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Antibacterial Activity of Two Phloroglucinols, Flavaspidic Acids AB and PB, from *Dryopteris crassirhizoma*

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The antimicrobial effect of solvent extracts from the rhizome of a thick-stemmed wood fern (*Dryopteris crassirhizoma*) was evaluated and its phloroglucinol components, flavaspidic acids PB and AB. Flavaspidic acids PB and AB were isolated from the *D. crassirhizoma* rhizomes by methanol extraction, followed by silica gel and Sephadex LH-20 column chromatography. The chemical structures were characterized by spectral techniques, including ESI-MS, UV, ^{1}H- and ^{13}C-NMR spectrum analysis. When the antimicrobial activity of the extracts and compounds was tested by the paper disc method, the extracts and compounds were highly active against Gram-positive bacteria, such as methicillin-resistant *Staphylococcus aureus* KCTC 1928 (a MRSA bacterium), *Streptococcus mutans* and *Bacillus subtilis*. The extracts and compounds were not active against fungi and chlorella. Our study revealed that the antibacterial activity of samples from *D. crassirhizoma* was mainly related to the flavaspidic acids.

Key words: Antibacterial activity, Phloroglucinols, Flavaspidic acids, *Dryopteris crassirhizoma*

INTRODUCTION

The thick-stemmed wood fern (*Dryopteris crassirhizoma* Nakai, Dryopteridaceae) is a semi-evergreen plant that grows on the deciduous forest floor as a pteridophyte. Two ferns, *Dryopteris crassirhizo*ma and *Osmunda japonica,* are commonly used as anti-infection agents, especially for the common cold and flu, and are frequently collectively referred to as the fern (Dharmananda, 2003). Recently, the fern has also been used as the major plant with six Chinese herbs (*Astragalus*, *Atractylodes*, Red *Atractylodes*, *Pogostemon*, *Adenophora*, *Lonicera*), a combination that was recommended as a prescription formula to prevent SARS. Its rhizomes have also been used in a vermicide (Namba, 1993). In the search for natural products with antimicrobial activity, methanol extracts of the *D. crassirhizoma* rhizome exhibited antimicrobial activity against some bacteria. Phloroglucinols (albaspidin, aspidin, flavaspidic acids and dryocrassin) and kaempferol acetyl rhamnosides (crassirhizomosides AC and sutchuenoside A) have been isolated from *D. crassirhizoma* (Noro et al., 1973; Widen et al., 1996; Min et al., 2001). Recently, acylphloroglucinols isolated from *D. crassirhizoma* were reported to inhibit fatty acid synthase (Na et al., 2006). In addition, the compound showed a cytoprotective effect against oxidative stress-induced cell damage via catalase activation (Kang et al., 2006). The phloroglucinol composition of 18 species (including subspecies) that belong to *Dryopteris* Adanson sect. *Fibrillosae* Ching has been investigated on a world-wide basis (Widen et al.,, 1996). Phloroglucinols were observed to have anti-tumor-promoting activity (Govind et al., 1996), nitric oxide inhibitory effect (Rie et al., 2001), anti-reverse transcriptase activity (Hideo et al., 1991) and antioxidant activity (Lee et al., 2003). Since Namba (1982) mentioned that some Chinese medicinal plants, including *D. crassirhizoma,* have an effect on dental care, the only report of ether-extracted plant fractions with antibacterial activity was an *in vitro* assay against

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Streptococcus mutans OMZ 176 (Do, 1993). Antimicrobial activity of phloroglucinols was once reported by Abbey et al. (2000). However, these phloroglucinols were acylated phloroglucinols from *Helichrysum caespititium*. The objectives of this study were to evaluate the antimicrobial potential of rhizome extracts and flavaspidic acids PB and AB from *D. crassirhizoma* against Gram-positive and -negative bacteria, fungi and chlorella.

