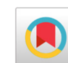
J Appl Biol Chem (2017) 60(3), 213–218
https://doi.org/10.3839/jabc.2017.034



Online ISSN 2234-7941
Print ISSN 1976-0442

Article: Bioactive Materials

Antifungal activity of pinosylvin from *Pinus densiflora* on turfgrass fungal diseases

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Received: 11 May 2017 / Accepted: 10 July 2017 / Published Online: 30 September 2017
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Abstract The objective was to examine the antifungal activity of *Pinus densiflora* extract for the control of turfgrass fungal diseases. Antifungal activities of the various fractions of *n*-hexane, methylene chloride (Ch), ethyl acetate (EtOAc), and *n*-butanol from *P. densiflora* were evaluated against *Rhizoctonia solani* AG1-1B*, R. solani* AG2-2IV*, Sclerotinia homoeocarpa*, *R. cerealis*, *Pythium* spp., and *Colletotrichum graminicola*. The Ch and EtOAc fractions showed antifungal activity against *Pythium* sp. and *C. graminicola* in paper disc assay. The effective concentration to produce 50% mycelial inhibition (EC_{50}) using five discriminatory concentrations of pinosylvin (**1**) from the Ch fraction of *P. densiflora* was evaluated on *R. solani* AG1-1B*, R. solani* AG2-2IV*, R. cerealis,* and *S. homoeocarpa*. *S. homoeocarpa* showed the highest sensitivity with the lowest mean EC_{50} value (8.426 μg/mL) among the four pathogens. Among the three *Rhizoctonia* pathogens*, R. cerealis* had the highest mean EC_{50} value (99.832 μg/mL) and *R. solani* AG2-2IV, with the lowest sensitivity, had the lowest EC_{50} value (39.696 μg/mL). These results suggested that pinosylvin (**1**) from *P. densiflora* could be a valuable lead compound in the improvement of a novel antifungal agent.

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Keywords Antifungal activity · Constituent · Pathogen · Pinosylvin

Introduction

The conversion of land across Korea into turfgrass has increased. These areas have been developed to become residential places, leisure parks and golf courses (Lee 2002). According to their temperature tolerance and photosynthetic strategies, turfgrasses are grouped into two type species; C4 or C3 species (Beard 1973; Turgeon 2005). Turfgrass is the most popular plant resource used by humans for glare reduction and recreational function, its uniform green color valued for either purpose (Lee 2002). Owing to its well-developed shoot and root systems, turfgrass both contribute benefits to human for recreation and the soil and ecosystem, having the potential to partially offset atmospheric carbon dioxide increase (Qian and Follett 1994; Huh *et al.* 2008).

The cool-season turfgrass species have been using in golf courses fairways and putting greens in temperate regions. The cool-season pathogens including *Rhizoctonia solani*, *Sclerotinia homoeocarpa*, *Rhizoctonia cerealis*, *Pythium* spp., and *Colletotrichum graminicola* are responsible for the disease in cool-season turfgrass species. The diseases are very destructive, even mild outbreak ruins the uniformity, appearance, and quality of turf surface. Diseases have also affected golf course management like Dollar spot caused by *Sclerotinia homoeocarpa* and *Rhizoconia* spp. in which more money is being allocated compared to other turfgrass diseases in USA (Vargas 1994). The disease of turfgrass has been managed with traditional cultural practices and fungicides. However, increasing fungicide resistance promises new management strategies (Golembiewski *et al.* 1995; Burpee 1997).

Pesticides have a wide range of applications to golf course, residential lawns, and the sports grounds to improve the turfgrass quality. Increased interest in the environmental effects of such chemical application to turfgrass field is because of the public's

increased attention for the environment (Vargas 1994).

The increased concern highlights the need for developing alternative methods of selective species control. In the search for more cost-effective, efficacious, selective, and environmentally safe pesticides, new strategies for discovery are being utilized. This trend partly is due to diminishing returns of traditional synthetic organic pesticides and screening methodologies and also due to the promise of more rational methods to find new pesticide chemistries (Duke and Lydon 1987). Industry is becoming aware that many natural compounds have good potential as commercially successful pesticides.

