed by Abdel-Rhman et al. (2015) showed that neither farnesol nor tyrosol at their subinhibitory concentrations had any effect on the activity of antibiotics on *Pseudomonas aeruginosa*.

Bacterial biofilm is a microbial derived sessile community, where cells are embedded in extracellular polymeric matrix. Cells in biofilm show an altered phenotype in growth, gene expression and protein production. Biofilm formation requires first adherence of organisms to surfaces, followed by proliferation and finally biofilm formation (Jabra-Rizk et al., 2006; Unnanuntana et al., 2009). In the current study, subinhibitory concentrations of tyrosol reduced biofilm formation significantly. This result is consistent with that of Arias et al. (2016) who demonstrated that tyrosol reduces single and mixed biofilms formed by *C. albicans* and *Streptococcus mutans*. Also, Jabra-Rizk et al. (2006) found that farnesol has the ability to inhibit biofilm formation by *S. aureus*.

Moreover, the effect of tyrosol on the production of some virulence factors of *S. aureus* was studied. Tyrosol subinhibitory concentrations (3.5-14.3 mM) reduced protease enzyme production in 100, 50 and 37.5% of the tested isolates, respectively. This result coincides with that of Abdel-Rhman et al. (2015) who reported a similar result against *P. aeruginosa*.

Another virulence factor of *S. aureus* is lipase that

contributes to skin colonization through hydrolyzation of human sebum. Moreover, the effect of tyrosol on lipase production was investigated. Tyrosol (14.3 mM) caused 25-40% reduction of lipase production in six isolates, 7.1 mM resulted in 20-30% reduction in four isolates and 3.5 mM resulted in about 20% reduction in only three isolates. These results are consistent with what reported by Kuroda et al. (2007) who found that subinhibitory MIC of farnesol inhibited lipase activity in *S. aureus*. On the contrary, lecithinase was significantly increased by the tested concentrations of tyrosol. None of the previous studies have investigated the effect of either tyrosol or farnesol on lecithinase production. So, this result requires further study to explain this increase by studying its effect on *agr* (accessory gene regulator) quorum-sensing system of *S. aureus* which controls the expression of many secreted virulence factors.

Conclusion

The present study results demonstrate the beneficial effects of tyrosol in relation to antimicrobial susceptibility and virulence factors production of *S. aureus*. This sheds more light on the therapeutic use of tyrosol against *S. aureus* infections. However, more studies are required to reveal its effect on quorum sensing system that controls virulence gene expression.

Conflict of Interests

The authors have not declared any conflict of interest.

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