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Investigations on the Inhibition Effects of 1,8-cineole against Microbial Biofilms

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Bacteria living as biofilm are frequently reported to exhibit the resistances to the common antimicrobial agents. Hence, the *in vitro* antimicrobial activities of the compound 1,8-cineole were evaluated to explore the novel agent. The bioactivities were estimated by the MIC and MBC determinations on 8 species of bacteria and fungi, as well as the confocal laser scanning on 3 types of bacterial biofilms. It was found that the essential oil has good inhibiting activities against fungi rather than bacteria, especially *Candida albicans*, with the lowest MIC value (0.156 %). Both of gram-positive and gram-negative bacterial biofilms are sensitive to 1,8-cineole. In addition, there is linear relationship between inhibition activities and concentration of 1,8-cineole. Further analysis revealed that 1,8-cineole can prompt the biofilm-surface bacteria to die off or live as planktonic, in order to help the immune system to remove the bacteria. Thus, 1,8-cineole could be judged as a kind of potential drug with rather antimicrobial activities.

Key words: 1,8-cineole; Antimicrobial activities; Bacterial biofilms; Confocal laser scanning.

It has become clear that bacterial biofilms are communities of unicellular organisms attached to the surface. The properties of biofilm-grown and planktonic cells are observably distinct, one of which is the increased resistance to antimicrobial agents[1]. Previous reports have revealed that there are great alterations on the structure of exopolysaccharides or other aspects (*rpoS*, multiple drug resistance pumps and *et. al.*) of biofilm architecture, with a biofilm-specific biocide-resistant phenotype[1]. Thus, great efforts have been devoted to the exploration of novel antimicrobial agents directed on the bacterial biofilms[2-4].

Recently, the essential oils and various extracts of plants have provoked interest as sources of natural products. They have been screened for their potential uses as alternative remedies for the treatment of many infectious diseases, such as the microbial infections[5-7]. Indeed, their antimicrobial activities have ever been reported and some of them are already used in topical applications against bacteria and fungi infections[7-9]. Recently, the major ingredient of essential oil extracted from the needles of *Pinus koraiensis* Sieb. et Zucc. has been determined as the α-pinene (10.49 % of overall proportion)[6].

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Simultaneously, agar diffusion assay revealed that the oil from *Pinus koraiensis* Sieb. et Zucc. needles have mild antimicrobial properties, especially fungi *Candida albicans* (28.9-31.5% vs. nystatin)[6]. Particularly, 1,8-cineole (eucalyptol), a major component of camphor-scented essential oils, has present anti-inflammatory and pain release properties and may promote leukemia cell death throughout recent clinical researches[10-15].

To the best of our knowledge, the antibacterial and antifungal activities of 1,8-cineole in biofilms have not been evaluated yet. Therefore, in this work, the activities will be evaluated by the MIC and MBC determinations, as well as the exposure of biofilm to compound. Subsequently, the physiological active of cells in biofilms will be visualized and studied via the confocal laser scanning. We anticipate that the investigation will be of value in the development of agents against the bacterial biofilms.

MATERIALS AND METHODS

Materials

1,8-cineole was obtained from Sigma Chemical Co. (St. Louis, MO, USA) and was stored in glass vials with Teflon sealed caps at -20 ± 0.5 °C in the absence of light.

Microbial strains and culture conditions

The microorganisms used for testing antimicrobial sensitivity including *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 6538, *Staphylococcus epidermidis* ATCC 49134, *Escherichia coli* ATCC 11229, *Proteus vulgaris* ATCC 6380, *Pseudomonas aeruginosa* ATCC 9027, *Candida albicans* ATCC 10231 and *Aspergillus niger* ATCC 16404. They were obtained from the Center for Microbiology Research, Jiamusi Medical Research Institute. The strains were cultured in Luria-Bertani (LB) and Czapek-Dox broth. All microorganisms were grown at 37 °C, except *Aspergillus niger* at 25 °C (7, 16).