Pinus densiflora (Japanese red pine) is geographically located in countries of East Asia including China, Japan and Korea (Kim and Chung 2000). Anti-microbial, anti-nociceptive, anti-inflammatory, and anti-bacterial activities of *P. densiflora* extract have been reported (Choi 2007; Sultan *et al.* 2008; Lee *et al.* 2009; Jeong *et al.* 2014).

This study aims to investigate the antifungal activities of *P. densiflora* extract against *Rhizoctonia solani* AG1-1B, *R. solani* AG2-2IV, *Sclerotinia homoeocarpa, R. cerealis, Pythium* spp., and *Colletotrichum graminicola,* with the aim of developing a cost effective and environmentally friendly protective system.

Materials and Methods

Plant materials

Pinus densiflora, Japanese red pine, obtained from the Korean herbal market Samhong Medicinal Herbs, Seoul, Korea. A voucher specimen of the plant was kept at our department, Korea.

Apparatus and reagents

^{1}H-nuclear magnetic resonance (NMR) spectra were checked with a Bruker AVANCE 400 NMR (Rheinstetten, Germany) spectrometer in CD_3OD using TMS, an internal standard. Solvents such as methanol (MeOH), *n*-hexane, methylene chloride (Ch), ethyl acetate (EtOAc), and *n*-butanol (*n*-BuOH) were supplied by SamChun Pure Chemical Co. (Pyeongtaek, Korea). TLC plate (silica gel, 0.25-mm layer thickness) was obtained from Merck (Darmstadt, Germany). Compound checking was visualized by spraying with 10% H_2SO_4. Medium pressure liquid chromatography (MPLC) (Biotage, Uppsala, Sweden) equipped with cartridges (KP-SIL, 39×225 mm) was used. Analytical grade of other chemicals and reagents were used. High performance liquid chromatography (HPLC) chromatograms were checked on a Waters 1525 Binary HPLC Pump (Miami, FL, USA) equipped with a Waters 2489 UV/VIS detector (Miami, FL, USA).

Sample preparation

The dried and powdered leaves of *P. densiflora* were refluxed with MeOH at 65-75 °C. The filtrate was concentrated *in vacuo*. Other extracts of *Pinus* species such as *P. rigida*, *P. banksiana*, *P. parviflora, P. strobus, P. koraiensis*, *P. densiflora* for. *multicaulis*, and *P. thunbergii* were obtained from the Plant Extract Bank of Korea Research Institute of Bioscience and Biotechnology in Deajeon, Korea.

Extraction, fractionation, and identification

Dried stems (500 g) were extracted with MeOH (2.5 L×3) under reflux. The extracts were evaporated to produce a brown residue. The residue dissolved in H_2O (1 L) was partitioned successively with *n*-hexane (1 L×3), Ch (1 L×3), EtOAc (1 L×3), and *n*-BuOH (1 L×3). Among these, the Ch fraction was subjected to MPLC eluted with an *n*-hexane/EtOAc gradient (100:0 0:100). Fractions were mixed based on the pattern results of TLC (PDC-1 PDC-20). Sub-fraction PDC-13 was recrystallized in MeOH to give compound **1**.

Compound **1**: EI-MS *m/z*: 212 $[M]^+$ (100), 197 (12.8), 183 (7.4), 165 (26.6), 141 (10.9), 128 (7.1), 106 (3.1), 82 (4.7), 69 (4.2), 51 (3.1); ^{1}H-NMR (CD_3OD, 500 MHz) δ 7.38 (d, J=8.6 Hz, H-2' and -6'), 6.99 (d, J=16.3 Hz, H-8, vinyl), 6.84 (d, J =16.3 Hz, H-7, vinyl), 6.74 (d, J=8.6 Hz, H-3' and -5'), 6.57 (d, J=8.6 Hz, H-2 and -6), 6.39 (t, H-4); ^{13}C-NMR (CD_3OD, 50.3 MHz) δ 158.8 (C-3), 158.5 (C-5), 139.7 (C-4'), 137.5 (C-1), 128.8 (C-1'), 128.8 (C-8), 128.4 (C-2' and -6'), 127.4 (C-7), 126.3 (C-3' and -5'), 106.5 (C-4) 103.8 (C-2 and C-6).

