academicJournals

Vol. 7(24), pp. 3078-3084, 11 June, 2013
DOI: 10.5897/AJMR12.1582
ISSN 1996-0808 ©2013 Academic Journals
http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Antimicrobial effects of essential oil from *Pinus koraiensis* Sieb. et Zucc. needles in the bioflims

Junxing Liu[1,2], Zhiwei Yang[2], Fei Wu[2], Lin Wang[3] and Hongwei Ni[1]*

[1]Institute of Natural Resources, Heilongjiang Academy of Sciences, Harbin 150040, China.
[2]School of Basic Medical Sciences, Jiamusi University, Jiamusi 154007, China.
[3]The First Affiliated Hospital of Jiamusi University, Jiamusi 154003, China.

Accepted 26 April, 2013

This study was designed to explore the *in vitro* antimicrobial activities of the essential oil derived from *Pinus koraiensis* Sieb. et Zucc. needles obtained throughout the steam distillation. The antimicrobial effectiveness was estimated by the minimum inhibition concentration (MIC) and minimum bacterial concentration (MBC) determinations on 8 species of bacteria and fungi, as well as the confocal laser scanning on 3 types of bacteria bioflims. It was found that the essential oil had good inhibitory activities against Gram-positive bacteria and fungi rather than Gram-negative bacteria, especially *C. albicans*, with the lowest MIC (0.780%) and MBC (1.560%) values. The Gram-positive bacteria biofilms were sensitive to the essential oil, whereas not the Gram-negative ones. Besides, there is no linear relationship between inhibition activities and oil concentrations within the bacteria biofilms. Further analysis revealed that the essential oil can prompt the biofilm-surface bacteria to die off or live as plankton, to help the immune system to remove the bacteria. Thus, *P. koraiensis* needle oil could be judged as a kind of potential agent with antimicrobial activities effectively.

Key words: *Pinus koraiensis Sieb. et Zucc.* Needles, essential oil, antimicrobial activities, bacterial biofilms, confocal laser scanning.

INTRODUCTION

Pinus koraiensis Sieb. et Zucc. belongs to the genus *Pinus* and the family of *Pinaceae*. It is a kind of evergreen and valuable conifer, found across northeast China, as well as some areas in Japan, Korea and Russia. In China, it is mainly located in the Changbai Mountains and listed as a national secondary key protected species (Xu and Yan, 2001). The seeds of *P. koraiensis* have been used as a food supplement and traditional herbs medicine for a long time (Hong et al., 2004; Lee et al., 2008). Besides, it was found that the extracts from various parts of *P. koraiensis*, including seeds, cones and barks, exhibit antimicrobial activities and antitumor abilities by the immunity enhancing (Asset et al., 1999; Hong et al., 2004; Lee et al., 2008; Li et al., 2007).

Essential oils, the volatile secondary metabolism of plants, have a wide application in medicine. Currently, the applicability of essential oil has been largely expanded as their antioxidant, antimicrobial, antibiotic, anticarcinogenic and sedative properties, with less toxicity and side effect (Cowan, 1999; Hong et al., 2004; Wang et al., 2011). Indeed, their antimicrobial activities have ever been reported and some of them are already used in topical applications against bacteria and fungi infections (Hammer et al., 1999; Harkenthal et al., 1999; Wang et al., 2011). Recently, the major ingredient of essential oil from the needles of *P. koraiensis*, through hydrodistillation, has been determined as the α- pinene, with 10.49% of overall proportion (Hong et al., 2004). Simultaneously,

*Corresponding author. E-mail: yzws-123@163.com. Tel: +086-0454-6166452. Fax: +086-0454-8618355.

agar diffusion assay revealed that the preparation has mild antimicrobial properties, especially C. albicans (28.9-31.5% vs. nystatin) (Hong et al., 2004). However, little is known about the action spectrum of oil from P. koraiensis needles against microbial cells composing the artificial biofilms.

Steam distillation has been employed in the manufacture of essential oils for many years, related in *P. koraiensis* needle oil (Hong et al., 2004). Using this method, steam is passed through the plant material containing the desired oils. On the industrial scale, the obtained oils are widely use in food preservation and fragrance industries, such as the eucalyptus oil and orange oil. Besides, steam distillation has also been widely used to separate intermediate or final products in petrochemical plants or complex organic compounds, referred to "steam stripping" (Hammer et al., 1999; Harkenthal et al., 1999; Wang et al., 2011).

