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Antioxidant and Antimicrobial Activities of Fermentation and Ethanol Extracts of Pine Needles (*Pinus densiflora*)

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Abstract The antioxidant and antimicrobial activity of the fermentation extract (PFE) and the 50 and 80% ethanol extracts (PE 50, PE 80) of *Pinus densiflora* pine needles were evaluated. Electron donating ability, superoxide dismutase (SOD) ability, and antimicrobial activity were observed in PFE; those abilities differed in PE 80 and PE 50, depending on the ethanol concentration used for the extraction. PFE had the highest electron donating ability with a value of 92.20%, while PE 80 and PE 50 had values of 74.66 and 53.47%, respectively. For SOD activity, PE 80 exhibited a slightly higher value of 31.11% compared to that of PFE and PE 50, which were 29.65 and 25.43%, respectively. PFE, PE 50, and PE 80 were all found to inhibit bacteria, and the effectiveness of this inhibition was strongly related to the type of extracts used. PFE showed good antimicrobial effects for all of the tested Gram-positive strains and for most of the tested Gram-negative strains. These results suggest that PFE has superior functionality compared to the ethanol extracts (PE 80, PE 50), in terms of antioxidant and antimicrobial activity. On the basis of these results, pine needle fermentation extracts can be used for industrial applications as a functional material.

Keywords: *Pinus densiflora,* fermentation extract, antioxidant activity, antimicrobial activity

Introduction

In recent years, much interest has focused on oxygen-centered radicals, such as the superoxide and hydroxyl radicals. A wide variety of oxygen-centered free radicals and other reactive oxygen species (ROS) are often formed as unwanted by-products in the human body and in food systems. Their formation also increases in human disease states, sometimes contributing significantly to the severity of the disease. ROS-induced damage occurs constantly in the human body and must be repaired. Antioxidant defenses protect the body against oxidative damages, but they are not 100% efficient; as a result, free-radical damage must be constantly repaired. On the other hand, the food matrix cannot repair itself, and so excessive oxidative damage, especially to lipids, must be prevented by the addition of antioxidants (1, 2).

The pine needle (*Pinus densiflora* Siebold et Zucc.) is widely distributed throughout Korea, Japan, northeastern China (eastern Jilin), and the extreme southeast of Russia (southern Primorsky Krai). The leaves are needle-like, 8-12 cm long, and clustered with two per fascicle. The cones are 4-7 cm long. This tree typically grows to 15.0 m high by 7.0 m wide and prefers sandy loam soil with a pH ranging from acidic to neutral and full sun with little to moderate amounts of moisture (3). While sometimes referred as a miracle medicinal plant, it has long been used as a folk remedy and health food to treat liver diseases, gastrointestinal diseases, nervous system diseases, circulatory diseases, and skin problems (4). Various parts of the pine tree, including the pine needles, cones, cortices, and pollen have been widely used as folk medicine or as food to promote health (5).

One study reported that dried pine needle powder and ethanol extracts of pine needles decreased total lipids and liver cholesterol concentrations and were effective in reducing body weight gain in rats (6). Supplementing rats with pine needle powder also improved liver function (7). In rats fed with a high cholesterol diet, pine needle powder was also shown to reduce total serum cholesterol levels and liver thiobarbituric acid-reactive substance (TBARS) (8). Ethanol extracts of pine needles have also been shown to act synergistically with anti-tumor substances to enhance the anti-tumor effects (3, 9). Furthermore, it has been shown that the ethanol extracts of pine needles suppress mutagenicity (10, 11) and exhibit antibiotic properties (12, 13). Also ameliorative effect of pine needle oil on liver protection and lipid metabolism of alcohol fed rats was reported. The pine needle oil treatment significantly reduced the levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), and triglyceride (TG) compared to the control rats (14).

