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Effect of Clove Essential Oil (*Syzygium aromaticum*) on Some Virulence Factors of *Staphylococcus aureus*

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ABSTRACT

Background and objectives: Clove (Syzygium aromaticum) essential oil is a food additive with proven antimicrobial and antioxidant properties. Thus, it may be a good candidate for controlling foodborne pathogens, such as Staphylococcus aureus. The aim of the present study was to evaluate effects of sub–minimum inhibitory concentrations (MICs) of clove oil on some virulence factors of S. aureus.

Methods: The standard strain and 12 field isolates of S. aureus were obtained from our microbial collections. The broth tube dilution method was used to determine the MIC of clove oil against the isolates. Sterile 96-well flat bottom polystyrene microtiter plates were used for planktonic growth and biofilm formation assays. Slide coagulase test was used for assaying effect of clove oil on clumping factor production. Production of α- and β-hemolysins was assessed by culture on 5% bovine blood agar.

Results: The results showed that sub-MIC concentrations of clove oil inhibited α- and β-hemolysins and biofilm production and planktonic growth of the examined isolates. However, clumping factor was not affected by sub-MIC concentrations of clove oil.

Conclusion: Our results indicate the favorable inhibitory effects of sub-MIC concentrations of clove oil against growth and biofilm and hemolysins production of S. aureus isolates.

Keywords: Clove Oil, Staphylococcus, Inhibitory Concentration, Virulence Factors.

INTRODUCTION

Foodborne diseases are a health problem worldwide, particularly in developing countries (1). Therefore, food production with minimum pathogenic bacterial contamination is a major public health concern. Estimations by the World Health Organization indicate that each year, about 30% of people suffer from foodborne diseases in developed countries (2). Now, there is a worldwide need to reduce the prevalence and negative effects of foodborne diseases (3). Clove essential oil has been tested for inhibitory activity against important spoilage microorganisms of intermediate moisture foods (4). Reports show that eugenol (an effective ingredient of clove essential oil) can inhibit growth of some foodborne pathogens, such as *Staphylococcus aureus* (5), a gram positive bacterium able to produce various virulence factors contributing to a spectrum of illnesses (6). This pathogen causes various diseases including localized and systemic infections, food intoxications and produce exotoxins such as hemolysins, leukocidin and toxic shock syndrome toxin 1 (7, 8). In addition, ability to produce enzymes such as coagulase and biofilm-producing capacity are other important virulence factor of *S. aureus* (9, 10).

Biofilm is a community of microorganisms living together in amorphous extracellular matrix composed of polysaccharides, extracellular DNA and proteins (11).

Alpha toxin, the major cytotoxic agent produced by *S. aureus*, was the first bacterial exotoxin to be identified as a pore former (12). Beta toxin is a neutral sphingomyelinase secreted by certain strains of *S. aureus*. This virulence factor lyses erythrocytes in order to evade the host immune system as well as to scavenge nutrients (13). Smith et al. showed that sub-inhibitory concentrations of clove essential oil can decrease the production of α-toxin, enterotoxin A and enterotoxin B by *S. aureus* (14). In this regard, a report indicated that sub-inhibitory concentrations of thymol (found in thyme, oregano and tangerine peel) decreases the production of α-hemolysin by *S. aureus* (9). Qiu et al. found that sub-minimum inhibitory concentrations (MICs) of eugenol can reduce the production of hemolysins in *S. aureus*, with no significant effect on bacterial growth (15). Yadav et al. reported that eugenol can decreases the biofilm biomass of S. aureus isolates by more than 50% (16).

In a study on species other than S. aureus, Husain et al. showed that biofilm forming capability of Pseudomonas aeruginosa and Aeromonas hydrophila WAF-38 was reduced at sub-MIC concentrations of clove essential oil (17). Khan et al. also found that sub-MIC concentrations of clove essential oil can inhibit quorum sensing activity of Chromobacterium violaceum and P. aeruginosa (18). In this study, we examine effect of sub-MIC concentrations of clove essential oil on some virulence factors of S. aureus, a foodborne pathogen.

