J. Microbiol. Biotechnol. (2015), 25(9), 1429–1432
http://dx.doi.org/10.4014/jmb.1506.06031

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A Phenylpropanoid Glycoside as a Calcineurin Inhibitor Isolated from *Magnolia obovata* Thunb.

Won Jeong Lee[1], Jae Sun Moon[1], Sung In Kim[1], Yong-Sun Bahn[2], Hanna Lee[3], Tae Hoon Kang[3], Heung Mook Shin[3], and Sung Uk Kim[1]*

[1]*Division of Biosystems Research, Korea Research Institute of Bioscience and Biotechnology, Daejeon 305-806, Republic of Korea*
[2]*Department of Biotechnology, Yonsei University, Seoul 120-749, Republic of Korea*
[3]*Korea Promotion Institute for Traditional Medicine Industry, Gyeongsan 712-260, Republic of Korea*

Received: June 12, 2015
Revised: June 23, 2015
Accepted: June 24, 2015

First published online
July 13, 2015

**Corresponding author*
Phone: +82-42-860-4554;
Fax: +82-42-861-2675;
E-mail: kimsu@kribb.re.kr

pISSN 1017-7825, eISSN 1738-8872

To identify plant-derived cell signaling inhibitors with antifungal properties, a two-component screening system using both wild-type *Cryptococcus neoformans* and a calcineurin mutant was employed owing to their counter-regulatory actions on the Hog1 mitogen-activated protein kinase and calcineurin pathways. Of the 2,000 plant extracts evaluated, a single bioactive compound from *M. obovata* Thunb. was found to act specifically on the calcineurin pathway of *C. neoformans*. This compound was identified as magnoloside A, and had potent antifungal activities against various *Cryptococcus* strains with minimum inhibitory concentration values ranging from 1.0 to 4.0 μg/ml.

Keywords: Signaling pathway, *Cryptococcus neoformans*, two-component system, calcineurin inhibitor, antifungal activity

Cryptococcus neoformans is an opportunistic fungal pathogen that can cause life-threatening meningoencephalitis primarily in immunocompromised [6], but also in immunocompetent, hosts [23]. Infection occurs *via* inhalation of basidiospores from environmental reservoirs (*i.e.*, pigeon guano, soil, and trees) into the lung, followed by dissemination *via* the blood stream into the central nervous system [6, 8, 25]. About 1 million cases of crypotococcal meningitis are diagnosed each year worldwide, and over half of the infected patients die [16]. *C. neoformans* represents a significant threat to human health, particularly to AIDS patients [15] and organ transplant recipients [22]. However, available drugs for the treatment of invasive fungal infections are limited to polyenes (amphotericin B and its derivatives), azoles, echinocandins, and flucytosine. Among them, only the azoles, flucytosine, and polyenes are reportedly effective therapeutic options against *C. neoformans* infections [18]. Therefore, the discovery of novel antifungal agents with low toxicity and broad-spectrum activity is crucial to combat increasingly drug-resistant strains of human fungal pathogens.

Calcineurin is a highly conserved, Ca^{2+}-dependent serine-threonine phosphatase and has a crucial role in mediating cell stress responses [10]. It is a heterodimer comprising a 60 kDa Ca^{2+}-calmodulin-binding catalytic subunit (calcineurin A) and a 19 kDa Ca^{2+}-binding regulatory subunit (calcineurin B) [14]. Calcineurin is also the target of immunosuppressant drugs such as cyclosporine A and tacrolimus (FK506), which bind cyclophilin A and immunophilin FKBP12, respectively, to inhibit the phosphatase function of calcineurin [19]. Moreover, calcineurin is known to have significant physiological roles in various fungal species [1, 20, 21]. The calcineurin pathway is intimately involved in the growth and pathogenesis of many fungi responsible for human disease, including *Aspergillus fumigatus*, *Candida albicans*, and *C. neoformans* [24]. Exploitation of the fungal calcineurin pathways could hold great promise in developing novel antifungal agents in the future [24].

The bark of *Magnolia obovata* Thunb. (Magnoliaceae), also known as "Hu-Bak," has been widely used as a traditional herbal remedy for various disorders (*e.g.*, gastrointestinal disorders, anxiety, and allergic disease) for hundreds of years in Asian countries [13, 26]. The genus *Magnolia* is known to contain at least 255 chemically distinct components,

such as alkaloids, coumarins, flavonoids, lignans, neolignans, phenylpropanoids, and terpenoids [13]. Three neolignans (magnolol, honokiol, and obovatol) have been the focus of studies examining the pharmacological effects of *Magnolia* [26]. Magnolol and honokiol (both isolated from *M. obovata*) have various pharmacological properties, including antiplatelet, anti-inflammatory, anti-anxiety, antidepressant, antispasmodic, antioxidative, anxiolytic, and neuroprotective activities, and inhibitory activities on periodontopathic bacterial growth [2, 4, 13]. Moreover, obovatol was found to enhance cognitive function; it has antitumor, antifungal, and antiplatelet properties [7, 11, 17].