It has become clear that microbial biofilms are communities of unicellular organisms attached to the surface and the properties of biofilm-grown and planktonic cells are distinct significantly, one of which is an increased resistance to antimicrobial agents (Mah and O'Toole, 2001). Recent work has indicated that there are great alterations on the structure of exopolysaccharides or other aspects (*rpoS*, multiple drug resistance pumps and so on) of biofilm architecture, with a biofilm-specific biocide-resistant phenotype (Mah and O'Toole, 2001). Thus, great efforts have been devoted to the exploration of novel antimicrobial agents directed on the bacterial biofilms, with the aid of artificial microbial biofilms and confocal laser scanning microscopy (Lu et al., 2012; Muranaka et al., 2012; Pamp et al., 2008).

The needle oil of *P. koraiensis* possesses the potential as high-quality edible oil that is beneficial to health and valuable natural antimicrobial agent in cosmetic and pharmaceutical industries. To the best of our knowledge, the effects of *P. koraiensis* needle oil against the microbial biofilms have not been evaluated yet. In this work, the oil will be extracted by the steam distillation and its activities will be evaluated by MIC and MBC determinations. At the same time, the antimicrobial effects of oil against the microbial biofilms will be studied, with the aid of confocal laser scanning. We anticipate that the investigation will be of value in the development of antimicrobial agents against the microbial biofilms.

MATERIALS AND METHODS

Essential oil extraction

The needles of *P. koraiensis* were collected in July from the forest farm of Northeast Forestry University, Harbin, China, and was authenticated by Prof. Shao-Quan Nie from the Key Laboratory of Forest Plant Ecology, Ministry of Education. Voucher specimens were deposited in the herbarium of laboratory and 200 g were subjected to steam distillation with a British-type clevenger apparatus at 100°C for 3 h. Then, the extracts were filtered and concentrated in vacuum at 4°C, yielding the soil in yellow.

Microbial strains and culture conditions

The microorganisms used for testing antimicrobial sensitivity included *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 (Wang et al., 2011; Yu et al., 2004).

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 (NCCLS, 2002). The essential oil was dissolved in sterilized physiological saline solution (0.9% w/v) supplemented with Tween 80 (Sigma) (Wang et al., 2011; Yu et al., 2004) . Serial doubling dilutions of the oils 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 (Wang et al., 2011; Yu et al., 2004). After activation, the MICs and MBCs were determined, with the positive controls of *Streptomycin* and *Amphotericin B* (Tianjin Chemical Reagents Co., Tianjin, China), respectively. The MIC was defined as the lowest concentration of oil at which the microorganism did not show visible growth. The MBCs, defined as the lowest concentration of essential oil at which incubated microorganisms were completely killed, were confirmed by reinoculating on agar plates with 10 µL of each culture medium from the microplates. 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 (Karpanen et al., 2008; Niu and Gilbert, 2004; Nostro et al., 2007). 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 *S. aureus, S. epidermidis* and *E. 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 essential oil of *P. koraiensis* needles, 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 (CLSM)

Image acquisition was performed with an Olympus FV1000 confocal laser scanning microscope (Olympus, Japan) equipped with an argon and a NeHe laser and detectors and filter sets for

Table 1. Antimicrobial activity of essential oil from P. koraiensis needles.

Bacterial strain	MIC (%, v/v)	MBC (%, v/v)
Bacillus subtilis, ATCC 6633	2.500	>5
Staphylococcus aureus, ATCC 6538	5.000	5.000
Staphylococcus epidermidis, ATCC 49134	1.250	2.500
Escherichia coli, ATCC 11229	>5	>5
Proteus vulgaris, ATCC 6380	>5	>5
Pseudomonas aeruginosa, ATCC 9027	>5	>5
Candida albicans, ATCC 10231	0.780	1.560
Aspergillus niger, ATCC 16404	2.500	>5

simultaneous monitoring (excitation, 488 nm). Before observation, the specimens were stained with propidium iodide (PI, sigma) and fluorescein diacetate (FDA, sigma) referred to previously literatures (Gabi et al., 2011; Li et al., 2012; Wu et al., 2009).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.

