Arch Pharm Res Vol 29, No 9, 746-751, 2006

Archives of Pharmacal Research
http://apr.psk.or.kr

Antifungal Effect of Amentoflavone derived from *Selaginella tamariscina*

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(Received February 6, 2006)

Amentoflavone is a plant biflavonoid that was isolated from an ethyl acetate extract of the whole plant of *Selaginella tamariscina* (Beauv.) spring. 1D and 2D NMR spectroscopy including DEPT, HMQC, and HMBC were used to determine its structure. Amentoflavone exhibited potent antifungal activity against several pathogenic fungal strains but had a very low hemolytic effect on human erythrocytes. In particular, amentoflavone induced the accumulation of intracellular trehalose on *C. albicans* as a stress response to the drug, and disrupted the dimorphic transition that forms pseudo-hyphae during pathogenesis. In conclusion, amentoflavone has great potential to be a lead compound for the development of antifungal agents.

Key words: Biflavonoid, Amentoflavone, Antifungal activity, Dimorphic transition

INTRODUCTION

Selaginella tamarisina (Korean name: Keoun Back) belongs to the family Selaginellaceae, and has been used in traditional oriental medicine to treat blood excrement, hematuria, prolapse of the anus and stanching (Carlo *et al.*, 1999). *Selaginella* species contain a large number of active compounds, the most important being biflavonoids (Silva *et al.*, 1995; Lin *et al.*, 1999). Biflavonoids are naturally occurring compounds that are ubiquitous in all vascular plants and have many favorable biological and pharmacological effects (Lee *et al.*, 1996; Baureithel *et al.*, 1997; Lobstein-Guth *et al.*, 1998). One such naturally occurring biflavonoid is amentoflavone, which was isolated from *Selaginella tamariscina* in this study. The anti-inflammatory (Kim *et al.*, 1998; Gil *et al.*, 1997; Gambhir *et al.*, 1987; Kim *et al.*, 1998; Lin *et al.*, 2000; Woo *et al.*, 2005), anti-urcerogenic (Gambhir *et al.*, 1987) activities on various animal models and cytotoxicity against human tumor cell lines (Lin *et al.*, 2000) have been reported as the pharmacological effects of amentoflavone. However, only limited studies have examined the antimicrobial effect of amentoflavone. Amentoflavone had been reported antiviral activity against influenza, herpes, and respiratory syncytial virus (RSV) (Lin *et al.*, 1999; Ma *et al.*, 2001). It had been also reported antifungal activity with the main focus being on phytopathogens; amentoflavone was isolated from three different plants, including *Cupressocyparis leylandii*, *Taxus baccata*, and *Ginkgo biloba*, and it exhibited antifungal activity against *Alternaria alternate*, *Cladosporium oxysporum*, *Fusarium culmorum*, and *Fusarium avenaceum* (Krauz-Baranowska *et al.*, 1999; 2003). This study investigated its antifungal activity against human pathogenic fungi as well as its effect on dimorphism in *C. albicans*. Additionally, to investigate the cytotoxicity, it also examined the hemolytic effect of amentoflavone on human erythrocytes.

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MATERIALS AND METHODS

Extraction and isolation of amentoflavone

The whole plant of *Selaginella tamariscina* (600 g) was extracted with MeOH at room temperature yielding 50.54 g of residue. The methanol extract was re-suspended in water and partitioned sequentially with dichloromethane, ethyl acetate, and *n*-butanol. The EtOAc fraction (3.0 g) was placed a silica gel (300 g, 4.8 x 45 cm) column and eluted using a $CHCl_3$-MeOH-H_2O (12:1:0.1→8:1:0.1→5:1:0.1→2:1:0.1→1:1:0.1→MeOH only) gradient system. Based on their TLC pattern, the fractions were combined to yield subfractions, which were designated E1-10. Subfraction E7 (296.33 mg) was finally purified by repeated column chromatography over a silica gel, RP-18, and Sephadex LH 20, yielding amentoflavone (82.23 mg). The UV, IR, ^{1}H NMR, ^{13}C NMR data for amentoflavone were identical to those reported in the literature (Markham *et al.*, 1978; Silva *et al.*, 1995).

Fungal strains

The *Saccharomyces cerevisiae* (KCTC 7296), *Trichosporon beigelii* (KCTC 7707) and *Aspergillus flavus* (KCTC 1375) strains were obtained from the Korean Collection for Type Cultures (KCTC) at the Korea Research Institute of Bioscience Biotechnology (KRIBB) Taejon, Korea. The *Candida albicans* (TIMM 1768) strain was obtained from the Center for Academic Societies, Osaka, Japan.

Antifungal activity

The three fungal strains, *Candida albicans*, *Saccharomyces cerevisiae* and *Trichosporon beigelii* were seeded on 96-well microtiter plates at a density of 2×10^3 cells per well in 100 μl of YPD (2% Dextrose, 1% Peptone, 0.5% Yeast extract) media. Ten μl of the serially diluted compound solutions were added to each well and the cell suspension was incubated for 16 h at 28°C. The minimum inhibitory concentration (MIC) was defined as the lowest amentoflavone concentration that completely inhibited the growth of fungal cells (Lee *et al.*, 2004). The results were the average of triplicate measurements in three independent assays.

