

Negative Effects of Water Extracts from *Pinus densiflora* Sawdust on Mycelium Growth of the Shiitake Mushroom *Lentinula edodes*

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ABSTRACT : Extracts from sawdust of *Pinus densiflora* were showed antifungal activity against *Lentinula edodes*. It was extracted by hot water and then successively extracted by *n*-hexane, ethyl acetate, and methanol. The yields of the *n*-hexane-soluble, ethyl acetate-soluble, methanol-soluble and methanol-insoluble fractions of water extracts were 8.2%, 10.6%, 32.0%, and 49.2%, respectively. The ethyl acetate-soluble fraction showed the greatest antifungal activity against *L. edodes*: 41.5% inhibition at 1,000 ppm. However, there were not significant differences of antifungal activities between *n*-hexane-soluble fraction and methanol-soluble fraction at a concentration of 1,000 ppm. The hot water extracts showed 23.5% of antifungal activity against *L. edodes* at a concentration of 1000 ppm. The four antifungal compounds were separated from ethyl acetate fraction by thin layer chromatography.

Key words : antifungal activity, *Lentinula edodes*, organic solvent fraction, *Pinus densiflora*, sawdust

INTRODUCTION

Lentinula edodes (Shiitake) is a mushroom which is eaten in Korean, Chinese and Japanese and is nowadays the second most commonly produced edible mushroom in the world. It is useful to its nutritional value and the possibility of its medical application (Jong & Nirmingham, 1993). Today, it has used not only for its culinary value but also for its medicinal antitumor properties. Among its medicinal, attributes are antitumor activity (Flynn, 1991), antiviral activity (Tochikura *et al.*, 1987) and cholesterol-lowering activity (Tokuda & Kaneda, 1979). Lentinan, a beta-glucan derived from *L. edodes*, is known to work positively against cachexia in patients with malignant tumors (Tamura *et al.*, 1997).

Most *L. edodes* cultivation occurs on hardwood tree logs, but its cultivation on synthetic logs (sawdust supplemented by nutrients) can result in higher mushroom production efficiency and could develop into an economical and cost-effective method of *L. edodes* cultivation. Several factors affect mushroom production efficiency on sawdust media, including the spawn run time and substrate formulation. In the sawdust media, wood cell wall materials are intimately associated in the form of a complex matrix where the lignin may act as a barrier to polysaccharide degradation by the mycelium and so restrict the availability of nutrients required for fungal growth.

Softwood has been investigated for the cultivation of the *L. edodes* mushroom, but the *L. edodes* mushroom has grown

poorly on softwoods such as *P. densiflora* (Kim *et al.*, 2002; Yang *et al.*, 2003). Some compounds of the softwood that are inhibitory to mycelial growth must be removed in order to utilize the softwood for the cultivation of *L. edodes* mushroom. Matsui *et al.* (2001) reported that extracts from the *P. densiflora* sawdust inhibited mycelium growth of *L. edodes* and that these inhibitory compounds could be removed by methanol extraction. They suggested that the antifungal activity of the mycelium growth of *L. edodes* was probably due to terpenoids, a major compound of the extracts of *P. densiflora*, and possibly to synergy with another inhibitory compound, cedrol. Antimicrobial compounds for *P. densiflora*. it has been extracted by cold water, hot water, steam and organic solvent to reduce the inhibitory components in the extracts of sawdust (Nakajima *et al.*, 1980; Kawachi *et al.*, 1991; Carmel *et al.*, 1995; Kishino *et al.*, 1995; Matsui *et al.*, 2001; Kofujita *et al.*, 2001). However, inhibitory mechanism and concentration of antifungal fraction of *P. densiflora* not yet known. This study was undertaken to determine inhibitory compounds and antifungal activity against the mycelial growth of *L. edodes* from the extracts of the *P. densiflora* sawdust.

MATERIALS AND METHODS

Fungal strain and sawdust preparation

The sawdust of *Pinus densiflora* which contained bark was obtained from the Forest Research Institute, in Seoul, Korea.

