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Allelopathic Potential and Substances from Cork Tree (*Phellodendron amurense* Rupr.)[1]

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ABSTRACT

Allelopathic effects of the cork tree (*Phellodendron amurense* Rupr.) on several crops and soil miro-organisms were assessed using germination bioassay and antimicrobial assay, and allelochemicals were identified. In a germination bioassay, extract of cork tree inhibited at high concentration on germination of several crop seeds such as cabbage, lettuce, and cucumber. However, aqueous extracts inhibited powerfully growth of test organisms such as *Streptococus aureus*, *S. aureus*, *S. typhimurium*, and *E. coli* as bacteria, and *Candida albicans* as yeast, and *Botrytis cineria* and *Alternata alternaria* as fungi.. The cork tree extract showed strong antimicrobial activities against isolated soil fungi. The allelochemicals were separated using Silica gel, Sephadex LH-20 gel column chromatography and HPLC. The substances were analyzed by UV spectrometry and EI-mass spectrometry. The active allelochemicals were identified as isoquinoline alkaloids, berberine and palmatine.

Keywords : Allelopathy, berberine, secondary metabolites, cork tree, antimicrobial compound

1. INTRODUCTION

Plant secondary metabolites have been known to play a major role in the quality of food, flavor and dye, and insecticide (Vepoorte, 1993). Plants release a variety of secondary metabolites into the soil by way of leaf leachates, root exudates, and through decomposition of litter. Several classes of compounds such as sugars, amino acids, proteins, and organic acids were also released from plant (Prikryl and Vancura, 1980). Certain classes of these compounds were found to be allelopathic to seed germination and seedling growth (Rice, 1984). When these compounds are released into the soil environment, a number of processes may affect directly with plants (Muller, 1966). Rice (1974) reported that direct or indirect harmful effect by one plant (including microorganisms) on another through the production of chemical compounds that relaesed into the soil.

Many allelochemicals exhibit narrow host range (Cheng and Lynn, 1987). Chemical activities on microorganisms are important in determining the distribution and growth of higher plants (Rice, 1984). Production of

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phytotoxic pathogens and allelopathic effects of microorganisms on nitrogen fixer and nutrifiers are categolized here. The responsible phytotoxins reported so far are phenolics, flavonoids, alkaloids and other unidentified compounds (Chou, 1987). The purpose of this research is identifying the allelochemicals of cork tree, which affect on plant and microorganisms.

2. MATERIALS and METHODS

2.1. Plant materials

Twigs (average diameter, 2~4 cm) at 1.5 m height were collected from cork trees in their habitats on plantation of Korea Forest Research Institute (Suwon, Korea). The bark, leaves, and root were separated from twig, were pulverized using milling machine. *Chamaecyparis obtusa* was chosen as indicator on the antimicrobial species and *Populus alba* X *P. glandulosa* was used as negative controls.

2.2. Germination bioassay of allelochemicals

For germination test, averaging 10~30 seeds of cabbage, lettuce and cucumber were placed on petri dish containing 10 ml distilled water with plant extract. Concentrations of extract is 0, 1.5, 15, 150, 1,500 and 7,500 μg/ml. Deionized water was used as a control for the comparisons of extracts influence on bioassay. The plates were under fluorescent lamp 3,000 lux at 25℃ culture room. After 3 weeks, the germination rate per plate on three crops was determined.

2.3. Isolation and culture of soil microorganisms

A litter and soil under 20 year-old cork tree were ground through 20-mesh Willey mill and 100 g aliquots mixed with 100 ml distilled deionized water and allowed to imbibe at 25℃ for 24 hr. Soil and litters were then gravity and vaccum-filtered, the filtrate was cultured on microbial media. These include LB medium for bacteria, YPD medium for yeast, MEA medium for fungi culture. After 24 hr in culture, petri dish was subcultured, and then bioassayed.

2.4. Antimicrobial assay of test microorganisms

Bioassay for bacteria was done by disc diffusion method (Zaika, 1988). Test microbial stains were purchased from Genetic Resources Center, Korean Research Institute of Bioscience and Biotechnology(KRIBB). Stock cultures of test bacteria (*Streptococus aureus* IFO12732, *S. aureus* R-209, *S. typhimurium* SL1102 and *E. coli*), and yeast (*Candida albicans* KCTC7901) were inoculated onto the LB medium and YPD medium without agar, and then cultured for overnight, respectively. Culture of bacteria was centrifuged 10,000 g and then gathered microorganisms. Five hundred ml of LB and YPD medium with 1.5% agar autoclaved, cooled to 50℃, and mixed with bacteria broth. Twenty ml of the mixture was poured into a petri dish (90 cm × 20 mm) to prepare a solid agar plate, and then stored at 4℃ cold room. For antimicrobial assay, paper disc (8 mm ϕ) containing various concentrations of plant extract were put on the agar plate. After 12 hr of incubation at 37℃ overnight, the diameters of inhibitory zones were measured by using autocaliper (CD-15B, Mitutoyo d Corp., Japan). Bioassay for fungus was done by the modified method of Yin and Cheng (1998). Stock cultures of test fungus (*Botrytis cineria,* KTCC1937 and *Alternata alternaria,* KTCC6005) were inoculated onto the Malt Extract Agar

