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Potent in Vivo Antifungal Activity against Powdery Mildews of Pregnane Glycosides from the Roots of *Cynanchum wilfordii*

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ABSTRACT: Two new pregnane glycosides, kidjoranine 3-*O*-β-D-glucopyranosyl-(1→4)-β-D-glucopyranosyl-(1→4)-α-L-cymaropyranosyl-(1→4)-β-D-cymaropyranosyl-(1→4)-α-L-diginopyranosyl-(1→4)-β-D-cymaropyranoside (**5**) and caudatin 3-*O*-β-D-glucopyranosyl-(1→4)-β-D-glucopyranosyl-(1→4)-α-L-cymaropyranosyl-(1→4)-β-D-cymaropyranosyl-(1→4)-α-L-diginopyranosyl-(1→4)-β-D-cymaropyranoside (**6**), were isolated from the roots of *Cynanchum wilfordii* along with four known compounds (**1**−**4**). The antifungal activities of the six compounds against barley powdery mildew caused by *Blumeria graminis* f. sp. *hordei* were compared to the antifungal activity of polyoxin B. The caudatin glycosides (**1**, **4**, and **6**) showed stronger antifungal activities than polyoxin B, whereas kidjoranine glycosides (**2**, **3**, and **5**) had weaker activities than polyoxin B. A wettable powder-type formulation (*C. wilfordii*-WP20) of the ethyl acetate extract from *C. wilfordii* roots prohibited the development of barley powdery mildew much more effectively than the commercial fungicide polyoxin B-WP10. In addition, *C. wilfordii*-WP20 effectively controlled strawberry powdery mildew caused by *Sphaerotheca humuli* under greenhouse conditions. Thus, the crude extract containing the pregnane glycosides can be used as a botanical fungicide for the environmentally benign control of powdery mildews.

KEYWORDS: pregnane glycosides, *Cynanchum wilfordii*, powdery mildew, caudatin glycosides, kidjoranine glycosides

INTRODUCTION

The yield and quality of major food crops worldwide are reduced nearly 20% by pathogenic fungi.[1] Among these, powdery mildew is one of the most widespread and easily recognized. Powdery mildew diseases are caused by many different species of fungi in the order Erysiphales,[2] and they affect virtually all plants. Infected plants display spots or patches of white or grayish talcum powder-like growth. Although the lower leaves are affected most, the mildew can appear on any above-ground part of the plant, including leaves, young stems, buds, flowers, and young fruit. Traditionally, powdery mildew is controlled using two main strategies: the application of synthetic fungicides and the use of resistant cultivars.[3] These methods help sustain production and crop quality by protecting crops from powdery mildew fungi.[4] Despite the benefits, however, the misuse and excessive application of synthetic fungicides have caused problems such as residual toxicity and environmental pollution.[5] In addition, pathogen populations resistant to synthetic fungicides have appeared in many crop-growing areas.[6]

Many studies have focused on the control of powdery mildew fungi, with the aim of reducing the input of synthetic fungicide, and the trend, at present, is toward safer and more benign compounds.[7] For the control of powdery mildews, several attempts have been made to develop antifungal compounds from varied sources, such as free water, sodium bicarbonate, plant-derived natural compounds, and biocontrol microorganisms.[8] Among these, medicinal plants have long been recognized as a rich source of antifungal agents.[9] Plant products remain the principal source of fungicidal agents used in traditional medicine, and they may lead to the development of new classes of safer disease-control agents.

Cynanchum wilfordii (Asclepiadaceae) roots have been used in Korean folk medicine for the prevention and treatment of various geriatric diseases involving vascular disorders, including diabetes mellitus, ischemia-induced diseases, and aging progression.[10] The dried roots of this plant have been used as a tonic to promote renal function. *C. wilfordii* roots have been studied extensively and have been found to contain steroidal alkaloids, including gagaminine,[11] pregnane glycosides, cynanchone, acetophenones such as cynandione A,[12] and anthraquinones such as emodin, chrysophanol, rhein, and physcion.[13] Gagaminine has been reported to have potent inhibitory effects on aldehyde oxidase activity and lipid peroxidation.[14] Pregnane glycosides such as wilfoside K1N have been proven to have antiangiogenic, anti-invasion, and antitumor activities.[15,16] Cynandione A has been demonstrated to protect cultured cortical neurons from toxicity

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induced by oxidative stress.[17] However, the antifungal activity of *C. wilfordii* roots against powdery mildews has not been assessed in vivo.

In a search for plant extracts with potent in vivo antifungal activity against *Blumeria graminis* f. sp. *hordei* in barley, we found that treatment with the methanol extract of *C. wilfordii* roots highly reduced the development of barley powdery mildew. The goals of this study were to isolate and identify antifungal substances from *C. wilfordii* roots and to assess their antifungal activities using an in vivo test. Additionally, the antifungal activity of *C. wilfordii* roots against strawberry powdery mildew caused by *Sphaerotheca humuli* was evaluated under greenhouse conditions.