MATERIALS AND METHODS

The study was carried out from April 2016 to December 2017. Clove oil was purchased from Kashan Barich essence Co., Iran. Gas chromatography (GC) and GC- mass spectrophotometry (GC/ MS) were used to determine chemical composition of clove oil. An Agilent 7890 GC (CA, USA) was used to conduct GC analysis. An Apolar HP-5 capillary column (30 m×0.25 mm, 0.25 µm film thickness) was used. Initial column temperature was 60 °C, increasing 4 ºC/ min to reach a final temperature of 280 °C. The temperature of the injector was set at 300 ºC and a ratio split of 1:100 was applied. First, 0.1 µl of clove oil was directly injected into the injection port of the system. GC-MS analysis was done by a gas chromatograph combined with mass selective detector and quadrupole EI mass analyzer (Agilent technologies 7890, Agilent 5975 C and Agilent Technologies, Palo Alto, CA respectively). For stationary phase, an HP-5MS 5% column (30 m×0.25 mm, 0.25 µm film thickness) coated with methyl silicone was used. Helium at a 0.8 ml/min flow rate was used as the carrier gas. By setting a 4 °C/min ramp rate, the temperature was programmed from 60 °C to 280 °C. The injector and the GC-MS interface temperatures were retained at 290 °C and 300 °C, respectively.

A 70 eV over a mass range of 50-550 (m/z) was used to record all mass spectra. The detector and ion source temperature was maintained at 150 °C and 250 °C respectively. Composition of clove oil was determined based on retention indices (determined by a homologous series of C5 - C24 of n-alkanes) and comparing mass spectral pattern of the oil with published data (19).

One standard strain (ATCC 19095) and 12 field isolates of *S. aureus* from human skin infections, all positive for production of α- and β-hemolysin as well as coagulase, were selected from the microbial collection of the microbiology laboratory of the College of Veterinary, Shahrekord University. Before examinations, all isolates were subcultured on blood agar (Micromedia, EU) for purity confirmation. After incubating at 37 ^{o}C for 24 h, the obtained pure cultures were inoculated in Trypticase Soy broth (TSB, Merck, Germany) and incubated at the same conditions for further examinations.

To determine the MIC of clove oil, 1g of the filtered sterilized clove oil was mixed with 10 ml of dimethyl sulfoxide to obtain a stock solution of 100 mg/ml. Further dilutions were made by adding 1 ml of stock solution to a tube containing 9 ml Muller-Hinton broth (MHb, Merck, Germany). The process continued to obtain a double serial dilution ranging from 5.0 to 0.078 mg/ml of clove oil. Tubes containing different dilutions (seven tubes for each isolate) were inoculated with 10 µl of S. aureus suspension (10-fold dilution of 0.5 McFarland standard concentration) so that each tube contained about 105 bacteria (20). Two tubes containing MHb with and without bacteria were used as controls. All tubes were incubated at 37 °C for 24 h. The lowest concentration of the clove oil that inhibited bacterial growth was determined as the MIC.

Four tubes each containing 5 ml of MHb were chosen. The standard strain of *S. aureus* (ATCC 19095) was cultured in TSB and after incubation as above, diluted to reach 10^5 cfu/ml. In each of the above tubes, 0.1 ml of the mentioned diluted culture was inoculated. Clove oil was added to obtain 0 (control), 1 MIC, ½ MIC and ¼ MIC concentrations. The prepared tubes were then placed at 37 °C. Absorbance of the samples at 600 nm was measured at 0, 2, 4, 8, 12 and 24 h using a spectrophotometer (Jenway, OSA, UK). Subsequently, the bacterial growth curve was plotted using Microsoft Excel 2013 (21).

Sterile 96-well flat bottom polystyrene microtiter plates were used for assays. Ten µl of fresh TSB containing each isolate (100-fold diluted from the 0.5 McFarland standard concentration) of *S. aureus* were inoculated into wells containing 200 µl of sterile MHb. A serial dilution of clove oil was prepared in order to obtain 1 MIC, ½ MIC and ¼ MIC concentrations in each row of the microtiter plates. Two tubes containing MHb with and without bacterial inoculation were used as controls (22). After incubation at 37 °C for 24 h, the absorbance of planktonic growth of bacteria at 492 nm was measured using a ELISA reader (Bio-Tek, Winooski, USA).

