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Inhibition of the Calcineurin Pathway by Two Flavonoids Isolated from *Miliusa sinensis* Finet & Gagnep.

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In order to discover plant-derived signaling pathway inhibitors with antifungal properties, a two-component screening system utilizing the calcineurin and Hog1 mitogen-activated protein kinase pathways responsible for the virulence networks of *Cryptococcus neoformans* was employed, owing to the counter-regulatory actions of these pathways. Of the 1,000 plant extracts tested, two bioactive compounds from *Miliusa sinensis* were found to act specifically on the calcineurin pathway of *C. neoformans*. These compounds, identified as pashanone and 5-hydroxy-6,7-dimethoxyflavanone, exhibited potent antifungal activities against various human pathogenic fungi with minimum inhibitory concentration values ranging from 4.0 to >128 μg/ml.

Keywords: *Miliusa sinensis* Finet & Gagnep., *Cryptococcus neoformans*, virulence network, two-component system, calcineurin pathway

Cryptococcus neoformans is a dimorphic human fungal pathogen that proliferates as budding yeast during vegetative growth but switches to hyphal filamentation during mating [16]. *C. neoformans* can cause life-threatening respiratory and neurological infections by the inhalation of basidiospores from pigeon guano, soil, or tree hollows [8, 11, 25]. The fungus is a major pathogen in patients with AIDS [19] and transplant recipients [22], causing meningoencephalitis in immunocompromised [11] and immunocompetent hosts [23]. Only a few available antifungals can treat invasive fungal infections including those caused by *C. neoformans*; moreover, drug resistance is on the rise. Therefore, the need for the development of novel antifungal agents with low toxicity, broad-spectrum activity, and different modes of action for use in combination therapies is greater than before.

Calcineurin, a calcium-calmodulin-dependent serine-threonine phosphatase, is critical for the growth of *C. neoformans* at 37°C and its virulence [14]. Calcineurin has crucial roles in various physiological processes, including morphogenesis, cell cycle progression, cytokinesis, cell wall biogenesis, ion homeostasis, and in mediating cell stress responses [5–7, 14, 18]. Calcineurin is the common target of the immunosuppressant agents tacrolimus (FK506) and cyclosporine A [20]. *C. neoformans* also uses the stress-activated Hog1 MAPK pathway for adaptation to a variety of environmental stressors, including osmotic shock, heat shock, oxidative damage, UV irradiation, toxic metabolites, and antifungal agents [1–3, 15]. Fludioxonil, which induces the activation of the Hog1 pathway, is a unique fungicide that targets signal transduction, and is now used to control various plant pathogenic fungi, including *Botrytis cinerea* and *Collectotrichum lagenarium* [15]. Exploitation of such fungal signaling pathways could potentially offer a great promise in developing novel antifungal drugs in the future [24].

The genus *Miliusa* Lesch. ex A.DC. (Annonaceae) consisting of 30~40 species is found across India and southern China as well as northern Australia [21]. In China, *Miliusa balansae* Finet & Gagnep. is traditionally used for the treatment of gastropathy and glomerulonephropathy [27]. Aporphine alkaloids, terpenoids, flavonoids, phenylpropanoids, strylpyrones, bis-styryls, and homogentistic acid derivatives are reported constituents of plants in this genus [9, 12, 13, 27, 28]. *Miliusa sinensis* Finet & Gagnep., found in southern Asia including Vietnam and southern China, has been

found to contain miliusane derivatives, which exhibit cytotoxic activities [28].

In the search for plant-derived substances that modulate fungal signaling pathways, inhibitors of the *C. neoformans* calcineurin pathway were found in the methanol extract from *M. sinensis* Finet & Gagnep. Although extracts of *M. sinensis* have been found to have potent cytotoxic activities against various cancer cell lines [28], there are no reports for their use as a specific inhibitor of the calcineurin signaling pathway in *C. neoformans*. Here, we describe the isolation and structure determination of two flavonoid compounds, pashanone and 5-hydroxy-6,7-dimethoxyflavanone, and examine their inhibitory activities against various human pathogenic fungi.

Utilizing the calcineurin and Hog1 pathways that control the virulence networks of *C. neoformans*, owing to the counter-regulatory actions on these pathways, plant-derived fungal signaling modulators were found using the agar diffusion method as described previously [17].