Although artificial food preservatives are generally used to inhibit the growth of food-spoilage microorganisms, antimicrobial substances that inhibit microbial growth exist in various plants (15, 16). Accordingly, a variety of research has been conducted to investigate the antimicrobial activities in plants and their application to food preservation (17-19). Within the many plants used as spices or flavoring materials, thymol (20), the essential oils of thyme and oregano; cinnamic aldehyde (19), the extract of cinnamon; eugenol (21) from cloves; and vanillin, found in vanilla beans, are all known as principal antimicrobial components. The flavonols, proanthocyanins, and anthocyanins present in the flowers of higher plants (22) are classified as nontoxic antimicrobial substances and are widely used as food colorants. Marwan and Nagel (23) reported that the antimicrobial activity of cranberries against *Saccharomyces*

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boyancis could be attributed to flavonols and proanthocyanins.

Recently, there has been a great deal of interest in the applications of plant-based extracts, specifically that of pine needles, to both the food and medicinal industries (24). For example, Lee *et al.* (25) reported the quality of vaporized liquid from water-boiled pine needles as it relates to drinking and aroma characteristics, and Jeon *et al.* (26) evaluated the anti-obesity effects of pine needle in rats and overweight people. In addition, there is a trend to limit the use of artificial preservatives, which prevent food deterioration due to their stability. Nevertheless, very little information currently exists concerning the nutritional value, functional effects, and systematic evaluation of pine needle. Therefore, it is important to investigate these natural antimicrobial materials and their applications to food, in addition to their reaction mechanisms. The objective of this study was to investigate the antioxidant and antimicrobial activities of the fermentation extract (PFE), the 50% ethanol extract (PE 50), and the 80% ethanol extract (PE 80) of pine needles.

Materials and Methods

Pine needle fermentation extract Pine needles were obtained from pine trees (*Pinus densiflora* Siebold et Zucc.) at Chungbuk in October 2001. Samples were then immediately washed with water, air dried, and cut into pieces prior to further use. Pine needles were mixed with water and sugar (1:1:1.5, w/w/w) and fermented naturally for 70 days at 20°C. After separating the supernatant, the extraction was carried out at 100°C for 6 hr by adding pine needles (1:0.5, w/w) to the fermentation remnants and then adding water to the mixture (1:3, w/w). The soluble-solids content of the extracted material was adjusted to 30 °Bx after concentration at 40°C. A second fermentation was then carried out with a mixture of the supernatant from the first fermentation and the concentrated sample with a ratio of 1:1.5 for 30 days at 20°C (27). The fermented extract was concentrated using a rotary vacuum evaporator (Model-NE; Rikakikai Co. Ltd., Tokyo, Japan) at 10 mmHg and then freeze-dried at -60°C using Freeze Dryer (Bondiro, Ilsin Eng. Co., Korea).

Pine needle ethanol extracts Ethanol-extracted samples were prepared by adding 1.5 L ethanol (50 and 80%) to 250 g of pine needles and soaking for 10 days at room temperature; the samples were then centrifuged for 10 min at 955×g (HRT-620v; Hanil Industrial Co., Korea). The extraction was subsequently repeated with the pelleted precipitates. The supernatants from each extraction were collected, concentrated, and freeze-dried as above.

DPPH free radical scavenging activity In order to measure antioxidant activity, 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging assay was carried out according to the procedure descried by Blois (28). Sample solutions were prepared by mixing 10 mg of each freeze-dried sample (PFE, PE 80, PE 50) with 1 mL of ethanol. After mixing 0.1 mL of each sample with 0.9 mL of 0.041 mM DPPH in ethanol for 10 min, the absorbance of the sample was measured at 516 nm with a spectrophotometer (Unikon 860; Kontron Instruments, USA). The control solution was prepared using equal amounts of ethanol alone instead of the sample extract solutions. The free radical scavenging activity was obtained from following equation:

$$\text{Radical scavenging activity (\%)} = \{1 - (OD_{sample}/OD_{control}) \times 100\}$$