medium (MEA), and then cultured for 16 hr at 25℃. Various concentrations (0, 1.5, 15, 150, 1,500 and 7,500 μg/ml) of extracts were poured into a 96 microwell (Nunc, USA), and then air dried under 4℃ cold room. After 24 hr of inoculation, a broth of fungus was diluted with MEA liquid medium, and then added into 96 microwell with concentration at 10 μℓ. After 5 day of inoculation, the growth of fungus was measured as increase in optical density at 600 nm wavelength and visual estimation.

2.5. Extraction and identification of allelochemicals

The twigs of cork tree (dry weight, 1 kg) were powdered and then extracted with 70% MeOH for 3 times and then evaporated. The viscous concentrates were percolated with various organic solvents (hexane, CH_2Cl_2, EtOAc and 70% EtOH) and distilled water, and then antimicrobial activities were estimated.

For purification, the filterate was evaporated under *in vacuo*, and the viscous concentrate was percolated with hexane, CH_2Cl_2, EtOAc, and distilled water, successively, and aqueous phase was chromatographed on silica column using EtOAc-C_6H_6-n-PrOH-MeOH-$EtNH_2$ (4 : 8 : 2 : 1 : 1) and MeOH - 25% NH_4OH - H_2O (8 : 1 : 1), and gave five fractions (I-V). TLC (Silica gel 60 F_{254}, 0.25 mm, Merck) was done by the above solvent systems.

Antimicrobial active fraction (II, 39.2 g) was also subjected to Sephadex LH-20 column chromatography with MeOH-H_2O (8 : 2). The active fractions separated by HPLC (Dionex BioLC) system equipped with a UV detector. Separation was accomplished using μ-bondapak (300×3.9 mm) column; solvent 1 mM tetrabutylammonium phosphate (pH 2.0) : acetonitrile (40 : 60); flow rate 0.5 ml/min; wavelength 265 nm. Active fraction was collected several times and concentrated. Compound II (1.5 g) was purified by prep. TLC with the solvent system as mentioned above. Active fractions were identified by comparing its R_f values, UV spectra and co-chromatography with authentic compounds. The qualitative analysis of active compound I and II were done by EI-MS (VG 7070) under high resolution mass spectra (70 eV).

3. RESULTS and DISCUSSION

3.1. Germination bioassay

When the extract of cork tree extracts was treated to seeds in petri dish, the germination of seeds was showed variable different concentrations (Table 1). Germinated seedling was decayed and showed chlorosis after 2 weeks in culture. Extract of cork tree was showed about 50% germination of lettuce seeds at 7,500 μg/ml concentrations. Cucumber seeds was observed about 50% germination at 150 μg/ml, and completely inhibited at 7,500 μg/ml concentrations. Germination of cabbage seeds showed ED_{50} at 7,500 μg/ml level. There was a strong correlation between extract concentration and increased toxicity to the test species. These concentrations which inhibited on seed germination are very high dosage compared to *Larix* previously reported Park *et al.* (1988). However, this level was higher than that of white clover, in which showed inhibited 25～100% inhibition at 200 μg/ml concentration. The cork tree was contained in high amount of alkaloids. It is a non-toxic compound among a number of alkaloids (Moon, 1984).

The bioassay results showed that cork tree possessed the weak phytotoxicity for lettuce, cucumber, and cabbage seeds. Many reports have been shown the inhibitory activity of plant extracts. Rasmamussen and Rice (1971) identi-

Table 1. Phytotoxicity of various seeds on aqueous extracts from cork tree.

Test seeds	Concentration (μg/ml)											
	0		1.5		15		150		1,500		7,500	
	G	RL	G	RL	G	RL	G	RL	G	RL	G	RL
Cabbage	100	2.5	96	2.5	98	1.2	98	0.5	73	0.6	50	0.5
Lettuce	100	2.0	96	2.1	97	1.0	95	0.8	70	0.8	48	0.2
Cucumber	98	3.0	98	2.8	96	2.5	50	2.2	30	0.8	0	0.3

* G : Rate of Germination (%), RL : Growth of root radicle

fied ferulic acid, *p*-coumaric acid from *Sporobus pyramidatus* and found allelopathic effects on associated species, resulting in either reduce grown or eliminating them from the stand.