HPLC condition

The analysis of compound **1** was subjected to reverse phase HPLC system. Waters Spherisorb 5 ìm ODS2 (4.6 mm×250 mm, 5 µm thickness) column was used, with a mobile phase consisting of 0.2% acetic acid in water and MeOH. The gradient solvent system was 60:40 and increased in linear gradients to 40:60 for 40 min. And then to 0:100 for 45 min, and finally, to 60:40 for 65 min. UV detection was measured at 330 nm and the flow rate was 1 mL/min. The injection volume was 10 µL and all injections were done three times.

Limit of detection (LOD) and limit of quantification (LOQ)

LOD and LOQ were determined to validate compound **1**. The linearity of the method was made by injecting three times at 0.005–1 range. Different concentrations (1.0, 0.5, 0.25, 0.125, 0.06, 0.03, and 0.005 µg/mL) were prepared to have calibration standard solutions. The calibration curve was consisted of Y axis (linear regression of the peak area-ratios) and X axis (concentration in mg/mL). Signal to noise ratio of 3 for LOD and 10 for LOQ was used to confirm their values.

Collection of fungal pathogens

The antifungal activity of *P. densiflora* extract was investigated against six turfgrass pathogens (*R. solani* AG1-1B*, R. solani* AG2-2IV*, S. homoeocarpa, R. cerealis, Pythium* spp. and *C. graminicola*). The previously isolated fungal species*, R. cerealis, Pythium* spp. and *C. graminicola* were collected from Turfgrass

Environment Research Institute Gyeonggi, South Korea. *S. homoeocarpa* was isolated from the diseased turfgrass leaves showing typical symptoms of dollar spot in BlueOne Sangju Country Club, Sanju, South Korea. Sodium hypochlorite (3%) was used to disinfect diseased leaf tissue surface for 1 min, and was washed with sterile water. Then, it was transferred on acidified potato dextrose agar with 85% lactic acid (0.75 mL) (Fisher Scientific, Fair Lawn, NJ, USA) for everyone liter of potato dextrose agar (PDA) (Difco Laboratories, Detroit, MI, USA) after autoclaving (Chang and Lee 2013). The plates for cultures were incubated at 25 °C. *S. homoeocarpa* was purified by making repeated hyphal tip transfers and stored as stock cultures (Chang and Lee 2013).

R. solani AG1-1B and *R. solani* AG2-2IV were isolated from the diseased Zoysia grass (*Zoysia matrella*) leaves showing typical symptoms of brown patch and large patch disease in Anseong Benest Golf Club and Golf Club Q Anseong, Korea, respectively. Isolation was conducted by transferring fragments of infected leaves and sheath tissues to selective medium plates (Chang and Lee 2013) and incubating in the dark at 25 °C. Pure cultures of *R. solani* were achieved by transferring hyphal tips in PDA and incubating in the dark condition at 25 °C for 48 h. The isolated fungal species were identified at species level by analyzing morphology and DNA sequence data of internal transcribed spacer (ITS) in 5.82 rDNA region using ITS1 and ITS4 primers.

Antifungal activity using paper disc method

Anti-fungal activity assay was done in a solid media containing PDA (20 mL) by disc diffusion method (Paik *et al.* 1998; Duru *et al.* 2003). Sterile 8-mm diameter Whatman paper discs was placed near the side of borders in the agar plates, where the extract at 5 mg/50 µL was used separately, and dried at room temperature for 30 min. A disc of 8-mm diameter fungal inoculum was removed from pre-grown fungal strain cultures, and positioned upside down in the center of the Petri dish. The disc was incubated at 25 °C for 6 days.

Inhibition concentration of relative mycelial growth by 50% (EC_{50} values)

Effective concentration (EC_{50} values) four fungal pathogens species were determined as described by Detweiler *et al.* (1983) and Golembiewski *et al.* (1995). Plugs of 5 mm in diameter, contain actively growing mycelium of each fungal species were transferred to PDA amended with compound **1** and in addition to non-amended PDA as control. PDA amended by using serial dilutions of compound **1** at 10, 50, 100, 250, and 500 µg/mL concentrations.

