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Furfural from Pine Needle Extract Inhibits the Growth of a Plant Pathogenic Fungus, *Alternaria mali*

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The antifungal effect of pine needle extract prepared by a distinguishable extraction method and the dry distillation method, was examined. The effect of this extract itself was insignificant. The chemical components of pine needle extract were then investigated by gas chromatographic analysis, and four chemical components, acetol, furfural, 5-methyl furfural, and terpine-4-ol, were identified. The antifungal effects of those four chemical components against *Alternaria mali* (*A. mali*), an agent of Alternaria blotch of apple, were then examined. It was observed that the minimum inhibitory concentrations (MICs) were 6.25, 0.78, 0.78, and 12.5 (mg/*ml*) of acetol, furfural, 5-methyl furfural, and terpine-4-ol, respectively. MICs of furfural and 5-methyl furfural had the same order of magnitude as that of an antifungal agrochemical, chlorothalonil. Although furfural itself can not be completely substituted for an antifungal agrochemical, a partial mixture of furfural and antifungal agrochemical may be used as a substitute. The use of agrochemicals for the prevention of plant disease caused by pathogenic fungus such as *A. mali* could be partially reduced by the application of this mixture.

KEYWORDS: Alternaria blotch, *Alternaria mali*, Antifungal activity, Furfural, Pine needle extract

Volatile components in pine needle extract have been identified by GC-MS analysis (Kim and Chung, 2000; Lee *et al.*, 2005; Ucar and Balaban, 2004), and it was reported that there existed many volatile compounds such as α-pinene, myrcene, β-thujene, terpinene-4-ol, δ-cadinene, isoamyl alcohol, *trans*-caryophyllene, α-terpineol, and δ-cadinene, particularly from pine needle tea and pine sprout tea (Kim and Chung, 2000). Lee *et al.* (2005) investigated volatile components in pine needles by GC-MS and the thermal desorption method. Using this method, it was revealed that monoterpenes (pinenes, camphene, limonene, and b-phellandrene) and sesquiterpenes (β-caryophyllene, germacrene D, and β-cubebene) are prominent flavor components. Ucar and Balaban (2004) reported that the major volatile components of pine needle extract were α-pinene, germacrene D, β-caryophyl, and β-pinene. Other volatile components of pine needles include alcohols, carbonyl compounds, esters, and carboxylic acids (Kim and Chung, 2000; Lee *et al.*, 2005; Ucar and Balaban, 2004). However, these findings depended on what kind of pine tree was used, and which extraction and analytical methods were utilized. Because consistent methods were not used, data regarding the major components of pine needle extract were not consistent.

Duffy and Power (2001) demonstrated the antimicrobial effect of pine needle extract against Gram(+) and Gram(−) bacteria. The MIC (minimum inhibitory concentration) of pine needle extract against both *Escherichia coli* and *Bacillus subtilis* was 50 mg/*ml*. However, antifungal activity was not detected in pine needle extract. Kim *et al.* (2000) found that the pine needle extract had an antimicrobial activity on foodborne illnesses caused by *E. coli* O157, *Staphylococcus aureus*, *Salmonella typimurium*, and *Listeria monocytogenes*, with MICs ranging from 25 to 45 mg/*ml* depending on the microorganism.

Additionally, it was shown that the mutagenic effect of cyclophosphamide was inhibited by pine needle extract (Zhiming *et al.*, 1995). An anticancer effect against several cancer cell lines by pine needle extract was also shown by Chung *et al.* (2002) and Moon *et al.* (1993). It was interesting that pine needle extract is effective for the reduction of glucose levels of diabetic rats (Kim *et al.*, 2005). Although there have been several reports on the biological functions of pine needle extract, the extraction methods differed greatly in these experiments, and their major components and component levels were also very diverse. Therefore, it is not clear which component of pine needle extract produced which effects in each experiment testing the biological functions.