MATERIALS AND METHODS

Samples, extraction and isolation of phloroglucinols

Rhizome of *Dryopteris crassirhizoma* was collected in Mt. Sulak, Korea in July 2002 and identified by Prof. Bae, College of Pharmacy, Chungnam National University. A voucher specimen (CNU 1011) was deposited in the herbarium of the College of Pharmacy, Chungnam National University.

As shown in Fig. 1, the dried rhizomes (1 kg) of *Dryopteris crassirhizoma* were extracted with methanol (3 L, 48 h×2) at room temperature, and the extract was concentrated to dryness *in vacuo* to yield a dark brown syrupy residue (150 g). The methanol extract (150 g) was suspended in H_2O (1 L) and then partitioned successively with hexane (1 L×2), ethyl acetate (1 L×2), and BuOH (1 L×2). The dry ethyl acetate extract (80 g) was subjected to column chromatography on silica gel (70-230 mesh, Merck, Germany). A step gradient was used for elution; each step constituted a 10% increase in acetone (in 1 L volumes) with hexane (10% acetone in 1 L volume), up to 80% acetone. Eleven 1-L fractions were collected. These fractions were tested by *in vitro* antimicrobial assays, and the active fractions (fr. 5, 6) were combined. Using methanol as a solvent, the active fraction (8 g) was subjected to column chromatography on Sephadex LH-20 to afford two active compounds: flavaspidic acid PB (**1**, 300 mg) and flavaspidic acid AB (**2**, 150 mg), which were characterized by spectral methods (Noro et al., 1973; Do, 1993).

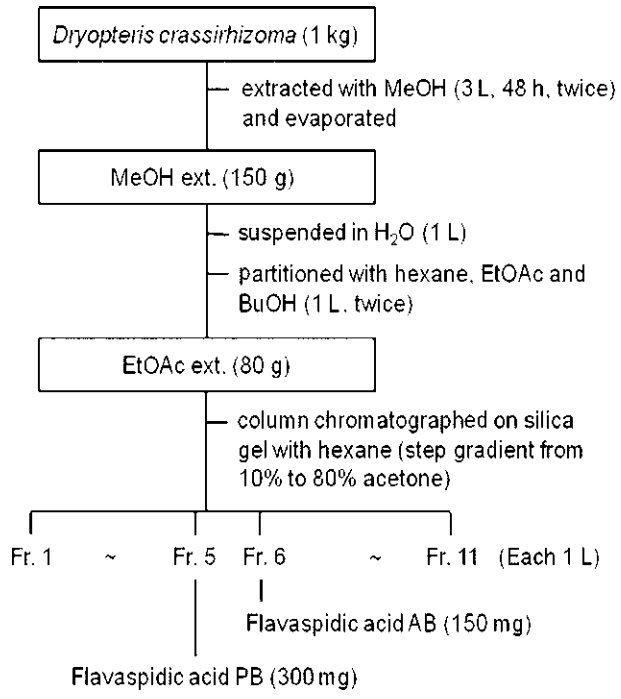

Fig. 1. Purification scheme for two phloroglucinols from *Dryopteris crassirhizoma*

Flavaspidic acid PB (**1**): Yellow needles ($CHCl_3$); mp 148°C; UV λ_{max} ($CHCl_3$) nm (log ε): 241 (3.81), 289 (3.78). ESI-MS m/z: 431.2 $[M-H]^-$, 455.2 $[M+Na]^+$, ^{1}H-NMR (300 MHz, $CDCl_3$) 0.99 [3H, t (*J*=7.5 Hz), H-11'], 1.10 [3H, t (*J*=7.5 Hz), H-11], 1.40 [6H, s, H-7,8], 1.66 [2H, m, H-10'], 2.05 [3H, s, H-7'], 3.05 [2H, t (*J*=7.5 Hz), H-9'], 3.10 [2H, q (*J*=7.5), H-10], 3.55 [2H, s, H-1"]. ^{13}C-NMR (100 MHz, $CDCl_3$) 198.3 (C-1), 107.4 (C-2), 187.6 (C-3), 44.1 (C-4), 171.7 (C-5), 111.2 (C-6), 24.7 (C-7, 8), 206.3 (C-9), 35.2 (C-10), 8.1 (C-11), 105.5 (C-1'), 159.8 (C-2'), 103.0 (C-3'), 156.4 (C-4'), 102.0 (C-5'), 161.2 (C-6') 7.4 (C-7'), 206.7 (C-8'), 45.8 (C-9'), 18.1 (C-10'), 8.5 (C-11'), 16.2 (C-1").