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The *Pinus densiflora* sawdust was collected in the spring, 2002 and was stored at constant condition. The moisture content of the air-dried sawdust was approximately 10~13% on the based oven-dried weight. The sawdust was screened to a 10~60 mesh size for water extraction.

The strain of *Lentinula edodes* (Berk.) Sing (Sanlim No. 5) was obtained from the Forest Research Institute, in Seoul, Korea. The *L. edodes* strain was cultured on a potato dextrose agar (PDA, Difco, USA) medium at 4°C

Extraction of antifungal fraction

The water extracts of *P. densiflora* sawdust was obtained by hot water extraction. For the extraction, 500 g of the sawdust was soaked in 3 l of distilled water and heated at 100°C for 3 hours using a soxhlet apparatus. The mixture was then filtered through filter paper No. 2 (Toyo roshi kaisha, Japan) and the water evaporated to near dryness and then freeze-dried.

For separation of the antifungal compounds, the powdered water extracts was treated serially using organic solvents as *n*-hexane, ethyl acetate, and methanol (Fig. 1).

One gram of the powdered water extracts and 20 ml of organic solvent were mixed in a 100 ml falcon tube and then the mixture was extracted by ultrasonicator (Jinwoo, JAC 2010, Korea) at 25°C for 60 minutes and the supernatant was separated by centrifugation at 3,000 rpm for 10 minutes. The supernatant was collected as the *n*-hexane-soluble fraction and then powdered. The *n*-hexane-insoluble fraction was suspended in the ethyl acetate, and treated by the same ultrasonicator and centrifugation to gain ethyl the acetate-soluble and ethyl acetate-insoluble fractions. The ethyl acetate-insoluble fraction was re-suspended in the methanol and extracted by the same ultrasonicator and centrifugation, and the supernatant was collected as the methanol-soluble fraction, and the last powder was collected as the methanol-insoluble fraction (Fig. 1).

The yields of each fraction from the water extracts by the

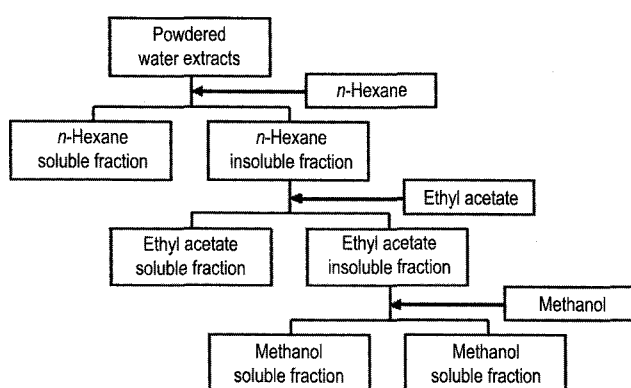


Fig. 1. Separation of antifungal fraction using various organic solvents from water extracts of *P. densiflora* sawdust.

organic solvents were obtained by measuring the dry weight of soluble parts. The yields of the fractions were expressed as a percentage of the dry weight of the organic extracts per dry weight of the non-extracted powder. Data were expressed as average of three separate experiments.

The *n*-hexane, ethyl acetate and methanol extract were further separated by silica gel (Kiesel gel 60, Merck) column chromatography and thin layer chromatography (Kiesel gel 60 F254, 0.5 mm, Merck). The silica gel column (6.0 cm × 60.0 cm) was filled with 250 g of the silica gel and eluted with three different mixed elution solvents of the *n*-hexane and ethyl acetate (v/v) in 5:1, 2:1 and 1:1 ratio. The column with silica gel in the *n*-hexane to ethyl acetate (5:1) was used for the chromatography of *n*-hexane extract, the 2:1 *n*-hexane to ethyl acetate for the ethyl acetate extract and the 1:1 *n*-hexane to ethyl acetate for the methanol extract. For the separation of the *n*-hexane, ethyl acetate and the methanol extract by the column chromatography, the elution solvents of the column with silica gel were used in 5:1, 2:1 and 1:1 *n*-hexane to ethyl acetate (v/v), respectively.

