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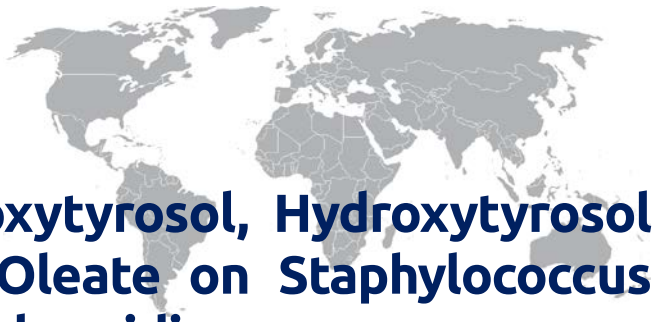

Antimicrobial effect of Hydroxytyrosol, Hydroxytyrosol Acetate and Hydroxytyrosol Oleate on Staphylococcus Aureus and Staphylococcus Epidermidis

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ABSTRACT

Introduction: Increasing microbial resistance to antibiotics is one of the main contributors for replacing the appropriate compounds to treat microbial infections. Staphylococcus is one of the most important genera of the bacteria as some strains present on the skin as normal flora and opportunistic infectious agents**.** Common resistance to antibiotics has been observed in infections caused by this genus from bacteria. Plants have different chemical compounds with antimicrobial properties. The aim of this study was to evaluate the effect of antimicrobial activity of hydroxytyrosol, hydroxytyrosol acetate and hydroxytyrosol oleate which are derived from the leaves of the olive on S. epidermidis and S. aureus.

Methods: Hydroxytyrosol, hydroxytyrosol acetate and hydroxytyrosol oleate that are two lipophilic derivatives of hydroxytyrosol, which have been prepared at Magna Graecia University, Italy. The antibiogram test was carried out according to the CLSI guidelines and the Kirby-Bauer method using well assay and Microplate broth dilution for S. aureus and S. epidermidis.

Results: The highest Zone of inhibition for the HT, HTA and HTO compounds for Staphylococcus aureus was 30, 19 and 15 mm at a concentration of 100 mg/ml respectively. The highest Zone of inhibition for the HT, HTA and HTO compounds for Staphylococcus epidermidis was 25, 19 and 14 mm at a concentration of 100 mg/ml respectively. The MIC for the above compounds for Staphylococcus aureus was 3.125, 12.5 and 25 mg/ml respectively. The MIC for the above compounds for Staphylococcus epidermidis was 6.25, 12.5 and 50 mg/ml respectively.

Conclusion: Respectively, the highest antimicrobial activity against S. aureus and S. epidermidis was evaluated in hydroxytyrosol, hydroxytyrosol acetate and hydroxytyrosol oleate compounds.

Keywords: hydroxytyrosol, staphylococci, antimicrobial activity

INTRODUCTION

Increasing microbial resistance to antibiotics is one of the main contributors for replacing the appropriate compounds to treat microbial infections (1). Staphylococcus is one of the most important genera of the bacteria as some strains present on the skin as normal flora and opportunistic infectious agents, and some strains act as human and animal pathogens (2). Staphylococci exist in the environment; and because of this feature, they might be found in food and water, as well as on the skin and mucous membranes of humans and mammals (3).

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These organisms are isolated in humans from wet and folded areas of the body such as the nasal passages, armpits and groins (4). Staphylococcus epidermidis is a strain of Staphylococcus genus that lives in symbiosis on the skin. The role of this bacterial strain has been proven in opportunistic infections (5, 6). The main concern for infection with opportunistic bacteria is the prevalence of resistance to glycopeptide antibiotics (7, 8), which has the potential for transferring resistance genes to Staphylococcus aureus (9). *S.* epidermidis is considered as one of the most important agents of hospital-acquired infection (HAI). The use of catheters, prostheses or artificial heart valve increases the risk of infection (10).

