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# In Vivo Disease Control Efficacy of Isoquinoline Alkaloids Isolated from *Corydalis ternata* against Wheat Leaf Rust and Pepper Anthracnose [S]

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Phytochemicals have been considered as alternatives for synthetic fungicides because of their biodegradability and low toxicity. In this study, we found that the methanolic extract of *Corydalis ternata* suppressed the development of plant diseases caused by *Puccinia triticina* and *Colletotrichum coccodes*. As the antifungal substance, three isoquinoline alkaloids (dehydrocorydaline, stylopine, and corydaline) were isolated from *C. ternata.* These active compounds also exhibited in vivo antifungal activity against *P. triticina* and *C. coccodes*. Taken together, our results suggest that *C. ternata* and its active compounds can be used to control plant diseases.

**Keywords:** *Corydalis ternata*, isoquinoline alkaloids, *Puccinia triticina, Colletotrichum coccodes,* in vivo antifungal activity

The agriculture industry faces constant threats from plant diseases, causing an annual yield loss of around 20% [1]. During the past decades, synthetic fungicides have been used extensively to reduce the yield loss [2]. Overuse of synthetic chemicals for pest management has led to several issues, such as potential toxicity in humans, pollution of the environment, and development of fungicide resistance [3]. Natural resources such as microbes and plant materials have been considered as an alternative to synthetic fungicides [4, 5]. In particular, plant materials are a rich source of bioactive compounds with chemical novelty and have attracted much attention for the development of novel fungicides [6, 7].

*Corydalis ternata* Nakai (Papaveraceae) is a native plant found throughout Korea, Japan, and China [8]. The tubers of this plant have long been used as a Korean folk medicine for the relief of pain and spasms [9]. Furthermore, several active compounds isolated from the *C. ternata* tuber exhibit pharmacological activity for Alzheimer disease, diabetes complications, and cancers [9–12]. However, despite the various biological activities of *C. ternata,* there are no reports on the potential of *C. ternata* for the control of plant diseases. Here, we examined the in vivo antifungal activity of a *C. ternata* extract and isolated three active compounds.

Dried tubers of *C. ternata* were purchased from the Kyung-dong herbal market (Korea), and voucher specimens were deposited in the laboratory. Dried tubers (2.5 kg) were extracted with 4 L of methanol for 3 days, yielding a methanol extract (311 g). The extract was sequentially partitioned with *n*-hexane, chloroform, and ethyl acetate to yield 23, 34, and 19 g, respectively. The chloroform layer was subjected to silica gel (230–400 mesh; Merck, Germany) liquid chromatography with a gradient elution of chloroform-methanol to yield 20 fractions. The no. 8 fraction (0.9 g) was further subjected to Sephadex LH-20 (Sigma-Aldrich, USA) column chromatography and recrystallized in methanol to yield compound **1**. The no. 10 and 11 fractions (3.1 g) were further fractionated by a second round of silica gel (70–

230 mesh; Merck) liquid chromatography with a gradient elution of chloroform-methanol. Compounds **2** and **3** were finally purified with the LC-6AD HPLC system (Shimadzu, Japan) equipped with Polaris C18-A column (250 × 21.2 mm, 10 μm; Agilent, USA). The column was eluted at a flow rate of 5 ml/min with 30% aqueous acetonitrile (containing 0.1% formic acid) to 80% aqueous acetonitrile (containing 0.1% formic acid) at a linear gradient over a 50 min uninterrupted interval. The effluent was monitored with the SPD-M10Avp photodiode array detector (Shimadzu).