In this report, the effect of pine needle extract on the pathogenic fungus, *Alternaria mali*, was examined in terms of fungal growth inhibition. It has been reported that Alternaria blotch of apple is caused by a pathogenic fungus, *A. mali* (Bulajic *et al.*, 1996). Lesions of Alternaria blotch in apple first appear on leaves or fruits in late spring or early summer as small, round, purplish or black-

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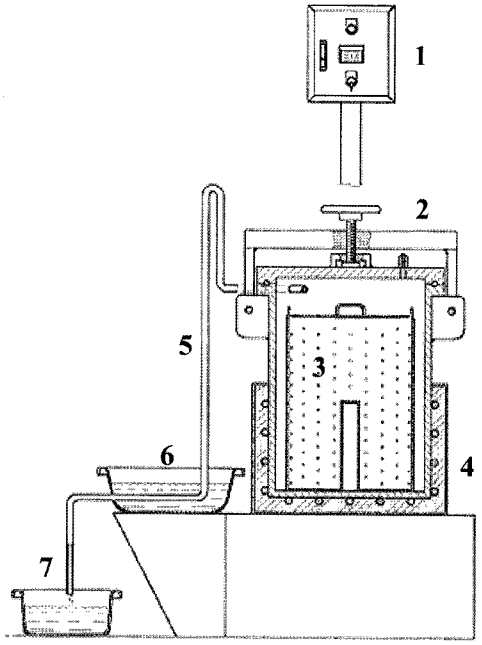

Fig. 1. Dry distillation apparatus for preparing pine needle extract. Dimensions of the inner region of the apparatus were 50(H) × 30(ID) cm. **1**, Temperature control box; **2**, Tight-sealed cover; **3**, Mesh bucket; **4**, Heating coil; **5**, Vapor outlet pipeline; **6**, Cooling water; **7**, Distillate (pine needle extract).

ish spots, gradually enlarging to 1.5~5 mm in diameter, with a brownish-purple border (Sawamura *et al.*, 1990). However, it was reported that chemical control of *A. mali* could be achieved through the use of fungicides such as iprodione, mancozeb, and captan (Kim *et al.*, 1986; Lee and Kim, 1986). The use of difenoconazole (Gullino *et al.*, 2000; Reuveni *et al.*, 2002), tebuconazole (Reuveni *et al.*, 2002), chlorothalonil (Reuveni *et al.*, 2002), and polyoxin B (Gullino *et al.*, 2000) as antifungal agents against Alternaria blotch have been reported. In this report, the pine needle extract was prepared by the dry distillation method, which is similar to the method used for the production of pyroligneous acid (wood vinegar). The composition and amounts of components isolated differ greatly from those of other extraction methods such as the hot water or solvent extract method. It was expected that pine needle extract or its active component could be used effectively for the environmentally-friendly control of Alternaria blotch of apple.

Pine needles harvested from a pine tree (*Pinus densiflora*) were washed with tap water and dried at room temperature. For obtaining pine needle extract, approximately 3 kg of the washed pine needles was used, and the dry distillation apparatus, shown in Fig. 1, was operated for 8 hr at 210°C. The antibacterial effect was tested by using *S. aureus* subsp. *aureus* ATCC 25923 and *E. coli* ATCC 11775 as Gram(+) and Gram(−) bacteria, respectively. Liquid cultures for seed preparation and antibacterial testing were prepared in 3% (w/v) tryptic soy broth (TSB) (Becton Dickinson, USA) at 37°C and pH 7.0. The pH of pine needle extract was adjusted to 7.0 by the addition of 5 M NaOH. Liquid culture media were prepared by mixing both pine needle extract and 3% (w/v) TSB; media were then sterilized using a 0.22 mm filter. Five milliliters of the sterilized media were dispensed into a sterilized 15 *ml* test tube. For seed preparation of *S. aureus* subsp. *aureus* ATCC 25923 and *E. coli* ATCC 11775, two shake flask cultures with volumes of 100 *ml* were carried out at 37°C and 150 rpm overnight, in which one colony of each of the bacteria that had been grown on TSB agar plates was used as seed for seed flask culture. Five *μl* of the seed cultures were then inoculated into 5 *ml* of TSB media containing pine needle extract. After 24 hr culturing of these test tubes in a shaking incubator at 37°C and 150 rpm, the OD at 600 nm of each test tube was measured.