The TLC of ethyl acetate fraction was performed using mixtures of *n*-hexane and ethyl acetate (v/v) in 2:1. The separated fractions were read under ultraviolet light at 254 nm and then the values of *R_f* were measured from each fraction. And then, the separated spots of *n*-hexane, ethyl acetate and methanol were regrouped into three basic fractions (I, II and III) for each extract. And then, separated fractions were repeat TLC using above mentioned mobile phase. These three fractions were scrape up by scapel, gathered and then eluted by ethyl acetate, and determined growth inhibition against the *L. edodes* by antifungal methods.

Antifungal activity test

The water extracts and organic solvent extracts were added to the PDA medium at 250 ppm, 500 ppm, 750 ppm and 1,000 ppm and without extracts. A ten mm diameter mycelium plug was cut from the margin of an actively growing fungal culture and placed fungus side down at the center of the agar in petri plates containing each extract. The plates were wrapped with Parafilm (Pechiney plastic packaging, Menasha, WI) randomized and incubated in a growth chamber at 25°C under dark. Radial growth (mm) of the cultures was recorded after three days. All experiments were done five replicate. To determine mycelial growth against each extract concentration, percentages of the antifungal activity were calculated (% antifungal activity = (control-treatment)/control × 100). The percentage data were transformed to arcsin of square root of the value and analyzed using Proc Glim of SAS (Statistical Analysis System, ver. 6.12). The statistical significance between con-

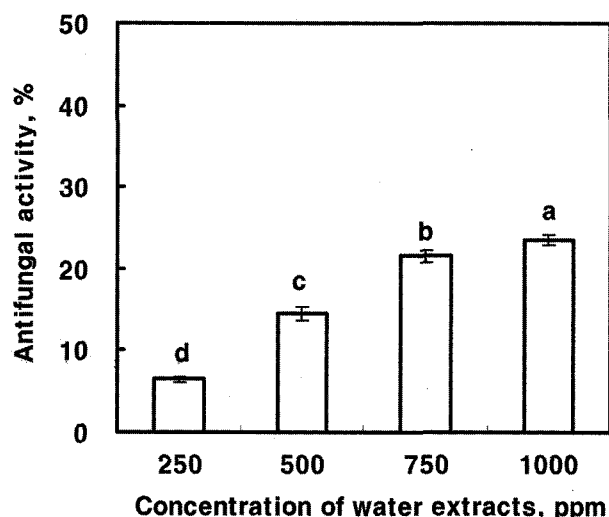


Fig. 2. Antifungal activity of water extracts from *P. densiflora* against *L. edodes* in the PDA medium at 25°C for 10 days. The vertical bars represents the standard errors of individual means. The bars followed by the same letter are not significantly different at $P < 0.05$ according to the Duncan's new multiple range test.

trasting treatments was assessed by Duncan's multiple range test ($p = 0.05$).

RESULTS AND DISCUSSION

Antifungal activity of water extracts

The antifungal activity of the water extracts against *L. edodes* was proportional to the concentration of the extracts. The average percentages of the antifungal activities at 250, 500, 750, and 1,000 ppm were 6.4%, 14.5%, 21.6% and 23.5%, respectively (Fig. 2). The antifungal activity of the water extracts at 1,000 ppm was highest at the tested concentration against *L. edodes* mycelium. The antifungal activity were significantly different as according as concentration of the water extracts.

The antifungal effects of the water extracts on the growth of *L. edodes* mycelium are shown in Fig. 3. The *L. edodes* mycelium grown on potato dextrose agar medium without water extracts presented strong growth rate of hyphae. The treatments of the water extracts in the culture medium induced reduction of growth diameter and dense of mycelium, which showed severe inhibition at high water extracts concentration. Greater reduction of growth diameter was observed with 750 ppm and 1,000 ppm of water extracts, which may be caused by mycelium growth inhibition and eventually, growth stopped. Growth of *L. edodes* mycelium was disturbed when the concentration of water extracts increased in the culture medium.