Chemical structures of the isolated active compounds were determined with the Q-Tof Micro mass (Waters, UK) and Bruker DPX-300 (Rheinstetten, Germany) spectrometers. $^{1}H$ and $^{13}C$ nuclear magnetic resonance (NMR) data were measured in chloroform-*d* (99.8 atom% D; Sigma-Aldrich). For the in vivo antifungal activity assay, five phytopathogenic fungi were used in this study: *Botrytis cinerea* for tomato gray mold, *Phytophthora infestans* for tomato late blight, *Puccinia triticina* for wheat leaf rust, *Blumeria graminis* f. sp. *hordei* for barley powdery mildew, and *Colletotrichum coccodes* for pepper anthracnose. As hosts for the pathogens, tomato (*Solanum lycopersicum* cv. Seokwang), wheat (*Triticum aestivum* cv. Eunpa), barley (*Hordeum sativum* cv. Dongbori), and pepper (*Capsicum annuum* cv. Bugang) were grown in a greenhouse at 25 ± 5°C for 1–5 weeks. All the samples were dissolved in methanol (60 mg/ml) and suspended in 0.025% Tween 20 solution. The potted plant seedlings were treated with the spray method, and then the treated plants were placed under laboratory conditions for 24 h. The control plants were treated with 0.025% Tween 20 solution containing 5% methanol. After the sample treatments, the plants were inoculated with their respective fungal pathogen and incubated as previously described [13, 14]. Fenhexamide, dimethomorph, flusilazole, benomyl, and dithianon were used as positive controls. The experiment was conducted twice with four replicates for each treatment. The percentage of disease control was determined with the following equation: control value (%) = 100 × [1–$B/A$], where $A$ = the diseased area (%) on the leaves of the control plants, and $B$ = the diseased area (%) on the leaves of the treated plants. Data were subjected to one-way ANOVA, and the means of the treatments were separated by Duncan's multiple range test ($p < 0.05$) with the R software.

As shown in Table 1, the treatment with the *C. ternata* tuber extract (3,000 μg/ml) showed control values of 67% and 40% against wheat leaf rust and pepper anthracnose, respectively. These activities were enhanced in the partitioned fractions. The hexane, chloroform, and ethyl acetate fractions

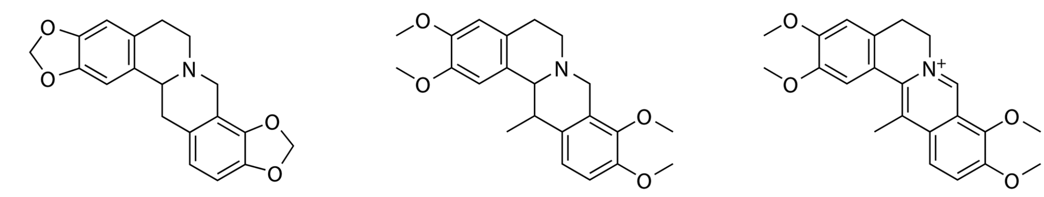
**Table 1.** Control efficacies of the *Corydalis ternata* extracts for five plant diseases.

| Treatment[a] | Conc. (μg/ml) | Disease control (%)[b] | | | | |
|---|---|---|---|---|---|---|
| | | TGM[c] | TLB | WLR | BPM | PAN |
| Methanol extract | 3,000 | 14 ± 7w | 14 ± 11x | 67 ± 0x | 0y | 40 ± 0w |
| Hexane layer | 2,000 | 36 ± 10xy | 36 ± 10y | 100z | 0y | 55 ± 7x |
| Chloroform layer | 2,000 | 21 ± 10wx | 21 ± 10xy | 90 ± 5y | 8 ± 12y | 75 ± 7yz |
| Ethyl acetate layer | 2,000 | 43 ± 0y | 7 ± 10x | 90 ± 5y | 0y | 20 ± 0v |
| Fenhexamide | 20 | 88 ± 3z | - | - | - | - |
| | 100 | 100z | - | - | - | - |
| Dimethomorph | 2 | - | 91 ± 2z | - | - | - |
| | 10 | - | 100z | - | - | - |
| Flusilazole | 2 | - | - | 83 ± 5y | - | - |
| | 10 | - | - | 100z | - | - |
| Benomyl | 20 | - | - | - | 90 ± 0z | - |
| | 100 | - | - | - | 100z | - |
| Dithianon | 10 | - | - | - | - | 65 ± 7xy |
| | 50 | - | - | - | - | 86 ± 0z |

[a]Each compound was dissolved in 5% methanol and 0.025% Tween 20 and then sprayed to run off on the following seedlings with fully expanded leaves: 2-leaf stage of tomato, 1-leaf stage of barley, 1-leaf stage of wheat, and 2-leaf stage of pepper. After 24 h, the treated seedlings were inoculated with the spores of each pathogen.

