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Evaluation of antimicrobial activity and total phenolic content of three *Pinus* species

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Abstract

This study compared the antimicrobial activity and total phenolic content of three *Pinus* plants (*Pinus densiflora*, *P. thunbergii*, *P. rigida*) for the first time. The antimicrobial activity of the water fraction of methanol extract of fresh leaves was stronger than that of fallen leaves at any concentrations. The water fraction of crude methanol extract from fresh leaves of *P. thunbergii* showed a higher growth inhibitory activity against gram-positive and gram-negative bacteria than that of *P. densiflora* and *P. rigida*. The results from the disc diffusion method followed by measurements of minimal inhibition concentration (MIC) indicate that *Bacillus subtilis* was the most sensitive microorganism with the lowest MIC value. The highest total phenolic content was found in fresh leaves of *P. rigida* and *P. thunbergii*. The assay showed that the fresh leaves of the three *Pinus* plants contained higher total phenolic content than fallen leaves of the three plants. The antimicrobial activity was related with the total phenolic content.

Key words: plant natural compound, antimicrobial activity, total phenolic content, *Pinus densiflora*, *Pinus thunbergii*, *Pinus rigida*

INTRODUCTION

The antimicrobial activity of plant natural compounds has been reviewed a number of times. Different aspects such as phytochemical diversity, involvement in mechanisms of resistances and constitutive have been extensively analyzed. The phytochemical diversity of antimicrobial compounds include terpenoids, saponins, phenolics and phenylpropanoids (Bonanomi et al., 2009). For a long time plants have played a very important role for human life. Nowadays, the use of plants as a way of treatment is still very important for human beings (Kultur, 2007). Many plants also play an important role as aromatic herbs and spices, and they have been found to have antimicrobial activity (Yang et al., 1995) and antioxidant activity (Rim et al., 2000). It is a Korean custom to steam rice cake with *Pinus* plant leaves to preserve for a long time. Food-borne illnesses continue to be a serious threat to public health all over the world (Yossa et al., 2010). Food borne diseases are still a major concern for consumers, food industry and food safety authorities (Al-Zoreky, 2009). Today there is consumer's demand for foods that are minimally processed and free from synthetic chemical preservatives (Weerakkody et al., 2010).

Pinus densiflora, Pinus thunbergii and *Pinus rigida* belong to the Pinaceae family. These plants are evergreen

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needle-leafed tree and sweet-scented. Pine (*P. densiflora*) is a representative coniferous tree and indigenous to Asia. The constituents of essential oil of pine are α-pinene, β-pinene, camphene, phellandrene, limonene, borneol, and bornyl acetate (Im, 1998). It has been shown that volatile chemicals of *P. densiflora* have growth-inhibiting effects on human intestinal bacteria (Jeon et al., 2001) and antimicrobial effects on lactic acid bacteria (Lim et al., 2001). Black pine (*P. thunbergii*) is distributed along Korean, Chinese, and Japanese shoreline where costal forests have become established (Hwang et al., 2000; Ogawa, 1979; Satake et al., 1989). Pitch pine (*P. rigida*), native to the northeastern region of USA, has been introduced into Korea in 1906 for the purpose of reforestation. However, this pine grows very poorly and produces low quality wood (Kim and Moon, 2007). Ku et al. (2007) reported that *Pinus rigida* bark was a usable polyphenol-rich source, whereas *P. densiflora* bark had a low yield of hot water extract.

The needles of different species of the genus *Pinus* are widly used in folk medicine and food additive due to numerous pharmacological properties, such as antiaging and antiinflamatory (Watanabe et al., 1995). Much concern has been focused on plant-derived growth inhibitors against harmful bacteria such as *Clostridium perfringens* and *Escherichia coli*, because plants constitute a rich source of bioactive chemicals and many of them do not have largely harmful adverse effects (Hwang and Lee, 2002).