The antifungal effect of pine needle extract was investigated against five plant pathogenic fungi (Table 1), which were kindly donated by KACC (Korean Agricultural Culture Collection). For seed cultures of these fungi, a loop of fungi mycelium cultured on a malt extract (Becton Dickinson, USA) agar (MEA, 1.7% (w/v) malt extract and 2% (w/v) agar) plate (60 × 15 mm) was inoculated into 10 *ml* of malt extract liquid medium in a sterile 50 *ml* conical tube (Greiner Bio-One, USA). These five seed cultures were cultured in a shaking incubator at 25°C and 150 rpm for 2~3 days. After the mycelium growth was confirmed visually, a sterilized filter paper disc (8 mm, Advantec, Japan) was soaked in 10 *ml* of malt extract liquid medium, followed by gentle shaking for one minute in order to allow effective absorption of fungi mycelium. Filter paper was then placed on the center of the pine needle extract-containing MEA plate (60 × 15 mm). The MEA

Table 1. Pathogenic fungi used in this study

Pathogenic fungi	Targets	Diseases	Abbreviations
Alternaria mali KACC 40026	Apple	Alternaria leaf spot	AM
Valsa ceratosperma KACC 40331	Apple	Valsa canker	VC
Glomerella cingulata KACC 40299	Apple, Peach	Bitter rot	GC
Phomopsis mali KACC 40839	Apple, Peach	Die-back	PM
Botrytis cinerea KACC 40573	Apple, Peach	Gray mold	BC

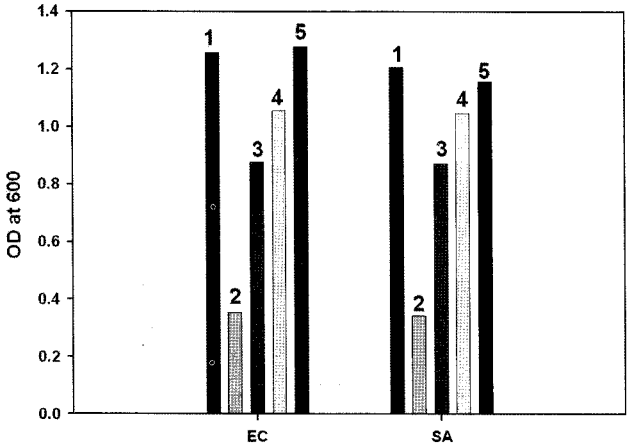

Fig. 2. Growth inhibitions of Gram(+) and Gram(−) bacteria in tryptic soy broth (TSB) with pine needle extract. **1**, TSB was prepared with distilled water (control); **2**, TSB was prepared only with pine needle extract; **3**, TSB was prepared with pine needle extract diluted 1 : 2; **4**, TSB was prepared with pine needle extract diluted 1 : 4; **5**, TSB was prepared with pine needle extract diluted 1 : 8; **EC**, *Escherichia coli* ATCC 11775. **SA**, *Staphylococcus aureus* subsp. *aureus* ATCC 25923. OD at 600 nm was measured after culturing for 24-hr.

plates were incubated at 25°C for 6 days, and the degree of mycelia growth on MEA plate was observed. In particular, the antifungal activities of some antifungal agrochemicals such as difenoconazole (Kyung Nong Co., Korea), chlorothalonil (Kyung Nong Co., Korea), tebuconazole (Bayer CropScience, Korea) were examined against *A. mali* in order to compare them with activities of pine needle extract. The activities of five identified chemical components of pine needle extract, acetol (Aldrich), furfural (Aldrich), 5-methyl furfural (Aldrich), terpine-4-ol (Acros),