Flavaspidic acid AB (**2**): Yellow needles ($CHCl_3$); mp 188-189°C; UV λmax ($CHCl_3$) nm (log ε): 240 (3.85), 285 (3.79). ESI-MS m/z: 417.3 $[M-H]^-$, 441.3 $[M+Na]^+$, ^{1}H-NMR (300 MHz, $CDCl_3$) 0.91 [3H, t (*J*=7.5 Hz), H-11'], 1.16 [6H, s, H-7, 8], 1.60 [2H, m, H-10'], 1.87 [3H, s, H-7'], 2.38 [3H,s, H-10], 3.04 [2H, t (*J*=7.5 Hz), H-9'], 3.55 [2H, s, H-12].

Determination of the chemical structure of phloroglucinols

Melting point was measured on an Electrothermal instrument (Dubuque, IA, USA). UV spectra were obtained on a Milton Roy 3000 spectrometer (Ivyland, PA, USA). ^{1}H- and ^{13}C-NMR spectra were recorded on a DRX 300 MHz (Bruker, Karlsruhe, Germany) with $CDCl_3$ as a solvent. ESI-MS spectra were measured on JMS 700 mass spectrometer (JEOL, Tokyo, Japan).

Determination of antimicrobial activity

The antimicrobial activity of the crude extracts and purified materials (flavaspidic acids AB and PB) was tested by the paper disc method. All samples were dissolved in trace ethanol. Sterile filter paper discs (Whatman No. 1, 8 mm diameter) were impregnated with 200 μg of each sample (50 μL, 4 mg mL^{-1}) per paper disc and dried under the laminar flow cabinet

overnight. Seeded agar plates were prepared and inoculated with 0.1 mL of each inoculum, and the paper discs were placed on the plates. The test microorganisms used in this study were *Bacillus subtilis* KCTC 1914 (Korean Culture Type Collection, KRIBB, Daejeon), *Escherichia coli* KCTC 1924, *Staphylococcus aureus* KCTC 1916, *Staphylococcus aureus* KCTC 1928 (as a MRSA bacterium), *Streptococcus mutans* DSM 6178 (Deutsche Sammlung von Mikroorganismen, Braunschweig, Germany), *Aspergillus flavus* EML-AF01 (Environmental Microbiology Lab, Chonnam National University, Gwangju, Korea), *Candida albicans* KCTC 1940, and *Chlorella regularis* EML-CR02. The medium for *Bacillus subtilis* and *Staphylococcus aureus* was Nutrient agar (pH 7.0) with 5 g peptone, 3 g meat extract and 15 g agar per liter. The medium for *Candida albicans* was YMPG agar with 3 g yeast extract, 3 g malt extract, 5 g soybean peptone, 10 g glucose and 15 g agar per liter. The medium for *Aspergillus flavus* was YpSs agar consisting of 4 g yeast extract, 15 g soluble starch, 1 g K_2HPO_4, 0.5 g $MgSO_4 \times 7\ H_2O$ and 15 g agar per liter. The medium for *Chlorella regularis* was Arnon's A5 medium (pH 6.5) consisting of 1 mL Arnon's A5 solution, 1 g KH_2PO_4, 1 g $MgSO_4 \cdot 7H_2O$, 0.005 g $FeSO_4 \cdot 7H_2O$, 5 g yeast extract, 20 g glucose and 20 g agar per liter. All assays were performed in duplicate, so that four inhibition zone measurements were obtained for each test combination. These values were averaged to obtain the final inhibitory activity results. For each assay, two control plates were inoculated with ethanol, but without actual extracts, and were treated in the same manner as the test plates.

