Evaluation of Antibiofilm Activity of Dentol

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Abstract: Biofilm is a community of bacteria which are attaching to a surface. They are responsible for many of catheter related or dental plaque related infectious diseases. Different approaches have been used for preventing biofilm related infections in health care settings. Many of these methods have disadvantages such as chemical based complications, and emergent antibiotic resistant strains. Therefore antibiofilm effect of Dentol was evaluated on two important medical bacterial biofilms in this study. Methods: Minimum inhibitory concentrations (MIC) of Dentol was calculated by serial dilution method by microtiter plates against standard bacteria. Antibiofilm effect of Dentol was evaluated by microtiter plate assay and after measuring absorbance in 550 nm, the difference between groups was calculated by Cruskal-wallis and Mann-withney tests. Results: 1:80 (12.5 µl/ml) & 1:20 (50 µl/ml) dilutions of commercially available Dentol were measured as MICs for *S.aureus* and *P.aeruginosa*, respectively. Two-fold dilutions of Dentol MICs completely eradicated and inhibited biofilm formation. Statistical analysis between test groups revealed significant differences. Disscussion: Dentol is a medicinal herbal essential oil derived from *Satureja khuzestanica* (SKEO). Several in vitro studies have reported its antidiabetic, analgesic, anti-inflammatory, and anti-hyperlipidemic effects. So because of complications that have been encountered in health care settings from routine methods of catheter processing, and biofilm related problems, efficacy of Dentol for destruction of bacterial biofilms as a safe material was studied.

Introduction

In many ecosystems, bacteria growing in surfaces as a layer that is called biofilms (1). Biofilms have important roles in many diseases. Prosthetic device colonization, dental plaque formation, infection of the cystic fibrosis, food spoilage, and unusual resistance to antibiotics are important conditions originating from biofilms (2-6). This kind of infections compromise over 70 % of all nosocomial blood stream infections (3).Traditional catheters locking with normal saline or heparin solutions have little protection against catheter related infections. On the other hand using of antibiotics lock solutions (such as vancomycin- heparin) have restricted because of emerging antibiotic resistant strains. Some studies have used EDTA salts and EDTA- minocycline combinations as catheter surface decontaminant for controlling catheter related infections (3). As we know scientists have been studied the potential of many materials as

inhibitors of biofilm formation with several approaches (2-12).

Since chemical intervention in many therapeutic procedures associated with side effects and complications, we assessed the efficacy of Dentol as a biofilm destroying agent or safe remover material in an in vitro model with microtiter plate assay. It should be considered that antiseptic properties of plants have been discovered for a long time. (13).

Our main purpose in this study is that whether Dentol (indigenous mountain plant in Lorestan province of Iran) can destroy or remove the biofilm layers from micropipette wells. Dentol is an essential oil of *Satureja Khuzistanica*(SKEO), that have indicated anti-inflammatory, anti-nociceptive, anti-diabetic, anti-oxidant, and anti-hyperlipidemic effects in several studies (14-17). The main component of this essential oil is Carvacrol (16). Carvacrol has a broad spectrum antimicrobial activity against bacteria. Bacterial exposure to

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carvacrol, resulting in leakage of intracellular ATP and potassium from cell membrane, and ultimately cell death. However, its antibiofilm activity has not been studied extensively (18).

With many clinical biofilm related problems in all countries, it is obvious that finding an effective and safe material with good biofilm destructive and antimicrobial properties is one of the interesting subjects in medical microbiology and infectious disease. So for this purpose we used *P.aeruginosa ATCC 27853* and *S.aureus ATCC 25923* as a representatives of more resistant and well known biofilm forming gram negative and gram positives respectively.

Patients and Methods

Biofilms studied by 96 well microtiter plate (Nunc) assay as a simple, inexpensive and scientific method (19) *P.aeruginosa ATCC 27853* & *S. aureus ATCC 25923* were used as in study. Bacterial strains were obtained from Scientific & Industrial Research Center of Iran (Microbial Collection Department) as lyophilized. *P.aeruginosa ATCC 27853* made large quantities of alginate.