Statistical analysis

All results were expressed as mean values ± standard deviations (SDs) (n = 3). The significance of difference was calculated by one-way analysis of variance via SPSS (Release 12.1; SPSS Inc., Chicago, IL), and values $p < 0.01$ were considered to be significant.

RESULTS AND DISCUSSION

MICs and MBCs of essential oil

Results of the MIC and MBC studies are presented in Table 1. It was found that the essential oil exhibited significant ($P < 0.01$) antifungal activities, especially *C. albicans* ATCC 10231, with MIC and MBC values of 0.780 and 1.560% respectively. It is consistent with the results of Hong et al. (2004). Besides, the oil exhibited rather high inhibitory effects on *B. subtilis* ATCC 6633, *S. aureus* ATCC 6538 and *S. epidermidis* ATCC 49134. As to the three testing microorganisms, the MICs and MBCs of the oil ranged from 1.250 to 5.000% and 2.500% to more than 5.000%, respectively. Growth inhibition of *E. coli* ATCC 11229, *P. vulgaris* ATCC 6380 and *P. aeruginosa* ATCC 9027 was also observed; while as to the MIC values for these strains were above 5.000%. The MBCs were similar or even higher than the corresponding MIC values. Confirmed by both MICs and MBCs data, it was indicated that the Gram-positive instead of Gram-negative bacteria were sensitive to the oil.

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 bioflims(Davey and O'Toole G, 2000; Hancock and Sahl, 2006; Pamp et al., 2008). Therefore, consistent efforts have been devoted to new potential antimicrobial therapeutics, such as antimicrobial peptides and essential oils (Davey and O'Toole G, 2000; Hancock and Sahl, 2006; Pamp et al., 2008). The latter is produced by almost all living plant organisms and till a matter of debate. As the MICs and MBCs, the essential oil from *P. koraiensis* needles is attracting increasing interest as a novel potential agent. Thus, the Gram-positive bacteria *S. aureus* ATCC 6538 and *S. epidermidis* ATCC 49134, as well as the Gram-negative bacterium *E. coli* ATCC 11229 were selected and used to further evaluate the bioactivities of oil against the artificial biofilms.

CLSM image analysis demonstrated that the essential oil from *P. koraiensis* needles inhibited the Gram-positive bacteria bioflims at each concentration in the range of 1.25 -10.00% (v/v), (Figures 1 and 2). Confirming the results from Figures 1 and 2, surface rather than other planes bacteria are relatively sensitive to the oil, as the density of dead bacteria in the middle layer is smaller than the surface one, under the same concentrations (Figures 1 and 2). There are a large number of dead bacteria at the surfaces of *S. aureus* and *S. epidermidis* bioflims with the treatment of 2.50% essential oil, associated as the fluorescence intensity (Alive /Dead) ratios of 0.116 ± 0.052 and 0.236 ± 0.020, respectively (Table 2). With the increase in the concentration of oil, the significant inactivation of *S. aureus* and *S. epidermidis* was not observed, such as those of 5.00 and 10.00% (Table 2). Reaching 10.00% essential oil, a certain number of bacteria were still survived, with the fluorescence intensity (Alive /Dead) ratios of 0.155 ± 0.022 and 1.884 ± 0.188, respectively. It was indicated that there is no linear relationship between bacterial (*S. aureus* and *S. epidermidis*) biofilm inhibition activities and concentration of oil. 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 *S. aureus* and *S. epidermidis* bioflims treated by various concentration oils, with the significant alterations of