3.2. Allelopathic effects for test microorganisms

Allelopathic effects were investigated using antimicrobial activities against *E. coli* were found in a fraction, which have a high polarity. Among the tested organic solvents, a 70% EtOH fraction showed high activity against test microorganisms (Figure 1).

Cork tree extracts inhibited the growth of various bacteria tested at a concentration of 625 μg/ml (Table 2). *Chamaecyparis obtusa* was chosen as indicator on the antimicrobial species which contained antimicrobial compounds, Hinokitiol and essential oil. In addition, poplar hybrid was used as negative controls, which have been not known antimicrobial activity as previously experiment. In this result, cork tree extract showed very strong antibacterial activity compared to that of *Chamaecyparis obtusa* extract.

Of 4 tested bacteria, *S. aureus* 12732 and *S. aureus* R-209 were sensitive on cell growth. MIC values for *S. aureus* 12732 and *S. aureus* R-209 represents 625 μg/ml, respectively. However, poplar hybrid not showed antibacterial

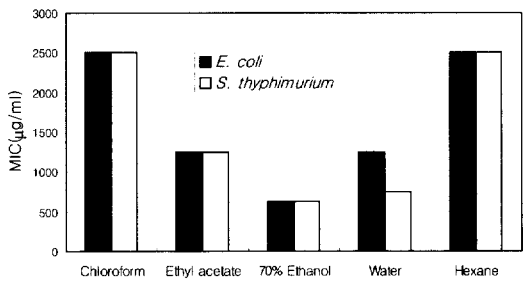

Fig. 1. MIC values of microbials based on various extraction solvents. The dried samples (1 kg) were extracted with 70 MeOH, and evaporated. The viscous concentrates were percolated with various organic solvent and distilled water, and then antimicrobial activities were bioassayed.

activity. Extract of cork tree was also inhibited growth of yeast, *Candida albicans.*

Cork tree and *Chamaecypris* extracts severely inhibited on fungus growth (Table 2). Cork tree extract showed very strong inhibition for *Alternaria alternata.* However, poplar hybrid are not observed antifungal activity.

A soil microorganisms acts degradation of the phytotoxic compounds exudated from root and fallen leaves. During the decomposition of plant residues in soil, microbial activities are involved. It is either improve or suppress on growth of plants. When the *Fusarium* produced fusarin, antibiotics with Murashige and Skoog medium treated sugarcane, leaves of its wilted and showed chlorosis (Chou, 1987).

Table 2. Minimum inhibition concentration (MIC) values of plant extracts for bacteria, yeast and fungi. (μg/ml)

Species	*S. aureus* IFO 12732	*S. aureus* R-209	*E. coli*	*Stryptococus typhimurium*	*Candida albicans* IFO 1594	*Botrytis cinerea*	*Alternaria alternata*
Phellodendron amurense	625	625	1,250	625	625	625	625
Chamaecyparis obtusa	625	1,250	1,250	1,250	1,250	1,125	1,125
P. alba X P. glandulosa	−*	−	−	−	−	−	−

* No activity

3.3. Antimicrobial activity for soil microorganisms

Microorganisms were isolated on selective culture media. When soil supernatant was cultured on each culture media, numerous microbials were obtained. For antimicrobial assay, repeated subculturing isolated growth dominant strains. M1 and M2 line revealed obtained from cultures on LB medium, whereas M3 line isolated from fungi culture medium. When extracts of cork tree treated to these microorganisms, antimicrobial activity showed same as test microorganisms (Table 3). The extracts showed strong antimicrobial activity against isolated fungi. The extract of cork tree have an MIC of 100, 150, and 200 μg/ml against isolated bacteria, yeast, and fungi, respectively. The active fraction of extracts has a high polarity. These active compounds may be leaching out to environment. The extracts could possibly active on allelopathy for neighboring soil microorganisms.

Table 3. MIC values of various plant tissues of cork tree (μg/ml)

Plant tissues	Microbial strains*			
	E. coli	M-1	M-2	M-3
Bark	625	312	312	625
Seed	1,250	1,250	1,250	1,250
Leaf	−**	−	−	−
Root	625	312	312	625

* A litter and soil(100 g) growing 20 year old tree and 100 ml distilled water were mixed filtered, and then cultured on microbial media (LB, YPD and MEA). The microbials isolated by repeated subculturing. M1 and M2 were obtained from cultures on LB medium, M3 was isolated from MEA medium.