MIC and MBC determination

The minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) were measured by the broth micro-dilution method according to the National Committee for Clinical Laboratory Standards[17]. The 1,8-cineole was dissolved in sterilized physiological saline solution (0.9 % w/v) supplemented with Tween 80 (Sigma)[7,16]. Serial doubling dilutions were prepared in a 96-well microtiter plate in the range of 5.000 % to 0.039 %. The final concentration of each strain was adjusted to 1.0×10^5 CFU/ml. All microtiter plates against all microorganisms were incubated at 37 °C for 24 h, except for *Aspergillus niger* that was incubated at 25 °C for 5 days (7, 16). After activation, the MICs and MBCs were determined, with the positive controls of *Streptomycin* and *Amphotericin B* (Tianjin Chemical Reagents Co., Tianjin, China), respectively. Each experiment was repeated in triplicate.

Cultivation of biofilms

The conditions and incubation period for the production of the bacterial biofilms were established according to previous reports[18-20]. Bacterial biofilms were prepared by aliquotting 200 ml of the bacterial suspension containing 1.0×10^5 CFU/ml into the wells of white walled, clear bottom, tissue culture-treated 96-well microtitre plates. Four wells in the last column of each plate were left blank to serve as bioluminescence negative controls. Suspensions of *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Escherichia coli* were prepared in the LB supplement. Microtitre plates containing bacterial suspensions were incubated in air at 37 °C for 48 h.

Exposure of biofilms to essential oil

Mature biofilms were exposed to the 1,8-cineole, under the final concentrations of 1.25, 2.50, 5.00 and 10.00 % (v/v), respectively. The saline (0.9 % w/v) and ethanol solutions (5.0 % v/v) were treated as blank and positive control, respectively. In these experiments, the spatial distribution of dead and live cells was observed, after 24 h of exposure at 37 °C and washed three times with PBS (pH 7.4).

Confocal laser scanning microscopy

Image acquisition was performed using an Olympus FV1000 confocal laser scanning microscope (Olympus, Japan) equipped with an argon and a NeHe laser and detectors and filter sets for simultaneous monitoring (excitation, 488 nm). Before observation, the specimens were stained with propidium iodide (PI, sigma) and fluorescein diacetate (FDA, sigma) refereed to the previous literatures (21-23). The alive cells will be stained with the FDA dye and visualized with a diffusely distributed green fluorescence, whereas those with damaged membranes (dead) will be

stained with PI and with fluorescent red. Thus, the viability of cells could be assessed by this way. Each assay was performed in quadruplicate and repeated at three times.

RESULTS AND DISCUSSION

MICs and MBCs of essential oil

Results of the MIC and MBC studies are shown in Table 1. It was found that 1,8-cineole exhibited significant antifungal activities, especially against *Candida albicans* ATCC 10231, with the MIC value of 0.156 %. It is consistent with the results of Hong *et. al.*,[6]. Besides, the compound exhibited rather high inhibitory effect on *Bacillus subtilis* ATCC 6633, with the MIC and MBC values ranging from 1.250 % to 5.000 % and 2.500 % to more than 5.000 %, respectively. Growth inhibition of other six microorganisms was also observed; with the MIC values of these strains being larger than 5.000 %. The situation of MBCs was similar as the case of MIC values. Confirmed by both MICs and MBCs, it was indicated that the bacteria were slightly sensitive to 1,8-cineole.

Table 1. Antimicrobial activity of 1,8-cineole

Bacterial strain	MIC (%)	MBC (%)
Bacillus subtilis, ATCC 6633	1.250	2.500
Staphylococcus aureus, ATCC 6538	5.000	>5
Staphylococcus epidermidis, ATCC 49134	>5	>5
Escherichia coli, ATCC 11229	5.000	>5
Proteus vulgaris, ATCC 6380	5.000	>5
Pseudomonas aeruginosa, ATCC 9027	>5	>5
Candida albicans, ATCC 10231	0.156	>5
Aspergillus niger, ATCC 16404	5.000	>5

Table 2. The ratios of fluorescence intensity with the treatment of various concentrations of 1,8-cineole

