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Inhibitory Activity against *Helicobacter pylori* of Isolated Compounds from *Pinus koraiensis* Siebold et Zucc Leaves

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Abstract A phenol substance was extracted from *Pinus koraiensis* Siebold et Zucc leaf extracts and its biological efficacy was measured. The highest content of the phenol substance contained in *Pinus koraiensis* Siebold et Zucc leaves was 13.5 mg/g, which was obtained when it was extracted with 80% ethanol. At a concentration of 200 mg/mL, the phenolic substances extracted with 80% ethanol and water showed antimicrobial activities against *Helicobacter pylori*, producing clear zones of 10 and 12 mm diameter, respectively. *Pinus koraiensis* Siebold et Zucc. leaf extracts were separated using a Sephadex LH-20 column and 4 fractions were obtained (fractions A-D). Fractions C and D showed the greatest inhibitory activity against *Helicobacter pylori* producing 10.1 and 12.3 mm clear zones, respectively. These two fractions were purified using a Sephadex LH-20 and MCI-gel column (H_2O→100% ethanol). Purified compounds A and B were identified as syringic acid and compound C was identified as *p*-coumaric acid based on ^{1}H-nuclear magnetic resonance (NMR), ^{13}C-NMR, and fast atom bombardment mass spectrometry spectra. When two or more purified compounds were mixed, a synergistic effect of anti-*Helicobacter pylori* activity was evident. This result indicates that extracts of *Pinus koraiensis* Siebold et Zucc leaves could be considered a functional food because of their high antimicrobial properties.

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Keywords *Helicobacter pylori* · identification · inhibitor · *Pinus koraiensis* leaves · purification

Introduction

As many food materials have clear benefits for health promotion and disease prevention, consumers are no longer satisfied with basic food characteristics of nutrition and taste preference. Instead, consumers are increasingly seeking so-called functional foods (Park et al., 2004) such as those regulating biophylaxis and biorhythms.

Pinus koraiensis Siebold et Zucc. is an evergreen tree species belonging to the *Pinaceae* family. Lee et al. (2003) reported that *P. koraiensis* contains substances such as 5-hydroxy-7-methoxy flavone, chrysin, pinocembrin, galangin, 3-hydroxy-5-methoxy stilbene, and pinosylvin. In a study of its antioxidative potency and antimicrobial effects, You (2010) reported that all *P. koraiensis* substances had an antimicrobial effect >99.9% against species such as *Escherichia coli, Staphylococcus aureus,* and *Candida albicans*. Bae and Kim (2003) identified gallic acid, protocatechuic acid, vanillic acid, syringic acid, *p*-coumaric acid, scopoletin, (+)-catechin, and other substances in extracts of *P. koraiensis* leaves. Kim et al. (2010) found that kaempferol-3-*O*-arabonoside (juglanin) isolated from *P. koraiensis* leaf extract had antimicrobial activity against *Propionibacterium acnes*, *Staphylococcus aureus, Pityrosporum ovale,* and *E. coli.*

Helicobacter pylori is the principal cause of chronic gastritis, leading to peptic and duodenal ulcers, as well as gastric cancer (Suerbaum and Michetri, 2002; Park et al., 2003; Kang and Lee, 2005). Since the World Health Organization designated *H. pylori* as the first carcinogenic factor of gastric cancer, research on *H. pylori* has increased and much progress has been made in understanding its effects (Kim, 2006; Park et al., 2010; Yun et al., 2010; Kim and Cho, 2011a, 2011b; Yoon et al., 2011; Park et al., 2012). The inflammation rate caused by *H. pylori* is high in

normal adults, at 60–75% (Kang and Lee, 2005). *H. pylori* agglutinates on gastric epithelial cells and forms a mucus membrane layer. Increased *H. pylori* accumulation can drive at rophic changes resulting in gastritis (Hunt, 1996). There are 4 main methods for eliminating *H. pylori in vivo*: bismuth, proton pump inhibitor, and ranitidine bismuth citrate based triple therapies (Korean *H. pylori* study group, 1998; Kil et al., 2004), and antibiotics. Although antibiotic-based methods can be an efficient way to remove bacteria, long-term antibiotic administration may cause side effects and select for antibiotic-resistant bacteria strains; therefore, methods for bacterial removal with natural substances are required (Cho et al., 2005; Cho et al., 2006; Kim et al., 2006; Cho et al., 2007; Cho et al., 2009; Ju and Cho, 2009).

