

Comparison of the Effects of Hydrosol Extracted from Turmeric (*Curcuma longa*) and Cinnamon (*Cinnamomum verum*) on *staphylococcal* biofilm

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ABSTRACT

BACKGROUND AND OBJECTIVE: The biofilm of bacteria, especially pathogenic bacteria, endanger food safety due to their high resistance to disinfectants; use of herbal extracts, essential oils and hydrosol (aqueous residual from extraction of essential oils) is one of the ways to combat the resistance microbial biofilms. Therefore, the effect of hydrosol extracted from turmeric (*Curcuma longa*) and cinnamon (*Cinnamomum verum*) on *staphylococcal* biofilm was investigated in this study.

METHODS: In this experimental study, the hydrosol of turmeric and cinnamon was extracted using steam distillation method. After separation of essential oil, 10, 30 and 50% concentrations were used to investigate the anti-biofilm effects. Anti-biofilm effect against *Staphylococcus aureus* (PTCC 1112), *Staphylococcus saprophyticus* (PTCC 1440) and *Staphylococcus epidermidis* (PTCC 1435) was evaluated using ELISA reader. The efficacy of both hydrosols in removing bacterial biofilms formed on different surfaces (glass, steel and polyvinyl chloride) was determined by microbiological culture method.

FINDINGS: The results showed that optical density in control samples of *Staphylococcus aureus*, *epidermidis* and *saprophyticus* was 0.254 ± 0.03 , 0.138 ± 0.019 , and 0.146 ± 0.017 , respectively, but after exposure to 50% cinnamon hydrosol reached to 0.072 ± 0.011 , 0.096 ± 0.021 , and 0.064 ± 0.01 , and after exposure to 50% turmeric hydrosol reached to 0.074 ± 0.02 , 0.098 ± 0.021 , and 0.057 ± 0.011 , respectively. Investigation of the efficiency of hydrosol in removal of formed biofilms showed that the highest decrease was in the case of *Staphylococcus saprophyticus* biofilm formed on glass and steel surfaces, and the logarithm of bacterial population declined from 4 to 1 in the presence of 50% hydrosol ($p \leq 0.05$).

CONCLUSION: The results of this study showed that the hydrosol extracted from cinnamon and turmeric is effective in preventing biofilm formation of *staphylococcal* bacteria and eliminating the formed biofilm.

KEY WORDS: Cinnamon, Turmeric, Biofilm, *Staphylococcus*.

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Introduction

Widespread use of antibiotics and antimicrobial compounds has led to the occurrence of microbial resistance. The resistance of microorganisms increases in the condition of biofilm formation. This has moved the attention of researchers to new antimicrobial compounds with few side effects. The herbal extract and essential oil are among such compounds (1).

Some pathogenic bacteria such as *Escherichia coli*, *Listeria monocytogenes*, *Salmonella typhimurium*, *Campylobacter jejuni*, and *Yersinia enterocolitica* can easily create biofilm or a part of biofilm population, making disinfection and cleaning of some surfaces extremely hard (2). Some studies have focused on the anti-biofilm effect of herbal hydrosol. Hydrosol is the byproduct of the extraction of herbal essential oil. To separate hydrosol from essential oil, the liquid that is obtained from distillation of the plant is poured into a separate funnel. This liquid is divided into two parts of upper phase and lower phase; the essential oil is in the upper phase and the remaining liquid in the lower phase is called hydrosol.

Hydrosol is the byproduct in the production of herbal essentials that contain bioactive compounds. Cid-Pérez et al. reported the antimicrobial and antioxidant effects of *Poliomintha longiflora* (3). Acheampong et al. reported the antimicrobial effects of hydrosol of *Cymbopogon nardus*, *Ocimum gratissimum*, and orange peel on *Escherichia coli*, *Bacillus subtilis*, and *enterococcus faecalis* (4). The strong antimicrobial effect of hydrosol extracted from *Nepeta nepetella* against *Klebsiella pneumoniae*, *Enterococcus faecalis*, *Staphylococcus aureus*, *Bacillus subtilis*, and *Listeria monocytogenes* has been reported. According to this research, gram positive bacteria showed more susceptibility compared with gram negative bacteria and *Bacillus cereus* showed highest level of susceptibility (5).