Determination of Minimum Inhibitory Concentration (MIC)

The minimum inhibitory concentration of Dentol against bacteria was determined according following method. A stock solution of commercially Dentol was purchased from pharmacies of Khoram Abad as a 10 % Carvacrol. Two rows of 10 sterile 7.5 x1.3 cm capped tubes arranged in the rack. Two ml of mixed Dentol was transferred to the first tube in each row. Using a fresh pipette, 1 ml of broth (TSB; Difco Laboratory) was added to the remaining 2 ml in the bottle, mixed and transferred 1 ml to the second tube in each row. Serial dilutions in this way continued to 9th tube in each row. One ml of Dentol free broth poured to the last tube in each row. One drop of an overnight broth cultures (diluted to 0.5 McFarland turbidity in TSB) of the *P. aeruginosa ATCC 27853* & *S. aureus ATCC 25923* inoculated to two rows respectively. Tubes were incubated for 18 hours at 37°C. A tube containing 1ml broth with the test organisms kept at +4°C in a refrigerator overnight to be used as standard for the determination of complete inhibition. Growth was visualized by turbidity and MIC is expressed as the lowest dilution, which inhibited growth of tested bacteria(20).

Microtiter plate biofilm assay

Biofilm study was performed by the method published by Christensen (19). In brief, Bacterial strains inoculated in 2-5 ml of Tripticase soy broth (TSB) and growed up to stationary phase respectively. Cultures diluted to 1:100 in TSB, and 100 µl of each dilution pipeted to four wells in a sterile flat bottom micotiter plate. After incubation for 48 h in 37c°, planktonic bacteria removed from all of the wells and washed with distilled water twice. 125µl of 0.1 % crystal violet solution (Sigma Chemical Co) added to each well, then washed with distilled water and poured out extracted stains and additional washes performed until unstaining. Microplates inverted and vigorously tap on paper towels to remove any excess liquid and air dried.200 µl of 95% ethanol and 33% glacial acetic acid (Sigma Chemical Co) poured in *P.aeruginosa* and *S. aureus* wells respectively. Biofilm stains solubilized at room temperature. After shaking and pipetting of wells, 125 µl of the solution from each well transferred to a sterile tube and volume reached to 1 ml with distilled water. Finally optical density of each well measured at wavelength of 550 nm (Spectronic 20 Spectrophotometer). It is noteworthy that, we performed experiment with 3 control groups: groups 1 as negative control (only culture media) groups 2 and 3 as positive for *P.aeruginosa ATCC 27853*, and *S. aureus ATCC 25923* respectively. In groups 4 and 6 bacteria inoculated simultaneously with two fold of Dentol MIC for each of the bacteria. In groups 5 and 7 Dentol added after 48 h incubation and biofilm formation.

We used Dentol as 10 % (w/w) Carvacrol (commercially available) solution of *Satureja Khuzistanica*. This essential oil has been used as dental anesthetic and antiseptic.

Statistical analysis

Significant differences in 7 groups were assessed by Cruskal-Wallis test. And nonparametric Mann-Whitney tests used for evaluating between two separate groups.

Results

Concentration of 1:80 and 1:20 dilutions of Dentol were calculated as MICs for *S.aureus* and *P.aeruginosa* respectively. Exposure of bacteria to two fold dilution of MICs completely eradicated the biofilms. (Figure 1).

In many cases Dentol had significantly ($P<0.05$) reduced the biofilm.

Figure 1. Microtiter plate assay of biofilms formation by *S.aureus*(first 2th rows) and *P.aeruginosa*(3th,4th, rows)

Our in vitro studies revealed that Dentol could easily release biofilm layer from microtiter wells and its effect greater on *P.aeruginosa* than *S.aureus* ($P<0.05$).

In group1 (negative control without Dentol) there was not any biofilm and absorbance in our experiments.

Cruskal-Wallis test analyses between means of all treatment groups indicate that there was significant dif-ference in 7 tested groups. Mann-Whitney analysis also indicates that there is significant difference between 2 separate groups respectively. Figure 2 summarized re-peated data mean values of three independent experi-ments with standard deviations in 7 treated groups that were described in previous sections.
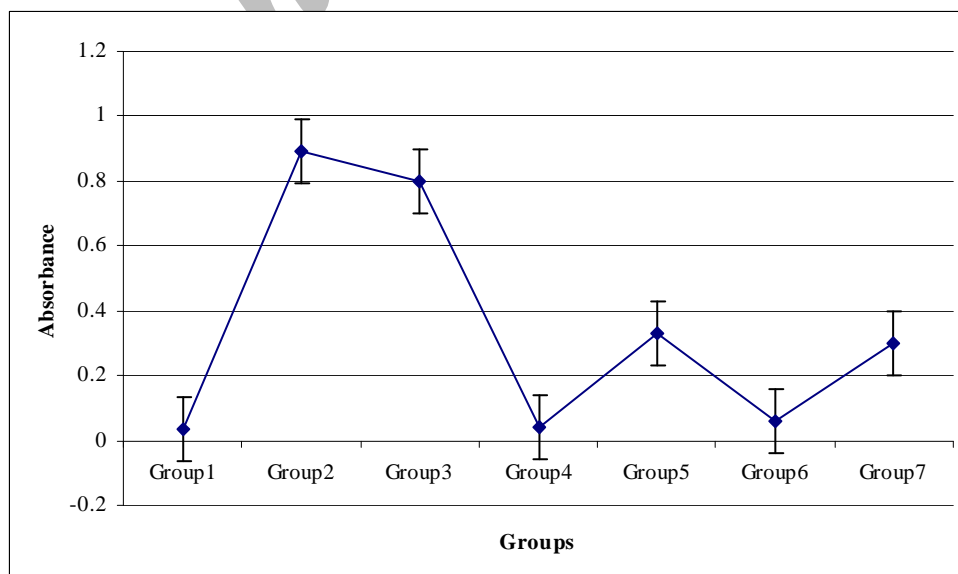