In order to assess biofilm formation, broth cultures were removed and the empty wells were washed by sterile phosphate buffered saline (PBS). To quantify biofilm formation, after drying the microplates at room temperature, 200 µl of 0.2% crystal violet solution were poured in each well and the microplates were placed at 37 °C for 15 min. After three times washing with PBS and drying, 200 µl of 80:20 (v:v) mixture of ethyl alcohol and acetone were used to determine biofilm-producing bacteria. Absorbance at 492 nm was determined using an ELISA reader (Bio-Tek, Winooski, USA). Results of biofilm production were analyzed according to a protocol described previously (23).

Isolates were inoculated into tubes containing sterile TSB and different clove oil concentrations (1 MIC, ½ MIC and ¼ MIC). A tube without bacteria was considered as the negative control. After incubation at 37 °C for 24 h, samples were streaked on 5% bovine blood agar and incubated at 37 °C for 24 h. Colonies were checked for α- and β-hemolysin production as well as coagulase production (8). Slide coagulase test was carried out to assess effects of clove oil on clumping factor production. Arabbit plasma drop was added and gently mixed with bacterial suspension to evaluate plasma coagulation. All experiments were done in triplicate. Statistical analyses of data were performed in SPSS (version 23) using T-test and Mann-Whitney U tests at significant of 0.05.

RESULTS

Table 1 shows the chemical compositions of clove oil according to results of GC and GC/MS. The examined clove oil contained eugenol, β-caryophyllene (9.54%) and α-humulene (1.33%).

Except for one isolate, clumping factor production was not affected by sub-MIC concentrations of clove oil.

We examined the effects of sub-MIC concentrations of clove oil on hemolysin.

production in 10 field isolates of *S. aureus*. As shown in table 2, ½ MIC concentration of clove oil inhibited production of α- and β-hemolysin in two (20%) and four (40%) isolates, respectively. Our data for standard and two field isolates are not shown because of some technical problemsThe results showed that MIC of clove oil significantly inhibited planktonic growth of all examined isolates.

In addition, ½ and ¼ MIC concentrations of clove oil inhibited planktonic growth of 10 (76%) and two (15%) isolates, respectively. Moreover, MIC and ½ MIC concentrations of clove oil inhibited biofilm production in two (15%) and three (23%) isolates, respectively (Table 3).

Table 1. The composition of clove oil detected by GC and combined GC/MS

Main compounds	%	RI	Retention time
Eugenol	77.63	1365.335	18.175
Iso-eugenol	0.65	1387.06	18.86
β- Caryophyllene	9.54	1421.934	19.937
α-Humulene	1.33	1456.262	20.984
delta-Cadinene	0.2	1524.656	23.07
Eugenol acetate	0.07	1587.019	24.852
Caryophyllene oxide	0.28	1587.019	24.852

Table 2. Effects of sub-MIC concentrations of clove oil on α- and β-hemolysin production by *S. aureus* isolates

Strain	**1/2 MIC**		**1/4 MIC**		**Positive Control**	
Hemolysin	α	B	α	β	α	β
Isolate 1	+	+	+	+	+	+
Isolate 2	+	+	+	+	+	+
Isolate 3	+	-	+	-	+	+
Isolate 4	+	+	+	+	+	+
Isolate 5	+	+	+	+	+	+
Isolate 6	+	+	+	+	+	+
Isolate 7	+	+	+	+	+	+
Isolate 8	-	-	+	-	+	+
Isolate 9	-	-	-	-	+	+
Isolate 12	+	-	+	-	+	+

Table 3. Effect of clove oil on planktonic growth and biofilm formation by *S. aureus* isolates (me ans of three repetition ± standard deviation) (Data represents optical density at 492 nm).
b in each row denotes differences are significant (p<0.05).