Phenolic compounds are widely distributed as secondary metabolites of plants as well as some edible plants (Hagerman et al., 1998; Soong and Barlow, 2004). In recent years, polyphenols have received a great deal of attention, due to their diverse biological functions (Xia et al., 2010). Phenolic compounds were found to have effect on antioxidative and antimicrobial activity (Lee et al., 2005; Ribeiro et al., 2008). The mechanisms are thought to be responsible for phenolic toxicity to microorganisms including adsorption and disruption of microbial membranes, interaction with enzymes, and metal ion deprivation (Fattouch et al., 2007).

The aim of this study was to assess the antimicrobial activity of water extracts from fresh and fallen leaves of *Pinus densiflora*, *P. thunbergii* and *P. rigida*, along with the relationship between its microbial inhibition and total phenolic content.

MATERIALS AND METHODS

Plant materials

The fresh leaves of *Pinus densiflora*, *P. thunbergii* and *P. rigida,* were collected from Suncheon (34°54′27″N, 127°34′52″E), Korea in June 2009. The fallen leaves of the three *Pinus* species were collected in November 2009. The collected samples were air-dried for 14 days for antimicrobial activity test and determination of total phenolic content.

Test microorganisms

The tested microorganisms included two gram-positive bacteria (*Bacillus subtilis* ATCC 9327 and *Staphylococcus aureus* ATCC 13301), two gram-negative bacteria (*Escherichia coli* ATCC 15489 and *Pseudomonas fluorescens* ATCC 11250). The gram-positive and gram-negative bacteria were cultured on a nutrient broth agar.

Extract preparation for antimicrobial activity of three *Pinus* species

We soaked 200 g samples of air-dried fresh leaves and fallen leaves of the three *Pinus* plants in 1,000 ml of methanol and ground the mixture for 20 min. The solution was kept at room temperature for 30 min and then filtered through Whatman No.2 paper.

The crude methanol extract was partitioned with 500 ml of hexane and then the top layer was concentrated (comprising the hexane fraction). The remaining layer was successively fractionated with 500 ml of diethyl ether and then ethyl acetate (forming the ether and ethyl acetate fractions). The remaining residue was the water fraction. Each fraction was concentrated *in vacuo* to 30 ml at 30°C and tested for antimicrobial activity. Antimicrobial activity was measured only with the water fraction. The other fractions revealed no activity.

Determination of antimicrobial activity

Each bacterial strain was grown in a nutrient broth at 30 °C for 18-24 hr prior to testing and subcultured three times for another 18-24 hr. The turbidity of bacterial cell suspensions was brought to 0.3 optimal density (OD) at 660 nm by adding sterile broth and was then used for the tests. We poured 0.1 ml of the bacterial cell suspensions uniformly on nutrient broth agar plates. The paper disks containing the extract (water fraction) was carefully

placed on the seeded Petri dishes. The diameters of the resulting inhibition zones were measured in mm after the cultures were incubated at 30 °C for 24 hr or 48 hr (Kumar, 2006). The antimicrobial activity was calculated as the net zone of inhibition estimated from the growth inhibition zone measurements (Magasneh and El-Oqlah, 1999). The minimal inhibition concentration (MIC) was determined as the lowest concentration that caused an inhibition zone.

Extract preparation for total phenolic content

Methanol/water (80:20 v/v, 50 ml) was mixed with air-dried and powdered samples (5 g), and the phenolic substances were extracted using a vortex at 40 Hz for 3 min. The mixture was centrifuged (1200 g, 10 min) and the resultant clear solution was separated. Each extraction was conducted in duplicate. The final volume of clear supernatant was made to 10 ml with 80 % methanol and analyzed for total phenolic contents.

Determination of total phenolic content

Total phenolic content was determined by a slight modified Folin–Denis method (Padda and Picha, 2008). A sample volume of 0.5 ml was placed in a 25 ml test tube and mixed with 8mL of distilled water followed by the addition of 0.5 ml of Folin-Denis reagent. After 3 min, 1 ml of sodium carbonate (10 % in distilled water) was added and the solution was allowed to stand for 2 hr at 22℃ in darkness. The absorbance was measured at 700 nm with a UV–vis. spectrophotometer (HP-8453, USA). A standard curve with tannic acid (50-300 mg / l) was used for quantification and the total phenolic content was expressed as milligrams tannin per gram dry weight.