Although *P. koraiensis*is known for its excellent antimicrobial efficacy, research on its biological activity related to its potential as a healthy functional food is scarce. In this study, we evaluated the potential of *P. koraiensis* as a medicinal food by testing its anti-microbial activity against *H. pylori*.

Materials and Methods

Materials. *Pinus koraiensis* Siebold et Zucc. leaves were purchased at a local oriental medicine store, dried in a dry oven at 50°C, ground in 40 mesh, and then stored at 4°C temperature until use.

Total phenolic assay. Total phenolic contents were assayed using the Folin-Denis method (Folin and Denis, 1912). One milliliter of sample and 95% ethanol were added to 5 mL of distilled water. Then, 0.5 mL 1 N Folin-Ciocalteu reagent was added. After 5 minutes, 1 mL 5% Na_2CO_3 solution was added and the reacted mixture was allowed to stand for 60 minutes, and then absorbance was measured at 725 nm. The phenolic content was determined using a standard curve established with gallic acid as reference.

Antimicrobial assay against *H. pylori*. The strain used in this experiment was *Helicobacter pylori* ATCC 43504, which is a references train. Optimal medium (0.5 g special peptone, 0.75 g agar, 0.25 g NaCl, 0.25 g yeast extract, 0.2 g beef extract, 0.025 g pyruvic acid) was used to incubate *H. pylori* in a 10% CO_2 incubator to maintain microaerophilic conditions. *H. pylori* were cultured on agar plates and incubated in a BOD incubator at ≥95% humidity and 37°C for 48–72 h (Gavidson and Parish, 1989). After inoculation, 100 mL of the *H. pylori* culture broth was placed on an optimal medium plate on sterilized ϕ8 mm disc paper and applied through a membrane filter to obtain phenolic concentrations of 50, 100, 150, and 200 μg/0.1 mL. The control sample was incubated for 24 h by absorbing 0.1 mL sterilized water. The antimicrobial activity was determined by measuring the diameter(include disc paper) of the clear zone around the disc (Stevenson et al., 2000).

Extraction, isolation and purification of inhibitory compounds against *H. pylori* from *P. koraiensis* leaves. Dried *P. koraiensis* leaves (5 kg) were added to 80% ethanol at a volume 10 times more than the sample volume. After a 24 h extraction, the supernatant was obtained and pelleted by centrifugation (10,000 rpm for 15 min). Ethanol (80%) was then added to the pellet and the extraction process was repeated 4 times. All supernatants were mixed and filtered through Whatman No. 1 filter paper and then condensed with a rotary evaporator. The ethanol extracts were used as the sample fractions. One hundred grams *P. koraiensis* leaf extract was solidified by freeze-drying, and compounds were separated based on their adsorbability properties using a Sephadex LH-20 column (4.5×50 cm). Ethanol (80%) was used as the effluent, and the phenolic content was measured based on the Folin-Denis method (Folin and Denis, 1912) from each fraction graduated on 15 mL effluent at a 1.0 mL/min eluting speed in each tube. The inhibitory activity against *H. pylori* of each fraction was tested with the disc method. MCI-gel CHP-20 was used owing to its adsorbability as a porous polystyrene gel; H_20 was eluted in to ethanol as a general reverse phase elution buffer. Then, the fraction's inhibitory activity was tested. The fraction eluted by the open column was spotted on a silicagel plate (5.0×5.0 cm), roaded by using toluene : ethyl acetate : formate acid (5:4:1, v/v/v) as the solvent, and the color was developed with $FeCl_3/K_3Fe(CN)_6$ at 70°C to identify the band (Gavidson and Parish, 1989; Stevenson et al., 2000).