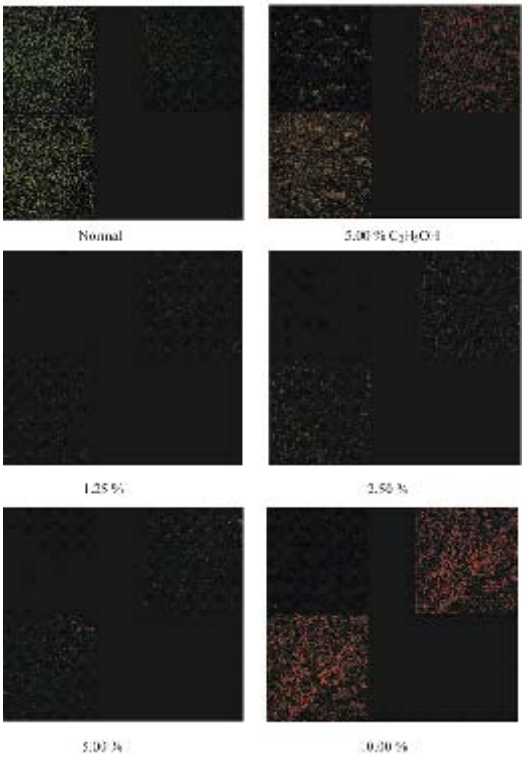
(Alive /Dead)	Negative	5%	1,8-cineole			
	Control	C_2H_5OH	1.25 %	2.50 %	5.00 %	10.00 %
Staphylococcus aureus, ATCC 6538	2.021± 0.113	0.490± 0.072	0.155± 0.004	0.098± 0.064	0.093± 0.037	0.025± 0.006
Staphylococcus epidermidis, ATCC 49134	4.308± 0.287	4.126± 0.172	2.011± 0.133	1.073± 0.096	0.853± 0.141	1.074± 0.033
Escherichia coli, ATCC 11229	40.959± 4.435	65.893± 1.841	2.144± 0.715	0.311± 0.147	0.905± 0.113	0.154± 0.016

Inhibitory effects of essential oil on bacteria biofilm

It has been confirmed that a number of human infections diseases are associated with the corresponding bacteria in nature living in spatially distinct communities, also as bacterial biofilms[2,24,25]. Therefore, consistent efforts have been devoted to new potential antimicrobial therapeutics, such as antimicrobial peptides and essential oils[2, 24, 25]. The latter is produced by almost all living plant organisms and till a matter of debate. As the MICs and MBCs, the Gram-positive bacteria *Staphylococcus aureus* and *Staphylococcus epidermidis*, as well as the Gram-negative bacterium *Escherichia coli* were selected and used to further evaluate bioactivities in the biofilms. Artificial biofilms were prepared and then exposed to the compounds of different concentrations, followed by the observations throughout confocal laser scanning microscopy (CLSM).

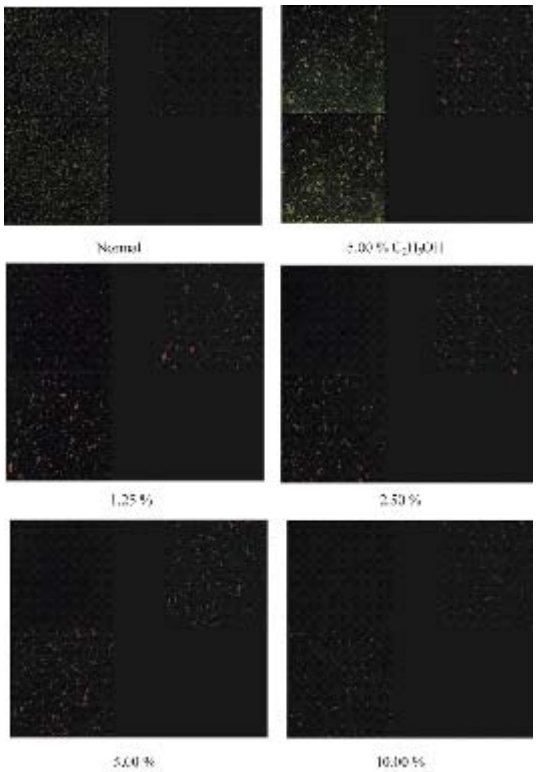
CLSM image analysis demonstrated that 1,8-cineole might inhibit the bacterial biofilms at each concentration in the range of 1.25 -10.00 % (v/v), see Figs. 1-3. Confirming the results from Figs. 1-3, surface rather than other planes bacteria are relatively sensitive to 1,8-cineole, as the density