Superoxide dismutase (SOD) activity According to the method of Marklund and Marklund *et al.* (29), the SOD activity of each sample was measured by the pyrogallol-mediated autoxidation of superoxide anions in the reaction system. To determine the SOD activity of a sample, 3 mL of Tris-HCl buffer (pH 8.5) was added to 0.2 mL of extract in ethanol (10 mg/mL); pyrogallol solution (7.2 mM) was then added to a final concentration of 0.2 mM. After the sample was incubated for 10 min at 25°C, 1 mL of 1 N HCl was added, and the absorbance at 420 nm was determined.

$$\text{SOD activity (\%)} = \{1 - (OD_{sample}/OD_{control}) \times 100\}$$

Antimicrobial activity measurement ***Microorganisms***: Three species of Gram-positive bacteria, three species of Gram-negative bacteria, two species of yeast and two species of mold were used for antimicrobial activity studies. The antimicrobial screening was performed using the media and conditions listed in Table 1 for each microorganism. All microorganisms were provided by the Korean Culture Center of Microorganisms, Korea.

Inhibitory zone formation activity: The antimicrobial activity of pine needle extracts was tested using the paper disc method (Ø 6 mm, Whatman) on appropriate medium as listed in Table 1. Strains were successfully grown in nutrient broth at their optimum temperature; bacteria and yeast for were grown for 24 hr and mold for 48 hr, then used as inocula. Sterilized media (15 mL) were made using the pour-plate method at 45°C, and 0.1 mL of the appropriate strain was added to the petri dishes (Ø 9 cm). The petri dishes were left at room temperature for 1 hr to allow the agar surface to dry. Sterile discs were impregnated with 10 mg/disc pine needle extracts, placed on the culture medium and incubated for 16 hr for bacteria and yeast and 48 hr for mold in an incubator (HB-201S; Hanbaek Science Co., Korea). The results were recorded by measuring the clear zone (mm) of growth inhibition surrounding the disc, which indicates the extent of antimicrobial activity (30).

Growth inhibiting activity: 0.1 mL of pine needle extracts (10 mg/mL in ethanol) were added to the nutrient broth (10 mL), which were then inoculated with the microbial culture (0.1 mL) and incubated in a shaking incubator (HB-201SL, Hanbaek Science Co.) at 150 rpm at optimum temperature. In the presence of different extracts, microbial growth was examined for 48 hr by monitoring the optical density at 620 nm at 12-hr intervals. In the control samples, only agar and microbial suspension were used (31).

Statistical analysis The statistical analysis was completed by the SAS Statistical Analysis System for Windows v8.1

Table 1. Microbial strains, media, and optimum temperature used for antimicrobial experiments

	Strains	Media	Optimum temp.
Gram-positive bacteria	*Bacillus cereus* KCCM 11204	NA[1)]	30±1°C
	Staphylococcus aureus KCCM 12214	TSA[2)]	37±1°C
	Micrococcus luteus KCCM 11211	NA	26±1°C
Gram-negative bacteria	*Salmonella typhimurium* KCCM 11862	NA	27±1°C
	Vibrio parahaemolyiticus KCCM 11965	NA	37±1°C
	Pseudomonas aeruginose KCCM 11328	NA	37±1°C
Yeast	*Saccharomyces cerevisiae* KCCM 11201	YM[3)]	26±1°C
	Candida tropicalis KCCM 50075	YM	26±1°C
Mold	*Aspergillus niger* KCCM 11239	MEA[4)]	28±1°C
	Penicillium chrysogenum KCCM 11609	PDA[5)]	28±1°C

[1)]Nutrient agar, [2)]Tryptic soy agar, [3)]Yeast malt extract agar, [4)]Malt extract agar, [5)]Potato dextrose agar.

(SAS Institute, Inc., Cary, NC, USA). All measurements were done in triplicate. The means were compared with Duncan's multiple range test at $p<0.05$.