Determination of intracellular trehalose

The *C. albicans* cell suspension containing amentoflavone was incubated for 1 h at 28°C. The negative control was incubated without amentoflavone, and the positive control was incubated with amphotericin B. The fungal cells were separated by centrifugation (12,000 rpm for 20 min) and dried. Fifteen mg (dry weight) of the dried fungal cells were destroyed by boiling in a 0.025 mM potassium-phosphate buffer (pH 6.6) for 15 min. The crude neutral trehalose-containing fractions were extracted by removing the cell debris. The trehalose residue was then digested with 100 mg of trehalase (sigma, T8778). After allowing the enzymatic reaction to proceed for 30 min at 37°C, the reaction suspension was mixed with H_2O, and a 16% DNS reagent (1% 3, 5-Dinitrosalicylic acid, 2% NaOH, 20% Sodium potassium tartrate) was added (Sengupta *et al.*, 2000). For the reaction between glucose and the DNS reagent, the mixture was boiled for 5 min and then cooled. The level of color formation was measured at a wavelength of 525 nm.

Effect of amentoflavone on the dimorphic transition of *C. albicans*

The *C. albicans* cells were maintained by periodic subculturing in a liquid YPD medium. Cultures of yeast cells (blastoconidia) were maintained in the liquid YPD medium at 37°C. Hyphal formation was induced by adding 20% fetal bovine serum. The dimorphic transition in *C. albicans* was examined after incubating the cultures with amentoflavone for 48 h at 37°C. The dimorphic transition to the hyphal forms was detected using phase contrast optical microscopy (NIKON, ECLIPSE TE300, Japan).

Hemolytic activity

The hemolytic activity of amentoflavone was determined by measuring the amount of hemoglobin released from a 4% suspension of fresh human red blood cells (hRBCs) at a wavelength of 414 nm (Shin *et al.*, 2000). The hRBCs were washed four times with phosphate-buffered saline (PBS: 35 mM phosphate buffer/0.15 M NaCl, pH 7.0). One hundred μl of the hRBCs suspended 8% (v/v) in PBS were plated onto a 96-well plate, and 100 μl of the serially diluted compound solution was added to each well. The plates were incubated for 1 h at 37°C and centrifuged at $150\times g$ for 5 min. One hundred μL of the aliquots was transferred onto 96-well plates. The level of hemolysis was measured by its absorbance at 414 nm using an ELISA plate reader (Molecular Devices Emax, Sunnyvale,

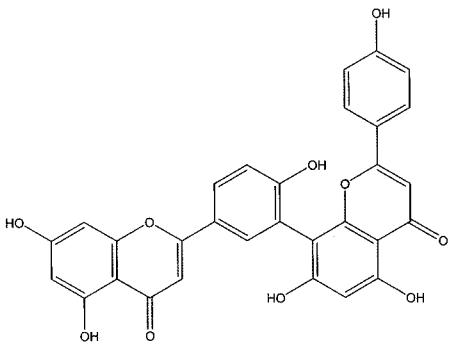

Fig. 1. Chemical structure of amentoflavone.

CA, USA). Zero 100% hemolysis was determined in PBS and 0.1% Triton X-100, respectively. The percentage hemolysis was calculated using the following equation:

% hemolysis=[(Abs_{414nm} in the compound solution - Abs_{414nm} in PBS)/(Abs_{414nm} in 0.1% Triton-X 100 - Abs_{414nm} in PBS)]×100.

RESULTS AND DISCUSSION

Amentoflavone is a plant biflavonoid found in several medicinal plants. It has been used in traditional medicine to treat human disease. Although, there have been many studies on the biological and pharmacological activities of amentoflavone, there are few reports on the antifungal effect of amentoflavone on phytopathogens, particularly on the effect against human pathogenic fungi. Therefore, the antifungal activities of amentoflavone against various fungal strains were examined, and are represented by the MIC values. Amentoflavone exhibited a significant antifungal activity at concentrations ranging from 5-10 μg/mL. This is slightly less potent than amphotericin B, which has a MIC of 2.5 μg/mL on all the strains (Table I). Amphotericin B, which was used as a positive control agent, has a high antifungal activity (Matsuoka *et al.*, 2002). In order to visualize its antifungal effect, *C. albicans* cells were treated with amentoflavone and spread on an agar plate. The growth of *C. albicans* cells which causes candidiasis, was also inhibited by amentoflavone (Fig. 2). This demonstrates that amentoflavone can serve as an antifungal agent against human infectious fungi. In addition, this study examined the extent of growth inhibition of amentoflavone using the agar hole method with filamentous fungus *Aspergillus flavus*. *A. flavus*, which is one of the causes of aspergillosis, was inhibited by amentoflavone. As shown in Fig. 3, amentoflavone inhibited filament growth at the circle of center on the plate.