By screening libraries of plant extracts for compounds that modulate cell-signaling pathways, a calcineurin inhibitor of *C. neoformans* was discovered from the ethyl acetate extract of the fruits of *M. obovata* Thunb. Although extracts of *M. obovata* have been widely used as folk remedies, there are no reports on their use as a specific inhibitor of the calcineurin signaling pathway in *C. neoformans*. Herein, we describe the isolation and structural determination of a phenylpropanoid glycoside, magnoloside A, and report its inhibitory activity against various human pathogenic fungi.

A two-component screening system, using both wild-type *C. neoformans* var. *grubii* H99 (*MAT*α) and a calcineurin *cna1* mutant of *C. neoformans* H99 (*MAT*α *cna1*Δ::*NAT-STM#117*), was employed to identify potential cell-signaling modulators using the agar diffusion method as described previously [12]. The YPD (1% yeast extract, 2% peptone, 2% dextrose) agar plates used in all bioassays were prepared as two separate layers: the base media containing solidified YPD agar were overlaid with each YPD medium containing *C. neoformans* H99 or *cna1* mutant strains. The solidified YPD plates seeded with each strain were labeled A–D for bioassays: A plates were cultured with *C. neoformans* H99 and sample; B plates were cultured with *C. neoformans* H99, sample, and fludioxonil; C plates were cultured with *C. neoformans cna1* mutant, sample, and fludioxonil; and D plates were cultured with *C. neoformans cna1* mutant and sample. Aliquots of samples, with or without fludioxonil (which induces activation of the Hog1 pathway) [9], were loaded onto 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 clear zones on each plate (A–D) were excluded, while samples with inhibitory activities on only B, C, or D plates were selected.

The *in vitro* minimum inhibitory concentrations (MICs) for test compounds against various human pathogenic fungi were determined by broth dilution performed according to the Clinical and Laboratory Standards Institute (CLSI, formerly NCCLS) [3].

A total of 2,000 plant extracts were screened for cell signaling pathway modulators. The diameter of inhibition zone for the same sample on each plate (A–D) was observed with the naked eye and compared with the diameter of inhibition zone for the fludioxonil control. A single candidate, which showed a clear zone on the B plate only, was selected from this screening. A bioactive compound obtained from *M. obovata* Thunb. was isolated and purified using ethyl acetate extraction, silica gel column chromatography, C_{18} silica gel column chromatography, and HPLC. The retention time of the purified bioactive compound on HPLC analysis was 21.5 min (Fig. 1). Structure analyses of the isolated compound with ESI-MS and various NMR techniques revealed that this compound had the molecular formula $C_{29}H_{36}O_{15}$ and the molecular weight of 624. From these data, the compound was identified as magnoloside A (Fig. 2). Magnoloside A: a yellow powder; ESI-MS (negative-ion mode), *m/z* 623.2 $[M-H]^-$; ^{1}H NMR (MeOH-d_4, 500 MHz): δ 7.59 (1H, d, *J* =

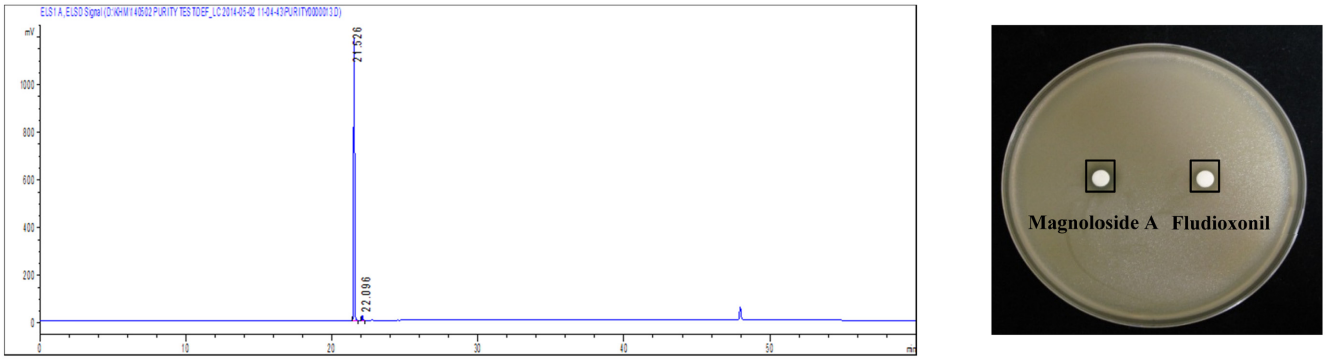

Fig. 1. HPLC chromatogram (left) and the biological activity (right) of magnoloside A isolated from *Magnolia obovata* Thunb.
Bioassy of magnoloside A (1 mg/ml) was performed on a plate containing *C. neoformans* var. *grubii* H99 and fludioxonil (0.1 mg/ml).