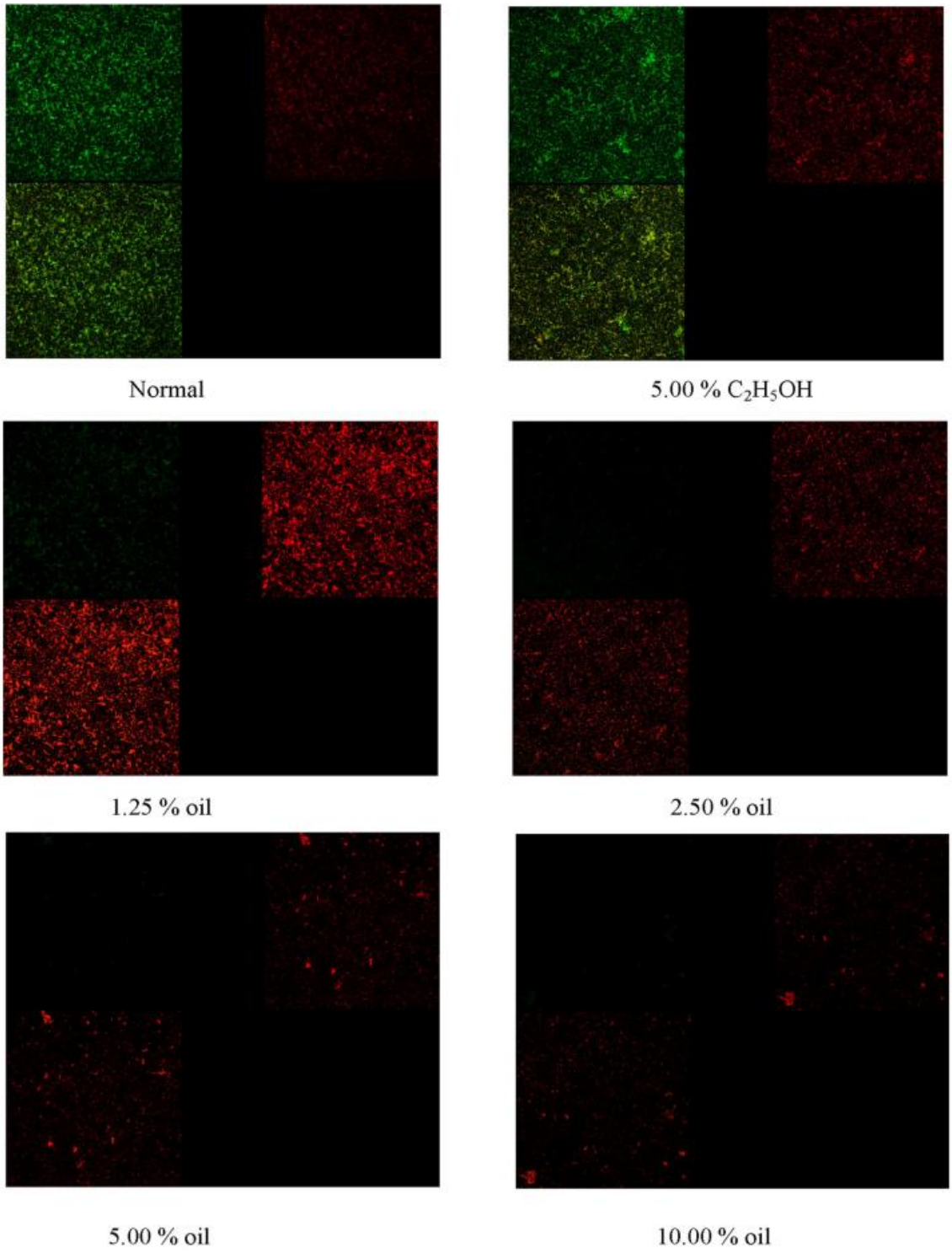

Figure 1. *S. aureus* biofilm after the treatment of essential oil from *P. koraiensis* needles.The 5.00 % (v/v) C_2H_5OH was treated as the positive control group. The confocal laser scanning micrographs show the section in the xz plane. Live cells appear green because of fluorescein diacetate; dead cells in red, staining with propidium iodide.

bacterial density (Figures 1 and 2). It might mean that the essential oil could prompt the Gram-positive bacteria to die off or live in the planktonic mode, which will help the body's immune system to remove the biofilm survive