Results and Discussion

DPPH free radical scavenging activity To evaluate the antioxidant activity of pine needles, DPPH radical scavenging activities were investigated. Measurement of radical scavenging activity via discoloration of DPPH radical scavenging assay has been widely used due to its stability, simplicity, and reproducibility. DPPH is a (stable) free radical that accepts an electron to become a stable molecule. In its free radical form, it has deep violet color with a strong absorption band at 516 nm; it becomes pale yellow when reacted with an antioxidant. Free radical scavenging capacities of PFE, PE 50, and PE 80 of pine needle are shown in Fig. 1. All samples demonstrated some level of free radical scavenging activity. The activity of PFE in DPPH radical scavenging was significantly higher than that of PE 50 and PE 80. PFE demonstrated the highest level of activity (92.2%), while PE 80 had an intermediate activity with 74.66%, and PE 50 exhibited the lowest activity with 53.47%. Kang *et al.* (32) reported that the hot water extract of pine needle and the 70% acetone extract contained 80.9 and 82.6% DPPH radical scavenging activity, respectively. Also, Kim *et al.* (33) reported that the electron donating ability of pine needle extracts differed depending on the extract concentration and were higher in ethanol extracts (84%) than in hot water extracts (55%). Furthermore, Kim *et al.* (34) reported that hot-air dried pine needle powder contained 81% activity, commercial pine needle powder showed 78% activity, and fresh pine needle powder exhibited 69% activity. These previously reported values indicate that PFE has the highest DPPH radical scavenging activity.

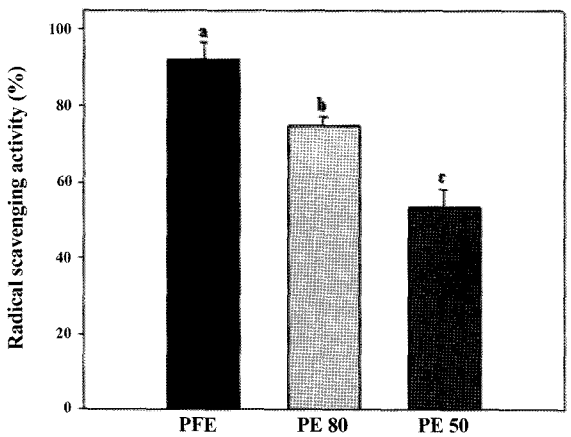

Fig. 1. Radical scavenging activity of pine needle fermentation and ethanol extracts. PFE, pine needle fermentation extract; PE 80, pine needle 80% ethanol extract; PE 50, pine needle 50% ethanol extract.

The radical-scavenging activities of antioxidants on the DPPH radical are thought to be due to their ability to donate hydrogen. In general, polyphenol concentrations are positively correlated with antioxidant activity (35). A nearly linear correlation between DPPH radical scavenging activity and concentrations of polyphenolic compounds in various vegetable and fruits has been reported (36, 37). These reports indicated that the radical scavenging capacity of extracts might be mostly affected by the presence and position of phenolic hydroxyl groups (38). DPPH radical scavenging ability of extracts from pine needles has also been related to various amounts of antioxidants. Kim *et al.* (39) successfully separated polyphenols from pine needle and Chung *et al.* (5) reported the soluble tannin content of pine needles, which increased with maturity. In addition, Boo *et al.* (40) isolated 4-hydroxy-5-methyl-3[2H]-furanone from pine needles and showed that it has antioxidant properties and exhibited inhibitory effects on the autoxidation of 3,4-dihydroxyphenylalanine and linolenic acid.

SOD activity ROS including both radicals, such as superoxide anion radical and hydroxyl radical, and non-radicals, such as singlet oxygen and hydrogen peroxide,

are various forms of activated oxygen (41). They are normally generated as by-products of metabolism. They cause oxidative damage to lipid, proteins, and DNA, which can lead to cell death. Free radical scavengers, including polyphenols, flavonoids, carotenoids, and dietary vitamins, play an important role in preventing oxidative damages (42). For example, free radicals formed during various biological reactions can accelerate cellular injury and the aging process. Antioxidants have been identified as free radical scavengers that protect the human body from free radicals, thus retarding the progress of aging and chronic diseases (43, 44).