MATERIALS AND METHODS

General Experimental Procedures and Apparatuses. The chemical structures of the purified compounds were determined by mass and nuclear magnetic resonance (NMR) spectroscopy. Electrospray ionization mass spectra (ESI-MS) of the purified compounds were recorded on an MSD1100 single-quadrupole mass spectrometer equipped with an ESI (Hewlett-Packard Co., Palo Alto, CA). ^{1}H and ^{13}C NMR spectra were recorded in CD_3OD (Merck, Darmstadt, Germany) with a Bruker AMX-500 spectrometer (Bruker Analytische Messtechnik Gmbh, Rheinstetten, Germany) operated at 500 MHz for ^{1}H and at 125 MHz for the ^{13}C nucleus. Chemical shifts (parts per million) are relative to tetramethylsilane (TMS), which was used as an internal reference. Coupling constants are reported in hertz. ^{13}C NMR resonance multiplicity was determined using distortionless enhancement by polarization transfer (DEPT) experiments. $^{1}H-^{1}H$ connectivity was determined using correlation spectroscopy (COSY). One-bond heteronuclear $^{1}H-^{13}C$ connectivity was determined with heteronuclear single-quantum coherence (HSQC) experiments. Two- and three-bond heteronuclear $^{1}H-^{13}C$ connectivity was determined using heteronuclear multibond connectivity (HMBC) NMR spectra. High-performance liquid chromatography (HPLC) was performed on a Waters 2690, equipped with a photodiode array detector and a corona detector (Waters Co., Ireland). The column used was a 150 mm × 4.6 mm i.d., 5 μm, Cosmosil C_{18}, with a 4 mm × 4 mm i.d. guard column of the same material (Nacalai Tesque Inc., Kyoto, Japan). The mobile phase was water/MeOH in a gradient of 70:30 to 0:100 over 30 min, and a 250 mm × 20 mm i.d., 5 μm, preparative Capcell Pak C_{18} column (Shiseido Co. Ltd., Tokyo, Japan) was used in the sample preparation. Column chromatography was performed with silica gel 60 (70−230 and 230−400 mesh, Merck), Sephadex LH-20 (25−100 μm, Sigma-Aldrich, Vienna, Austria), and reversed-phase C_{18} silica gel (ODS: 40−63 μm, Merck). Precoated silica gel (Merck 60 F-254, Merck) plates were used for thin-layer chromatography (TLC). The spots from TLC were detected under UV lamps (254 nm) and by spraying with an anisaldehyde/sulfuric acid solution followed by heating.

Chemicals. The solvents used in this study were of HPLC grade and purchased from Merck & Co., Inc., and SK Chemicals Co., South Korea. All other chemicals were of analytical grade and commercially available from Dr. Ehrenstorfer GmbH Co., Augsburg, Germany, unless otherwise stated.

Plant Material. The dried *C. wilfordii* roots were purchased from a folk medicine market, 'Yakryongsi', in Daegu, Republic of Korea. A voucher specimen has been deposited at the Plant Extract Bank, Korea Research Institute of Chemical Technology, Daejeon, Republic of Korea.

Extraction and Isolation. The dried powder of *C. wilfordii* roots (3.00 kg) was extracted twice with 80% methanol (10.0 L) for 24 h at room temperature. The methanol extracts were combined and concentrated using a rotary evaporator. The methanol extracts (175 g) were successively partitioned with *n*-hexane (Hex), ethyl acetate (EtOAc), *n*-butanol (BuOH), and water, and the four resulting fractions (including an aqueous fraction) were concentrated to dryness to give *n*-hexane- (12.9 g), EtOAc- (100 g), *n*-butanol- (333 g), and water-soluble fractions (208 g). The four fractions were bioassayed for in vivo antifungal activity against *B. graminis* f. sp. *hordei* in barley plants. Among the four fractions, the EtOAc fraction displayed the strongest activity against barley powdery mildew. The active EtOAc portion was subjected to column chromatography over silica gel (70−230 mesh, 5.0 × 60 cm) and eluted with $CHCl_3$/MeOH (95:5, v/v). Eight fractions (F1−F8) were obtained on the basis of TLC analysis, and fractions with similar TLC patterns were pooled. The combined F1−F3 fraction (46.0 g) exhibited antifungal activity against barley powdery mildew, and this product was rechromatographed on a silica gel column (230−400 mesh, 5.7 × 50 cm) and eluted with *n*-hexane/acetone (3:2, v/v) to give six fractions (F11−F16). F13 (8.40 g) and F14 (6.12 g) were separately subjected to Sephadex LH-20 column (2.0 × 30 cm) separation and eluted with methylene chloride/*n*-hexane/MeOH (5:5:1, v/v/v). Compound **1** (160 mg) was obtained from F13, and compound **2** (475 mg) was obtained from F14. F6 (2.90 g) was successively chromatographed on a reversed-phase ODS column (2.0 × 30 cm) using 70% MeOH and a Sephadex LH-20 column (2.0 × 30 cm) using 100% MeOH. The active fraction was finally purified using a preparative high-performance liquid chromatographic (prep-HPLC) system (Shimadzu, Kyoto, Japan) with a C_{18} reversed-phase column (Capcell Pak C_{18}, 20 × 250 mm; Shiseido, Tokyo, Japan). The antifungal substances were eluted with a linear gradient solvent system from water to MeOH (70% MeOH from 0 to 10 min and 100% MeOH from 10 to 40 min at a flow rate of 10 mL/min) and detected at wavelengths of 220 and 275 nm. The preparative HPLC gave the pure compounds **3** (99.0 mg) and **4** (107 mg) (Figure 1). F8 (10.6 g) was chromatographed on a Sephadex LH-20 column (2.0 × 30 cm) using 100% MeOH and a silica gel column (2.0 × 30 cm) using $CHCl_3$/MeOH (5:1, v/v). Further purification by preparative HPLC, as described above, was used to give compounds **5** (74 mg) and **6** (85 mg).