Statistical analysis

A randomized complete block design with three replications was applied in all the experiments. Each experiment was repeated three or four times. Statistical analysis was performed with the software program SPSS (Version 16.0). The data represent the mean ± standard deviation. The level of significance was set at $p < 0.05$.

RESULTS

Antimicrobial activity of three *Pinus* species

The antimicrobial activity and minimum inhibitory concentration (MIC) of water fractions of methanol extracts from fresh leaves and fallen leaves of the three *Pinus* plants are shown in Table 1, Table 2, Table 3 and Table 4.

Table 2. Antimicrobial activities of the water fraction of methanol extract against *Staphylococcus aureus*

Tested plant		Clear zone (± SD, mm) at various concentrations (mg/ml)					MIC (mg/ml)
		0.1	0.2	0.5	1.0	2.0	
P. densiflora	Leaves	-	9.0 ± 0.4bc	11.4 ± 0.3a	12.5 ± 0.4ab	14.6 ± 0.4b	0.2
	Fallen leaves	-	8.3 ± 0.1c	10.1 ± 0.6b	13.0 ± 0.5a	14.5 ± 0.4b	0.2
P. thunbergii	Leaves	-	9.9 ± 0.4a	10.6 ± 0.3ab	13.2 ± 0.4a	15.9 ± 0.2a	0.2
	Fallen leaves	-	9.2 ± 0.2b	11.0 ± 0.4a	12.0 ± 0.3b	13.1 ± 0.5c	0.2
P. rigida	Leaves	-	8.6 ± 0.4cc	10.1 ± 0.6b	12.1 ± 0.5b	14.4 ± 0.3b	0.2
	Fallen leaves	-	-	8.6 ± 0.3c	10.3 ± 0.2c	12.9 ± 0.3c	0.5

Values followed by the same letter in the same columns are not significantly different at $p < 0.05$.
-, not detected.

Table 1. Antimicrobial activities of the water fraction of methanol extract against *Bacillus subtilis*

Tested plant		Clear zone (± SD, mm) at various concentrations (mg/ml)					MIC (mg/ml)
		0.1	0.2	0.5	1.0	2.0	
P. densiflora	Leaves	-	9.4 ± 0.1a	11.2 ± 0.3a	13.4 ± 0.3a	14.4 ± 0.5a	0.2
	Fallen leaves	-	8.5 ± 0.2c	10.0 ± 0.2b	12.3 ± 0.3b	13.7 ± 0.5b	0.2
P. thunbergii	Leaves	-	8.8 ± 0.2c	10.9 ± 0.2b	14.0 ± 0.1a	14.6 ± 0.5a	0.2
	Fallen leaves	-	8.6 ± 0.3c	9.7 ± 0.1c	11.4 ± 0.2c	12.2 ± 0.2c	0.2
P. rigida	Leaves	-	9.1 ± 0.3b	9.4 ± 0.2c	11.3 ± 0.1c	13.7 ± 0.5b	0.2
	Fallen leaves	-	8.5 ± 0.2c	9.4 ± 0.1c	11.0 ± 0.4c	13.2 ± 0.4c	0.2

Values followed by the same letter in the same columns are not significantly different at $p < 0.05$.
-, not detected.

Table 3. Antimicrobial activities of the water fraction of methanol extract against *Escherichia coli*

Tested plant		Clear zone (± SD, mm) at various concentrations (mg/ml)					MIC (mg/ml)
		0.1	0.2	0.5	1.0	2.0	
P. densiflora	Leaves	-	8.6 ± 0.3b	9.1 ± 0.4c	13.1 ± 0.5a	13.7 ± 0.7ab	0.2
	Fallen leaves	-	8.4 ± 0.4b	9.3 ± 0.3c	9.6 ± 0.6c	9.8 ± 0.5d	0.2
P. thunbergii	Leaves	-	9.2 ± 0.5a	11.0 ± 0.5a	12.5 ± 0.4a	14.5 ± 0.4a	0.2
	Fallen leaves	-	8.4 ± 0.3b	9.2 ± 0.4c	11.4 ± 0.4b	11.9 ± 0.4c	0.2
P. rigida	Leaves	-	-	10.2 ± 0.4b	11.5 ± 0.5b	13.4 ± 0.3b	0.5
	Fallen leaves	-	-	8.6 ± 0.3c	10.0 ± 0.4c	11.1 ± 0.5c	0.5

Values followed by the same letter in the same columns are not significantly different at $p < 0.05$.
-, not detected.