Therefore, it can be concluded that water extracts of the *P.*

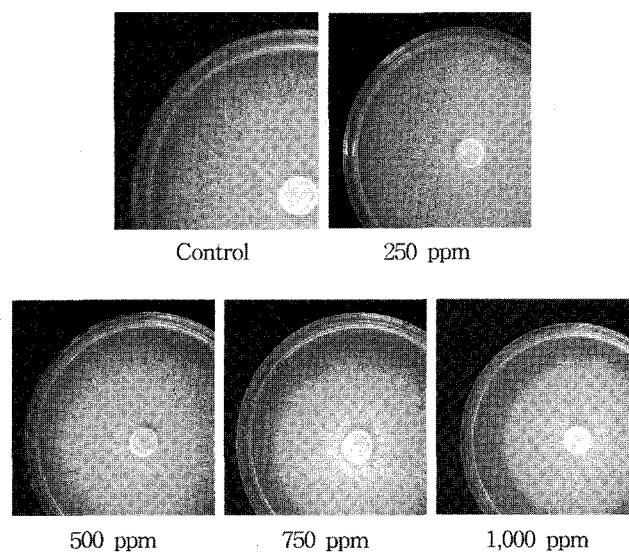


Fig. 3. Antifungal activity against *L. edodes* mycelium in various concentration of water extracts. The PDA medium plates with water extracts were incubated for 10 days 4 weeks at 25°C

densiflora sawdust have inhibitory compounds against the growth of the *L. edodes* mycelium. There may be fungistatic compound in the water extracts of the *P. densiflora* sawdust. In other aspect, the hot water extracts from the *P. densiflora* sawdust may be considered for development as environmental friendly antifungal agent which can be used in agrochemicals. The water extracts of the *P. densiflora* sawdust in this study had lower antifungal activity than that of methanol extracts from *C. japonica* against the mycelial growth of *L. edodes*. One of softwoods in Japan, *Cryptomeria japonica*, contains several terpenoids, such as ferruginol, suginol, thymol, sandaracopimarinol, phyllocladanol and β -sitosterol, that antifungal activity against *L. edodes* (Nakajima *et al.*, 1980; Kawachi *et al.*, 1991; Matsui *et al.*, 2001). Matsui *et al.* (2001) reported that ferruginol, suginol and sandaracopimarinol isolated from *C. japonica* were found to inhibit the mycelium growth of *L. edodes* at a concentration of $5,000 \mu\text{g cm}^{-3}$. Thus, these results demonstrated that the 50% inhibition concentration of ferruginol was determined at *ca.* $100 \mu\text{g cm}^{-3}$ and that of sandaracopimarinol at *ca.* $1000 \mu\text{g cm}^{-3}$. Antifungal compounds extracted from *P. densiflora* and *C. japonica* is different because these extracts were extracted by different organic solvents from different species of the softwood.

Yields and antifungal activity of fractions of water extracts

The powdered water extracts was further treated serially using the organic solvents in order to separate the antifungal active fractions of *n*-hexane, ethyl acetate, methanol and meth-

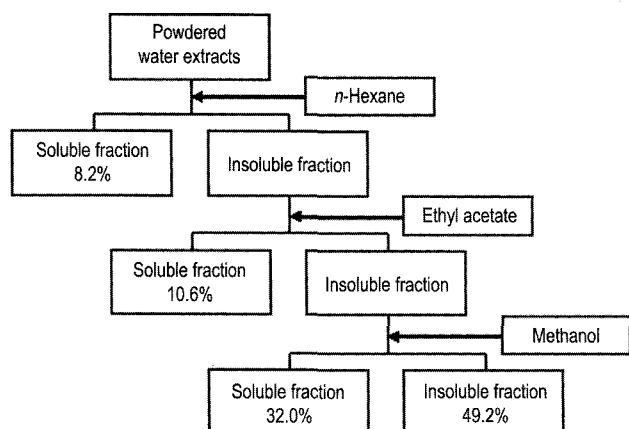


Fig. 4. Extraction yield of antifungal fraction and fractionation by various organic solvents of water extracts.