Chemical and physical properties of the purified phenolic compounds. The melting point of the isolated material was measured on a microelectrothermal actuator with 1 g of sample. The infrared spectrum was assayed using the halogenic alkalic tablet method. ^1H and ^{13}C-nuclear magnetic resonance (NMR) spectra were evaluated by melting 10 mg whole purified samples with dimethyl sulfoxide as the solvent and compared to a tetramethyl silane (TMS) standard using proton magnetic resonance (PMR, 400 MHz). The mass spectrum was generated using negative ion fast atom bombardment (FAB) mass spectrometry with 1 g sample under decompression (~10^{-4}–10^{-6} mmHg); thioglycerol was used as the solvent, and mass analysis was carried out with a 22–28 eV emitter current and a 6–7 kV accelerative pressure of the ion source. Element analysis was assayed with 1 mg sample that was dried by decompression for 48 h, and the hydrogen, carbon, and oxygen content was determined based on molecular conversion (Matsuo and Ito, 1978).

Results and Discussion

Extraction of phenolic compounds from *Pinus koraiensis* leaves. Among the possible edible organic solvents, ethanol was selected as the extraction solvent. Various concentrations of ethanol were used with *P. koraiensis* leaves and its phenolic compounds content was measured. As shown in Fig. 1, extraction with 70–100% ethanol resulted in a higher phenolic content than water extraction, and the highest content (13.5 mg/g) was obtained with 80% ethanol. This indicates that various phenolic compounds were eluted from different concentrations of ethanol due to differences in the solubility pattern caused by differences in polarity among the compounds.

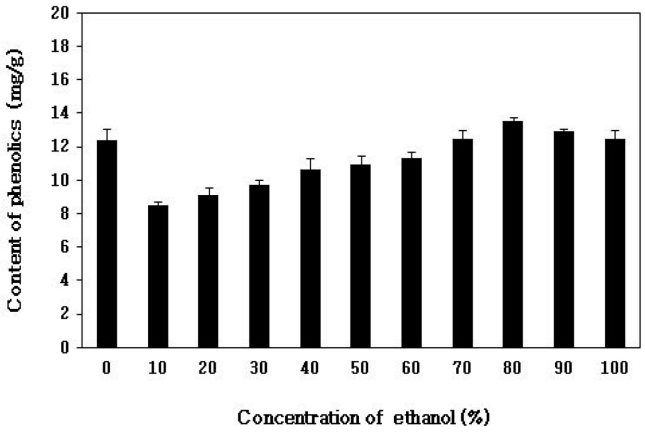


Fig. 1 Effect of ethanol concentration on extraction of phenolic from *Pinus koraiensis Siebold et Zucc* leaves. The data were expressed as the mean ± SD (n =3).

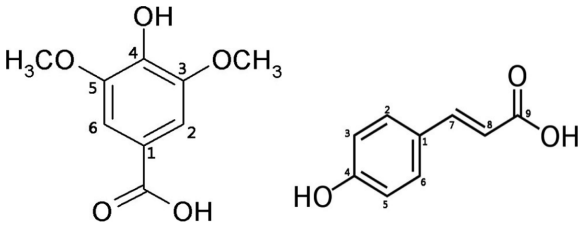
Antimicrobial assay against *Helicobacter pylori* of extracts. *H. pylori* infection is known to cause chronic gastritis and pepticulcers, and can lead to gastric cancer if infection is maintained over along time period. The gastric mucous membrane of infected patients shows symptoms of acute, chronic infection and degeneration of epithelial cells due to meronecrosis or elimination from the gastric mucous membrane. Epithelial cell damage is known to result from the direct effect of inflammatory response related secretions in response to *H. pylori* infection (Kang et al., 2006).

