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Extracts from *Pinus densiflora* Siebold et Zuccarini Sawdust Inhibit the Mycelial Growth of *Lentinus edodes* (Berk.) Sing., Edible Mushroom

Seong Cheol Hong*, Myung Suk Choi*, Bu Kug Lim**, Jong Yoon Lee**,
Danny L. Rinker***, and Jae Kyung Yang*†

*Faculty of Forest Sci. & Institute of Agriculture & Life Sci., Gyeongsang Natl. Univ., Korea.
**Department of Forest Science & Technology, Kyungpook Natl. Univ., Korea.
***Department of Plant Agriculture, University of Guelph, Ontario, Canada.

ABSTRACT : The extracts of *Pinus densiflora* sawdust by *n* hexane, ethyl acetate and methanol solvent were investigated to identify their mycelial growth inhibition against *Lentinus edodes*. The yields of n hexane soluble fraction, ethyl acetate-soluble fraction, and methanol soluble fraction from *P. densiflora* sawdust were obtained 1.36%, 2.21% and 4.03% using organic solvent, respectively. The mycelial growth inhibition of *L. edodes* was the greatest for *n* hexane extract, ranging from 36.5% to 47.6% at concentrations of 125 ppm to 1,000 ppm, with the values for all concentrations significantly different from one another. After direct extraction of *P. densiflora* sawdust using n hexane, ethyl acetate and methanol, each extract was separated into three fractions by silica gel column chromatography and then the fractions were isolated on the values of R_f by thin layer chromatography. The mycelial growth inhibition against *L. edodes* was recognized in the fractions II (33.5%) and III (37.6%) of *n* hexane extract, the fraction II (21.4%) of ethyl acetate extract and the fraction II (26.4%) of methanol extract. The fractions III of *n*-hexane extract showed the highest growth inhibition among the nine fractions of the organic solvent extract.

Key words : extracts, *Pinus densiflora*, sawdust, mycelial growth inhibition, *n* hexane

INTRODUCTION

Lentinus edodes (Shiitake) mushroom is important in Asian countries including China, Japan, Taiwan, and Korea. *L. edodes* mushroom is the second most popular edible mushroom on the global market. This is attributed to its nutritional value and the possibility of its medical application (Jong & Nirmingham, 1993). Today, *L. edodes* mushroom is consumed not only for its culinary value but also for its medicinal anti-tumor properties. Some compounds of softwood that are inhibitory to mycelial growth must be removed in order to utilize the softwood for the cultivation of *L. edodes*, and the removal method should be duly considered. Extracts from the pine tree contain essential oils (α-pinene, β-pinene, camphene, limonene and borneol), turpentione, rosin, tar, ascorbic acid, anthocyan, flavonoid, choline, tall oil, fatty acid and tannin (Kawachi *et al.*, 1991; Matsui *et al.*, 2001) which are fungistatic. Matsui *et al.* (2001) reported that extracts from sawdust of *P. densiflora* inhibited mycelial growth of *L. edodes* and that these inhibitory components could be removed by methanol extraction. They suggested that the antifungal activity of the mycelial growth of *L. edodes* was probably due to terpenoids, a major component of the extracts of *P. densiflora*, and possibly to synergism with another inhibitory compound, cedrol. Removal of inhibitory

† Corresponding author : (Phone) +82-55-751-5504 (E-mail) jkyang@gshp.gsnu.ac.kr
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compounds against mycelial growth of *L. edodes* is a potential means mushroom cultivation by using softwood. For example, sawdust of the softwood has been treated by extraction using cold water, hot water, steam and organic solvent to reduce the inhibitory compounds in the extracts of the 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). Although mushroom cultivation by various treatment of softwood has recently been studied extensively, reports on the *P. densiflora* have been few so far. The Korean forest locations are found in three major zones; the warm-temperate forest, the cool-temperate forest and the sub-frigid forest. Among the forests, *Pinus* sp. is distributed entire three zone of Korea. Thus, removal of growth inhibitory factors from *P. densiflora* is very attractive strategy for mushroom cultivation and multiple uses of logged and wasted woods. The present study was undertaken to determine inhibitory compounds on the mycelial growth of *L. edodes* from the extracts of *P. densiflora* sawdust.

MATERIALS AND METHODS

Plant material and mycelial strain

Pinus densiflora sawdust, which contained bark, was kindly produced from the Forest Research Institute, in Seoul, Korea. *Pinus densiflora* sawdust was collected in the 2002 spring and was stored in an enclosed building until it was used. The moisture content of the sawdust air-dried was approximately 10~13% by weight. The sawdust was screened to a 10~60 mesh size for extraction.

Strain of *Lentinus edodes* (Berk.) Sing used in this study were FRI Sanlim No. 5, a stock culture of the Forest Research Institute, in Seoul, Korea. The stock culture was maintained on potato dextrose agar (PDA) medium at 4℃. Mycelium of *L. edodes* was sub cultured on the PDA medium for 2 weeks at 25℃.