Lipid peroxidation refers to the oxidative degradation of lipids. Lipid hydroperoxides are non-radical intermediates derived from unsaturated fatty acids, phospholipids, glycolipids, cholesterol esters, and cholesterol itself. Their formation occurs in both enzymatic and non-enzymatic reactions involving ROS, which are responsible for toxic effects in the body via damage to various tissues. Lipid peroxide (LPO) has acute toxicity and can be produced by oxygen radicals like the hydroxyl radical or nitrite oxide. LPO can be detoxified by scavenger enzymes such as SOD. It is therefore important to measure SOD activity of pine needle extracts as another indicator of antioxidant activity. PFE exhibited 29.65% SOD activity, while PE 80 had the highest SOD activity with 31.11%, and PE 50 contained 25.43% SOD activity. Kim and Kim (6) reported that pine needle powder and 95% ethanol extract of pine needle exhibit increased liver SOD activity with values of 13.62 and 11.46%, respectively. In addition, Kim *et al.* (34) reported that the SOD-like activity of hot-air dried pine needle powder (HPNP) contained 44.30% SOD activity, while commercial pine needle powder (CPNP) and fresh pine needle (FPN) had 18.14 and 12.80% activity, respectively. Kang *et al.* (7) also reported that the 6% acetone extract of pine needles exhibited significantly higher SOD activity than control. These values indicate that pine needle extracts contain SOD activity that could reduce the damage of free radicals.

Inhibitory zone formation activity PFE, PE 50, and PE 80 exhibited different inhibition levels against the examined microorganisms (Table 2). PFE shows marked -inhibition activity against the Gram-positive bacteria, *Bacillus cereus* and *Staphylococcus aureus,* compared to the ethanol extracts. On the other hand, all three extracts exhibited strong inhibition activity against another Gram-positive bacterium, *Micrococcus Luteus*. When testing the Gram-negative bacteria, *Pseudomonas aeruginose* was found to be the most susceptible to PFE, whereas *Vibrio parahaemolyiticus* was not affected by any of the pine needle extracts. Of the three Gram-negative bacteria tested, PE 50 and PE 80 were only able to inhibit *P. aeruginose.* Antimicrobial activity is often related to the presence of phenolic compounds. Two of the major constituents of pine needles are cinnamic acid and benzoic acid, which are believed to contribute to the strong antimicrobial effects (45, 46). The mechanism of the antimicrobial action of essential oils has been well documented. Inhibition is caused by sensitization of the phospholipid bilayer by the phenolic components, resulting in an increase in permeability and leakage of vital intracellular constituents. PFE showed a broad and remarkable antimicrobial spectrum against both Gram-positive and Gram-negative bacteria, whereas PE 80 and PE 50 exhibited limited activities. With PFE, the greatest activity was observed toward *P. aeruginose*, followed by *M. luteus* and *S. aureus*. It is also interesting to note that the inhibition zone formation activity of PE 80 and PE 50 against *M. luteus*, 16 and 14 mm, respectively, was greater than previously reported values (8-12 mm) for

Table 2. Antimicrobial activity of *Pinus densiflora* extracts[1)]

Microorganisms	Strains	Diameter of zone of inhibition (mm)		
		PFE[2)]	PE 80[3)]	PE 50[4)]
Gram-positive bacteria				
Bacillus cereus	KCCM 11204	10.0±0.0	6.9±0.1	-[5)]
Staphylococcus aureus	KCCM 12214	11.0±0.0	6.8±0.2	-
Micrococcus luteus	KCCM 11211	16.2±0.2	16.1±0.1	14.4±0.1
Gram-negative bacteria				
Salmonella typhimurium	KCCM 11862	10.3±0.5	-	-
Vibrio parahaemolyiticus	KCCM 11965	-	-	-
Pseudomonas aeruginose	KCCM 11328	19.0±0.4	12.0±0.2	12.0±0.1
Yeast				
Saccharomyces cerevisiae	KCCM 11201	-		
Candida tropicalis	KCCM 50075	8.1±0.2		
Mold				
Aspergillus niger	KCCM 11239	-	-	-
Penicillium chrysogenum	KCCM 11609	-	-	-

[1)]Results are mean±SD values of three replicates, 10 mg/disc.
[2)]Fermentation extract, [3)]80% ethanol extract, [4)]50% ethanol extract.
[5)]- Indicates no sensitivity.