Inoculum under sterile conditions, agar plugs of 5-mm diameter with mycelia of *R. solani* AG1-1B (brown patch), *R. solani* AG2-2IV (large patch), *R. cerealis* (yellow patch) and *S. homoeocarpa* (dollar spot) were cut from the edge of 1-4 day old cultures using a sterile cork borer. After inverting each plug, the mycelium contacting the media surface the mycelium side placed in the center of PDA plate. This experiment was conducted with five replicates and repeated four times. Radial mycelial growth of was measured after 60 hr of incubation in 25 °C. The percent relative mycelial growth was calculated as radial growth on compound **1** amended PDA/radial growth on non-amended PDA multiply by 100. The regression analyses of EC_{50} values of compound **1** and relative mycelial growth were performed for 10, 50, 100, 250, and 500 µg/mL concentrations. The concentration of compound **1** yielded the highest coefficient of determination (*r*) value was selected as the discriminatory concentration (Chang and Lee 2013).

Statistical analyses

Relative mycelial growth values of the isolates of *R. solani* AG1-1B, *R. solani* AG2-2IV, *R. cerealis* and *S. homoeocarpa* were used for statistical analyses. EC_{50} and ANOVA of mean relative fungal growth were calculated using SAS 9.0 Proc Probit and SAS 9.0 Proc Mixed (SAS Institute, Cary, NC, USA), respectively. EC_{50} values of compound **1** were determined from the linear regression models. EC_{50} values were qualitatively estimated for *t* test based on growth (>50% relative mycelial growth). Significant effects were considered at $P = 0.05$.

Results and Discussion

Antifungal activity of *P. densiflora*

In a previous paper (Kang *et al.* 2013), the extract of *P. densiflora* exhibited the highest degree of inhibition (> 65% at 5 mg/50 µL) on six plant-pathogenic fungi. The *n*-BuOH and *n*-hexane fractions did not demonstrate an inhibitory effect on the growth of *Pythium* spp. or *C. graminicola.* However, the EtOAc and Ch fractions formed inhibition zones larger than 10 mm in the growth of *Pythium* spp. and *C. graminicola*. Among them, the Ch fraction exhibited the greatest antifungal activity against *Pythium* spp. and *C. graminicola* (Fig. 1).

HPLC analysis of pinosylvin (1)

Compound **1** was confirmed as pinosylvin (Fig. 2) by comparison of the previous data (Schultz *et al.* 1992). Pinosylvin (**1**) from plants has significant antiviral, antioxidant, antibacterial, and antifungal functions (Schultz *et al.* 1992; Lee *et al.* 2005). Content of pinosylvin (**1**) in *Pinus* species was conducted using HPLC analysis. After identifying the peak of pinosylvin (**1**) in HPLC chromatograms at concentrations of 500 µg/mL (330 nm), the calibration equations, linear range, and correlation factors (r^2) were shown in Table 1. The content of pinosylvin (**1**) in stem and root of *P. densiflora* was checked at 0.4286 and 0.3544 mg/g extract, respectively. In the chromatograms of *Pinus* species, pinosylvin (**1**) showed a wide range of 0.0000 and 0.4286 mg/g extract. There were no peaks corresponding to pinosylvin (**1**) in