Figure 2. Absorbance(550 nm) of biofilms in 7 groups

Discussions

This study indicates that Dentol has lower effects on biofilm of *P.aeruginosa* than *S.aureus*. One of the reasons for this difference was the production of large quantities of exopolysaccharide in *P.aeruginosa* that protects bacterium from bactericide effects of Dentol. This fact has been mentioned in other studies (21-25). As mentioned above, Carvacrol is main known component of Dentol. Knoles *et al* showed that Carvacrol selectively inhibited *S.aureus* during early stages of biofilm formation, in spite of resistance to higher concentrations(20mM) of Carvacrol (18).

In other study Nostro *et al* reported that low concentrations of Carvacrol (1-2 mg) have good antibacterial effects (26).

Nickel and colleagues showed that physically disrupting structures of the biofilms was sufficient to restore tobramycin susceptibility to the level seen in planktonic bacteria; they did not sterilize urinary catheter biofilms with 1 g of tobramycin/ml for 12 hour (27). It seems that copious quantity of alginate (slime) production by *P.aeruginosa* especially in cystic fibrosis patients has an important role in the resistance of *P.aeruginosa* to tobramycin, the primary therapy in cystic fibrosis patients (28).

Alginate production is affected by a number of environmental regulators. Two studies have shown the interesting result that the contact of bacterium with an abiotic surface can lead to up-regulation of the alginate biosynthesis genes (1).

Bhattacharya *et al* reported that extracts of hops plant (Humulus Lupulus L.) as a component of mouth washes has antibacterial effect on *S.mutans* and other oral streptococci plaques. They calculated MICs of four hops extracts at a pH of 7.5 from 2 to 5 mg/ml (29). In other study Michael discussed that addition of 0.5 % of slime polysaccharide of *S. epidermidis* to broth microdilution susceptibility plates increased the MIC of both vancomycin and teichoplanin approximately fivefold versus both slime positive and – negative strains. Slime also blocked the synergistic effects of both vancomycin and gentamicin on bacteria (30).

In our studies direct observation of Dentol treated *P.aeruginosa* biofilms indicated that this essential oil could have destructive physical deformities on biofilm. Dentol treatment in bacterial biofilms has lowered levels of jelly appearance, and this phenomenon indicates that Dentol may be blocks polysaccharide production. Knoles *et al* also indicated that Carvacrol has potent

antibiofilm effects in early stages of biofilm formation on *S.aureus* (18). So Dentol could be used as a factor for increasing sensitivity of biofilm in contact with bactericides such as ultra violet, antibiotics and other chemotherapeutic agents. In fact Dentol has detaching effects on biofilms and producing planktonic bacteria.

On the other hand *S.aureus* infections are different to treat with current antibiotics because of their natural resistance to antibiotics. Their resistance is higher in biofilm than planktonic forms.

Based on many researches, it is not clear, the mechanism by which staphylococci or enterococci detach from a biofilm (1) but in our experiments it was obvious that starvation is a leading factor for initiation of detaching process.

Because direct supernatant observation of 48 h cultures of biofilms without refreshing culture media indicate that bacteria are significantly in planktonic forms than biofilms with refreshing cultures Medias.

Direct enumeration of culture dilutions indicate that after addition of Dentol in biofilm wells of microtiter plates, planktonic bacteria increased in comparison with controls.

In conclusion it is noteworthy that Dentol like other known plant essential oils could be used as a safe chemical biocide for destroying different bacterial biofilms. based on microtiter plate assay(0.1 % crystal violet staining) it have a potential for preparing new lock solutions for catheter processes and as an additive for dental pastes (plaque inhibitor) ,or other biofilm based health problems. However final conclusions could be proposed after adequate in vivo experiments.

Acknowledgments

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