The anti-fungal (6) and anti-viral (7) effects of hydrosol of various plants have also been reported. Research has been done on the anti – biofilm effects of herbal hydrosol. For example, Karampoula et al. reported that the hydrosol of *Thymbra capitata* has anti-biofilm effects on *salmonella typhimurium* and reduced the logarithm of biofilm formation of this organism up to 7.5 (8). So far, no research has been done to study the effects of turmeric and cinnamon hydrosol on *staphylococcal* biofilms. The present study was conducted to investigate the effect of hydrosol extracted from Turmeric (*Curcuma longa*) and Cinnamon (*Cinnamomum verum*) on *Staphylococcus aureus*

(PTCC 1112), *Staphylococcus saprophyticus* (PTCC 1440) and *Staphylococcus epidermidis* (PTCC 1435) biofilm formation, and to evaluate the effect of these two types of hydrosol in the removal of biofilm formed on different surfaces (glass, stainless steel, and Polyvinyl chloride).

Methods

This experimental study was performed in the microbiology lab of the Islamic Azad University of Neyshabur with the code of ethics: IR.IAU.NEYSHABUR.REC.1397.012. Turmeric (*Curcuma longa*) and Cinnamon (*Cinnamomum verum*) were prepared from the local market and for the extraction of hydrosol, 100 g of each of them was grinded and heated in 2-liter flask containing one liter water (1:10 w/v) for two hours by Clevenger apparatus and steam distillation was performed. In the next step, the essential oil was separated from hydrosol by separatory funnel and the hydrosol was kept at 4 °C until use (8).

Hydrosol sterilization was performed using a membrane filtration with a pore size of 0.45 µm and the resulting hydrosol was considered as a 10% concentration (9). Oven was used for condensation of hydrosol and preparation of different concentrations of hydrosol. Microbial strains of *Staphylococcus aureus* (PTCC 1112), *Staphylococcus saprophyticus* (PTCC 1440), *Staphylococcus epidermidis* (PTCC 1435) were prepared from Iranian Research Organization for Science and Technology in lyophilized form. The bacterial vial was broken in sterile conditions and transferred to appropriate broth media (Trypticase Soy Broth for *Staphylococcus aureus* and Nutrient Broth for *Staphylococcus saprophyticus* and *Staphylococcus epidermidis*) and incubated for 24 h at 37 °C. Microbial cells were harvested by ALC 4232 centrifuge at 4000 rpm. The bacterial count was determined by the McFarland method and diluted to about 10⁶ CFU/ml (10).

Investigation of the effect of hydrosol on preventing biofilm formed by *Staphylococcus* strains: To evaluate biofilm formation, 200 µl of incubated broth media was transferred to 96-well Polystyrene Microplates. In order to investigate the effect of hydrosols on microbial biofilm formation, 200 µl of different concentrations of hydrosol extracted from cinnamon and turmeric (10, 30, 50%) were also added to each well of microplate, and incubated at 37 °C for 24 hours. Wells containing sterile broth medium were

used as control. After incubation, the broth medium was evacuated and each well was washed three times with 200 μ l phosphate-buffered saline (pH=7.4) to remove free cells and was placed upside down to dry. The biofilm layer was then fixed with 95% ethanol and stained with 100 μ l of 1% crystal violet for 5 min. The stain residue was washed three times with sterile distilled water and the microplate was dried for 30 min. Then, the optical density at 570 nm was read by ELISA Reader (AWARNES model) in each well. The level of biofilm formation was categorized as follows. Optical density greater than 1 indicates high biofilm formation; optical density between 0.1 and 1 indicates moderate biofilm formation and optical density less than 0.1 indicates no biofilm formation (11).

Investigation of the effect of hydrosol on removal of biofilm formed by *Staphylococcus* species on different surfaces (glass, stainless steel, polyvinyl chloride): To investigate the formation of biofilm on glass, stainless steel and polyvinyl chloride, 1.5 cm² pieces of these materials were first sterilized by immersion in 70% ethanol for 30 min and then rinsed with sterile distilled water and then placed in ultrasonic equipment (EUROSONIC model) for 20 minutes and then rinsed with sterile distilled water and dried. Then 100 μ l of bacterial suspension was placed on each one and incubated at 37 °C for 48 hours.