Table 2. Degree of growth inhibition of pathogenic fungi on malt extract agar (MEA) with pine needle extract

		Degree of growth inhibition[1]				
		AM[2]	BC	GC	VC	PM
Dilution of PNE[3]	1 : 2	−	−	−	−	−
	1 : 4	−	+	−	+	−
	1 : 8	+	+	+	+	+
	1 : 16	+	++	+	++	+
	1 : 32	+++	+++	++	+++	+
	1 : 64	+++	+++	+++	+++	++
Control[4]		+++	+++	+++	+++	+++

[1]−, No growth; +, Radial growth from disc filter paper was less than half of the radius of the MEA plate; ++, Radial growth from disc filter paper was more than half of the radius of the MEA plate and did not cover the entire MEA plate; +++, Growth from disc filter paper covered the entire MEA plate.
[2]Pathogenic fungi is described using the abbreviations in Table 1.
[3]PNE is an abbreviation of "pine needle extract", and PNE at dilutions from 1 : 2 to 1 : 64 were investigated in this experiment.
[4]MEA was prepared only with distilled water.

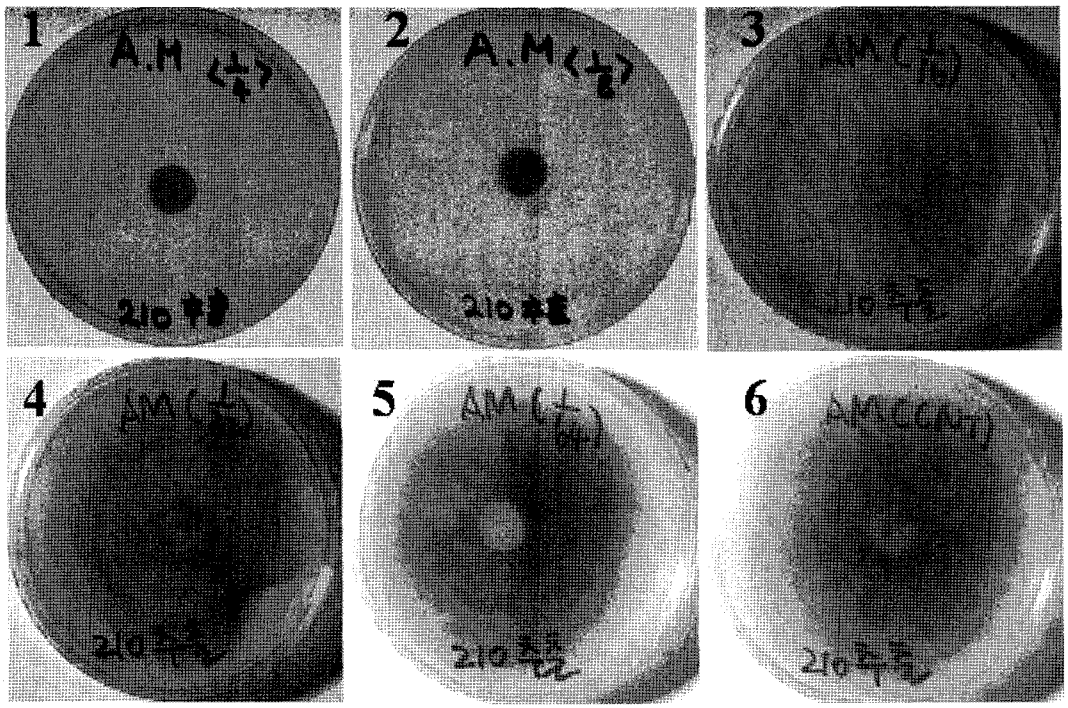

Fig. 3. Growth inhibition of a pathogenic fungus, *Alternaria mali*, on malt extract agar (MEA) with pine needle extract. **1**, MEA was prepared with pine needle extract diluted 1 : 4; **2**, MEA was prepared with pine needle extract diluted 1 : 8; **3**, MEA was prepared with pine needle extract diluted 1 : 16; **4**, MEA was prepared with pine needle extract diluted 1 : 32; **5**, MEA was prepared with pine needle extract diluted 1 : 64; **6**, MEA was prepared with distilled water (control).

and α-terpineol (Aldrich), were also investigated using the same methods.