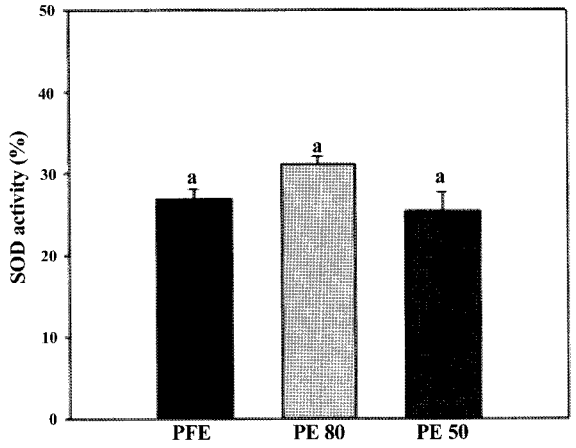

Fig. 2. SOD activity of pine needle fermentation and ethanol extracts. PFE, pine needle fermentation extract; PE 80, pine needle 80% ethanol extract; PE 50, pine needle 50% ethanol extract.

honey bee propolis extract (47). Also, PFE antimicrobial activity was similar to that of other pine needle extracts (12) and natural antimicrobial plant extracts toward food-spoilage microorganisms (48). Although the paper disc assay is a practical approach for studying potential anti-bacterial compounds, using the size of inhibition zone to indicate relative antibacterial activity of pine needle extracts is not adequate. The zone of inhibition may be affected by the extract's solubility and the rate of diffusion in the agar medium or its volatilization; this could, in turn, affect the results.

Antimicrobial activity of pine needle extracts Three extracts of pine needle were added to bacterial, yeast and mold cultures, and growth inhibition was examined at 12-hr intervals during a 48-hr incubation period by monitoring optical density at 620 nm; the results are shown in Fig. 3-6. *M. luteus* and *P. aeruginose* were found to be the most susceptible to pine tree extracts, whereas *V. parahaemolyiticus, Saccharomyces cerevisiae, Aspergillus niger,* and *Penicillium chrysogenum* were the least susceptible. PFE showed strong inhibitory effect against all Gram-positive bacteria, while the inhibitory effect of the ethanol extracts increased with increasing ethanol concentration used for extraction. The sensitivity of *S. aureus* to PFE and PE 80 was close to that of *B. cereus.* The growth of *M. luteus* slowed down with increased ethanol concentration used for extraction, and noticeable suppression was observed with PFE compared to control. However, while the inhibitory effect of PFE, PE 80, and PE 50 on *M. Luteus* was similar and strong, PE 50 exhibited no growth inhibition of *B. cereus* and *S. aureus.*

Of the Gram-negative bacteria, *P. aeruginose* was the most susceptible to PFE, whereas *V. parahaemolyiticus* was more resistant than any other strains, exhibiting no inhibition. The growth of *Salmonella typhimurium* was not effectively inhibited by ethanol extracts of pine needle, although it was markedly inhibited by PFE. Similarly, *P. aeruginose* was less inhibited by the ethanol extracts, PE 80 and PE 50, but PFE exhibited marked inhibition. In