S. aureus is a specific pathogen strain of Staphylococcus genus that is found in the anterior nasal region of adults (11), and carriers are the main contributor to S. aureus infections. This bacterium is a Pus-forming agent and also responsible for toxin-related diseases, for example, in food poisoning. This bacterium develops a wide range of skin infections, pneumonia, osteomyelitis and endocarditis (12). Over time, various strains of this bacterium have been resistant to penicillin and its derivatives by β-lactamase (penicillinase). This resistance has become wider; those strains resistant to all penicillins are called methicillin-resistant Staphylococcus aureus (MRSA) (13). The methicillin-resistant isolates are of HAI agents around the world. There is a strain of MRSA, whose infection is not associated with HAIs and causes minor and fatal diseases among healthy people (14, 15). Given the above-mentioned microbial resistance of two bacterial species, it seems necessary to find alternative drugs for the treatment of resistant bacteria. The researchers conducted a lot of research in this regard, including studies in the field of medicinal herbs. Plants have different chemical compounds with antimicrobial properties. For example, Nikrooze et al. (16) compared the effect of Jaft aqueous extract and silver sulfadiazine on Burn healing in male rat. The Jaft aqueous extract at a concentration of 7% had the best effect against pathogenic microorganisms in the skin such as S. epidermidis (16). In this regard, three compounds of hydroxytyrosol, hydroxytyrosol acetate and hydroxytyrosol oleate that can be derived from olive oil or olive leaf extract were studied in this study.

The phenolic compounds of the olive oil include the heterogeneous mixture of oleuropein, ligstroside or eridic acid (periodic acid) compounds that are related to hydroxytyrosol and tyrosol compounds. The majority of biochemical and medicinal effects in the olive plant include polyphenols, including hydroxytyrosol and its derivatives (17).

Hydroxytyrosol is a secondary metabolite derived from oleuropein through the enzymatic hydrolysis (18). It has antioxidant properties and beneficial features in the treatment of cardiovascular disease (19).

Hydroxytyrosol acetate and hydroxytyrosol oleate are two lipophilic derivatives of hydroxytyrosol, which have been prepared at Magna Graecia University, Italy (20-22).

The aim of this study was to evaluate the effect of antimicrobial activity (measurement of inhibition zone diameter, MIC and MBC) of hydroxytyrosol, hydroxytyrosol acetate and hydroxytyrosol oleate on S. epidermidis and S. aureus bacteria in comparison with standard antibiotics.

MATERIALS AND METHODS

Preparation of Antimicrobial Agents

Hydroxytyrosol was synthesized from 3,4- dihyodroxyphenilacetic acid using the method reported in the literature (20). Hydroxytyrosol peracetate and oleate were obtained from hydroxytyrosol by catalysis of $Er(OTf)_3$ according to the green synthetic procedures optimized in previous works. Briefly hydroxytyrosol peracetate was realized by reacting purified hydroxytyrosol in dry acetic anhydride, under nitrogen and at room temperature, in presence of 2%mol of $Er(OTf)_3$. (20, 21) Analougsly, hydroxytyrosol oleate was obtained by esterification of hydroxytyrosol with oleyl chloride in dry THF and at room temperature, in presence of 1%mol of $Er(OTf)_3$. (21) All the product were purified after work-up by flash chromatography and characterized by comparison with the data reported in the literature.

Measurement of Inhibition Zone Diameter

The standard strains of S. aureus and S. epidermidis were provided from the Pasteur Institute of Iran. The antibiogram test was carried out according to the CLSI guidelines and the Kirby-Bauer method using well assay. Initially, the bacterial suspensions were prepared equal to 0.5 McFarland turbidity standards (1.5×10^8 CFU/ml). The suspensions were then transferred by sterile swab onto the Muller Hinton Agar medium using a lawn culturing method. After that, the wells were created on the medium by Burier to pour different concentrations of the materials studied. Six concentrations of 3.125, 6.25, 12.5, 25, 50 and 100 mg/ml were prepared from each of the studied chemicals and poured into the created wells. After incubation for 24 hours at 37°C, the emerged inhibition zone diameter was measured using a ruler (in mm)

Table 1: *The mean (±standard deviation) inhibition zone diameter caused by the effect of hydroxytyrosol, hydroxytyrosol acetate and hydroxytyrosol oleate compounds on S. aureus*

Concentration (mg/ml)	Hydroxytyrosol (mm)	Hydroxytyrosol Acetate (mm)	Hydroxytyrosol Oleate (mm)
100	30±1.00	19±0.57	15±0.57
50	27±1.00	17±0.57	14±0.00
25	24±0.00	15±0.00	11±0.00
12.5	22±0.57	13±1.00	8±0.86
6.25	19±1.52	10±0.00	6±0.00
3.125	16±0.00	6±0.00	0±0.00

and recorded. The experiment was carried out with three replications; the results were compared with the antibiogram results for the standard antibiotics.