Then, pieces of stainless steel, glass and polyvinyl chloride containing biofilm were rinsed with sterile brine (0.85%) and placed in tubes containing hydrosol extracted from cinnamon or turmeric and were stirred at 90 rpm for 30 minutes. After performing other washing steps, they were transferred to appropriate culture medium (Trypticase Soy Broth for *Staphylococcus aureus* and Nutrient Broth for *Staphylococcus saprophyticus* and *Staphylococcus epidermidis*) and

incubated at 37 °C for 24 hours and then the cells were counted (12). The effect of different types of hydrosol and different concentrations of hydrosol on biofilm formation and biofilm removal of the studied bacteria were recorded as mean and standard error (mean \pm SD). Statistical analysis was performed by SPSS 14 using ANOVA test and $p < 0.05$ was considered significant.

Results

The results showed that *Staphylococcus aureus*, *Staphylococcus saprophyticus* and *Staphylococcus Epidermidis* were capable of biofilm formation and this property was significantly decreased with exposure to cinnamon and turmeric hydrosols ($p < 0.05$) (Table 1). The studied hydrosols at higher concentrations had more effect on preventing bacterial biofilms formation and the highest effect was at 50% concentration ($p < 0.05$) (Table 1).

Evaluations showed that cinnamon and turmeric hydrosol could remove biofilms composed of *Staphylococcus aureus*, *saprophyticus* and *epidermidis* on different surfaces of glass, stainless steel and polyvinyl chloride ($p < 0.05$). Among the studied *staphylococci*, *Staphylococcus aureus* biofilm was more resistant to hydrosol and the removal of this bacterial strain was insignificant against both hydrosols, but *Staphylococcus saprophyticus* biofilm was highly sensitive to the studied hydrosol; when exposed to 50% concentration of turmeric and cinnamon hydrosol, the logarithm of the population of this bacterial species reached to 1 and 1.3 in the case of steel and glass, respectively (Table 2). Among the biofilms formed on different surfaces (stainless steel, glass and plastic), the highest reduction was observed in the biofilm formed on steel and glass.

Table 1. Optical density at a wavelength of 570 nm

<i>Staphylococcus aureus</i>		<i>Staphylococcus epidermidis</i>		<i>Staphylococcus saprophyticus</i>	
alone	0.254 \pm 0.03 a	alone	0.138 \pm 0.019 a	alone	0.146 \pm 0.017 a
Cinnamon hydrosol (10%)	0.157 \pm 0.027 b	Cinnamon hydrosol (10%)	0.111 \pm 0.014 ab	Cinnamon hydrosol (10%)	0.139 \pm 0.019 ab
Cinnamon hydrosol (30%)	0.148 \pm 0.021 b	Cinnamon hydrosol (30%)	0.108 \pm 0.013 ab	Cinnamon hydrosol (30%)	0.134 \pm 0.020 ab
Cinnamon hydrosol (50%)	0.072 \pm 0.011 c	Cinnamon hydrosol (50%)	0.096 \pm 0.021 b	Cinnamon hydrosol (50%)	0.064 \pm 0.010 c
Turmeric hydrosol (10%)	0.154 \pm 0.01 b	Turmeric hydrosol (10%)	0.121 \pm 0.018 ab	Turmeric hydrosol (10%)	0.119 \pm 0.010 ab
Turmeric hydrosol (30%)	0.144 \pm 0.024 b	Turmeric hydrosol (30%)	0.112 \pm 0.013 ab	Turmeric hydrosol (30%)	0.114 \pm 0.016 b
Turmeric hydrosol (50%)	0.074 \pm 0.02 c	Turmeric hydrosol (50%)	0.098 \pm 0.021 b	Turmeric hydrosol (50%)	0.057 \pm 0.011 c

Numbers with different letters in each column indicate a significant difference at the level of $p < 0.05$ (numbers more than 1 indicate high biofilm formation; numbers between 0.1 and 1 indicate moderate biofilm formation; numbers less than 0.1 indicate the non-biofilm formation).