[b]Each value represents the mean ± standard deviation of two runs with four replications. Different small letters in each column indicate significant difference at $p < 0.05$ (Duncan's test).

[c]TGM, tomato gray mold (caused by *Botrytis cinerea*); TLB, tomato late blight (caused by *Phytophthora infestans*); BPM, barley powdery mildew (caused by *Blumeria graminis* f. sp. *hordei*); WLR, wheat leaf rust (caused by *Puccinia triticina*); PAN, pepper anthracnose (caused by *Colletotrichum coccodes*).



1 2 3

**Fig. 1.** Chemical structures of stylopine (**1**), corydaline (**2**), and dehydrocorydaline (**3**).

(2,000 μg/ml) showed a disease control value of more than 90% against wheat leaf rust compared with the non-treated control. These activities were comparable to the disease control values of the positive control flusilazole. Pepper anthracnose was also reduced 75% by the chloroform fraction. In contrast, the aqueous layer did not show any activity (data not shown).

The chloroform fraction was subjected to further purification because of its strong suppressive effects on wheat leaf rust and pepper anthracnose. On the basis of the activity-guided fractionation, three compounds (**1**–**3**) were isolated. The electrospray ionization-mass spectrometry (ESI-MS) of compounds **1**–**3** presented molecular ions at *m/z* 324 $[M+H]^+$, 370 $[M+H]^+$, and 366 $[M^+]$, respectively. The MS and NMR spectra of compounds **1**–**3** were in complete agreement with the literature data of stylopine, corydaline, and dehydrocorydaline (Fig. 1), respectively [15–20]. The $^1$H and $^{13}$C NMR spectra are summarized in Table S1. In tests of the control efficacy of the isolated compounds for five plant diseases, wheat leaf rust was reduced 60% by dehydrocorydaline at a concentration of 500 μg/ml (Fig. 2). Pepper anthracnose was reduced 65% and 50% by stylopine and corydaline, respectively (Fig. 2). No obvious phytotoxic effects were found from each compound (data not shown). The in vitro antifungal activity was evaluated by broth microdilution method against *Colletotrichum coccodes* (pepper anthracnose) only, since *Puccinia triticina* (wheat leaf rust) is an obligate parasite that is difficult to grow on artificial media. The fungal growth was restricted by the treatment with stylopine, corydaline, and dehydrocorydaline, with inhibition rates of up to 39%, 26%, and 16%, respectively

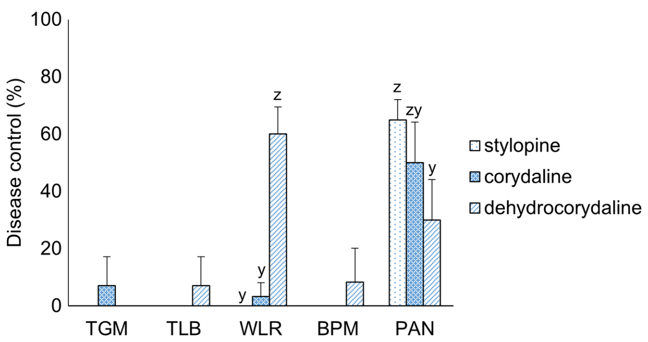



**Fig. 2.** In vivo disease control efficacy of three isoquinoline alkaloids isolated from *Corydalis ternata*.

The concentration of each compound was adjusted to 500 μg/ml and then treated onto plants. After 24 h of incubation, the treated plants were inoculated with spores or mycelial suspensions of pathogens. Each value represents the mean ± standard deviation of two runs with four replications. Different small letters in each column indicate significant difference at $p < 0.05$ (Duncan's test). TGM, tomato gray mold; TLB, tomato late blight; WLR, wheat leaf rust; BPM, barley powdery mildew; PAN, pepper anthracnose.