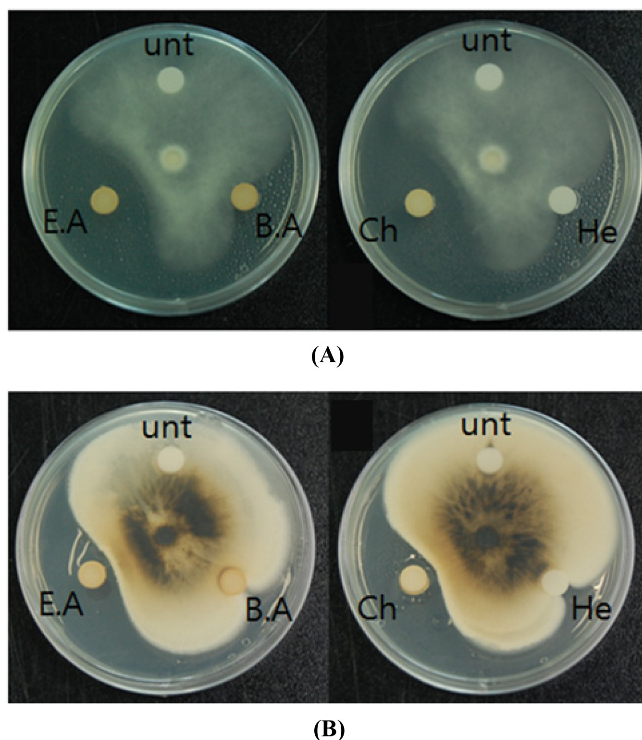

Fig. 1 Inhibition of mycelium growth of *Pythium* spp. (**A**) and *C. graminicola* (**B**) on the medium supplemented with the solvent fractions (E.A: ethyl acetate fraction; B.A: butanol fraction; Ch: methylene chloride fraction; He: hexane fraction) of Japanese red pine

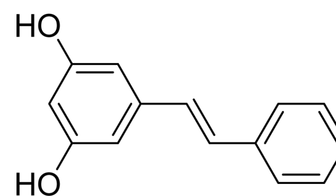

Fig. 2 Structure of pinosylvin (**1**)

Table 2 Contents of pinosylvin (**1**) in *Pinus* species

Scientific Name	Part	Pinosylvin (**1**) (mg/g extract)
Pinus densiflora	Leaf	0.4286±0.0613
	Stem bark	0.3544±0.0333
P. rigida	Leaf	0.0136±0.0010
	Stem	ND
P. banksiana	Leaf	0.0026±0.0018
	Stem	ND
P. parviflora	Leaf	0.3490±0.0524
	Stem bark	0.0104±0.0020
P. strobes	Leaf	0.2552±0.0182
	Stem bark	0.0021±0.0007
P. koraiensis	Leaf	0.1528±0.0307
	Stem bark	0.0759±0.0112
P. densiflora for. *multicaulis*	Leaf	0.2197±0.0016
	Stem	0.1383±0.0266
P. thunbergii	Leaf	0.3728±0.0284
	Stem	0.1392±0.0203

Data are represented as the mean ± SD (n =4) in mg/g of the MeOH extracts of samples
ND: Not detected

the chromatograms of *P. rigida* (stem) and *P. banksiana* (stem) (Table 2). The abundance of pinosylvin (**1**) in *P. densiflora* (leaf, 0.4286 mg/g extract) was the highest among the *Pinus* species extracts. The range of LOD and LOQ values are from 0.049 to 0.175 μg/mL, respectively (Table 3). We analyzed pinosylvin (**1**) in various *Pinus* species using HPLC. The quantitative method could be used for rapid evaluation of the quality control of pinosylvin (**1**) in *Pinus* species.

Inhibition concentration of relative mycelial growth

EC_{50} was estimated with the five discriminatory concentrations of pinosylvin (**1**). EC_{50} values of each fungal pathogen species isolates were significantly different (Fig. 3). Mean EC_{50} value was lowest for *S. homoeocarpa* (8.426 μg/mL) among four pathogens (Table 4). In case of three *Rhizoctonia* pathogens, mean EC_{50} value based on relative mycelial growth was highest for *R. cerealis* (99.832 μg/mL) and *R. solani* AG2-2IV (39.696 μg/mL) having the lowest sensitivity(Table 4).

The use of EC_{50} values were effective means for checking predictions about fungicide efficacy and insights about the potential development of fungicides in the future (Jo *et al.* 2006). It was a valuable method for screening of *S. homoeocarpa, R. solani* AG1-1B, *R. solani* AG2-2IV, and *R. cerealis* collected from golf courses. The consistency of the fungicide in both *in vitro* sensitivities and efficiency in the field may be suggested by the population dynamics of *S. homoeocarpa* and the mode of action of this fungicide (Jo *et al.* 2006). Pinosylvin (**1**) is considered a stilbenoid found in nature and commonly located in leaves and heartwoods

Table 1 Linearity of standard curves of pinosylvin (**1**)