The composition of pine needle extract was analyzed by gas chromatography (HP 6890, Agilent technologies, USA) with a flame ionization detector (FID), and an HP INNOWax column (Agilent 19091N-113, film thickness; 0.25 μm, length; 30 m, inner diameter; 0.32 mm) was used. The initial temperature, maximum temperature, and temperature increase rate in the oven were 75°C, 200°C, and 3°C/min, respectively. The initial temperature was maintained for 8 min after sample injection, and then the oven temperature began to increase. Both the injector and FID temperatures were controlled at 270°C. Nitrogen was used as a carrier gas, and its flow rate was controlled at 50 *ml*/min. α-Pinene (Aldrich), acetol (Aldrich), furural (Aldrich), 5-methyl furfural (Aldrich), terpine-4-ol (Acros), and α-terpineol (Aldrich) were used as standard chemicals as characteristic markers of pine needle extract.

For the preparation of pine needle extract, a batch of approximately 3 kg of pine needles was used. 1.2 *l* of pine needle extract was finally obtained from the operation of a dry distillation apparatus. This extract was examined for its antibacterial and antifungal activity. As shown in Fig. 2, the growth of Gram(+) and Gram(−) bacteria was not effectively inhibited by pine needle extract. Furthermore, these bacteria grew in the pine needle extract itself. The growth inhibition of pine needle extract against the plant pathogenic fungi was observed only at dilution of 1 : 2 or 1 : 4 (Table 2). Figure 3 shows one of those tests for antifungal effects. However, these antifungal effects of pine needle extract were much weaker than those of pyroligneous acid, wood vinegar. The growth of *A. mali* was completely inhibited at a 1 : 32 dilution of pyroligneous acid and the growths of *Glomerella cingulata* and *Phomopsis mali* occurred at a dilution of 1 : 16 (Jung, 2007).

Because a distinguishable extraction method, dry distillation, was used in this work, the chemical components of pine needle extract were investigated by gas chromatographic (GC) analysis. Therefore, we listed the major chemical components, previously reported in some research papers, of pine needle extract (Yoo *et al.*, 2001; Yoo and Day, 2002). Six chemicals were selected as follows: α-pinene, acetol, furural, 5-methyl furfural, terpine-4-ol, and α-terpineol. Acetic acid, methanol, and phenol, all of which were know to exist as major components of pine needle extract, were also added to the list (Lee *et al.*, 2005). As shown in Fig. 4, the existences of these chemicals in pine needle extract were verified by GC analysis. α-Pinene and α-terpineol were not found in the GC chromatogram of the pine needle extract. The peaks of acetol, furfural, 5-methyl furfural, terpine-4-ol, methanol, acetic acid, and phenol were only detected by comparing the retention times of the standard chemicals in the GC chromatogram. Among those chemicals, four chemical components, acetol, furfural, 5-methyl furfural, and terpine-4-ol, were tested for their antifungal effects because they were distinctive components that we found in the pine needle extract. Among five kinds of plant pathogenic fungi (Table 1), an antifungal effect against *A. mali* was examined, particularly because this fungus is an agent of Alternaria blotch of apple and apple is a representative fruit of Chungju, Chungbuk, Republic of Korea.