C. neoformans var. *grubii* H99 (*MAT*α) and *C. neoformans cna1*Δ (*MAT*α *cna1*Δ::*NAT-STM#117*) mutant strains [15, 17] were cultured on YPD (1% yeast extract, 2% peptone, 2% dextrose) medium at 30°C, and various human pathogenic fungi were cultured in Sabouraud broth at 30°C for 24 h. The YPD agar plates used in all bioassays were prepared as two separate layers: base medium containing solidified YPD agar overlaid with YPD medium containing either *C. neoformans* H99 or *cna1*Δ mutant strains. Solidified YPD plates, seeded with either strain, were labeled A–D for bioassays as described previously [17]. A plates were cultured with *C. neoformans* H99 and test sample; B plates were cultured with *C. neoformans* H99, test sample, and fludioxonil; C plates were cultured with *C. neoformans cna1*Δ mutant, test sample, and fludioxonil; and D plates were cultured with *C. neoformans cna1*Δ mutant and test sample. Aliquots of samples, with or without fludioxonil, were loaded onto sterile paper disks placed on the surface of the agar plate from each of the four groups (A–D) and incubated for 24 h at 30°C. Test samples showing a large clear zone on A, B, and D plates were primarily selected, whereas those with a smaller clear zone on the C plate compared with that of fludioxonil used as a control were chosen. Test samples simultaneously exhibiting clear zones on all plates (A–D) were excluded.

The in vitro minimum inhibitory concentrations (MICs) for the isolated compounds against various human pathogenic fungi were determined by the broth microdilution method from the Clinical and Laboratory Standards Institute (CLSI, formerly NCCLS) for yeast [4].

One thousand foreign plant extracts supplied by the International Biological Material Research Center, Korea Research Institute of Bioscience and Biotechnology, were screened to identify modulators of fungal signaling pathways. The change in the inhibition zone of the same sample on the four bioassay plates containing wild-type *C. neoformans* and its calcineurin mutant was analyzed with the naked eye and compared with that of the control compound fludioxonil. Candidates showing inhibitory activities only on B or C plates were selected from the 1,000 plant extracts, resulting in the identification of two bioactive compounds from *M. sinensis* with a clear zone on the B plate only. Both compounds were isolated and purified using dichloromethane extraction, silica gel column chromatography, thin layer chromatography, and high-performance liquid chromatography (HPLC). The dried powder of *M. sinensis* Finet & Gagnep. (1 kg) was extracted four times with 80% methanol at room temperature for 12 h. The methanol extract was filtered and evaporated in vacuo, and then the crude extracts (145.7 g) were suspended in 5% methanol, followed by successive solvent partitioning with hexane, dichloromethane, and ethyl acetate. The active dichloromethane fraction (21.3 g) was subjected to silica gel column chromatography (Kiesel gel 60, 70–230 mesh, 5.6 × 35 cm; Merck) and eluted stepwise with a gradient of *n*-hexane/ethyl acetate/methanol (2:1:0, 1:1:0, 1:2:0, 1:5:0, 1:5:1, 0:5:1, 0:1:1, 0:0:1 (v/v), 0.8 L each). Each eluent was concentrated in vacuo, dissolved in methanol, and subjected to bioassays against *C. neoformans* H99 or *cna1*Δ mutant strains using the agar diffusion method. The active fractions (fraction 2 among 12 fractions, 5.75 g) were collected, evaporated, and chromatographed on a silica gel column (Kiesel gel 60, 70–230 mesh, 4.4 × 40 cm; Merck) by eluting with hexane/ethyl acetate/methanol (1:4:0, 1:10:0, 1:10:1, 1:10:2, 0:10:2, 0:2:1 0:1:1, 0:0:1 (v/v)). The methanol-insoluble fraction (1.65 g) from fraction 2 was dissolved in chloroform/methanol (10:1) for further purification by silica gel column (Kiesel gel 60, 230–400 mesh, 5.6 × 35 cm; Merck) eluting with chloroform/methanol (10:1, 5:1, 3:1, 2:1, 1:1, 1:2, 0:1). The eluents containing antifungal activity against B plate with *C. neoformans* H99 were collected and evaporated in vacuo and then crystalized (100% methanol) to give pashanone (301.5 mg). The purity of the compound was determined by HPLC with a C_{18} column (4.6 × 250 mm, 5 μm; YMC–Pack Triart). The column was eluted stepwise with a gradient of water containing 0.1% acetic acid/methanol (0:100) at a flow rate of 1 ml/min. In addition, the methanol-soluble fraction from fraction 2 was dissolved in chloroform/methanol (30:1) and subjected to silica gel