RESULTS AND DISCUSSION

Flavaspidic acid PB (**1**) had a molecular weight of 432, as identified by ESI-MS ($[M-H]^-$: m/z 431.2; $[M+Na]^+$: m/z 455.2). The characteristic ^{1}H-NMR signals δ_H 0.99 [3H, t (*J*=7.5 Hz)], δ_C 8.5 and δ_H 1.10 [3H, t (*J*=7.5 Hz)], and δ_C 8.1 were indicative of C-11' and C-11, respectively. δ_H 1.40 [6H, s] and δ_C 24.7 indicated gem-dimethyl at C-7, 8. In addition, δ_H 2.05 [3H, s], δ_C 7.4 and δ_H 3.55 [2H, s] were indicative of C-11' and C-12. Thus, the structure of **1** was determined to be flavaspidic acid PB (Fig. 2). This was confirmed by comparison of the physiochemical and spectral data with published data (Noro et al., 1973).

Flavaspidic acid AB (**2**) had a molecular weight of 418, as identified by ESI-MS ($[M-H]^-$: m/z 417.3; $[M+Na]^+$: m/z 441.3). The characteristic ^{1}H- and ^{13}C-NMR signals δ_H 0.91 [3H, t (*J*=7.3 Hz)] and δ_C 13.8 was indicative of C-11'. δ_H 1.16 [6H, s] and δ_C 25.6 corresponded to gem-dimethyl at C-7, 8. δ_H 1.87 [3H, s] and δ_C 7.9 indicated an aromatic methyl (C-7'). The spectral data mentioned above were similar to flavaspidic acid PB (**1**), but the secondary methyl (C-10) signals in **1** did not appear in **2**. Thus, the structure of **2** was determined to be flavaspidic acid AB (Fig. 2). This was confirmed by comparison of the physiochemical and spectral data comparison with published data (Noro et al., 1973; Do, 1993).

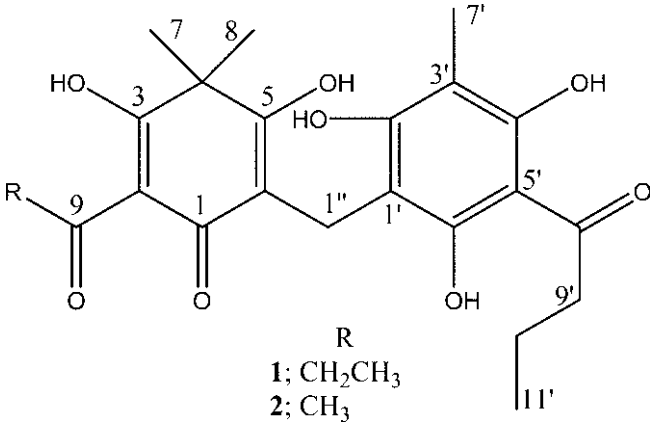

Fig. 2. Chemical structures of two phloroglucinols

The methanol and ethyl acetate extracts from the *Dryopteris crassirhizoma* rhizome were highly active against bacteria. As shown in Table I and Fig. 3, flavaspidic acids PB and AB from the *Dryopteris crassirhizoma* rhizome were active against Gram-positive bacteria, including *Bacillus subtilis* KCTC 1914, two strains of *Staphylococcus aureus* KCTC 1916 and 1928, and *Streptococcus mutans* DSM 6178, producing an inhibition zone of up to 19 mm at 200 μg. Minimal inhibitory concentrations (MICs) were approximately 12-20 μg mL^{-1} on paper disc depending

Table I. Antimicrobial activity of flavaspidic acids AB and PB against microorganisms

Microorganism	Inhibition zone (mm diameter)	
	Flavaspidic AB	Flavaspidic PB
Escherichia coli KCTC 1924	11[b]	11
Bacillus subtilis KCTC 1914	15	19
Candida albicans KCTC 1940	NA[c]	NA
Staphylococcus aureus KCTC 1916	16	16
Staphylococcus aureus KCTC 1928[a]	19	19
Streptococcus mutans DSM 6178	18	19
Aspergillus flavus EML-AF01	NA	NA
Chlorella regularis EML-CR02	NA	NA

[a]A MRSA bacterium.
[b]Relative inhibition zone (mm) at 200 μg per paper disc.
[c]No activity.