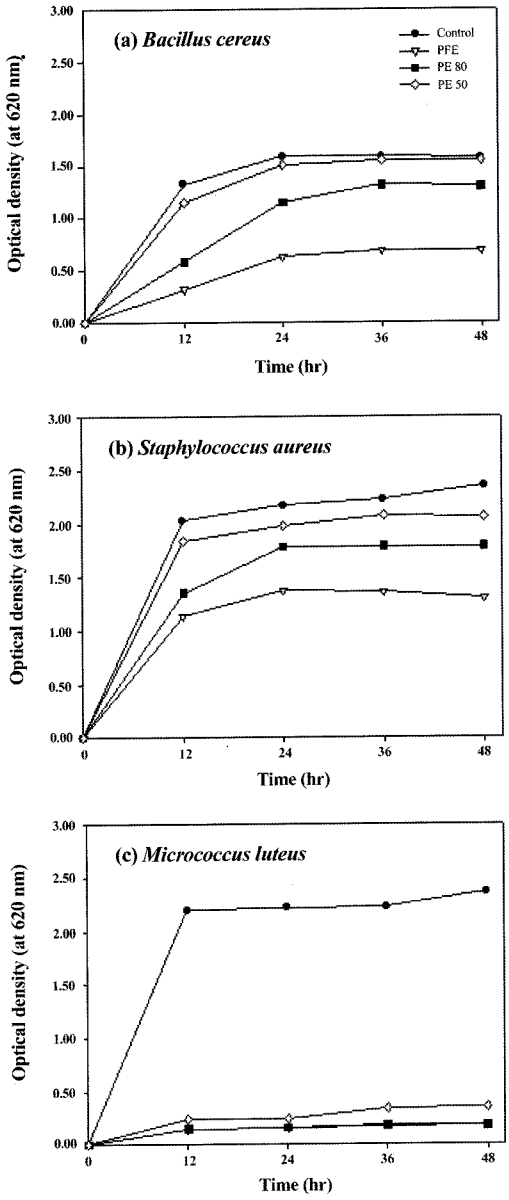

Fig. 3. Growth inhibition activity of pine needle fermentation and ethanol extracts against Gram-positive bacteria. PFE, pine needle fermentation extract; PE 80, pine needle 80% ethanol extract; PE 50, pine needle 50% ethanol extract.

terms of the antifungal activity of pine needle extracts, none of the extracts exhibited any inhibitory activity against yeast and mold, except PFE, which displayed an inhibitory effect against *Candida tropicalis.*

Each bacterial strain responded differently to the various pine needle extracts. Thus, the extracts may exhibit different modes of action against the bacterial strains. These include: interference with the phospholipid bilayer of the bacterial cell membrane, causing increased permeability and loss of cellular constituents; impairment of a variety of enzyme systems; and destruction or inactivation of genetic material. Essential oil constituents are, in general, more effective inhibitors of fungi than of bacteria, and are also believed to be more active against

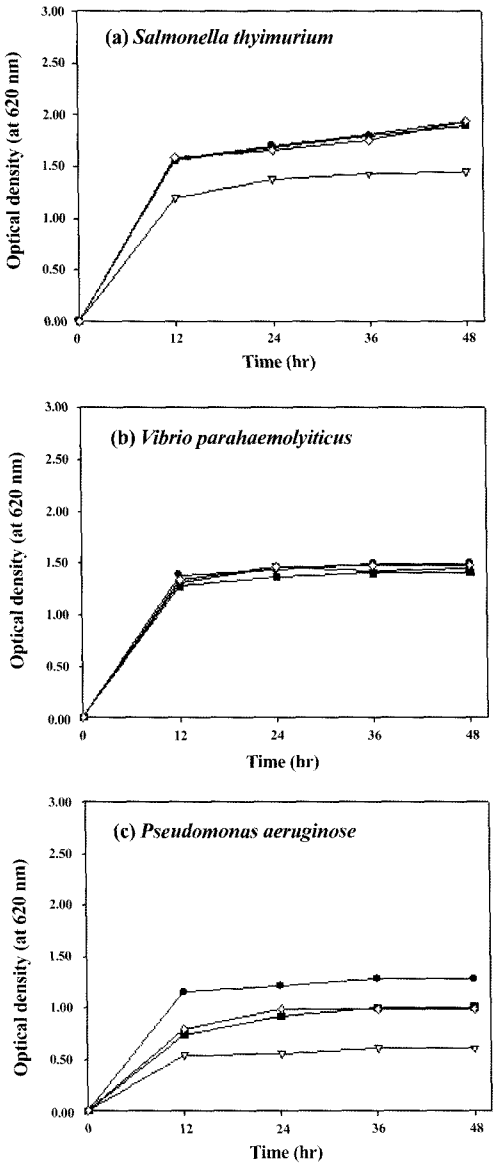

Fig. 4. Growth inhibition activity of pine needle fermentation and ethanol extracts against Gram-negative bacteria. PFE, pine needle fermentation extract; PE 80, pine needle 80% ethanol extract; PE 50, pine needle 50% ethanol extract.