Compound	R
1	H
4	β_D-glucose
6	α_L-glucose-β_D-glucose

Compound	R
2	H
3	β_D-glucose
5	β_L-glucose-β_D-glucose

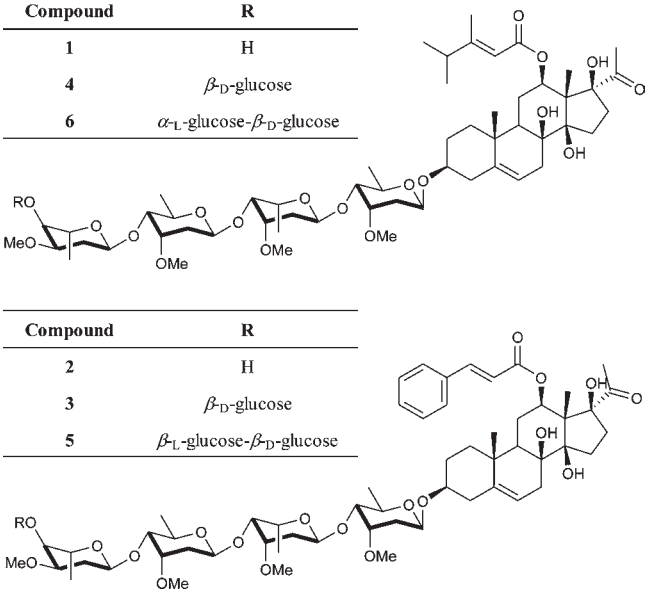

Figure 1. Chemical structures of antifungal compounds (**1**−**6**) isolated from *Cynanchum wilfordii* roots: **1**, wilfoside C1N; **2**, wilfoside K1N; **3**, cynauricuoside A; **4**, wilfoside C1G; **5**, wilfoside K1GG; **6**, wilfoside C1GG.

Wilfoside C1N (Compound 1). This compound was obtained as a white amorphous powder. In the ESI-MS spectrum, the $[M + Na]^+$ ion

Table 1. ^{13}C and ^{1}H NMR (500 MHz, CD_3OD) Spectroscopic Data for Compounds 5 and 6[a]

	5		6	
position	^{13}C	^{1}H (multi, J)	^{13}C	^{1}H (multi, J)
1	39.9	1.83 m, 1.12 m	39.9	1.83 m, 1.13 m
2	30.3	1.84 m, 1.61 m	30.3	1.87 m, 1.60 m
3	79.4	3.66 m	79.4	3.66 m
4	39.9	2.38 m, 2.22 m	39.9	2.37 m, 2.23 m
5	140.4		140.4	
6	119.8	5.36 t (2.4)	119.8	5.35 t (1.6)
7	35.3	2.21 m, 2.13 m	35.3	2.19 m, 2.13 m
8	75.1		75.1	
9	45.2	1.57 m	45.3	1.54 m
10	38.3		38.3	
11	25.6	1.98 m, 1.77 m	25.6	1.88 m, 1.71 m
12	74.5	4.65 dd (12.0, 4.0)	73.4	4.50 dd (12.0, 6.0)
13	58.9		58.8	
14	90.1		90.1	
15	33.5	2.87 m, 1.74 m	33.4	2.86 m, 1.70 m
16	34.4	2.04 m, 1.92 m	34.4	2.01 m, 1.89 m
17	93.2		93.1	
18	10.6	1.60 s	10.6	1.51 s
19	18.8	1.16 s	18.8	1.15 s
20	212.3		211.7	
21	27.9	2.22 s	27.7	2.21
1′	167.5		167.6	
2′	119.0	6.40 d (16.0)	114.4	5.54 s
3′	135.8	7.62 d (16.0)	167.5	
4′	135.8		39.5	2.39 m
5′	129.4	7.60 m	21.4	1.08 d (1.6)
6′	130.2	7.41 m	21.5	1.08 d (1.6)
7′	131.7	7.41 m	16.8	2.11 d (0.8)
8′	130.2	7.41 m		
9′	129.4	7.60 m		
D-cym[b]				
-1″	97.1	4.88 dd (9.6, 1.6)	97.1	4.87 dd (9.6, 1.2)
-2″	35.5[c]	2.17 m, 1.54 m	35.5	2.21 m, 1.55 m
-3″	78.4[d]	3.73 q (3.2)	78.4	3.71 q (3.2)
-4″	82.9[e]	3.27 m	82.9	3.22 m
-5″	70.4[f]	3.90 m	70.4	3.85 m
-6″	17.9	1.21 d (6.4)	17.9	1.20 d (6.4)
-3′-OCH_3	57.5	3.42 s	58.4	3.42 s
L-dig				
-1‴	101.8	4.97 d (3.2)	101.8	4.97 d (3.2), 1.92 m
-2‴	32.7	1.93 m, 1.75 m	32.7	1.75 dd (12.0, 4.8)
-3‴	75.6	3.62 m	75.6	3.61 m
-4‴	75.2	3.95 m	75.2	3.94 m
-5‴	68.4	3.98 m	68.4	3.97 m
-6‴	18.7	1.22 d (6.4)	18.7	1.22 d (6.4)
-3‴-OCH_3	56.9	3.40 s	56.9	3.40 s
D-cym				
-1⁗	100.5	4.80 dd (9.6, 1.6)	100.5	4.81 dd (9.6, 1.6)
-2⁗	35.9[c]	2.27 m, 1.64 m	35.9	2.23 m, 1.64 m
-3⁗	78.8[d]	3.75 q (2.4)	78.8	3.75 q (3.2)
-4⁗	83.3[e]	3.27 m	83.3	3.23 m
-5⁗	70.4[f]	3.88 m	70.4	3.89 dd (10.4, 2.4)
-6⁗	18.6	1.23 d (6.4)	18.6	1.23 d (6.4)
-3⁗-OCH_3	58.4	3.40 s	58.8	3.49 s
L-cym				
-1⁗′	100.1	4.88 dd (9.6, 1.6)	100.1	4.85 m
-2⁗′	32.4f	2.26 m, 1.80 m	32.4	2.28 m, 1.82 m
-3⁗′	74.3	3.81 q (3.2)	74.3	3.81 q (3.2)
-4⁗′	79.3	3.66 m	79.3	3.66 m
-5⁗′	66.2	4.29 dq (8.0, 6.4)	66.2	4.29 dq (8.8, 7.2)
-6⁗′	18.5	1.25 d (6.4)	18.5	1.25 d (6.4)
-3⁗′-OCH_3	56.2	3.39 s	56.2	3.39 s
D-glc				
-1⁗″	102.0	4.45 d (7.2)	102.0	4.45 d (8.0)
-2⁗″	75.1	3.27 m	75.1	3.28 m
-3⁗″	76.3	3.52 m	76.3	3.53 m
-4⁗″	81.0	3.57 m	81.0	3.57 m
-5⁗″	76.8	3.41 m	76.8	3.42 m
-6⁗″	62.2	3.91 m, 3.83 m	62.2	3.91 m, 3.84 m
D-glc				
-1⁗‴	104.8	4.42 d (8.0)	104.8	4.42 d (8.0)
-2⁗‴	75.1	3.23 m	75.1	3.23 m
-3⁗‴	78.0	3.36 m	78.0	3.37 m
-4⁗‴	71.5	3.31 m	71.5	3.32 m
-5⁗‴	78.3	3.34 m	78.3	3.34 dd (6.4, 2.4)
-6⁗‴	62.5	3.91 m, 3.66 m	62.5	3.88 m, 3.66 m