Extraction and isolation compounds

Organic solvent extractions were taken directly from the *P. densiflora* sawdust using *n*-hexane, ethyl acetate and methanol. For each organic solvent extraction from *P. densiflora* sawdust, a 300 g sample was mixed in 2 ℓ of the respective organic solvent and shaken at 120 rpm for 12 hours at 21~25℃. Supernatant from the mixture was filtrated by the filter paper (Whatman No. 2) and 50 mℓ of the supernatant was concentrated. Finally, concentrated solutions of *n* hexane extract, ethyl acetate extract and methanol extract were stored in −5℃ cold room. Yields of the organic solvent extracts were obtained by measuring the weight of the soluble parts. The yields were expressed as percentage of the oven dried weight extracts per oven dried weight of the non-extracted sawdust. Five replicates for each yield were used.

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 F_{254}, 0.5mm, Merck). The silica-gel column (6.0 ㎝×60.0 ㎝) 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 was performed using mixtures of *n*-hexane and ethyl acetate (v/v) in 1:1 and 2:1. The TLC plate with the 1:1 *n*-hexane to ethyl acetate was used for the chromatography of fractions from the *n*-hexane extract and methanol extract, and the plate with the 2:1 *n*-hexane to ethyl acetate for the chromatography of the fractions from ethyl acetate extract. The separated fractions were read under ultraviolet light at 254 ㎚ and then the values of R_f were measured from each fraction. The *n*-hexane, ethyl acetate and methanol fractions were divided on the values of R_f by the TLC for each of the organic solvent extracts. And then, the separated fractions of *n*-hexane, ethyl acetate and methanol were regrouped

into three basic fractions (I, II and III) for each extract. These basic fractions (I, II and III) were then determined growth inhibition against the *L. edodes*.

Growth inhibition against mycelial of *L. edodes*

Growth inhibition against *L. edodes* mycelium was represented antifungal assay. The organic solvent extracts and the fractions of the organic solvent extracts were dissolved by organic solvents and added to potato dextrose agar (PDA, Difco) before autoclaving. The same amounts of organic solvents in the PDA medium were applied for controls. All extracts from *P. densiflora* sawdust were used as an ingredient in the PDA medium for the evaluation of the growth inhibition against *L. edodes*. The growth inhibitions of the organic solvent extracts were tested as concentrations of 125, 250, 500, 750 and 1,000 ppm. And the growth inhibitions of the fractions of the organic solvent extracts were tested on 1000 ppm. The PDA was poured into petri dishes (20 mℓ/plate), and a 10 ㎜ diameter plug of *L. edodes*, excised from the actively growing edge in the PDA medium, was seeded at the center of PDA plate containing extracts. All cultures were inoculated at 25℃ for 10 days, by which time the growth of the control would have reached the edge of the plate. The diameter of the colonies on each plate was measured daily for 10 days. The growth inhibition of mycelium was calculated as the percentage of inhibition of radial growth relative to the control. Five replicates for test of growth inhibition were used.

Statistical analysis

Data were subjected to analysis of variance and Duncan's new multiple range test (Aidoo, 1981) using Proc Glm of SAS (Statistical Analysis System, ver. 6.12) to determine whether significant differences (P=0.05) existed between mean values of treatments and control.

RESULTS AND DISCUSSION

Growth inhibition against mycelium of *L. edodes* of the organic solvent extracts

The yields of *n*-hexane-soluble, ethyl acetate-soluble and methanol-soluble fractions from *P. densiflora* sawdust were obtained 1.36%, 2.21% and 4.03% using organic solvents, respectively (Table 1).

Table 1. Yield of extracts of *Pinus densiflora* sawdust by different organic solvents

Solvents	Yield of extracts† (%)
n-Hexane	1.36±0.12 c*
Ethyl acetate	2.21±0.08 b
Methanol	4.03±0.17 a

† Each value represents mean±standard deviation (n=5).
* Means followed by the same letter within the same column are not significantly different (P<0.05) according to Duncan's new multiple-range test.

The growth inhibition of *L. edodes* mycelium by *n*-hexane extract from *P. densiflora* sawdust at concentrations 125, 250, 500, 750, and 1,000 ppm were 36.5%, 41.2%, 43.1%, 45.4% and 47.6 %, respectively (Table 2). The growth inhibition of mycelium was the greatest for *n*-hexane extract, ranging between 36.5% and 47.6% at concentrations of 125 ppm to 1000 ppm, with the values for all concentrations significantly different from one another. The values for each concentration were significantly different for methanol as well. However, no significant differences in the growth inhibition of mycelium were observed for ethyl acetate from 500 ppm to 1,000 ppm.