Table 2. Effect of different concentrations of cinnamon and turmeric hydrosol on the logarithm of bacterial populations of *Staphylococcus aureus*, *Staphylococcus saprophyticus* and *Staphylococcus epidermidis* biofilms

Hydrosol	Density	Steel			Glass			Polyvinyl chloride		
		<i>Staphylococcus aureus</i>	<i>Staphylococcus epidermidis</i>	<i>Staphylococcus saprophyticus</i>	<i>Staphylococcus aureus</i>	<i>Staphylococcus epidermidis</i>	<i>Staphylococcus saprophyticus</i>	<i>Staphylococcus aureus</i>	<i>Staphylococcus epidermidis</i>	<i>Staphylococcus saprophyticus</i>
Cinnamon	10%	3.96 a	2.9 i	1.76 o	3.93 abc	3.6 de	1.76 o	3.93 abc	3.94 ab	3 hi
	30%	3.8 bc	2.7 jk	1.56 p	3.8 bc	3.4 f	1.56 p	3.8 bc	3.65 d	2.6 kh
	50%	3.79 c	2.5 h	1.3 q	3.79 c	3.16 g	1.3 q	3.79 c	3.56 de	2.3 l
Turmeric	10%	3.97 a	2.3 l	1.6 p	3.93 abc	3.5 ef	1.6 p	3.93 abc	3.93 abc	2.76 j
	30%	3.93 abc	2.2 lm	1.4 q	3.8 bc	3.1 gh	1.4 q	3.8 bc	3.6 de	2.1 mn
	50%	3.8 bc	2.1 mn	1 r	3.79 c	2.9 i	1 r	3.79 c	3.5 ef	2 n

(Similar letters indicate lack of significant difference based on Duncan test [5%]).

Discussion

According to the results of this study, all three bacteria (*Staphylococcus aureus*, *Epidermidis* and *Saprophyticus*) were able to form biofilm at moderate levels, and the amount of biofilm formation by this bacterial species decreased in exposure to hydrosol (turmeric and cinnamon). Various studies have confirmed the presence of bioactive compounds in hydrosol. The most important of these compounds are aldehydes and alcohols (13), which are antimicrobial agents in plant hydrosol. In the analysis of the constituents of cinnamon hydrosol, the presence of a variety of hydrosols, aldehydes and acids has been identified. Other bioactive compounds in cinnamon hydrosol include vulgarol, emersol, oleic acid and methyl palmitate (14). The antimicrobial effects and antiviral and antioxidant properties of cinnamic acid and its derivatives have been reported by Sova et al. (15).

The anti-biofilm properties of different plant species have been reported in other studies; Mohammadi et al. have investigated and reported the effect of the extract of Ajwain plant on the biofilms of different bacterial species. Extract of this plant reduced 98% biofilm formation of *Acinetobacter baumannii* and 19% in *Klebsiella pneumoniae* (1).

The difference in the efficacy of different herbal extracts on microbial biofilms depends on the type of microorganism, the degree of microbial resistance to the plant extracts as well as the type of bioactive constituents of the herbal extracts. The results of

evaluating the efficacy of cinnamon and turmeric extract hydrosol on the removal of bacterial biofilms formed on surfaces of different materials (stainless steel, glass, polyvinyl chloride) revealed that both studied hydrosols are effective in biofilm removal and this effect is enhanced by increasing the concentration of hydrosol. Biofilms from different strains of studied bacteria showed different sensitivity to herbal hydrosol, with the highest susceptibility of *Staphylococcus saprophyticus* and the least susceptibility of *Staphylococcus aureus*, respectively. The type of surface also had a significant effect on hydrosol efficiency in removal of bacterial biofilm; removal of bacterial biofilm by herbal hydrosol on polyvinyl chloride was the lowest and it was the highest on stainless steel and glass. The reason for this is the difference in the roughness of the three tested surfaces. The higher the surface roughness, the greater the number of microorganisms trapped in it, thereby reducing the cleaning efficiency (2).

The removal of biofilms formed by herbal extracts has also been reported by other researchers; Abeyundara et al. investigate the effect of cantaloupe extract on *Salmonella* biofilms on rubber, stainless steel, high-molecular-weight polyethylene and polyurethane reported that the type of biofilm formation surface was effective on the efficacy of the herbal extract and the effect of the herbal extract on the biofilm formed in rubber was less than stainless steel, polyethylene and polyurethane (16). According to the

results of this study, cinnamon and turmeric hydrosol are efficient both in preventing of staphylococcus bacteria biofilm formation and in removal of staphylococcus biofilm formed on glass, stainless steel and poly vinyl chloride and 50% concentration showed the most effect.

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