	Concentration	Bacterial isolates											
		Standard strain	1	2	3	4	5	6	7	8	9	10	11	12
MIC-µg/ml	MIC	1250	1250	1250	1250	1250	312	1250	1250	1250	1250	1250	1250	2500
Planktonic growth	MIC	0.423± 0.013[b]	0.388±0.015[a]	0.392±0.005[b]	0.399±0.008[b]	0.432±0.001[a]	0.457±0.038[a]	0.415±0.006[b]	0.400±0.006[b]	0.403±0.011[b]	0.457±0.059[b]	0.436±0.004[a]	0.428±0.011[a]	0.405±0.003[b]
	1/2 MIC	0.556±0.006[b]	0.753±0.118	0.673±0.033[b]	0.608±0.032[a]	0.551±0.045[b]	0.959±0.069[b]	0.715±0.041	0.563±0.155[a]	0.918±0.065[b]	0.473±0.040[b]	0.536±0.035[b]	0.712±0.264	0.414±0.005[b]
	1/4 MIC	0.831±0.051	0.883±0.049	0.755±0.018	0.615±0.073	0.693±0.038	1.139±0.088	0.777±0.129	1.057±0.143	0.775±0.051[a]	0.493±0.097	0.844±0.046[b]	1.049±0.042	0.586±0.095
	Positive control	0.833±0.151	0.673±0.026	0.783±0.012	0.716±0.244	0.676±0.117	1.217±0.027	0.777±0.078	1.088±0.121	1.195±0.002	0.642±0.053	1.181±0.124	1.079±0.061	0.604±0.048
Biofilm formation	MIC	0.369±0.008	0.363±0.011	0.374±0.006	0.365±0.011[a]	0.375±0.002	0.373±0.017	0.363±0.011	0.376±0.005	0.356±0.014[a]	0.369±0.012	0.359±0.011	0.361±0.013	0.376±0.006
	1/2 MIC	0.369±0.005	0.352±0.019	0.388±0.027	0.374±0.016	0.359±0.016	0.407±0.029	0.356±0.008	0.389±0.023	0.377±0.009[b]	0.363±0.007	0.344±0.011[a]	0.344±0.007[a]	0.356±0.008
	1/4 MIC	0.382±0.011	0.360±0.019	0.474±0.062	0.392±0.017	0.353±0.015	0.416±0.042	0.362±0.013	0.371±0.01	0.439±0.023[b]	0.427±0.048	0.359±0.018	0.349±0.011	0.397±0.016
	Positive control	0.396±0.026	0.358±0.007	0.379±0.003	0.403±0.011	0.362±0.008	0.408±0.024	0.357±0.005	0.375±0.004	0.386±0.039	0.365±0.019	0.379±0.004	0.363±0.007	0.413±0.048