[a] Assignments were based on distortionless enhancement by polarization transfer (DEPT), $^{1}H-^{1}H$ correlation spectroscopy (COSY), heteronuclear single-quantum coherence (HSQC), and heteronuclear multibond connectivity (HMBC) experiments. Due to signal overlapping, only detectable J in Hz are reported. [b] L-cym, α-L-cymaropyranosyl; D-cym, β-D-cymaropyranosyl; L-dig, α-L-diginopyranosyl; D-glc, β-D-glucopyranosyl. [c] Assignments may be interchangeable. [d] Assignments may be interchangeable. [e] Assignments may be interchangeable. [f] Assignments may be interchangeable.

peak appeared at m/z 1090 ($C_{58}H_{86}O_{19}$). The ^{1}H and ^{13}C NMR spectra were comparable to those previously reported for wilfoside C1N.

Wilfoside K1N (Compound 2). This compound was obtained as a white amorphous powder. In the ESI-MS spectrum, the $[M + Na]^+$ ion peak appeared at m/z 1105 ($C_{58}H_{86}O_{19}$). The ^{1}H and ^{13}C NMR spectra were comparable to those previously reported for wilfoside K1N.

Cynauricuoside A (Compound 3). This compound was obtained as a white amorphous powder. In the ESI-MS spectrum, the $[M + NH_4]^+$ ion peak appeared at m/z 1267 ($C_{64}H_{96}O_{24}$). The ^{1}H and ^{13}C NMR spectra were comparable to those previously reported for cynauricuoside A.

Wilfoside C1G (Compound 4). This compound was obtained as a white amorphous powder. In the ESI-MS spectrum, the $[M + NH_4]^+$ ion peak appeared at m/z 1247 ($C_{62}H_{100}O_{24}$). The ^{1}H and ^{13}C NMR spectra were comparable to those previously reported for wilfoside C1G.

Wilfoside K1GG (Compound 5). This compound was obtained as a white amorphous powder. In the ESI-MS spectrum, the $[M + NH_4]^+$ ion peak appeared at m/z 1429 ($C_{70}H_{106}O_{29}$). The ^{1}H and ^{13}C NMR spectra data are summarized in Table 1.

Wilfoside C1GG (Compound 6). This compound was obtained as a white amorphous powder. In the ESI-MS spectrum, the $[M + NH_4]^+$

ion peak appeared at m/z 1410 ($C_{68}H_{110}O_{29}$). The ^{1}H and ^{13}C NMR spectra data are summarized in Table 1.

In Vivo Antifungal Activity. The 1-day protective activities of the fractions and purified substances obtained from *C. wilfordii* roots were evaluated against barley powdery mildew. The in vivo antifungal bioassays were performed as previously described.[18] Polyoxin B, a fungicide of microbial origin discovered in cultures of *Streptomyces cacai* var. *asoensis*[19] and registered in Korea for use against powdery mildews on ornamental and vegetable crops, was applied as a positive control. The pots were arranged in a randomized complete block design, with three replicates per treatment. All experiments for in vivo antifungal activities of the purified substances were performed twice, and treatment values were converted into disease-control percentages (± standard deviation) using the equation

$$\% \text{ control} = 100[(A - B)/A]$$

where A = area of infection (%) on leaves sprayed with Tween 20 solution alone and B = area of infection (%) on treated leaves.

Formulation of the Crude Extract of C. *wilfordii* Roots. To evaluate the potential of a crude extract of *C. wilfordii* roots as a natural fungicide, 2 g of the dried EtOAc extract was formulated into wettable powder (WP) as previously described.[20] One commercial fungicide product, with 10% (by weight) polyoxin B (Dongbu Hannong, Seoul, Korea) as the active ingredient (ai), was used as a positive control. The experiment was repeated twice and performed in triplicate each time.