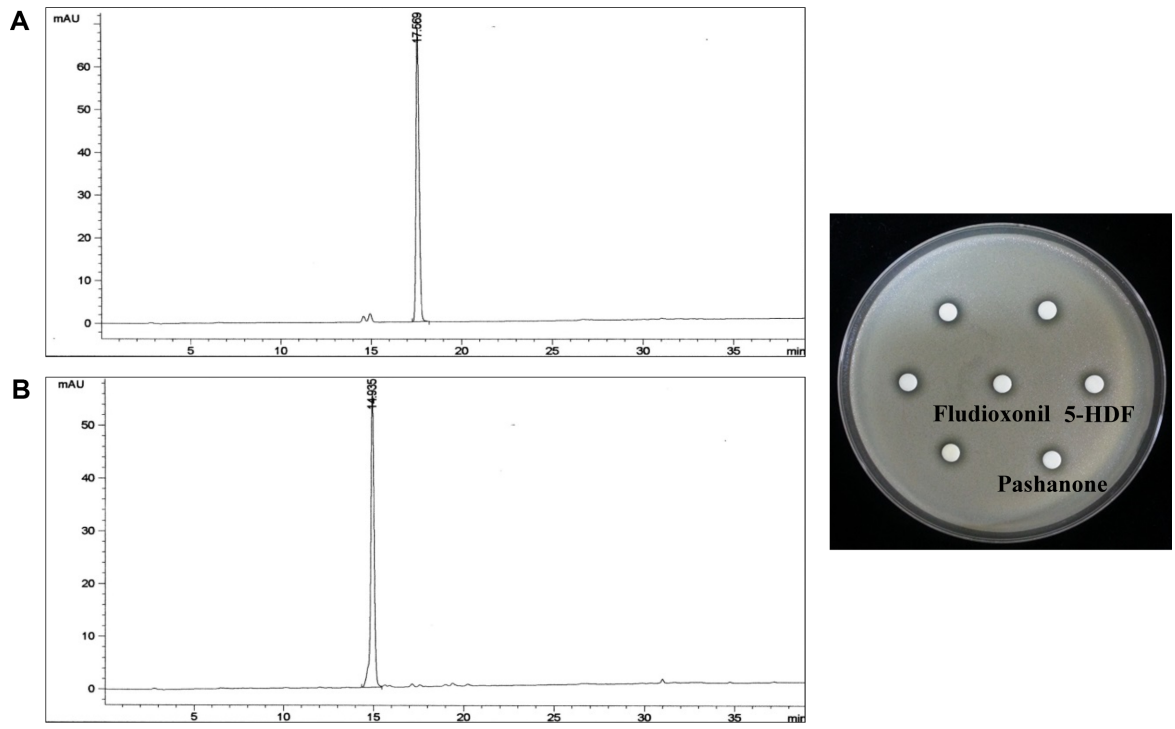

Fig. 1. HPLC chromatograms (left) and the biological activities (right) of pashanone (**A**) and 5-hydroxy-6,7-dimethoxyflavanone (5-HDF; **B**), isolated from *Miliusa sinensis* Finet & Gagnep.

Bioassays of pashanone and 5-hydroxy-6,7-dimethoxyflavanone at the concentration of 50 μg/ml were performed on plates containing *C. neoformans* var. *grubii* H99 and fludioxonil (100 μg/ml).

column chromatography (Kiesel gel 60, 230–400 mesh, 5.6 × 35 cm; Merck) eluting with chloroform/methanol (50:1, 20:1, 10:1, 5:1, 1:1, 0:1 (v/v)). The resulting fraction (fraction 3 among 7 fractions, 474.5 mg) was dissolved in 30% dichloromethane and then crystalized (dichloromethane/methanol, 3:7) to give 5-hydroxy-6,7-dimethoxyflavanone (155.8 mg). The purity of the compound was determined by HPLC with the same condition as described above. The retention times in HPLC analysis of the purified compounds were 14.9 and 17.5 min for 5-hydroxy-6,7-dimethoxyflavanone and pashanone, respectively (Fig. 1). Structural analyses with electrospray ionization mass spectrometry (ESI-MS) and various nuclear magnetic resonance (NMR) techniques, including ^{1}H-^{1}H COSY, HMQC, DEPT, and HMBC, revealed that both compounds had the same molecular formula $C_{17}H_{16}O_5$ and molecular weight of 300. Based on the data, the compounds were identified as pashanone and 5-hydroxy-6,7-dimethoxyflavanone (Fig. 2). The MS and NMR data for both compounds were in agreement with previously published spectral data [10, 26].