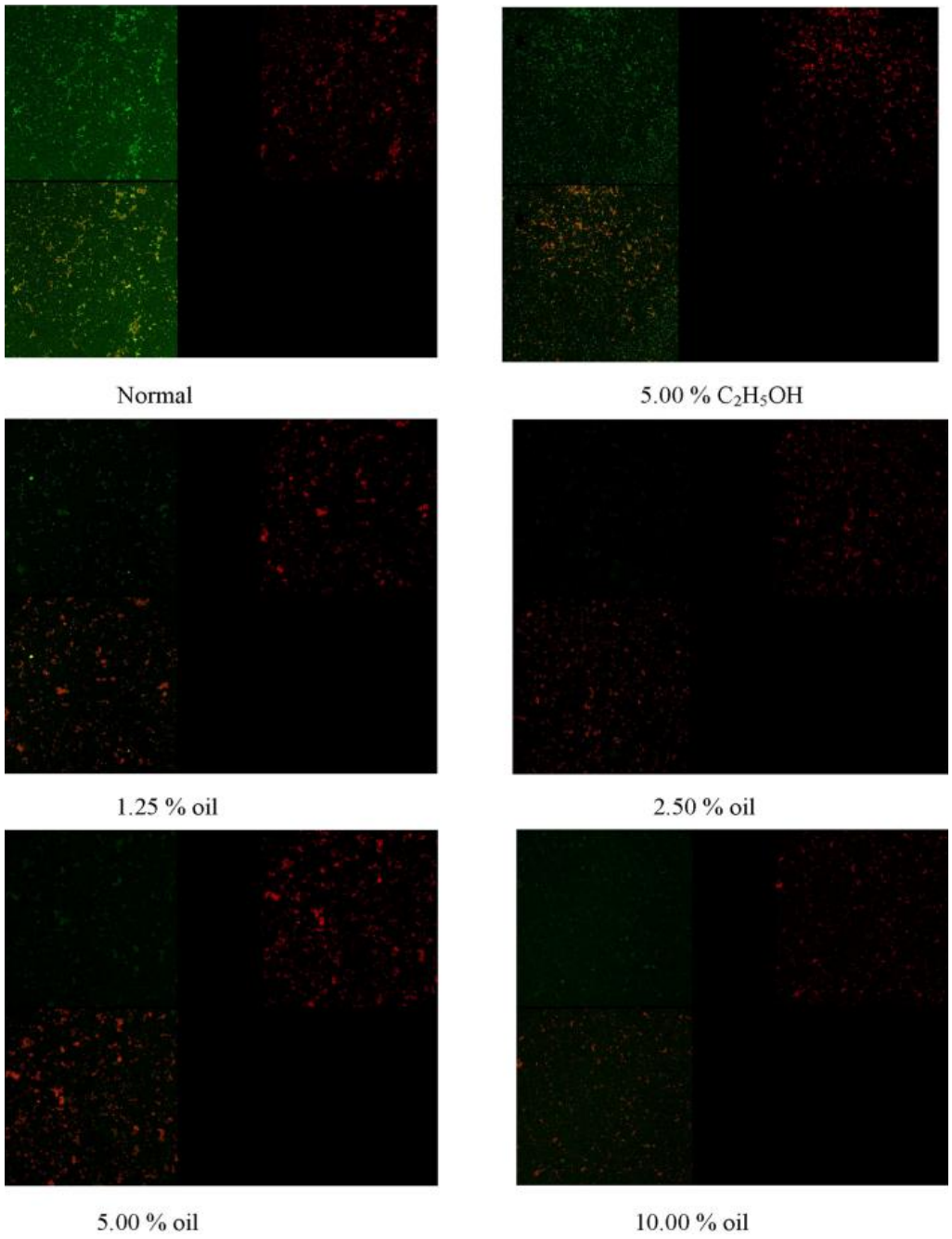

Figure 2. *S. epidermidis* biofilm after the treatment of essential oil from *P. koraiensis* needles. The 5.00 % (v/v) C_2H_5OH was treated as the positive control group. The confocal laser scanning micrographs show the section in the xz plane. Live cells appear green because of fluorescein diacetate; dead cells in red, staining with propidium iodide.

remained cell (Davey and O'Toole G, 2000; Mah and O'Toole, 2001; Pamp et al., 2008). As *E. coli* ATCC 11229, there were no statistically apparent dead bacteria since the treatment of 5.00% essential oil, which was 0.381 ± 0.185 for the fluorescence intensity ratio (Figure 3 and Table 2). The 10.00% essential oil, however, still less inhibited the growth of *E. coli,* with the value of 0.230 ± 0.012. It is consistent with the results of MIC and MBC, and further supported that the Gram-negative bacteria were not sensitive to the essential oil from *P. koraiensis* needles. It is generally comparable to the previous experimental results (Hong et al., 2004), although some disagreements have been detected, which may be caused by many potential reasons, such as the micro-organism type and the microbial cells composing artificial biofilms. As shown in Table 2, 10.00% essential oil can

Table 2. The ratios of fluorescence intensity with the treatment of various concentration of *P. koraiensis* needles' essential oil.