Antifungal Activity of *C. wilfordii* Roots under Greenhouse Conditions. The antifungal activity experiment was carried out in a commercial strawberry (*Fragaria ananassa* Duch. cv. Janghee) greenhouse in Nonsan City, Korea. Cold-stored strawberry runners were maintained at −2 °C until the day before planting and transplanted at the beginning of November. A general N, P, K, and Mg fertilizer mixture was applied twice a week without the addition of the fungicide active against powdery mildew. The experiment was conducted under normal conditions used for strawberry production with natural disease occurrence. We used a randomized complete block design with three rows, each containing three treatments. Each treatment plot was 4 m long, but data were recorded only from the middle 3 m, leaving 0.5 m on both sides as separation between treatment plots. The three treatments were as follows: (1) control, (2) *C. wilfordii*-WP20, and (3) triflumizole-WP30 (Kyungnong Co. Ltd.; 4000-fold, recommended rate). The treatments were sprayed according to conventional practice at 1-week intervals, and spraying was done three times with a hand-held sprayer until runoff. Prior to each spraying, ripe fruits from each plot were harvested, and the numbers of total and diseased fruits per plot were counted. The percentage of diseased fruits for each treatment was converted into a disease-control percentage relative to the negative control.

Statistical Analysis. Analysis of variance was performed using the PROC GLM procedure (SAS Institute, Cary, NC). If $P > F$ was l<0.01, mean values were significantly different according to Duncan's multiple-range test at the $P = 0.05$ level. Data from disease-control values were arcsine transformed.

RESULTS AND DISCUSSION

Isolation and Identification of Pregnane Gylcosides from *C. wilfordii*. Powdery mildew diseases caused by many fungal species in the order Erysiphales are the most critical diseases affecting various crops worldwide. Despite extensive research on their pathogenesis, epidemiology, and control, biological control of powdery mildew disease remains a challenge for future research and development.[7] In recent years, attention has been given to the development of safer antifungal agents, such as those derived from plant-based extracts, to control powdery mildew in agriculture because they constitute very different kinds of bioactive chemicals. Some phytochemicals of plant origin, such as milsana and *Macleaya* extract, have already been formulated as botanical fungicides for the control of powdery mildews and other plant diseases and used successfully in integrated pest management programs.[21]

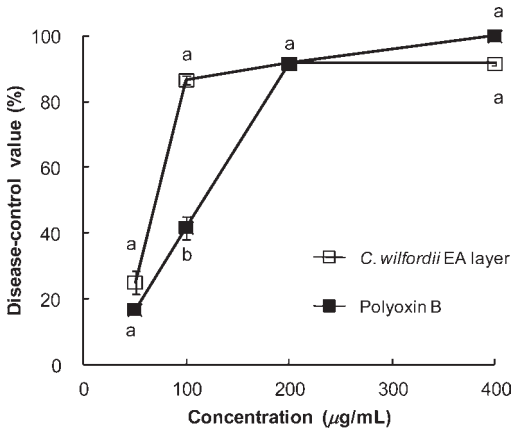

Figure 2. In vivo antifungal activities of the ethyl acetate fraction obtained from the methanol extract of *Cynanchum wilfordii* roots (*C. wilfordii* EA layer) and polyoxin B against barley powdery mildew. Mean values (± standard deviation) at each concentration followed by the same letter are not significantly different ($P = 0.05$) according to the least significant difference test.

In this study, the methanol extract of *C. wilfordii* roots exhibited potent in vivo antifungal activity against *B. graminis* f. sp. *hordei* in barley. At a concentration of 3000 μg/mL, the methanol extract of *C. wilfordii* roots highly suppressed the development of powdery mildew symptoms in barley plants with a disease-control value of 88% (data not shown). Until now, there have been no reports on the use of the crude extract of *C. wilfordii* roots as a fungicide to control plant diseases including barley powdery mildew. Here, we report the chemical structures of antifungal substances isolated from *C. wilfordii* roots for the first time and provide evidence of the efficacy of the crude extract from *C. wilfordii* roots as a botanical fungicide.

The methanol extract was then partitioned successively with *n*-hexane, EtOAc, and butanol. Three organic fractions and one aqueous fraction were obtained from the methanol extract of *C. wilfordii* roots, and of these, the EtOAc fraction was most effective at controlling barley powdery mildew; its disease-control value was 87%. The activities of the other fractions were ranked in the following order: BuOH, Hex, and aqueous fractions showing disease-control values of 80, 42, and 8%, respectively. The EtOAc fraction exhibited a dose-dependent response against barley powdery mildew (Figure 2). Although the EtOAc fraction showed a weaker activity than polyoxin B at a concentration of 400 μg/mL, it was more active than polyoxin B at 100 μg/mL. The EtOAc fraction did not show any phytotoxic symptoms on barley plants even at 400 μg/mL. Purification of the biologically active constituents from the EtOAc fraction of *C. wilfordii* roots was performed by repeated column chromatography under the guidance of the in vivo bioassay. Six compounds (**1**−**6**) were isolated using the process. The four known compounds were identified as wilfoside C1N (**1**),[22] wilfoside K1N (**2**),[22] cynauricuoside A (**3**),[23] and wilfoside C1G (**4**)[22] (Figure 1) by comparison of their MS and NMR spectra to published data. The

two remaining compounds, wilfoside K1GG (**5**) and wilfoside C1GG (**6**), were isolated from *C. wilfordii* roots for the first time.