Table 4. Antimicrobial activities of the water fraction of methanol extract against *Pseudomonas flurorescens*

Tested plant		Clear zone (± SD, mm) at various concentrations (mg/ml)					MIC (mg/ml)
		0.1	0.2	0.5	1.0	2.0	
P. densiflora	Leaves	-	8.3 ± 0.3a	9.9 ± 0.4b	13.2 ± 0.3ab	15.6 ± 0.7a	0.2
	Fallen leaves	-	8.3 ± 0.4a	9.3 ± 0.4bc	12.6 ± 0.4bc	14.8 ± 0.5a	0.2
P. thunbergii	Leaves	-	8.6 ± 0.5a	11.1 ± 0.5a	14.0 ± 0.7a	15.2 ± 0.4a	0.2
	Fallen leaves	-	-	9.0 ± 0.6bc	10.9 ± 0.7de	12.5 ± 0.6c	0.5
P. rigida	Leaves	-	-	9.3 ± 0.6bc	11.7 ± 0.3cd	15.0 ± 0.7a	0.5
	Fallen leaves	-	-	8.9 ± 0.4c	10.7 ± 0.6e	13.6 ± 0.6b	0.5

Values followed by the same letter in the same columns are not significantly different at $p < 0.05$.
-, not detected.

Table 5. Total phenolic contents (mean ± SD) of *Pinus densiflora*, *P. thunbergii* and *P. rigida*

Tested plants	Total phenolic content (mg/g of dry weight)	
	Leaves	Fallen leaves
Pinus densiflora	104.07 ± 4.26 b	20.52 ± 1.06 c
Pinus thunbergii	116.75 ± 2.62 a	24.53 ± 1.50 b
Pinus rigida	119.90 ± 2.00 a	33.03 ± 2.40 a

Values followed by the same letter in the same columns are not significantly different at $p < 0.05$.

The water fractions of methanol extracts from fresh leaves of *P. densiflora*, *P. thunbergii* and *P. rigida* showed strong inhibitory effect on bacterial growth. The diameters of the clear zones resulting from application of the water fractions of methanol extracts ranged from 8.3 mm to 15.9 mm (including the diameter of the disk, 8.0 mm). We classified the antimicrobial activity of the plant extracts into three classes as follows: weak (< 10 mm inhibition zone), moderate (10-15 mm inhibition zone), and good to very good (> 15 mm inhibition zone).

The antimicrobial activity of water fractions of methanol extracts from the three *Pinus* plants displayed moderate and good to very good results to gram-positive bacteria and gram-negative bacteria at higher concentrations than 0.5 mg / ml.

The results from the disc diffusion method followed by measurements of minimal inhibition concentration (MIC) indicate that *Bacillus subtilis* is the most sensitive microorganism with the lowest MIC value. Another sensitive microorganism is *Staphylococcus aureus*.

Total phenolic contents of three *Pinus* species

The total phenolic contents of the three *Pinus* plants measured by a slightly modified Folin-Denis method are shown in Table 5. The highest total phenolic contents was found in the fresh leaves of *P. rigida* (119.90 ± 2.00 mg / g dw) and *P. thunbergii* (116.75 ± 2.62 mg / g dw), followed by fresh leaves of *P. densiflora* (104.07 ± 4.26 mg / g dw). The least amount of total phenolic content was found in fallen leaves of *P. densiflora* (20.52 ± 1.06 mg / g dw). The fresh leaves of the three *Pinus* species had more total phenolic content than those of fallen leaves.