(Alive /Dead)	Negative Control	5% C_2H_5OH	Essential oil			
			1.25 %	2.50%	5.00%	10.00%
Staphylococcus aureus, ATCC 6538	2.037± 0.111	1.428±0.091	0.121±0.003	0.116±0.052	0.037±0.004	0.155±0.022
Staphylococcus epidermidis, ATCC 49134	4.179± 0.540	2.646±1.099	1.088±0.067	0.236±0.020	0.765±0.013	1.884±0.188
Escherichia coli, ATCC 11229	38.536± 2.608	30.162±3.317	4.479±1.011	6.200±0.839	0.381±0.185	0.230±0.012

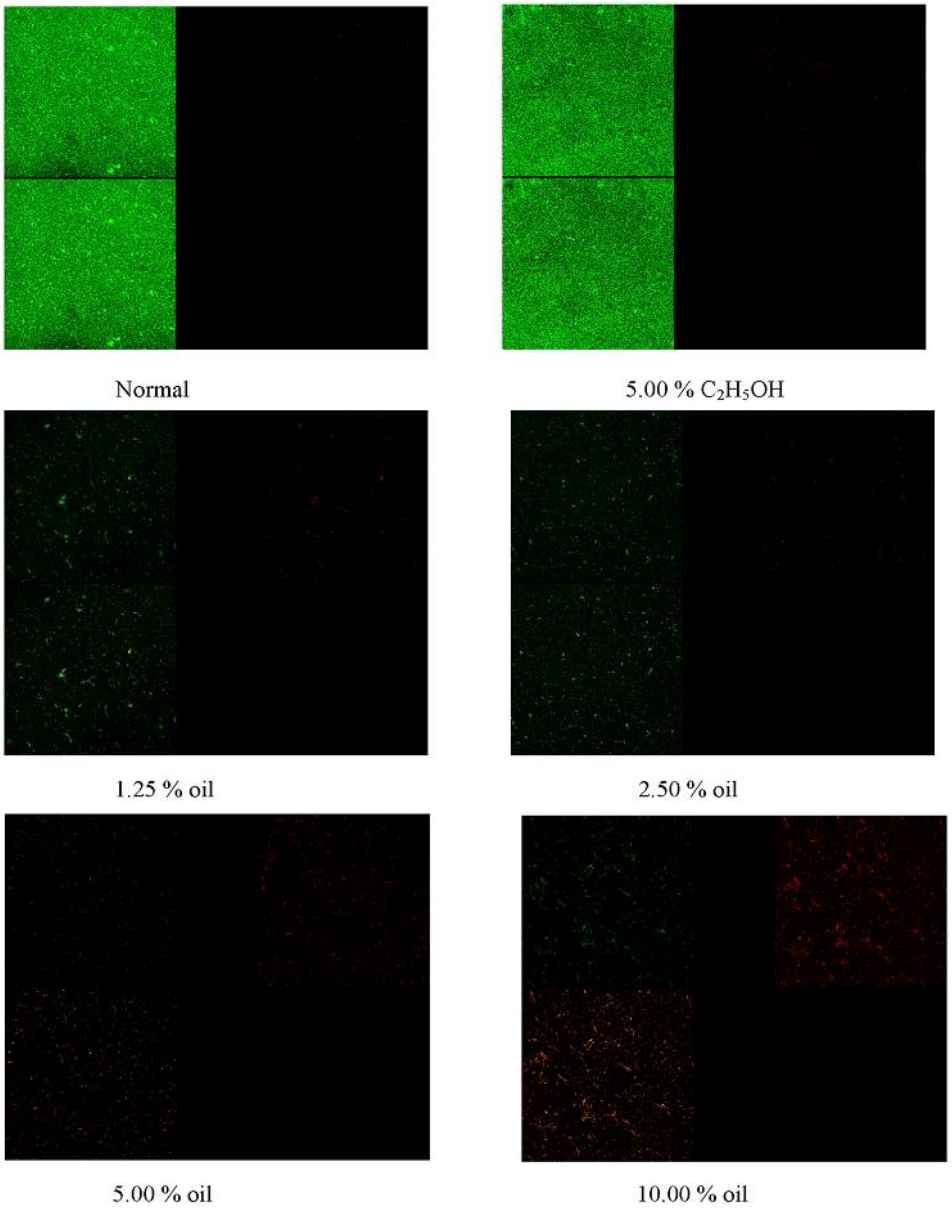

Figure 3. The *E. coli* biofilm after the treatment of essential oil from *P. koraiensis* needles. The 5.00% (v/v) C_2H_5OH was treated as the positive control group. The confocal laser scanning micrographs showed the section in the xz plane. Live cells appear green because of fluorescein diacetate; dead cells in red, staining with propidium iodide.

not completely kill all the three microorganisms tested. We thought that it could be attributed to the limitation by permeation rate and lack concentration of the effective antibacterial ingredient (Mah and O'Toole, 2001).

Taken together, it is likely that essential oil from *P. koraiensis* needles has good inhibitory activities against Gram-positive bacteria bioflims, whereas not for the Gram-negative ones. The oil may have a potential application in the treatment of bioflim-bacteria. Therefore, further related studies should be based on this point of view, and a certain effort are urgently needed to be devoted on its active ingredients in the fields of fine extraction and identification.

Conclusions

Due to the virulence of *Pinus koraiensis* needle oil, it can work as natural antimicrobial agent in pharmaceutical industry or food supplement. But there have been few studies on the activities of the oil. In this study, we evaluated its inhibiting activity against several common bacteria and fungi by MIC and MBC determinations, and then estimated its antimicrobial effectiveness on artificial bacteria bioflims, via the confocal laser scanning.