Compound **5** was obtained as a white amorphous powder, and the molecular formula $C_{70}H_{106}O_{29}$ was determined by positive ESI-MS (Figure 1). The compound displayed 70 carbon signals in its ^{13}C NMR spectrum, of which 30 were assigned to the aglycone and 40 to the sugar moiety (Table 1). Two tertiary methyl groups at δ_H 1.60 (H-18) and 1.16 (H-19) and one olefinic proton at δ_H 5.36 (H-6) observed in the ^1H NMR spectrum, coupled with the information from the ^{13}C NMR spectrum [two methyl carbons at δ_C 10.6 (C-18) and 18.8 (C-19), two olefinic carbons at δ_C 140.4 (C-5) and 119.8 (C-6), and one ketone carbonyl carbon at δ_C 212.3 (C-20)], indicated that the aglycone possessed an unknown derivative of a C/D-*cis*-polyoxypregnane ester-type skeleton.[24] Two acyl substituents were assigned to one cinnamoyl group and one acetyl group on the basis of the ^1H NMR and ^{13}C NMR data shown in Table 1. In the HMBC spectrum, the carbonyl signal of the cinnamoyl group at δ_C 167.5 was correlated with the signal of a methane proton (H-12) at δ_H 4.65 (dd, J = 12.0, 4.0 Hz) on an oxygen-bearing carbon (C-12) at δ_C 74.5. The signal of the methyl group at δ_H 2.22 (H-21) was correlated with the signals δ_C 212.3 (C-20) and 93.2 (C-17). Taken together, these data establish that the cinnamoyl group is located at C-12 in **5**. The aglycone was thus identified as kidjoranine and confirmed by comparison of its spectroscopic data to those in the literature.[24] The NMR spectroscopic data of **5** revealed that it contained six anomeric carbon signals at δ_C 97.1 (C-1″), 101.8 (C-1‴), 100.5 (C-1⁗), 100.1 (C-1⁗′), 102.0 (C-1⁗″), and 104.8 (C-1⁗‴), correlating with anomeric proton signals at δ_H 4.88 (dd, J = 9.6, 1.6 Hz, H-1″), 4.97 (d, J = 3.2 Hz, H-1‴), 4.80 (dd, J = 9.6, 1.6 Hz, H-1⁗), 4.88 (dd, J = 9.6, 1.6 Hz, H-1⁗′), 4.45 (d, J = 7.2 Hz, H-1⁗″), and 4.42 (d, J = 8.0 Hz, H-1⁗‴), respectively, which indicated that there were six sugars with α- and β-linkages in **5**.[22−24] The existence of one L-diginopyranosyl, two D-cymaropyranosyl, one L-cymaropyranosyl, and two D-glucopyranosyl units was confirmed by comparison of its spectroscopic data with those in the literature.[22−24] Compared with wilfoside K1N, the glycosidation shifts were observed at C-2 (+0.4 ppm), C-3 (+1.7 ppm), and C-4 (+0.9 ppm) in the aglycone moiety. Therefore, the sugar moiety was linked to the C-3 hydroxyl group of the aglycone. The sugar sequence of **5** was confirmed by the HMBC spectrum, in which distinct correlations were observed for each of the following: H-1⁗‴′ of the β-D-glucopyranosyl (δ_H 4.42) to C-4⁗′ of the β-D-glucopyranosyl (δ_C 81.0); H-1⁗′ of the β-D-glucopyranosyl (δ_H 4.45) to C-4⁗′ of the outer α-L-cymaropyranosyl (δ_C 79.3); H-1⁗′ of the outer α-L-cymaropyranosyl (δ_H 4.88) to C-4‴ of the inner β-D-cymaropyranosyl (δ_C 83.3); H-1‴ of the inner β-D-cymaropyranosyl (δ_H 4.80) to C-4‴ of the α-L-diginopyranosyl (δ_C 75.2); H-1‴ of the outer α-L-diginopyranosyl (δ_H 4.97) to C-4″ of the inner β-D-cymaropyranosyl (δ_C 82.9); and H-1″ of the β-D-cymaropyranosyl (δ_H 4.88) to C-3 (δ_C 79.4). Thus, the structure of **5** was established as kidjoranine-3-*O*-β-D-glucopyranosyl-(1→4)-β-D-glucopyranosyl-(1→4)-α-L-cymaropyranosyl-(1→4)-β-D-cymaropyranosyl-(1→4)-α-L-diginopyranosyl-(1→4)-β-D-cymaropyranoside (**5**), and compound **5** was named wilfoside K1GG.

Compound **6** was obtained as a white amorphous power, and the molecular formula $C_{68}H_{110}O_{29}$ was determined by positive ESI-MS (m/z 1408.7 $[M + NH_4]^+$) (Figure 1). In the acid hydrolysis experiment, the same sugar component as that of **5** was obtained by TLC comparison with authentic samples. Comparison of the NMR data of compound **6** with those of **5** showed that the signals for one ikemaoyl group were absent in **6**, based on the signals at δ_H 1.08 (each 3H, d, J = 1.6 Hz, 5′, 6′-CH_3), 2.11 (3H, d, J = 0.8 Hz, 7′-CH_3), 2.39 (1H, m, 4′-CH), and 5.54 (1H, s, 2′-CH) (Table 1). Thus, the aglycone was identified as caudatin, which was confirmed by comparison of its spectroscopic data with those in the literature.[24] Furthermore, the HMBC showed the long-range correlations between two proton signals at δ_H 4.50 (H-12) and 5.54 (H-2′) and the carbon signal at δ_C 167.6 (C-1′), suggesting that the ikemaoyl group was located at C-12. Complete ^1H and ^{13}C NMR resonance assignments for the saccharide units were carried out unambiguously on the basis of $^1H-^1H$ COSY, HSQC, and HMBC experiments. The existence of one L-diginopyranosyl, two D-cymaropyranosyl, one L-cymaropyranosyl, and two D-glucopyranosyl units was confirmed by comparison of the spectroscopic data with those in the literature.[22−24] On the basis of the above evidence, compound **6** was shown to be caudatin-3-*O*-β-D-glucopyranosyl-(1→4)-β-D-glucopyranosyl-(1→4)-α-L-cymaropyranosyl-(1→4)-β-D-cymaropyranosyl-(1→4)-α-L-diginopyranosyl-(1→4)-β-D-cymaropyranoside (**6**) and named wilfoside C1GG.