** No activity

3.4. Identification of allelochemicals

The two active fractions were isolated by column chromatography. The active compound (Compound I) was identified by comparing its fluorescent under UV light, R_f value, its color with Dragendorff's reagent and MS data. The active compound was chromatographed on silica plate using solvent system as EtOAc-C_6H_6-n-PrOH-MeOH-$EtNH_2$ (4 : 8 : 2 : 1 : 1). The R_f values of the active fractions were 0.24 and 0.69. A colorimetric test using dragendorff reagent gave a dark-yellow color for the compound with R_f 0.24. The color of this compound revealed fluorescence under long wavelength UV in 365 nm. The color reaction of the compounds was close to that of isoquinoline alkaloids. The active compound gave yellow needles from H_2O, mp 192~194℃ (decomp.) and comparing of the UV spectrum

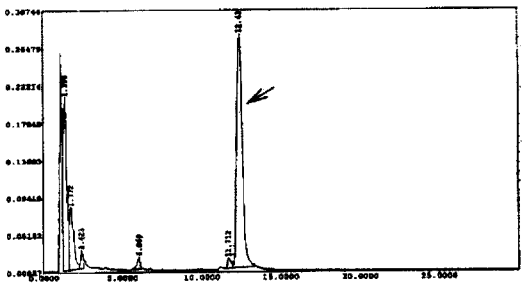

Fig. 2. HPLC chromatogram of allelochemicals (compound I) of cork tree. HPLC condition: HPLC (Younglin) system, column; μ-bondapak (300×3.9 mm) column; solvent 1 mM tetrabutylammonium phosphate (pH 2.0) : acetonitrile (40 : 60); flow rate 0.5 ml/min; wavelength 265 nm UV and injection volume 10 μl.

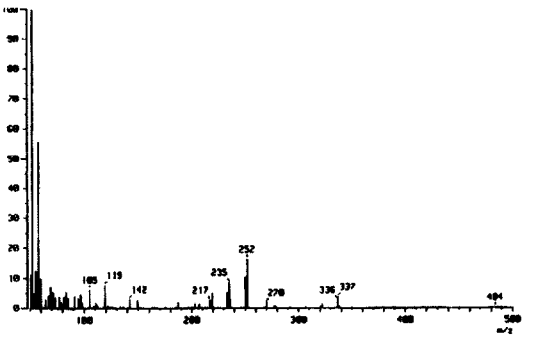

Fig. 3. EI-MS ion spectrum (70 eV) of the allelochemical (Compound I) obtained from cork tree.

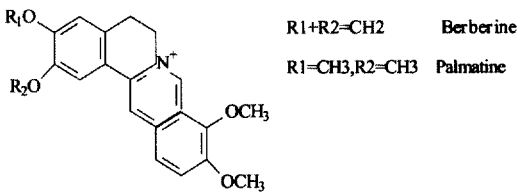

Fig. 4. Structures of allelochemicals obtained from cork tree.

and library spectra of Mass spectrometry elucidated its structure. The retention time of isolated compound was 12.43 min (Figure 2).

The retention times of isolated compound was the same as that as that of the authentic berberine-Cl. Ultraviolet absorption spectra of the R_f 0.24 compound showed major peak located at approximately 228, 264, 347 and 427 nm. Mass spectrum of compound I as followed, m/z(%) 337(M+, 2), 336(18), 270(12), 252 (100), 235(47) 217(23.5), 142(29), 119(41.2), and 105(6), in which can be presumed as isoquinoline alkaloid, berberine (Figure 3 and 4). The amount of berberine from cork tree was calculated 5.5 mg per gram based on dry weight (data not shown). Compound II also identified by direct comparisions (UV, HPLC and EI-MS) with authentic sample. The compound II identified as palmatine by above analysis (Figure 4). The compound II showed lower antimicrobial activities than that of compound I against isolated soil microorganisms.

Many alkaloids have been implicated in plant-plant or plant - animal chemical interactions but few have been associated with allelopathy. Several alkaloids were to be strong inhibitors of seed germination. Few work have been done on alkaloids except for caffeine (Robinson, 1983). The inhibitory effect of plant and soil organisms within plantation of cork tree is now being investigated. Soil microorganism such as mycorrhizae affected plant growth. The soil microorganisms protect seedling from disease, and degrade toxic substance like phenolics, and improve the nutrient uptake. Therefore, allelopathic effects on soil microorganism may have direct, negative effects on seedling performance. Aqueous compounds leached from litter and root tissues will inhibit on the plant growth. Although cork tree has a weak allelopathic against plant, its effects against soil microorganisms may be very important. These isoquinoline alkaloid compounds isolated from cork tree come into pharmaceutical for anti-HIV and bio-herbicide for plant

pathogens as well as allelochemical. Also, our report that revealed antimicrobial activities may useful in the silviculture and nursery control.

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