Investigation of Inhibition Zone Diameter for Standard Antibiotics

The susceptibility of studied bacteria to standard antibiotics was tested according to the CLSI protocol and disc diffusion method. The bacterial suspensions were prepared equal to 0.5 McFarland turbidity standards and then transferred by sterile swab on separate plates containing the Muller Hinton Agar medium using a lawn culturing method. Two antibiotic discs of vancomycin and oxacillin were used for antibiogram testing. After 24 hours of incubation at 37°C, the inhibition zone diameter (mm) was measured for each antibiotic. The experiment was carried out with three replications and the results were presented as mean.

The MIC and MBC values were evaluated by microplate dilution method in 96-well microplate at 8 consecutive dilutions of 0.78.1.56, 3.125, 6.25, 12.5, 25, 50 and 100 mg of each material, including hydroxytyrosol, hydroxytyrosol acetate and hydroxytyrosol oleate, separately for each bacterium. The bacterial suspensions were separately prepared equal to 5×10^5 CFU/ml turbidity in the sterile test tubes, and then 10 µl of each suspension was added in separate microplate wells. Next, optical density of each well before the incubation was read using an ELISA Microplate Reader at 620 nm and recorded.

After 24 hours of incubation at 37°C, the optical density of each well was examined for turbidity using the ELISA Microplate Reader at 620 nm. The wells with no appeared turbidity were determined. The lowest concentration of the study materials with no turbidity was considered as the MIC value. To confirm the outcome, a sample was taken from wells with no turbidity and then cultured onto Blood Agar medium and incubated at 37°C for 24 hours. The lowest concentration of the well that showed no increased turbidity after incubation but was positive for growth of post-culture microorganism was reported as the MIC value and the lowest concentration that caused 99.9% death of bacteria and revealed no growth of microorganisms after cultivation was the MBC value. The results of this study were analyzed by descriptive statistics and ANOVA test.

RESULTS

The results obtained from well assay to examine the inhibition zone created by the antimicrobial effect of hydroxytyrosol were 16, 19, 22, 24, 27 and 30 mm on *S. aureus* and 11, 13, 16, 19, 22, 25 mm on *S. epidermidis*. The results are reported for three replications as mean and standard deviation.

The results of the well assay for the evaluation of the inhibition zone caused by the antimicrobial effect of hydroxytyrosol acetate at the concentrations of 3.125, 6.25, 12.5, 25, 50 and 100 mg/ml were 6, 10, 13, 15, 17 and 19 mm on *S. aureus* and 6, 9, 13, 15, 17 and 19 mm on *S. epidermidis*. The results are presented for three replications as mean and standard deviation.

The results of the well assay for three replications to test the inhibition zone appeared by the antimicrobial effect of hydroxytyrosol *oleate* at the concentrations of 3.125, 6.25, 12.5, 25, 50 and 100 mg/ml were 0, 6, 8, 11, 14 and 15 mm on *S. aureus* and 0, 0, 5, 8, 12 and 14 mm on *S. epidermidis*. The results are shown as mean and standard deviation.

The results of these tests are reported in **Table 1**.

The results of measuring the inhibition zone diameter in standard antibiogram with vancomycin and oxacillin antibiotics for *S. aureus* were 39 mm and 22 mm respectively, which were within the sensitive range.

The results of measuring the inhibition zone diameter in standard antibiogram with vancomycin and oxacillin antibiotics for *S. epidermidis* were 38 mm (within the sensitive range) and 9 mm (within the resistant range) respectively, which were in the sensitive range.