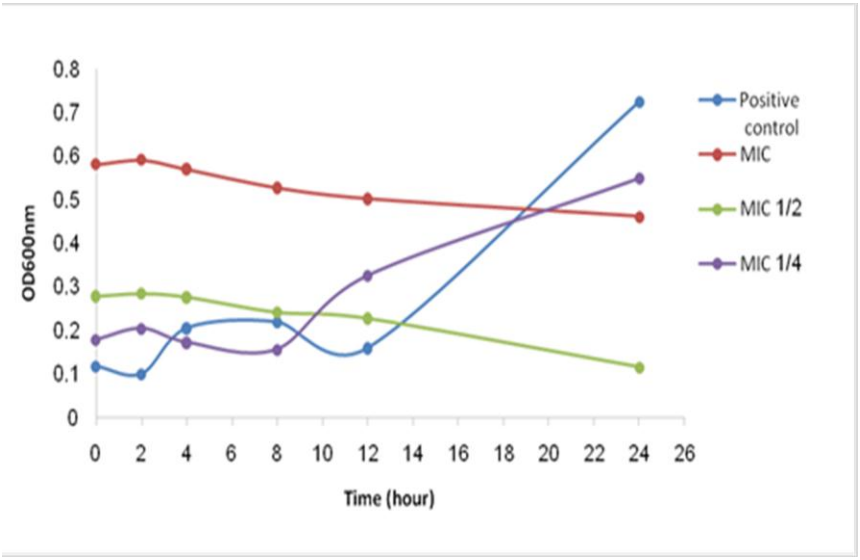

Figure 1. Effects of sub-MIC concentrations of clove oil on growth curve of standard strain of *S. aureus*
As shown in figure 1, the MIC and ½ MIC concentrations of clove oil inhibited growth of standard strain of *S. aureus* after 24 h. However, ¼ MIC concentration of clove oil did not inhibit growth of *S. aureus* from eight hours incubation onward.

DISCUSSION

According to the GC and GC-MS analyses, the most abundant components of clove oil were eugenol (77.63%), followed by β-caryophyllene (9.54%). These results are similar to results of a study by Khan et al. (18). It has been demonstrated that eugenol can have potential beneficial properties, such as antimicrobial activity (24). B-caryophyllene is used as a food additive and fragrance in cosmetics. It has been reported that ≥MIC concentrations of β-caryophyllene can have rapid bactericidal effects (25). The broth tube dilution method is one of the basic techniques used for assessing antimicrobial activity of essential oils. Kalemba and Kunicka suggested that only MIC and minimum bactericidal concentration values should be reported for evaluating essential oils' antimicrobial activity (26). The MIC of clove oil against *S. aureus* isolates was 1250 µg/ml. Growth curve of *S. aureus* isolates was affected by MIC and sub-MIC concentrations of clove oil. Statistical analysis showed that ½ MIC concentration of clove oil had significant inhibitory effects on planktonic growth of *S. aureus* isolates.

Qiu et al. reported that sub-MIC concentrations of eugenol (an effective component of clove oil) had no significant influence on growth curve of *Staphylococcus* spp. (15). This may indicate that in vitro antimicrobial activities of clove oil may be related to the synergistic effect of clove oil components (25). Moreover, the growth kinetics of bacteria can vary greatly between species and strains.

The biofilm production of 30% of isolates decreased significantly after treatment with sub-MIC concentrations of clove oil. This finding is in line with results of Yadav et al. that showed that ½ MIC concentration of eugenol can decrease the biofilm biomass of *S. aureus* isolates by more than 50%. The mentioned study suggested that eugenol might eradicate *S. aureus* biofilms, primarily via bacterial lysis within biofilms and breaking connections between cells (16).

Similar to our findings, another study demonstrated that some plant-derived compounds are able to inhibit biofilm production in *S. aureus* strains (27). Clove oil can strongly inhibit quorum sensing in *P. aeroginosa* (18). Husain et al. showed that biofilm forming capability of *P. aeruginosa* and *Aeromonas hydrophila* WAF-38 was reduced after treatment with sub-MIC concentrations of clove oil (17).

Production of α- and β-hemolysin by *S. aureus* decreased significantly after exposure to ½ and 1/4 MIC concentrations of clove oil. Consistent with this finding, Smith et al. showed that sub-MIC concentrations of clove oil can reduce α-toxin production in *S. aureus* (14). In another study, Qiu et al. indicated that sub-MIC concentrations of thymol (found in thyme, oregano and tangerine peel) can decreases the production of α-hemolysin by *S. aureus* (9).

In our study, sub-MIC concentrations of clove oil had no significant impact on production of clumping factor by the isolates. Hammer et al. reported that tea tree oil increased level of coagulase production in *S. aureus* (28). A study reported that sub-MIC concentrations of linezoloid was able to decrease production of hemolysins and coagulase in *S. aureus* (29).

CONCLUSION

We demonstrated that sub-MIC concentrations of clove oil can exert antibacterial activity against *S. aureus* isolates, inhibit biofilm formation and decrease production of α- and β-hemolysins. Thus, it may prevent *S. aureus* toxicities if used as a food additive. Further studies are required to evaluate the effects of clove oil on expression of the examined exotoxins.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest regarding publication of this article.

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