Table 2. Inhibition of *Lentinus edodes* mycelial growth by organic solvent extracts from *Pinus densiflora* in the PDA medium at 25℃ for 10 days

Concentration of extracts (ppm)	Inhibition of growth† (%)		
	n-Hexane extracts	Ethyl acetate extracts	Methanol extracts
125	36.5±1.5 e*	27.0±1.7 c	25.2±0.4 e
250	41.2±1.0 d	38.3±0.4 b	35.7±0.4 d
500	43.1±0.4 c	39.3±0.8 a	37.2±0.7 c
750	45.4±0.6 b	38.5±0.5 a	41.6±1.0 b
1,000	47.6±0.8 a	38.9±0.4 a	44.6±0.3 a

† Inhibition of mycelial growth represents percentage of control and each value represents mean ± standard deviation (n=5).
* Means followed by the same letter within the same column are not significantly different at P<0.05 according to Duncan's new multiple-range test.

The growth inhibition of mycelium of the extracts from *n*-hexane, ethyl acetate and methanol at 125 ppm were 36.5%, 27.0% and 25.2%, respectively. The methanol extract at 125 ppm showed the lowest growth inhibition of mycelium at 25.2%, but increased proportionally to 44.6% at 1,000 ppm.

It was thought that the growth inhibition against *L. edodes* mycelium would not be influenced by the different extraction methods because the content and sorts of extracts were different depending on the organic solvent. In this experiment, the growth inhibition by *n*-hexane extract was the highest. This result supposed that the *n*-hexane extract has not only many more inhibitory compounds against *L. edodes* than the ethyl acetate and the methanol extracts but also the inhibitory compound may be more easily dissolved by *n*-hexane than by ethyl acetate or methanol.

Growth inhibition against mycelium of *L. edodes* of the fractions

The fractions were isolated from each solvent by silica-gel column chromatography and then the fractions were confirmed and divided on the values of

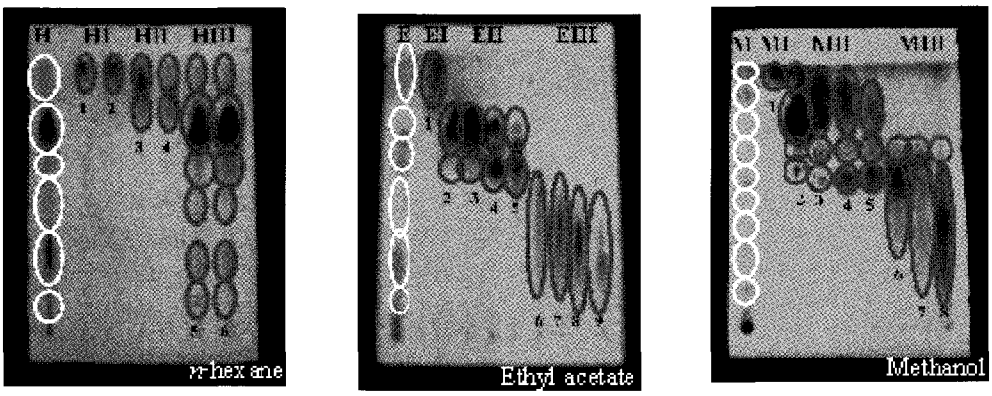

Fig. 1. Separation of compounds from organic-soluble fractions by column chromatography (column: 6.0 cm×60.0 cm, silica gel 60: 2,000 g) and thin layer chromatography (TLC plate: silica gel 60 F_{254}, 0.5 mm, MERCK). The eluent solvents for column of *n*-hexane-soluble, ethyl acetate-soluble and methanol-soluble fraction were used as 5:1, 2:1 and 1:1 of *n*-hexane: ethyl acetate. The TLC was performed using mixture of *n*-hexane and ethyl acetate (v/v) in 1:1 and 2:1. The 1:1 *n*-hexane to ethyl acetate was used for *n*-hexane extract and methanol extract and the plate with the 2:1 *n*-hexane to ethyl acetate for ethyl acetate extract.

Table 3. Separation of compound fractions by thin layer chromatography

Fraction	Solvent (v/v)	Compound number†	Range of R_f of compound	
n Hexane	*n*-Hexane:Ethyl acetate	HI (1, 2)	0.85	1.00
	= 1:1	HII (3, 4)	0.72	1.00
		HIII (5, 6)	0.07	1.00
Ethyl	*n*-Hexane:Ethyl acetate	EI (1)	0.79	0.98
acetate	= 2:1	EII (2, 3, 4, 5)	0.48	0.81
		EIII (6, 7, 8, 9)	0.08	0.52
Methanol	*n*-Hexane:Ethyl acetate	MI (1)	0.89	1.00
	= 1:1	MII (2, 3, 4, 5)	0.51	1.00
		MIII (6, 7, 8)	0.09	0.72

† H I, H II and H III, fractions of two (No. 1 and 2), two (No. 3 and 4) and two (No. 5 and 6) compounds of six from *n*-hexane extraction, respectively: E I, E II and E III, fractions of one (No. 1), four (No. 2, 3, 4 and 5) and four (No. 6, 7, 8 and 9) from ethyl acetate extracts, respectively: M I, M II and M III, fractions of one (No. 1), four (No. 2, 3, 4 and 5) and three (No. 6, 7 and 8) compounds from methanol extracts, respectively.