Table 1. *In vitro* antifungal activities of magnoloside A against various human pathogenic fungi.

Test organism	Minimum inhibitory concentration (μg/ml)	
	Magnoloside A	Amphotericin B
Candida albicans ATCC 10231	>128	0.12
Cryptococcus gatti R265	4	<0.12
Cryptococcus gatti WM276	2	<0.12
Cryptococcus neoformans JEC21	2	<0.12
Cryptococcus neoformans ATCC 36556	1	0.12
Cryptococcus neoformans var. *grubii* H99	2	0.12
Aspergillus fumigatus ATCC 16424	>128	0.5
Trichophyton mentagrophytes ATCC 9533	>128	0.25

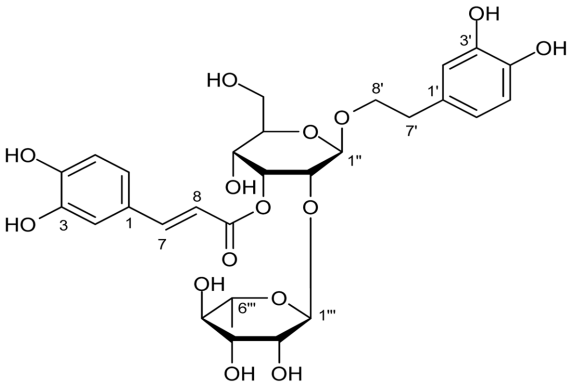

Fig. 2. Structure of magnoloside A isolated from *Magnolia obovata* Thunb.

15.8 Hz, H-7), 7.07 (1H, d, *J* = 1.8 Hz, H-2), 6.97 (1H, dd, *J* = 1.7, 8.0 Hz, H-6), 6.79 (1H, d, *J* = 8.0 Hz, H-5'), 6.69 (1H, d, *J* = 2.0 Hz, H-2'), 6.66 (1H, d, *J* = 8.0 Hz, H-5), 6.57 (1H, dd, *J* = 2.0, 8.0 Hz, H-6'), 6.36 (1H, d, *J* = 16.1 Hz, H-8), 4.90 (H-1'', overlapped in H_2O peak), 4.73 (1H, d, *J* = 8.0 Hz, H-1'''), 4.03 (2H, m, H-8', 5'''), 3.87 (1H, dd, *J* = 11.8 Hz, H-6''), 3.79 (1H, m, H-5''), 3.69 (5H, m, H-4'', 6'', 5', 2'', 8'), 3.59 (1H, m, H-3'''), 3.39 (1H, t, *J* = 9.5 Hz, H-4'''), 2.77 (2H, t, *J* = 7.8 Hz, H-7'), and 1.24 (3H, d, *J* = 6.3 Hz, H-6'''). The collected MS and NMR data for the purified compound were both in good agreement with the previously published spectral data for magnoloside A [5].

Magnoloside A displayed potent antifungal activity against various *Cryptococcus* species (MICs ≤ 1.0−4.0 μg/ml), but only modest activity against *Aspergillus*, *Candida*, and *Trichophyton* strains (MICs > 128 μg/ml) (Table 1).

Although a diverse array of pharmacological effects have been reported for *M. obovata* Thunb. isolates [2, 4, 7, 11, 17], information regarding their antifungal properties is lacking. In particular, little is known about the *in vitro* antifungal activity of phenylpropanoid glycosides against *Cryptococcus* species, which are known to be zoonotic pathogens. This is the first study to report that magnoloside A has potent antifungal activity against various *Cryptococcus* species, stemming from its inhibition of the calcineurin pathway. Thus, magnoloside A may serve as a useful tool in the development of signaling pathway inhibitors of *C. neoformans*. However, the detailed mechanism of action of this compound warrants future research.

Acknowledgments

We thank the International Biological Material Research Center (IMBRC), Korea Research Institute of Bioscience and Biotechnology and Korea Promotion Institute for Traditional Medicine Industry (KOTMIN) for providing various plant extracts.

This work was supported by a grant from Procurement and Development of Foreign Biological Resources funded by the Ministry of Science, ICT and Future Planning of the Korean government (NRF-2011-00495).

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