Table 2: *MIC values (mg/ml) of hydroxytyrosol, hydroxytyrosol acetate and hydroxytyrosol oleate compounds against S. aureus and S. epidermidis*

Bacterial strains	Hydroxytyrosol	Hydroxytyrosol acetate	Hydroxytyrosol oleate
S. aureus	3.125	12.5	25
S. epidermidis	6.25	12.5	50

Table 3: *MBC values (mg/ml) of hydroxytyrosol, hydroxytyrosol acetate and hydroxytyrosol oleate compounds against S. aureus and S. epidermidis*

Bacterial strains	Hydroxytyrosol	Hydroxytyrosol acetate	Hydroxytyrosol oleate
S. aureus	6.25	25	50
S. epidermidis	12.5	25	100

The value of MIC evaluated by the microplate dilution method obtained from different concentrations of hydroxytyrosol for *S. aureus* was 3.125 mg/ml.

The value of MBC evaluated by the microplate dilution method obtained from different concentrations of hydroxytyrosol for *S. aureus* was 6.25 mg/ml.

The MIC and MBC values of hydroxytyrosol assessed by the microplate dilution were 6.25 mg/ml and 12.5 mg/ml for *S. epidermidis*.

The MIC and MBC values of hydroxytyrosol *acetate* assessed by the microplate dilution were 12.5 mg/ml and 25 mg/ml for *S. aureus*.

The MIC and MBC values of hydroxytyrosol *acetate* assessed by the microplate dilution were 12.5 mg/ml and 25 mg/ml for *S. epidermidis*.

The MIC and MBC values of hydroxytyrosol *oleate* assessed by the microplate dilution were 25 mg/ml and 50 mg/ml for *S. aureus*.

The MIC and MBC values of hydroxytyrosol *acetate* assessed by the microplate dilution were 50 mg/ml and 100 mg/ml for *S. epidermidis*.

The overall results of the microplate dilution method for evaluating MIC and MBC values of hydroxytyrosol, hydroxytyrosol acetate and hydroxytyrosol oleate compounds for *S. aureus* and *S. epidermidis* are presented in **Tables 2** and **Tables 3**.

DISCUSSION AND CONCLUSION

Microorganisms play an important role in the development of human diseases. In this regard, the infectious diseases cause mortality and impose a huge cost. Hence, human beings have always been thinking about ways to deal with microorganisms. Since the discovery of antibiotics, these compounds have been responsible for controlling microorganisms in human and animal infections. With the advent and spread of microbial resistance, therapeutic systems have been considering various alternatives to manage the microbial resistance. The compounds derived from plant extracts are an important category of these materials that have been most recently regarded. It is believed that the plant compounds can be largely free of side effects. Therefore, the investigations conducted in the recent years have been further developed in this area. In this study, researchers have examined the antimicrobial properties of extractable materials from the olive leaf, such as hydroxytyrosol, and its synthesized derivatives including hydroxytyrosol acetate and hydroxytyrosol oleate on *S. aureus* and *S. epidermis* bacteria. In the present study, the inhibition zone created by different concentrations of hydroxytyrosol for *S. aureus* was obtained between 16 mm and 30 mm. In addition, the inhibition zone diameter was also reported from 11 mm to 25 mm for *S. epidermidis* at the same concentrations of hydroxytyrosol. The present results showed that the effect of hydroxytyrosol on *S. aureus* was greater than that of *S. epidermidis*, which could be somewhat indicative of the resistance mechanisms of this substance against *S. epidermidis*. The conditions for the transfer of this substance into the bacterium should also be considered. Comparing these results with the standard antibiotics demonstrated that the susceptibility of *S. aureus* was higher to vancomycin and oxacillin antibiotics and was associated with the inhibition zone within the sensitivity of both antibiotics ($P \leq 0.05$). However, the results were different for *S. epidermidis* and this bacterium is resistant to oxacillin. The comparison of the inhibitory zone created by the effect of different concentrations of hydroxytyrosol and standard antibiotics for *S. aureus* showed that most of the tested hydroxytyrosol concentrations had antimicrobial activity within the sensitive range similar to that of both standard antibiotics ($P \leq 0.05$). Nevertheless, the inhibition zone diameter of the lowest concentration of

hydroxytyrosol in exposure to *S. epidermidis* was within the resistant range. By increasing the concentration, this range tends toward the sensitive that was lower to the vancomycin and higher to the oxacillin.

The antimicrobial effect of two compounds derived from hydroxytyrosol suggested that the hydroxytyrosol acetate compared with hydroxytyrosol has less antimicrobial effect on both bacteria but its antimicrobial effect enhances with increasing the concentration. The antimicrobial effect of hydroxytyrosol acetate on *S. aureus* and *S. epidermidis* was approximately the same.