R_f by thin layer chromatography (Fig. 1). The fractions were produced six band of *n*-hexane, nine band of ethyl acetate and eight band of methanol extract. The *n*-hexane, ethyl acetate and methanol fractions were used for their antifungal activity against *L. edodes* after being regrouped into three basic fractions (I, II and III) for each extract (Table 3).

The growth inhibition against *L. edodes* mycelium of the fractions HI (compound number: 1 and 2), HII (compound number: 3 and 4) and HIII (compound number: 5 and 6) of the *n*-hexane extract were 19.4%, 33.5% and 37.6%, respectively; the effects of the fractions EI (compound number: 1), EII (compound number: 2, 3, 4 and 5) and EIII (compound number: 6, 7, 8 and 9) of the ethyl acetate extract were 5.9%, 21.5% and 10.5%, respectively; and the effects of the fractions MI (compound number: 1), MII (compound number: 2, 3, 4 and 5) and MIII (compound number: 6, 7 and 8) of the methanol extract were 5.6%, 26.4% and 11.2%, respectively, at 1,000 ppm (Figure 2). The *n*-hexane III fraction showed the highest growth inhibition against *L. edodes* mycelium among the nine fractions of the organic solvent extracts.

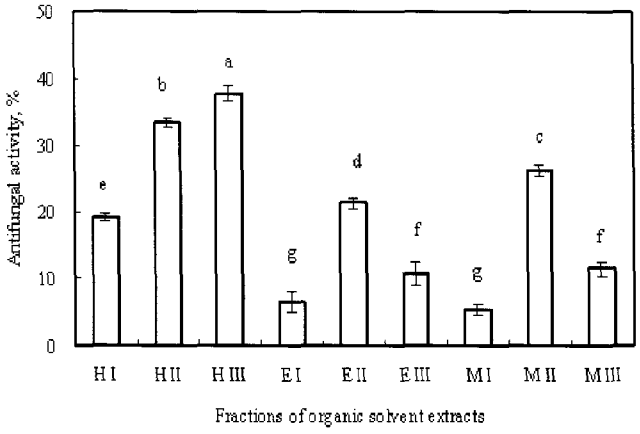

Fig. 2. Growth inhibition by each fraction of organic solvent extracts (1,000 ppm) from *Pinus densiflora* on the mycelial growth of *Lentinus edodes* on PDA medium after 25℃ for 10days. The vertical bars represent the standard errors of individual means. Bars followed by the same letter are not significantly different at P<0.05 according to Duncan's new multiple range test.

In the results of our study, the HIII fraction from *n*-hexane extract showed the highest growth inhibition against *L. edodes* mycelium (Fig. 3). In general, the *n*-haxane-soluble compounds from *P. densiflora* were contained non-polar compounds such as essential oil etc. Terpenoids is considered to be involved in sawdust of *P. densiflora*. Nakajima *et al.* (1980) suggested that the antifungal activity for mycelial growth of *L. edodes* was probably due to a synergistic effect of terpenoids. Matsui *et al.* (2001) also reported that the terpenes such as ferruginol, suginol, sandaracopimarinol, sandaracopimarinal, phyllocladanol, and β-sitosterol, can be obtained from methanol extracts of *Cryptomeria japonica*. The antifungal activity observed by *Pinus densiflora* softwood extracts was probably caused by a synergistic effect of various monoterpenes and sesquiterpenes. In near future, further work should be needed to find out precise mechanism, and identification of bioactive compound. Additionally, the applications of mushroom cultivation should be developed.

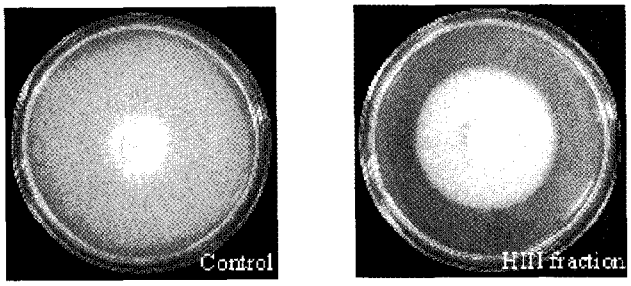

Fig. 3. The growth inhibition of HIII fractions from *n*-hexane extract against the *L. edodes*.

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