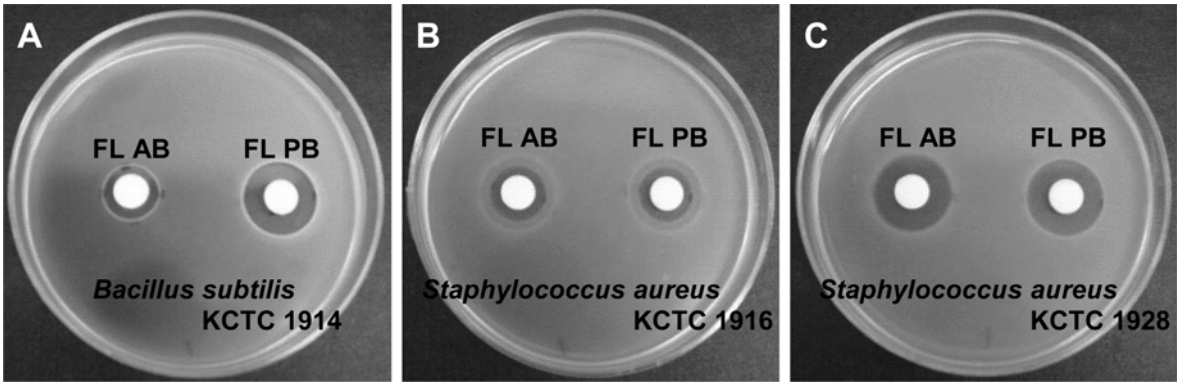

Fig. 3. Antibacterial effect of flavaspidic acids AB (FL AB) and PB (FL PB) against bacteria [**A**: *Bacillus subtilis* KCTC 1914, **B**: *Staphylococcus aureus* KCTC 1916, **C**: *Staphylococcus aureus* KCTC 1928 (a MRSA bacterium)]. Each inhibition zone was measured 24 hrs after treatment with 200 μg per paper disc.

on kinds of microorganisms tested (data not shown). Interestingly, the flavaspidic acids were considerably more active against the MRSA bacterium, *Staphylococcus aureus* KCTC 1228, than against *Staphylococcus aureus* KCTC 1916. Also, flavaspidic acid PB was somewhat more active against *Bacillus subtilis* than flavaspidic acid AB. However, both compounds were moderately to slightly active against a Gram-negative bacterium, *E. coli*, and were not active against a fungus, *Aspergillus flavus* or an alga, *Chlorella regularis*.

This study reports the antibacterial activity of plant-derived phloroglucinols. We found that the ethylacetate fraction of the *Dryopteris crassirhizoma* rhizome exhibited antimicrobial activity and yielded two phloroglucinols, flavaspidic acid PB and flavaspidic acid AB. The identity of the compounds was first confirmed through interpretation of their spectral characters in comparison with reported data (Noro et al., 1973). Do (1993) once reported that flavaspidic acids PB and AB had an MIC value of 12.5 μg per ml toward *Streptococcus mutans* OMZ 176. However, detailed antimicrobial activities against other bacteria were not investigated.

Recently, MRSA bacteria have become more resistant to vancomycin antibiotics. Interestingly, the flavaspidic acids were highly active against Gram-positive and MRSA bacteria including *Staphylococcus aureus*, but not against fungi. Our study revealed that thick-stemmed wood fern extracts may be applied to development of natural functional products with antibacterial activity. More studies on the antibacterial spectrum, the susceptibility of various bacteria to the compounds, and the mode of action are now under way.