DISCUSSION

The fresh pine leaves have been used as foods and medicines in Korea (Kim et al., 2006). The antimicrobial activity of water fractions of methanol extracts from the three *Pinus* plants showed moderate and good to very

good results at higher concentrations than 0.5 mg / ml.

Similar results have been reported antibacterial activity of *P. densiflora* leaves: Hwang and Lee (2002) observed moderate activity against *E. coli* at a dose of 10 mg / disk. And the antibacterial activity of pinosylvin from *Pinus densiflora* exhibited more potent growth inhibitory activity against *Saccharomyces cerevisiae* (Lee et al., 2005).

Our result revealed that increasing concentrations of the three *Pinus* plant extracts led to increasing inhibition of bacterial growth. Antimicrobial activities of water fractions of methanol extracts from fresh leaves were stronger than those of fallen leaves extracts at any concentrations. The water fractions of fresh leaves of *P. thunbergii* showed higher antimicrobial activity than the other two *Pinus* extract in case of *Bacillus subtilis, Escherichia coli* and *Staphylococcus aureus*. The tested fractions of fresh leaves appeared more active against the tested gram-positive bacteria than gram-negative bacteria. Some gram-negative bacteria are less sensitive than gram-positive bacteria to the action of plant extracts and compounds (Boussaada et al., 2008; Yun et al., 2008), but gram-negative bacteria are often more susceptible than gram-positive bacteria to the inhibitory effects of essential oils (Smith-Palmer et al., 1998). In the present study, the antimicrobial activity of extract of fresh leaves from *P. densiflora* is shown moderate activity against *Escherichia coli*. Previous work has shown that the antimicrobial effect of *P. rigida* extracts had moderate inhibition activity against the *Staphylococcus aureus* (Jang et al., 2008).

The results from the disc diffusion method followed by measurements of minimal inhibition concentration (MIC) indicate that *Bacillus subtilis* is the most sensitive microorganism with the lowest MIC value and another sensitive microorganism is *Staphylococcus aureus*. This observation may be explained by the fact that gram-negative bacteria possess an outer membrane and a periplasmic space, both of them are abscent in gram-positive bacteria. The periplasmic space contains enzymes which are capable of breaking down foreign molecules introduced from outside (Duffy and Power, 2001). Antimicrobial extracts from the three *Pinus* plants can be assumed to be useful against infectious disease. Our results allow us to conclude that the extracts of *P. thunbergii* exhibited significant antimicrobial activity and properties that support its food additive and medicinal use as an antimicrobial agent.

Phenolic compounds are ubiquitous in plants which collectively synthesize several thousand different chemical structures characterized by hydroxylated aromatic ring. Phenolic compounds represent the most studied phytochemicals and have been widely exploited as mode system in different areas of plant research (Boudet, 2007).

Most phenolics that display antimicrobial activity are phenolic acids or flavonoids. Phenolic acids are a major class of phenolic compounds occurring in a diverse range of plants (Wojdylo et al., 2007). Among the phenolic compounds, protocatechuic acid was the major phenolic compounds in *P. rigida* (Kim and Lee, 1996). Kujumgiev et al. (1993) concluded that phenolic moiety plays an important role in determining a plant's antimicrobial activity.

Antimicrobial activities and total phenolic contents of fresh leaves were higher than those of fallen leaves. The antimicrobial activity and the total phenolic content of the three *Pinus* species had a positive linear correlation. The strongest correlation between total phenolic contents and antimicrobial activities were observed in fresh leaves and fallen leaves of *Pinus thunbergii*. The correlation between the antimicrobial activity and total phenolic compounds was reported by other authors (Baydar et al., 2004; Lizcano et al., 2010; Rodríguez-Vaquero et al., 2010).

In this study, the water extracts of fresh leaves from the three *Pinus* species showed higher antimicrobial activity than those of fallen leaves. The extract of *P. thunbergii* was found to be the best extract for antimicrobial activity. The total phenolic content of fresh leaves did significantly differ with fallen leaves. According to the results of this study, the fresh leaves of the three *Pinus* species can be used as a potential source of natural antimicrobial resources. Nevertheless further studies are needed for enlightening the chemicals responsible for antimicrobial activity.

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