The antimicrobial effect of hydroxytyrosol oleate was very low and the inhibition zone of the bacteria was clearly observed at 100 mg/ml. Generally, by examining the inhibition zones created at different concentrations as comparison with the standard antibiotics, three compounds of hydroxytyrosol, hydroxytyrosol acetate and hydroxytyrosol oleate showed antimicrobial effects, respectively ($P \leq 0.05$). In different studies that have often investigated the effect of olive leaf extract and oil on various microorganisms, the measured inhibition zone diameter has been reported in the lower range of the inhibition zone diameter of standard antibiotics, which is consistent with the current research. However, these studies have confirmed the presence of hydroxytyrosol, tyrosol and hydroxytyrosol acetate in the olive leaf extract. Nevertheless, the extract or oil of this plant has shown antimicrobial activity as mg/ml (23). Smaller inhibition zones of extracts compared to standard antibiotics can be so justifiable that since hydroxytyrosol, hydroxytyrosol acetate and hydroxytyrosol oleate are compounds with a different hydrophobic nature, so their inhibition zone diameter in the MHA medium (which has a low diffusion aqueous phase) is lower compared to the standard antibiotics (which have a good solubility in water).

In this study, the MIC and MBC values of hydroxytyrosol for *S. aureus* were 3.125 mg/ml and 6.25 mg/ml, respectively. In this regard, the MIC and MBC values of this compound for S. *epidermidis* were 6.25 mg/ml and 12.5 mg/ml, which was lower than in the MIC and MBC values of hydroxytyrosol acetate and hydroxytyrosol oleate respectively, indicating higher antimicrobial activity of hydroxytyrosol ($P \leq 0.05$) . The MIC and MBC values of hydroxytyrosol acetate were respectively 12.5 mg/ml and 25 mg/ml for *S. aureus*, as well as 12.5 mg/ml and 25 mg/ml for *S. epidermidis*. The MIC and MBC values evaluated for hydroxytyrosol oleate were respectively 25 mg/ml and 50 mg/ml on *S. aureus*, as well as 50 mg/ml and 100 mg/ml on *S. epidermidis*. A general review of the results here also shows that the MIC and MBC values of hydroxytyrosol were higher than hydroxytyrosol acetate, and hydroxytyrosol oleate had the lowest MIC and MBC values for the bacteria studied. In addition, with regard to bacterial resistance, *S. epidermidis* was more resistant than *S. aureus* to all three compounds. At higher concentrations of these compounds, the MIC and MBC are seen for the bacteria mentioned.

In a study of M. Martínez et al. (24) in 2015, the MIC value of hydroxytyrosol was higher than 1000 µg/ml in the studied strains, as well as the MIC value of hydroxytyrosol was reported to be 400 µg/ml for *S. aureus*. It was also reported that the increasing concentrations of hydroxytyrosol reduced the bacterial growth in the kinetic curves (24), which is in line with the study.

Due to the fact that hydroxytyrosol and its derivatives are found in the olive leaf extract, some antimicrobial studies can be discussed in this regard.

In a study by M. Gökmen et al. (25) in 2015, the MIC value of olive leaves against *Listeria monocytogenes*, *E. coli*, and *Pseudomonas aeruginosa* was more than 32 mg/ml. This value was higher than 16 mg/ml for *Bacillus cereus* and *S. aureus* and *Enterococcus faecalis* and some other bacteria.

Furthermore, J. Wei et al. (26) in 2017 evaluated the antimicrobial activity of hydroxytyrosol acetate derived from *Olea europaea* olive leaf. In this study, the MIC and MBC values of hydroxytyrosol acetate against *Vibrio parahaemolyticus* were 39µg/ml and 78µg/ml. In general, hydroxytyrosol and its derivatives (mg/ml) have antimicrobial properties ($P \leq 0.05$). According to the previous studies and the present research, these compounds have relatively good antimicrobial properties against most bacteria. Additionally, the reported MIC and MBC values have been measured against different bacteria. There are differences in the susceptibility of different bacteria to the studied compounds. Given that the MIC and MBC values in the present study are also evaluated as mg/ml, the obtained results are consistent with the findings of most studies.

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