The antimicrobial activity of *P. koraiensis* leaf extracts against *H. pylori* can both indirectly and directly cause gastritis. Antimicrobial activity increased significantly with increased phenolic concentration from 50–200 μg/0.1 mL, as shown in Table 1; at the highest phenolic concentration, a clear zone of 12 and 13 mm appeared on water extract and 80% ethanol extract *H. pylori* plates, respectively. And Table 2; at the butanol layer among fraction by various solvents for isolation of antimicrobial compounds from 80% ethanol extracts, a clear zone of 12.5 mm appeared. Lee et al. (1999) reported that *Caesalpinia sappan* L., *Perllatrutescens* var. acuta, and *Coptisjaporiica* extracts formed 13, 13, and 8 mm clear zones, respectively, on *H. pylori* culture plates. Cho et al. (2008) reported similar antimicrobial activities with a 60% ethanol extract of *Salviaofficinalis*, which formed a 13 mm clear zone. Therefore, the *P. koraiensis* leaf extracts used in this study can be considered appropriate in industrial applications as an antimicrobial drug against *H. pylori*.

Table 2 Inhibition activity of fraction by various solvents from *Pinus koraiensis Siebold et Zucc* leaves ethanol extracts against *Helicobacter pylori*

Type of solvent	Clear zone (mm)
Control	ND
Buthanol layer	12.5±0.2[b]
Aqueous layer	8.3±0.1[a]
Ethylacetate layer	8.9±0.1[a]

ND was expressed no activity. Contents of phenolic contents was 200 μg/0.1 mL. The data were expressed as the mean ± SD (n =3).



(A) (B)

Fig. 2 Molecular structure of syringic acid (A) and *p*-coumaric acid (B).

Identification and structure of antimicrobial compounds. Antimicrobial fraction were separated by Sephadex LH-20 and MCI-gel CHP-20 column chromatography to obtain two pure antimicrobial compounds (compound A and B) against *H. pylori*. These compounds were roaded to thin layer chromatography with toluene : ethyl acetate : formate acid (5:4:1, v/v/v). The appearance of a single band indicated that these fractions were purified into a single compound. The chemical structure was identified based on ^{1}H-NMR, ^{13}C-NMR and negative ion FAB mass spectroscopic data and was confirmed using previously published literature. Compound A was a white powder with a melting point at 205–209°C and a molecular weight of 198, based on the FAB-MS spectrum. A 7.32-ppm (1H, s) benzene ring structure was predicted, and two 3.87-ppm (3H, s) methoxy species were identified in the ^{1}H-NMR spectrum. A carboxyl species carbon signal of δ 165.77 was found as well as a methoxy species carbon signal of δ 60.12 in the ^{13}C-NMR spectrum. In addition, a methine carbon of δ 115.36 was found, as shown in Table 3. These results corroborated the results of Park (2011). Compound A was therefore identified as syringic acid (Fig. 2A). Compound B was a yellow powder with a melting point of 210–213°C and a molecular weight of 164, based on the FAB-MS

Table 1 Antimicrobial activity *Pinus koraiensis Siebold et Zucc* leaves extracts against *Helicobacter pylori*

Helicobacter pylori	Clear zone (mm)									
	Phenolic contents (μg/0.1 mL)									
	Water extracts					80% EtOH extracts				
	0	50	100	150	200	0	50	100	150	200
	ND	8.3±0.1[a]	8.3±0.2[a]	9.5±0.1[b]	12±0.1[c]	ND	8.3±0.2[a]	8.4±0.1[a]	9.4±0.1[b]	13±0.3[c]

ND was expressed no activity. The data were expressed as the mean ± SD (n =6). Means with different superscripts (a-c) are significantly different at p <0.05 by Duncan's multiple range tests.