of dead bacteria in the middle layer is smaller than the surface one, under the same concentrations (Figs. 1-3). There are a large number of dead bacteria at the surfaces of *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Escherichia coli* biofilms with the treatment of 5.00 % concentration of 1,8-cineole, associated with the fluorescence intensity (Alive /Dead) ratios of 0.093 ± 0.037, 0.853 ± 0.141 and 0.905±0.113 respectively (Table 2). With the increasing concentration, the significant inactivation of *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Escherichia coli* was observed, such as the cases of 2.50%, 5.00% and 10.00% concentrations (Table 2). It was indicated that there is linear relationship between bacterial (*Staphylococcus aureus, Staphylococcus epidermidis* and *Escherichia coli*) biofilm inhibition activities and concentration of 1,8-cineole. With the aid of CLSM image analysis, it was found that the proportions of dead bacteria per unit area were distinct at the surfaces of *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Escherichia coli* biofilms treated by various concentration compounds, with the significant alterations of bacterial density (Figs. 1-3). It might mean that 1,8-cineole could prompt the bacteria to die off or live in the planktonic mode, which will help the body's immune system to remove the biofilm survive remained cell (1, 2, 24). Taken together, it is likely that 1,8-cineole has good inhibitory activities against bacterial biofilms. The compound may have a potential application in treatment of biofilm-bacteria. Therefore, further related studies should be based on this point of view.



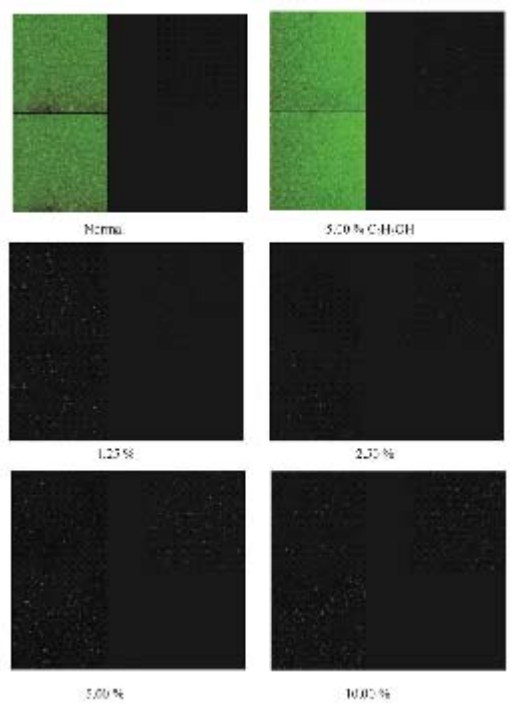
The 5.00 % (v/v) C_2H_5OH was treated as the positive control group. The confocal laser scanning micrographs show the section in the xz plane. Live cells appear green because of fluorescein diacetate; dead cells in red, staining with propidium iodide.

Fig. 1. The *Staphylococcus aureus* biofilms after the treatment of 1,8-cineole



The 5.00 % (v/v) C2H5OH was treated as the positive control group. The confocal laser scanning micrographs show the section in the xz plane. Live cells appear green because of fluorescein diacetate; dead cells in red, staining with propidium iodide.

Fig. 2. The *Staphylococcus epidermidis* biofilms after the treatment of 1,8-cineole



The 5.00 % (v/v) C_2H_5OH was treated as the positive control group. The confocal laser scanning micrographs show the section in the xz plane. Live cells appear green because of fluorescein diacetate; dead cells in red, staining with propidium iodide.

Fig. 3. The *Escherichia coli* biofilms after the treatment of 1,8-cineole

CONCLUSIONS

Due to the nature of 1,8-cineole, it can work as natural antimicrobial agent in pharmaceutical industry. Here, we evaluated its inhibiting activity against several common bacteria and fungi by the MIC and MBC determinations, and then estimated its antimicrobial effectiveness on artificial bacteria biofilms, via the confocal laser scanning.

Through the studies on the antimicrobial activity, it was found that 1,8-cineole has good inhibitory activities against fungus *Candida albicans*, with the lowest MIC (0.156 %) value; whereas not bacteria. CLSM image analysis further revealed that 1,8-cineole could inhibit both the Gram-positive and Gram-negative bacterial biofilms. In addition, there is linear relationship between bacterial biofilm inhibition activities and concentration of 1,8-cineole. It was indicated that 1,8-cineole can contribute to the biofilm surface bacteria died off or lived as planktonic, thereby reducing the density of bacteria within the bacterial biofilm surface per unit area, which will help the body's immune system to remove the biofilm survive remained cell. We hope that our results open the possibility of using 1,8-cineole for the antimicrobial agent against the bacterial biofilms.

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