Compound	Linear range (μg/mL)	Linear regression equation Y =ax+b		Correlation coefficient (r^2)
		Slope (a)	Intercept (b)	
Pinosylvin (**1**)	0.005-1	23,143	93.494	0.9994

r^2 =Correlation coefficient for thirteen data points in the calibration curves

Table 3 LOD and LOQ of of pinosylvin (**1**)

Compound	Linear range (μg/mL)	Linear regression equation Y =ax+b		Correlation coefficient (r^2)	LOD (μg/mL)	LOQ (μg/mL)
		Slope (a)	Intercept (b)			
Pinosylvin (**1**)	0.005-1	971.56	4.0136	0.9993	0.049±0.003	0.175±0.007

r^2 =Correlation coefficient for thirteen data points in the calibration curves

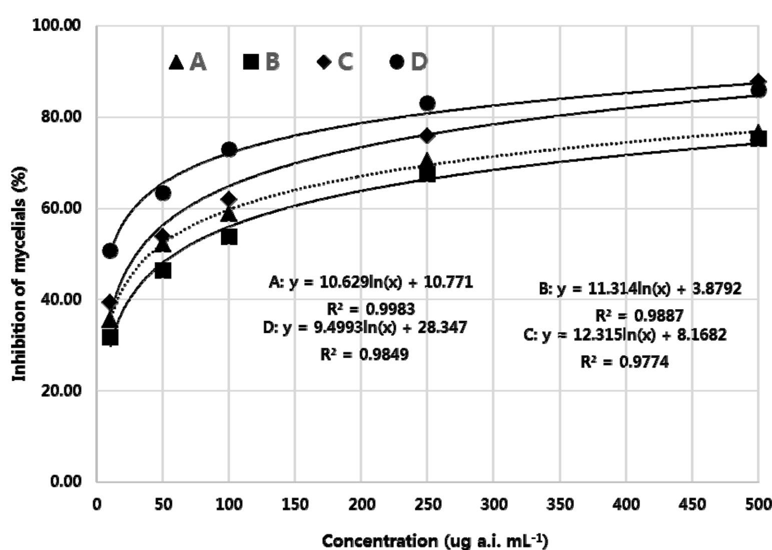

Fig. 3 Relative mycelial growth of *R. solani* AG2-2IV (A), *R. solani* AG1-1B (B), *R. cerealis* (C), and *S. homoeocarpa* (D) on PDA amended with five discriminatory concentrations of pinosylvin (**1**). RGM (Rapidly growing mycobacteria) values were collected from 72 and 120 hr (*R. cerealis* and *S. homoeocarpa*) after inoculation by dividing the mean colony diameter of PDA amended with pinosylvin (**1**) by the mean diameter of non-amended PDA

Table 4 EC_{50} and MIC of pinosylvin (**1**) on four turfgrass pathogens

Pathogens	EC_{50} (μg/mL)	MIC (μg/mL)
R. solani AG1-1B	58.814	44.601-75.318
R. solani AG2-2IV	39.696	27.621-53.244
R. cerealis	99.832	72.348-138.897
S. homoeocarpa	8.426	4.454-13.187

EC_{50} (inhibition concentration of relative mycelial growth by 50%) values were collected from 72 and 120 hr (*R. cerealis* and *S. homoeocarpa*) after inoculation by dividing the mean colony diameter of PDA amended with pinosylvin (**1**) by the mean diameter of non-amended PDA
MIC: minimum inhibitory concentration

of *Pinus* species. Stilbenoids (Pinosylvin) showed the strong antifungal activity against *Pyricularia grisea* and *Alternaria citri* (Pacher *et al.* 2002).

In conclusion, the efficacy in the field of pinosylvin (**1**) against *S. homoeocarpa* could not be determined by this study. This information can help to develop effective programs for disease control that may be applied to reduce severity of disease. The results did not completely verify fungicide efficacy in the field trials. However, the results suggest that pinosylvin (**1**) from *P. densiflora* could be a valuable lead compound in the development of a potential antifungal agent in the future.

Acknowledgments This work was supported by grants from SMBA Research Fund (No. C0038879), Republic of Korea. We thank the National Center for Inter-University Research Facilities (Seoul National University, Republic of Korea) for the measurement of spectroscopic data.

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