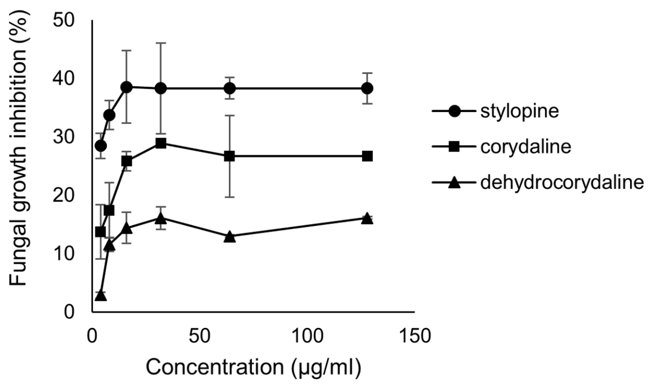



**Fig. 3.** In vitro antifungal activity of three isoquinoline alkaloids isolated from *Corydalis ternata* against *Colletotrichum coccodes* causing pepper anthracnose.

A 96-well microtiter plate was used for fungal growth in potato dextrose broth (100 μl). Each compound was added at final concentrations of 4–128 μg/ml per well containing $10^5$ conidia/ml of *C. coccodes*. Culture medium containing 4% methanol was used as the control. Two days after incubation, the optical density at 600 nm ($OD_{600}$) of each well was recorded using a microplate reader. Fungal growth inhibition (%) was calculated as [1 – ($OD_{600}$ of treatment/$OD_{600}$ of control)] × 100. Each value represents the mean ± standard deviation of three replicates.

(Fig. 3). These in vitro results agreed with the data from the in vivo assessment, and demonstrated the reason why dehydrocorydaline was less effective to control pepper anthracnose. In addition, it was found that the presence/absence of the methyl group at C-13 and the quaternary nitrogen atom seem to play important roles in the antifungal activity. These protoberberine-type alkaloids have attracted considerable attention as antifungal agents owing to their promising antifungal activity [21–27].

A number of isoquinoline alkaloids have been found in the genus *Corydalis*, including aporphine, protopine, protoberberine, tetrahydroprotoberberine, benzo[*c*]phenanthridine, phthalideisoquinoline, benzylisoquinoline, morphinan, and spirobenzylisoquinoline [25]. Although the pharmaceutical activities of these alkaloids have been extensively explored over the past few decades [9–12], few studies have shown antifungal activities against phytopathogenic fungi. Orhan *et al*. [21] described the inhibitory effects of isoquinoline alkaloids isolated from *Corydalis* spp. on the human pathogenic fungus *Candida albicans* (MICs = 4–8 μg/ml). In addition, corynoline, acetylcorynoline, tetrahydropalmatine, *N*-methylhydrasteine hydroxylactam, and 1-methoxyberberine chloride from *Corydalis* plants showed in vitro antifungal activities against phytopathogenic fungi, including *Alternaria*, *Curvularia*, *Cladosporium*, *Colletotrichum*, *Helminthosporium*, *Heterospora*, and *Ustilago* [21–25]. Here, we present evidence for the first time showing that the in vivo antifungal activities of stylopine, corydaline, and dehydrocorydaline can be used to control plant diseases, and suggest that the isoquinoline alkaloids in the present study could be used as an active ingredient to develop biocontrol agents or fungicides for the agriculture industry.

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## Conflict of Interest

The authors have no financial conflicts of interest to declare.

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