The antifungal activities of the isolated pashanone and 5-hydroxy-6,7-dimethoxyflavanone against various human

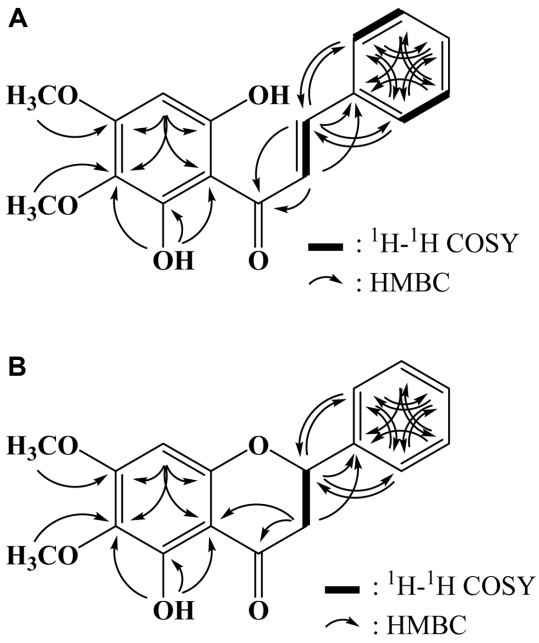

Fig. 2. Structures of pashanone (**A**) and 5-hydroxy-6,7-dimethoxyflavanone (**B**) isolated from *Miliusa sinensis* Finet & Gagnep.

Table 1. In vitro antifungal activities of pashanone and 5-hydroxy-6,7-dimethoxyflavone against various human pathogenic fungi.

Test microorganism	Minimum inhibitory concentration (μg/ml)		
	Pashanone	5-Hydroxy-6,7-dimethoxyflavanone	Amphotericin B
Candida albicans ATCC10231	16	8	0.25
Candida krusei ATCC6258	32	32	0.5
Candida lusitaniae ATCC42720	32	16	0.25
Candida tropicalis ATCC13803	32	16	0.25
Cryptococcus gatti R265	32	16	0.25
Cryptococcus gatti WM276	16	16	0.125
Cryptococcus neoformans JEC21	16	4	0.125
Cryptococcus neoformans ATCC36556	8	4	<0.125
Cryptococcus neoformans var. *grubii* H99	8	8	0.125
Aspergillus fumigatus ATCC16424	>128	>128	0.5
Trichophyton mentagrophytes ATCC9533	32	64	0.25

pathogenic fungi were determined by MIC values using the broth dilution method from the CLSI. 5-Hydroxy-6,7-dimethoxyflavanone showed potent antifungal activities against *C. albicans*, *C. neoformans*, and *C. neoformans* var. *grubii*, with MICs ranging from 4 to 8 μg/ml, whereas pashanone showed potent antifungal activities against *C. neoformans* and *C. neoformans* var. *grubii*, with an MIC of 8 μg/ml. Both compounds displayed relatively modest activities against other *Candida*, *Cryptococcus*, and *Trichophyton* strains (MICs of 16−64 μg/ml), but weak activity against *A. fumigatus* (MIC >128 μg/ml) (Table 1).

Although a number of compounds from *M. sinensis* Finet & Gagnep. have been identified [26, 28], information regarding their antifungal effects (except cytotoxic activities) is extremely limited. In particular, there is little knowledge about the in vitro antifungal activities of the two flavonoids identified in this study against various *Candida* and *Cryptococcus* strains. Taken together, to the best of our knowledge, this is the first report identifying the potent antifungal activities of pashanone and 5-hydroxy-6,7-dimethoxyflavanone against human pathogenic fungi via inhibition of the calcineurin pathway in the zoonotic fungus *C. neoformans*. Thus, both compounds may serve as useful tools in the development of signaling pathway inhibitors of *C. neoformans*. Their detailed mode of action remains to be investigated.

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