Table 3 shows the growth inhibition effect of those four chemical components against *A. mali*. The MICs of acetol, furfural, 5-methyl furfural, and terpine-4-ol were 6.25, 0.78, 0.78, and 12.5 (mg/*ml*), respectively. The antifungal effects of furfural and 5-methyl furfural were more effective compared to the other components. These MIC values were much higher than the commercially available antifungal agrochemicals against *A. mali* (Table 4). However, it could be concluded that MICs of furfural and 5-methyl furfural had the same order of magnitude as an agrochemical, chlorothalonil, and those MICs were much

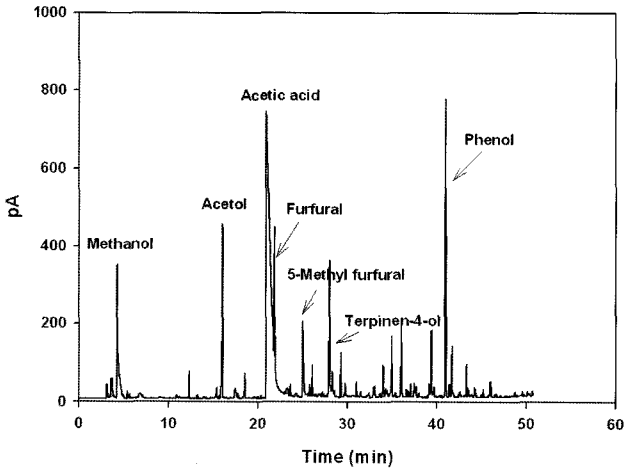

Fig. 4. Gas chromatogram of pine needle extract. Peaks were identified by the retention times of standard chemicals.

Table 3. Degree of growth inhibition of *Alternaria mali* on malt extract agar (MEA) with the components of pine needle extract

Acetol[2] (mg/*ml*)	12.5	6.25	3.12	1.56
DGI[1]	–	–	++	+++
Furfural[2] (mg/*ml*)	1.56	0.78	0.39	0.19
DGI	–	–	++	+++
5-Methyl furfural[2] (mg/*ml*)	1.56	0.78	0.39	0.19
DGI	–	–	+++	+++
Terpinen-4-ol[2] (mg/*ml*)	25	12.5	6.25	3.12
DGI	–	–	+++	+++

[1]DGI is an abbreviation for "degree of growth inhibition". Determinations of DGI were performed with the same criterion as in Table 2.
[2]Four chemicals were purchased, not purified from pine needle extract.

Table 4. Degree of growth inhibition of *Alternaria mali* on malt extract agar (MEA) with some antifungal agrochemicals

Difenoconazole (*μg/ml*)	1.952	0.976	0.488	0.244
DGI[1]	−	−	−	+
Chlorothalonil (*μg/ml*)	468.7	234.3	117.1	58.59
DGI	−	−	++	++
Tebuconazole (*μg/ml*)	9.765	4.882	2.441	1.22
DGI	−	−	+	++

[1]DGI is an abbreviation for "degree of growth inhibition". Determinations of DGI were performed with the same criterion as in Table 2.

lower than that of an antifungal antibiotic, polyoxin B, which had an MIC of 2 mg/*ml* against *A. mali* (Jung, 2007). The antifungal activities of furfural and its derivative have not been reported frequently, although some papers have discussed their feasibilities for antifungal treatment (Jouad *et al.*, 2001; Moon *et al.*, 1993).

Currently, furfural is produced from agricultural waste biomass (corn cobs, rice hulls, flax dregs, cotton hulls, sugar cane bagasse, wood) and is used in flavoring, pharmaceuticals, specialty polymers, and as a precursor for specialty chemicals (Win, 2005). It is reported that the prince rage of furfural is $500~1,100/ton, and furfural is now commercially available as a bulk chemical (Lee *et al.*, 2005). In view of its antifungal activity and price, furfural may be considered as a substitute for agrochemical, particularly antifungal reagents. Although furfural alone cannot be used as a substitute for an antifungal agrochemical, a partial mixture of furfural and antifungal agrochemical could be utilized, and this may act to reduce the effects of pathogenic fungus such as *A. mali* and prevent a number of plant diseases.

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