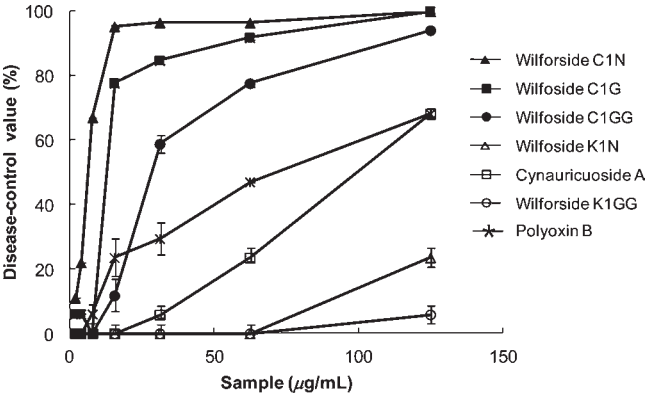

Figure 3. In vivo antifungal activities of the six pregnane glycosides isolated from *Cynanchum wilfordii* roots and polyoxin B against barley powdery mildew. Seedlings were inoculated with spore suspensions of the test organism 1 day after spraying with the chemical solutions.

C. wilfordii has been studied extensively by Mitsuhashi et al.,[25] who reported the isolation of C/D-*cis*-polyoxypregnane esters, including caudatin, kidjoranine, penupogenin, aglycone-D, and aglycone-E from the hydrolysate of the crude glycosides of this plant. They subsequently reported the structure of wilforibiose from the hydrolysate of the glycoside mixture.[26] Subsequently, they identified 13 other glycosides of wilfoside.[22,24] Gong et al.[27] reported the isolation of benzophenone from *C. auriculatum*. Pregnane glycosides are pharmaceutically relevant natural products that have attracted attention due to their bioactivities, including antitumor, cytotoxicity, antifungal, acetylcholine esterase inhibition, and antiosteoporosis activity. Hwang et al.[15] reported that wilfoside K1N, wilfoside C1N, and cynauricuoside A showed significant multidrug resistance-reversing activity. In addition, wilfoside K1N was reported to have strong antiangiogenic and anti-invasive activities both in vitro and in vivo.[15,16] Despite the various chemical investigations of *C. wilfordii*, however, the biological activity of *C. wilfordii* against plant pathogenic fungi had not previously been reported.

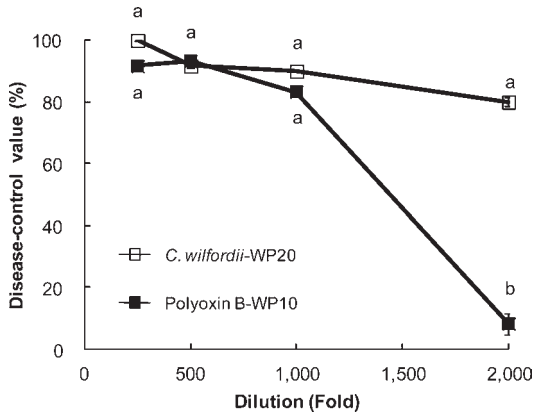

Figure 4. In vivo antifungal activities of the wettable powder-type formulation of the ethyl acetate fraction of *Cynanchum wilfordii* roots (*C. wilfordii*-WP20) and polyoxin B-WP10 against barley powdery mildew. Mean values (± standard deviation) at each dilution followed by the same letter are not significantly different ($P = 0.05$) according to the least significant difference test.

In Vivo Antifungal Activity against Barley Powdery Mildew. The antifungal activities of the six compounds isolated from *C. wilfordii* roots against barley powdery mildew were evaluated in vivo and compared with the antifungal activity of polyoxin B. The three caudatin glycosides **1**, **4**, and **6** exhibited potent in vivo antifungal activities and had disease-control values of >77% at a concentration of 63 μg/mL (Figure 3). In particular, compounds **1** and **4** were highly active even at a low concentration of 16 μg/mL, with disease-control values of 95 and 78%, respectively. The IC_{50} values (i.e., the concentration required for 50% inhibition) were determined as 3.24 μg/mL for **1**, 12.90 μg/mL for **4**, and 28.35 μg/mL for **6**. These compounds showed stronger in vivo antifungal activities than polyoxin B (IC_{50} value = 71.36 μg/mL), whereas the three kidjoranine glycosides **2**, **3**, and **5** had weaker activities than the commercial fungicidal agent. The only structural difference between the two groups is the substituent at C-12; caudatin glycosides (**1**, **4**, and **6**) have an ikemaoyl group, whereas kidjoranine glycosides (**2**, **3**, and **5**) have a cinnamoyl group. Among the three caudatin glycosides, the tetraglycoside (**1**) most effectively controlled barley powdery mildew, followed by the pentaglycoside (**4**) and the hexaglycoside (**6**). Our results suggest that the antifungal activities of pregnane glycosides may be attributed to the polarity of the glycosidic linkage and to the nature of the hydrophobic ester linkage at C-12. Moreover, we observed that the antifungal activities of pregnane glycosides increased with the removal of the C-3 glycone moiety. Similarly, Hwang et al.[15] reported that the tetraglycoside wilfoside K1N displayed much stronger cytotoxicity and multidrug resistance-reversing activity than the pentaglycoside cynauricuoside A. These results indicate that the sugar moiety plays a key role in imparting antifungal activity. Nevertheless, the structure−activity relationships of pregnane glycosides for antifungal activity are not well understood, and this investigation is the first to describe structure−activity relationships of pregnane glycosides from *C. wilfordii* roots against *B. graminis* f. sp. *hordei*.