Through the studies on the antimicrobial activity, it was found that the essential oil has good inhibitory activities against Gram-positive bacteria (*B. subtilis*, *S. aureus* and *S. epidermidis*) and fungi (*C. albicans* and *A. niger* V. Tiegh), especially *C. albicans* presents the lowest MIC (0.780%) and MBC (1.560%) values; whereas not the Gram-negative bacteria (*E. coli*, *P. vulgaris* and *P. aeruginosa*). Further CLSM image analysis revealed that the essential oil could inhibit the Gram-positive bacteria in the biofilm, rather well, but poor inhibitory effects to Gram-negative ones. While, there is no linear relationship between bacteria biofilm inhibition activities and concentration of essential oil. It was indicated that the essential oil 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 the essential oil from the *P. koraiensis* needles for the antibacterial agent against the bacteria biofilm.

ACKNOWLEDGEMENT

We are grateful for the financial supports from the Heilongjiang Province Science and Technology Research Program (GC08A309).

REFERENCES

Asset G, Staels B, Wolff R, Baugé E, Madj Z, Fruchart J-C and Dallongeville J (1999). Effects of Pinus pinaster and Pinus koraiensis seed oil supplementation on lipoprotein metabolism in the rat. Lipids 34: 39-44.

Cowan MM (1999). Plant products as antimicrobial agents. Clin. Microbiol. Rev. 12: 564-582.

Davey ME, O'Toole GA (2000). Microbial biofilms: from ecology to molecular genetics. Microbiol. Mol. Biol. Rev. 64: 847-867.

Gabi M, Hefermehl L, Lukic D, Zahn R, Voros J, Eberli D (2011). Electrical microcurrent to prevent conditioning film and bacterial adhesion to urological stents. Urol. Res. 39: 81-88.

Hammer KA, Carson C, Riley T (1999). Antimicrobial activity of essential oils and other plant extracts. J. Appl. Microbiol. 86: 985-990.

Hancock REW, Sahl H-G (2006). Antimicrobial and host-defense peptides as new anti-infective therapeutic strategies. Nat. Biotech. 24: 1551-1557.