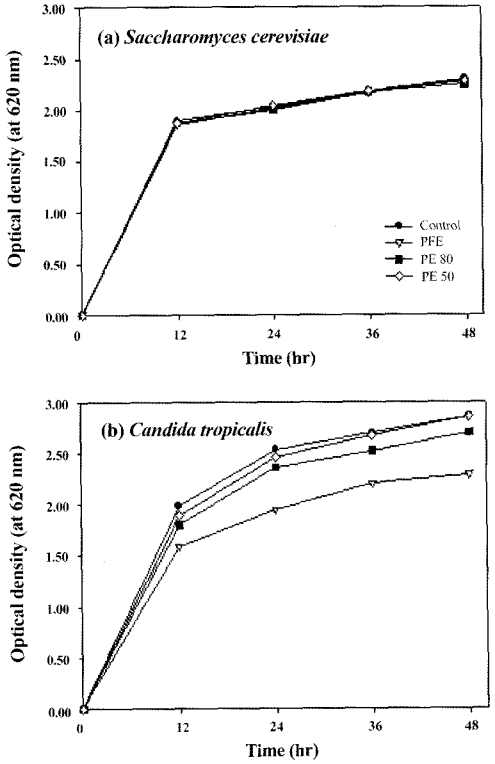

Fig. 5. Growth inhibition activity of pine needle fermentation and ethanol extracts against yeast. PFE, pine needle fermentation extract; PE 80, pine needle 80% ethanol extract; PE 50, pine needle 50% ethanol extract.

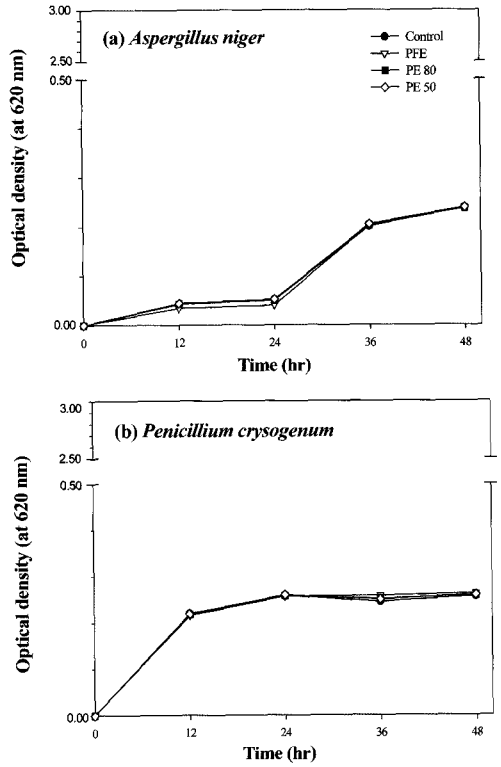

Fig. 6. Growth inhibition activity of pine needle fermentation and ethanol extracts against molds. PFE, pine needle fermentation extract; PE 80, pine needle 80% ethanol extract; PE 50, pine needle 50% ethanol extract.

Gram-positive than Gram-negative bacteria (49). The study of Kim *et al.* (49) showed *Vibrio vulnificus* (Gram-negative) was the most sensitive to essential oil components, while *Listeria monocytogenes* (Gram-positive) was the most resistant. In our studies, however, there was no discernible trend reflected in the type of bacteria strains inhibited; both Gram-negative and Gram-positive organisms were affected. In fact, in this study, *P. aeruginose* (Gram-negative) was the most sensitive to pine needle extracts.

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