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REFERENCES

Dharmananda, S., SARS AND CHINESE MEDICINE. How the Chinese People and Institutions Responded with Herbs: http://www.itmonline.org/arts/sars.htm (2003).

Do, D. S., Antibacterial components of *Dryopteris crassirhizoma* against a *Streptococcus mutans* OMZ 176. MS thesis, Chungnam National University. Daejeon, Korea (1993).

Govind, J. K., Harukuni, T., Takao, K., Midori, T., Junko, T., and Hoyoku, N., Anti-tumor promoting activity of *Dryopteris* phlorophenone derivatives. *Cancer Letters,* 105, 161-165 (1996).

Hideo, N., Munehisa, A., Akio, F., Saburo, K., and Katsuhiko. O., Inhibition of HIV-reverse transcriptase activity by some phloroglucinol derivatives. *FEBS Letters,* 286, 83-85 (1991).

Kang, K. A., Lee, K. H., Chae, S., Zhang, R., Jung, M. S., Ham, Y. M., Baik, J. S., Lee, N. H., and Hyun, J. W., Cytoprotective effect of phloroglucinol on oxidative stress induced cell damage via catalase activation. *J. Cell Biochem.,* 97, 609-620 (2006).

Lee, S. M., Na, M. K. An, R. B., Min, B. S., and Lee, H. K., Antioxidant activity of two phloroglucinol derivatives from *Dryopteris crassirhizoma*. *Biol. Pharm. Bull.,* 26, 1354-1356 (2003).

Mathekga, A. D. M., Meyer, J. J. M., Horn, M. M., and Drewes, S. E., An acylated phloroglucinol with antimicrobial properties from *Helichrysum caespititium*. *Phytochemistry,* 53, 93-96 (2000).

Min, B. S., Tomiyama, M., Ma, C. M., Nakamura, N., and Hattori, M., Kaempferol Acetylrhamnosides from the

rhizome of *Dryopteris crassirhizoma* and their inhibitory effects on three different activities of human immunodeficiency Virus-I reverse transcriptase. *Chem. Pharm. Bull.,* 49, 546-550 (2001).

Na, M., Jang, J., Min, B. S., Lee, S. J., Lee, M. S., Kim, B. Y. Oh, W. K., and Ahn, J. S., Fatty acid synthase inhibitory activity of acylphloroglucinols isolated from *Dryopteris crassirhizoma. Bioorg. Med. Chem. Lett.,* 16, 4738-4742 (2006).

Namba, T., Tsunezuka, M., and Hattori, M., Dental caries prevention by traditional Chinese medicines. *Planta Medica*, 44, 100-106 (1982).

Namba, T., The encyclopedia of Wakan-Yaku with Color Picture Vol. I. Osaka, Japan: Hoikusha Co., Ltd., pp. 142-144 (1993).

Noro, Y., Okuda, K., Hisada, S., Inagaki, I., Tanaka, T., and Yokohashi, H., Dryocrassin: A new acylphloroglucinol from *Dryopteris crassirhizoma. Phytochemistry,* 12, 1491-1493 (1973).

Rie, I., Masakazu, H., Koichi, S., Munehisa, A., and Susumu, K., Inhibitory effects of phloroglucinol derivatives from *Mallotus japonicus* on nitric oxide production by a murine macrophage-like cell line, RAW 264.7, activated by lipopolysaccharide and interferon-γ. *Biochimica et Biophysica Acta - General Subjects,* 1568, 74-82 (2001).

Widen, C. -J., Fraser-Jenkins, C. R., Reichstein, T., Gibby, M., and Sarvela, J., Phloroglucinol derivatives in *Dryopteris* sect. *Fibrillosae* and related taxa (*Pteridophyta, Dryopteridaceae*). *Ann. Bot. Fennici,* 33, 69-100 (1996).