anol. The yields of the fractions of *n*-hexane-soluble, ethyl acetate-soluble, methanol-soluble and methanol-insoluble from the powdered water extracts were 8.2%, 10.6%, 32.0% and 49.2%, respectively (Fig. 4). Extracts from sawdust contains a large number of different compounds which can be separated by means of polar and non-polar solvents. The extracts of hexane-soluble are compounds belonging to the group of fat, waxes and their components as well as of terpenes (Fengel & Wegner, 1983). In this results, there may be contained about 20% of low molecular weight polyphenols in the water extracts of the *P. densiflora* sawdust. Nakajima *et al.* (1980) also treated serially using organic solvents in order to separate the inner bark (2 g) into portions of methanol-soluble (1.674 g), *n*-hexane-soluble (0.067 g), ethyl acetate-soluble (0.019 g) and ethyl acetate-insoluble (0.238 g) from *C. japonica*.

The antifungal activities of the *n*-hexane-soluble, ethyl acetate-soluble, methanol-soluble and methanol-insoluble fractions against *L. edodes* were 26.2%, 41.5%, 25.4% and 2.5%, respectively, at 1,000 ppm (Fig. 5). The ethyl acetate-soluble fraction showed much higher antifungal activity than the other fractions. The antifungal activity of the ethyl acetate-soluble fraction against *L. edodes* at 1,000 ppm showed a statistically significant difference in the *n*-hexane-soluble, ethyl acetate-soluble, methanol-soluble, and methanol-insoluble fractions. And there was not significant difference between the antifungal activities of the *n*-hexane-soluble and methanol-soluble fractions at 1,000 ppm. It was supposed that the antifungal compounds of the ethyl acetate-soluble fraction on mycelial growth of *L. edodes* could be extracted more effectively using the ethyl acetate of middle polar solvent than the *n*-hexane as light polar solvent or methanol as heavier polar solvents. The fractions of *n*-hexane-soluble, methanol-soluble and methanol-insoluble contained much more non-inhibitory compounds

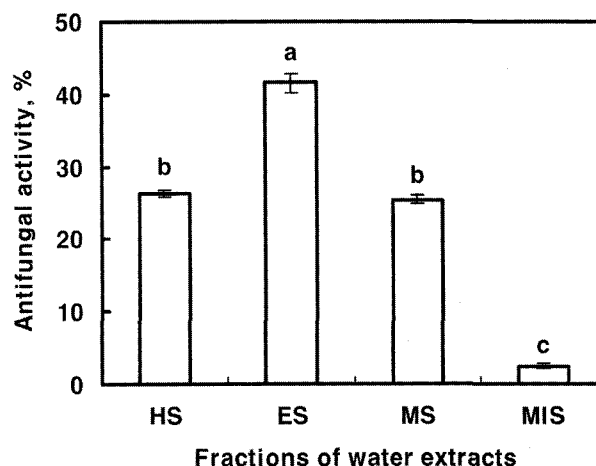


Fig. 5. Antifungal activity of various fractions separated from water extracts against *L. edodes*. The PDA medium plates with 1,000 ppm of water extracts were incubated for 10 days 4 weeks at 25°C HS, *n*-hexane-soluble fraction; ES, ethyl acetate-soluble fraction; MS, methanol-soluble fraction; MIS, methanol-insoluble fraction. The vertical bars represents the standard errors of individual means. The bars followed by the same letter are not significantly different at $P < 0.05$ according to the Duncan's new multiple range test.