Harkenthal M, Reichling J, Geiss H and Saller R (1999). Comparative study on the in vitro antibacterial activity of Australian tea tree oil, cajuput oil, niaouli oil, manuka oil, kanuka oil, and eucalyptus oil. Pharmazie 54: 460-463.

Hong EJ, Na KJ, Choi IG, Choi KC, Jeung EB (2004). Antibacterial and antifungal effects of essential oils from coniferous trees. Biol. Pharm. Bull. 27: 863-866.

Karpanen TJ, Worthington T, Hendry ER, Conway BR, Lambert PA (2008). Antimicrobial efficacy of chlorhexidine digluconate alone and in combination with eucalyptus oil, tea tree oil and thymol against planktonic and biofilm cultures of Staphylococcus epidermidis. J. Antimicrob. Chemother. 62: 1031-1036.

Lee JH, Yang HY, Lee HS, Hong SK (2008). Chemical composition and antimicrobial activity of essential oil from cones of Pinus koraiensis. J. Microbiol. Biotechnol. 18: 497-502.

Li K, Li Q, Li J, Zhang T, Han Z, Gao D, Zheng F (2007). Antitumor activity of the procyanidins from Pinus koraiensis bark on mice bearing U14 cervical cancer. Yakugaku. zasshi. 127: 1145-1151.

Li Y, Ma Y, Zhang L, Guo F, Ren L, Yang R, Lou H (2012). In vivo inhibitory effect on the biofilm formation of Candida albicans by liverwort derived riccardin D. PLoS One 7: e35543.

Lu X, Weakley AT, Eric Aston D, Rasco BA, Wang S, Konkel ME (2012). Examination of nanoparticle inactivation of Campylobacter jejuni biofilms using infrared and Raman spectroscopies. J. Appl. Microbiol. 113: 952-963.

Mah TF, O'Toole GA (2001). Mechanisms of biofilm resistance to antimicrobial agents. Trends Microbiol. 9: 34-39.

Muranaka LS, Takita MA, Olivato JC, Kishi LT, de Souza AA (2012). Global expression profile on biofilm resistance to antimicrobial compounds in a plant-pathogenic bacterium reveals evidence of persister cells. J. Bacteriol. 194: 4561-4569.

NCCLS (2002). In National Committee for Clinical Laboratory Standards. Reference method for broth dilution antifungal susceptibility testing of yeasts: proposed standard.

Niu C, Gilbert ES (2004). Colorimetric method for identifying plant essential oil components that affect biofilm formation and structure. Appl. Environ. Microbiol. 70: 6951-6956.

Nostro A, Sudano Roccaro A, Bisignano G, Marino A, Cannatelli MA, Pizzimenti FC, Cioni PL, Procopio F, Blanco AR (2007). Effects of oregano, carvacrol and thymol on Staphylococcus aureus and Staphylococcus epidermidis biofilms. J. Med. Microbiol. 56: 519-523.

Pamp SJ, Gjermansen M, Johansen HK, Tolker-Nielsen T (2008). Tolerance to the antimicrobial peptide colistin in Pseudomonas aeruginosa biofilms is linked to metabolically active cells, and depends on the pmr and mexAB-oprM genes. Mol. Microbiol. 68: 223-240.

Wang L, Yang Z, Wang S, Liu J (2011). Antioxidant and antibacterial activities of Camptotheca acuminate D. seed oil. Afr. J. Microbiol. Res. 5: 5854-5862.

Wu XZ, Cheng AX, Sun LM, Sun SJ, Lou HX (2009). Plagiochin E, an antifungal bis(bibenzyl), exerts its antifungal activity through mitochondrial dysfunction-induced reactive oxygen species accumulation in Candida albicans. Biochim. Biophys. Acta. 1790: 770-777.

Xu D, Yan H (2001). A study of the impacts of climate change on the geographic distribution of Pinus koraiensis in China. Environ. Int. 27: 201-205.

Yu JQ, Lei JC, Yu HD, Cai X, Zou GL (2004). Chemical composition and antimicrobial activity of the essential oil of *Scutellaria barbata*. Phytochemistry 65: 881-884.