Table 3 ^{1}H and ^{13}C-NMR spectral data for compound A and B

Carbon No.	Spectrum (ppm)			
	Compound A		Compound B	
	δ_C	δ_H	δ_C	δ_H
1	150.41	-	127.2	-
2	115.36	7.32 (1H, s)	131.0	7.45 (2H, *d*, *J*=8.6 Hz)
3	120.86	-	116.7	6.81 (2H, *d*, *J*=8.6 Hz)
4	145.10	-	161.1	-
5	120.86	-	116.7	6.81 (2H, *d*, *J*=8.6 Hz)
6	115.36	7.32 (1H, s)	131.0	7.45 (2H, *d*, *J*=8.6 Hz)
7	165.77	-	146.6	7.53 (1H, *d*, *J*=16.0 Hz)
8	-	-	115.5	6.29 (1H, *d*, *J*=16.0 Hz)
OCH_3	60.12	3.87 (3H, s)	-	-
COOH			171.0	-

‰ units in ppm down field from internal TMS in DMSO.

spectrum. As shown in Table 3, a 2-aromatic ring structure of *ortho*-coupling doublets was found at 7.45 ppm (2H, *d*, *J*=8.6 Hz) and 6.81 ppm (2H, *d*, *J*=8.6 Hz) following ^{1}H-NMR analysis, and δ 116.71 and δ 146.66 carboxyl species signals were found in the ^{13}C-NMR spectrum. Compound B was identified as *p*-coumaric acid, which corroborates the analysis of Yoon et al. (2011) (Fig. 2B).

Synergistic antimicrobial activity of the purified compounds against *Helicobacter pylori*. Based on the chemical structures identified from the ^{1}H-NMR and ^{13}C-NMR spectra, the antimicrobial activities of syringic acid and *p*-coumaric acid on *H. pylori* plates were evaluated using the disc method, and the results are shown in Table 4. The phenolic compound concentration was controlled at 50, 100, 150, or 200 μg/0.1 mL, and single compound experiments of syringic acid and *p*-coumaric acid showed a 13 and 12 mm clear zone at a concentration of 200 μg/0.1 mL, respectively. The synergistic effect of the two compounds was tested by mixing syringic acid and *p*-coumaric acid at a concentration of 50–200 μg/0.1 mL. A 17 mm clear zone appeared at a phenolic compound concentration of 150 μg/0.1 mL, which was greater than the 15 mm clear zone observed at 200 μg/0.1 mL. Therefore, 150 μg/0.1 mL is the optimal concentration of these two compounds for inducing maximal antimicrobial activity.

These results also show that although syringic acid and *p*-coumaric acid show antimicrobial activities against *H. pylori* separately, they show higher antimicrobial activity when mixed due to a synergistic effect. Therefore, as antimicrobial activities with extracts are more efficient than treatment with are fined single compound, extracted compounds are more suitable for industrial applications. These results are in line with previous studies demonstrating a synergistic effect of mixing several compounds of rosemary (Yoon et al., 2011), *Morus alba* L. (Yun et al., 2010), and *Rhododendron mucronulatum* (Ju and Cho, 2009) to increase the antimicrobial activity against *H. pylori*.

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Table 4 Synergy effect of inhibition activity against *Helicobacter pylori* by purified compounds from *Pinus koraiensis Siebold et Zucc* leaves

Helicobacter pylori	Clear zone (mm)				
	Phenolic content (μg/0.1 mL)				
	0	50	100	150	200
Syringic acid	ND	$12\pm0.5^{ab\beta}$	11 ± 0.1^{a}	12 ± 0.2^{ab}	$13\pm0.1^{b\beta}$
p-Coumaric acid	ND	$9\pm0.2^{a\alpha}$	$12\pm0.1^{b\beta}$	12 ± 0.4^{b}	12 ± 0.3^{b}
Syringic acid + *p*-coumaric acid	ND	$12\pm0.4^{a\alpha\beta}$	$13\pm0.3^{a\gamma}$	$17\pm0.3^{c\beta}$	$15\pm0.1^{b\gamma}$

ND was expressed no activity. Syringic acid:*p*-coumaric acid = 1:1 (w/w). Means with different superscripts (a-c) letters within level and different superscripts (α-γ) letters within column are significantly different at $p < 0.05$ by Duncan's multiple range tests.

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