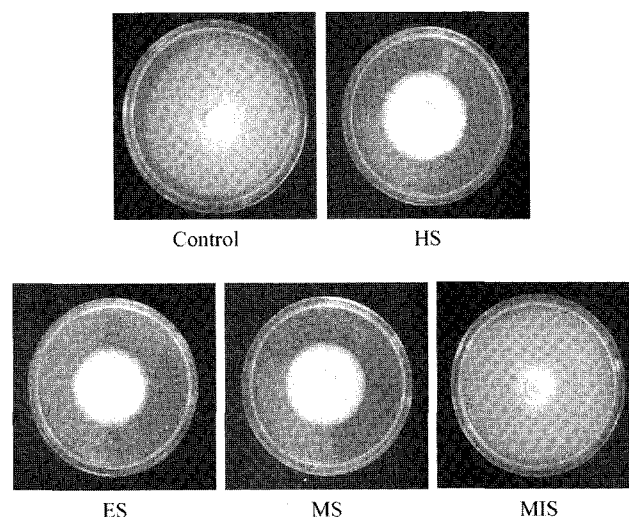


Fig. 6. Antifungal activity against *L. edodes* mycelium in various fractions extracted from *P. densiflora* sawdust. The PDA medium plates with 1,000 ppm water extracts were incubated for 10 days 4 weeks at 25°C HS, *n*-hexane-soluble fraction; ES, ethyl acetate-soluble fraction; MS, methanol-soluble fraction; MIS, methanol-insoluble fraction.

than the ethyl acetate-soluble fraction in addition to the inhibitory compounds.

L. edodes mycelial growth in the different organic solvent fractions of water extracts are presented in Fig. 6. The control medium showed that mycelium had a mean diameter of 8.0 cm

Table 1. Separation of antifungal compounds by TLC in ethyl acetate fractions.

Fraction	Solvent (v/v)	Compound	R_f value
Ethyl acetate	<i>n</i> Hexane:Ethyl acetate = 2:1	EI	0.79 0.98
		EII, EIII	0.48 0.81
		EIV	0.08 0.52

and homogeneous, equal growth form. At ethyl acetate-soluble fraction contained medium, the mycelial colony was smaller than that of the extract non-treatment medium and had mean diameter of 3.3 cm. In presence of *n*-hexane-soluble-fraction and methanol-soluble fraction, the diameter of mycelial colony became more decreased than that of the extract non-treatments. At methanol-insoluble fraction, diameter of mycelial growth was slightly lower than that of the extract non-treatments.

The methanol-soluble fraction may be contained catechin and cis-isomeric epicatechin which are the main components in the methanol extractives of bark of pine wood (Fengel & Wegner, 1983). The methanol-insoluble fraction in the same concentration showed a weak inhibitory effect. Nakajima *et al.* (1980) reported that the methanol, *n*-hexane and ethyl ether extracts from the inner bark and sapwoods of *C. japonica* showed inhibition against the mycelial growth of *L. edodes*. It was supposed that the inhibitory compounds of the ethyl acetate-soluble fraction from the powdered water extracts for the mycelial growth of *L. edodes* could be extracted much more using the ethyl acetate of middle polar solvent than the *n*-hexane of lower polar solvent or methanol of higher polar solvents. Further works is needed to determine compounds of the ethyl acetate-soluble fraction separated from the water extracts.

Separation of antifungal active compounds

The TLC was performed using mixtures of *n*-hexane and ethyl acetate (v/v) in 2:1. The separated fractions were detected under ultraviolet light at 254 nm and then the values of R_f were measured from fraction (Table 1). The ethyl acetate fractions which shows high antifungal active fraction, were separated to four compounds. These basic fractions (I, II and III) in ethyl acetate fraction were then determined growth inhibition against the *L. edodes*. Among the three fraction, fraction II was high in antifungal activity (Data not shown).

As further study, we make a plan the elucidation of antimicrobial compounds from *P. densiflora* sawdust. In this results, it is reasonable to expect that valuable and alternative of chemical synthetic antifungal compound. Also, it is softwood tree logs, especially sawdust which removed the antifungal com-

pounds can be result in higher mushroom production efficiency and could develop into an economical and cost-effective method of *L. edodes* cultivation.

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