The IC_{50} values of the three caudatin glycosides are much lower than the IC_{50} of polyoxin B. Among the natural compounds from plants, chrysophanol, parietin, and nepodin isolated from *Rumex crispus* roots were highly active against barley powdery mildew; the IC_{50} values of these compounds were 4.7 μg/mL for chrysophanol, 0.48 μg/mL for parietin, and 20 μg/mL for nepodin.[28] Although parietin is highly active against the powdery mildew diseases of barley and cucumber, use of the chemical or the crude extract of *R. crispus* roots as a natural fungicide has not been commercialized because it is phytotoxic to several crops including cucumber. In contrast, pregnane glycosides from *C. wilfordii* roots have not been found to have any phytotoxic effects in various crops, highlighting its potential as a natural fungicide for the control of powdery mildews.

Antifungal Activity of a *C. wilfordii* Root Formulation against Barley Powdery Mildew. A wettable powder-type formulation of the EtOAc fraction of *C. wilfordii* roots (*C. wilfordii*-WP20) was tested for its in vivo antifungal activity against barley powdery mildew and compared with polyoxin B-WP10. As shown in Figure 4, *C. wilfordii*-WP20 showed higher activity against barley powdery mildew than polyoxin B-WP10. The former more effectively reduced the appearance of powdery mildew symptoms on barley plants at all dilutions tested, except at the 500-fold dilution. *C. wilfordii*-WP20 prohibited barley powdery mildew by 80% even at a 2000-fold dilution. Polyoxin B-WP10 was generally effective in controlling powdery mildew on barley leaves, with a few lesions observed on barley leaves treated with 250-, 500-, and 1000-fold dilutions of polyoxin B-WP10. The adjuvant mixture without plant extracts had virtually no effect on barley powdery mildew (data not shown). Compared to the polyoxin B-WP, *C. wilfordii*-WP was more effective in barley powdery mildew at the 2000-fold dilution. This study shows that the crude extract of *C. wilfordii* roots and its wettable powder-type formulation can effectively control barley powdery mildew and that the major active antifungal substances are the six pregnane glycosides.

Antifungal Activity of *C. wilfordii*-WP20 against Strawberry Powdery Mildew under Greenhouse Conditions. In the greenhouse experiment performed in the Nonsan farmhouse, the antifungal activity of *C. wilfordii*-WP20 against strawberry powdery mildew caused by *S. humuli* was also evaluated (Table 2). Treatment of *C. wilfordii*-WP20 at a 1000-fold dilution showed control efficacy against strawberry powdery mildew statistically similar to that of triflumizole-WP30, a chemical fungicide, applied at a 4000-fold dilution. Both treatments highly suppressed the occurrence of disease symptoms on strawberry fruits with disease-control values of 81% for *C. wilfordii*-WP20 and 89% for triflumizole-WP30. We observed no harmful effects of *C. wilfordii*-WP20 on the strawberry plants tested in this study. Thus, *C. wilfordii*-WP20 was also highly effective against the infection of powdery mildew fungus in strawberry under greenhouse conditions.

For the control of powdery mildews, the most commonly used chemical agents are azoxystrobin, bitertanol, fenarimol, and hexaconazole.[29] Intensive use of these fungicides has resulted in resistance to powdery mildew fungi. Moreover, it has led to several problems including environmental pollution and phytotoxicity. Because natural products are generally recognized as safer control agents, the natural fungicide containing the six pregnane glycosides may contribute to increased yields for various crops, including barley and strawberry, the supply of high-quality food products, and the reduction of environmental pollution by synthetic fungicides. *C. wilfordii* is a perennial herb, and its roots are widely used for medicinal purposes and as a foodstuff in Korea. These practices suggest low toxic effects in mammals. Furthermore, we did not observe any phytotoxic symptoms in plants treated with the plant extract and pregnane glycosides. Because of the low toxicity, lack of phytoxicity, and

Table 2. Disease-Control Efficacy of the Wettable Powder-Type Formulation of the Ethyl Acetate Fraction of *Cynanchum wilfordii* Roots (*C. wilfordii*-WP20) and Triflumizole-WP30 against Strawberry Powdery Mildew in a Commercial Greenhouse[a]

treatment	dilution (fold)	diseased fruits (%)[b]	disease-control value (%)
C. wilfordii-WP20	1000	12.7 bc	81
triflumizole-WP30	4000	7.2 c	89
control		62.2 a	

[a] The chemical solutions were applied three times until runoff on strawberry plants with a 7 day interval in a commercial greenhouse. [b] Each value represents the mean of three replicates. Mean values followed by the same letter are not significantly different ($P = 0.05$) according to the least significant difference test.

potent in vivo antifungal activity, the crude extract containing the six pregnane glycosides may constitute a highly valuable natural fungicide for the control of powdery mildews.

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