Table I. Antifungal activity of amentoflavone

	MIC (μg/mL)		
	C. albicans	*S. cerevisiae*	*T. beigelii*
Amentoflavone	5	5	5 - 10
Amphotericin B	2.5	2.5	2.5

The fungal strains were grown at 28°C in YPD medium. The fungal cells were seeded on the well of a 96-microtiter plate of YPD medium at a density of 2×10^3 cells (100 μl per well). Microbial growth was determined using the microdilution method as described in materials and methods.

Recently, deeply invasive mycoses have emerged as a result of opportunistic infections with the advent of organ transplantation, cancer chemotherapy or the HIV infection. *C. albicans*, which causes a variety of superficial and deep-seated mycoses such as candidiasis, is an important pathogen in opportunistic infections by fungi and is becoming of increasing importance in human medicine. This organism exists as a yeast in the mycelial form in response different environmental conditions, and the conversion to the filamentous form is correlated with the pathogenesis of host tissue invasion (Calderone *et al.*, 2001; Mclain *et al.*, 2000). The effect of amentoflavone on the dimorphic transition of *C. albicans* was investigated by examining this transition in cultures containing 40 μg/mL amentoflavone and 20% fetal bovine serum for 48 h at 37°C (Lee *et al.*, 2002). As shown fig. 5, amentoflavone remarkably disrupted the serum-induced mycelial form of

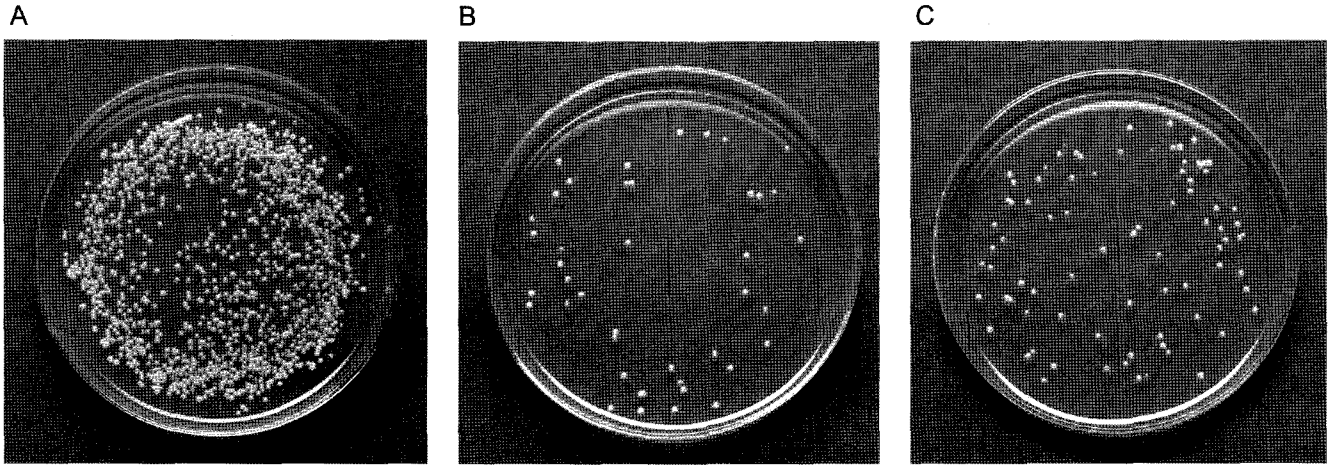

Fig. 2. Effect of amentoflavone on *C. albicans* colony formation. The fungal cells were suspended in YPD medium to a density of 2×10^3/mL and amentoflavone was added to make a concentration of 5 μg/mL. The reaction mixture was spread over a YPD agar plate after incubating the cells for 6 h at 28°C, and the plate was incubated for 20 h at 28°C. A, no treatment; B, Cells treated with 2.5 μg of amphotericin B; C, 5 μg of amentoflavone.

C. albicans cells. Therefore, amentoflavone is a potential therapeutic agent for human invasive fungi.

The intracellular trehalose level in *C. albicans* cells was measured in order to determine if amentoflavone induces a stress response in fungal cells as a toxic agent. Trehalose (α-D-glucopyranosyl-1,1-α-D-glucopyranoside) is widely believed to act as a carbon and energy reserve (Elbein et *al.*, 2003) or a stress protectant. Previous studies have reported that the level of trehalose increases after exposing these organisms to environmental stresses such as heat, cold, toxic agents, oxidants and organic solvents (Attfield *et al.*, 1987; Benaroudj *et al.*, 2001). Therefore, trehalose plays an important role in protecting the cellular constituents, including the membranes and proteins, from environmental stress (Paik *et al.*, 2003). The amount of intracellular trehalose from *C. albicans* after amentoflavone exposure was measured by hydrolyzing the resulting glucose that had been degraded from trehalose in the presence or absence of amentoflavone. The level of glucose was measured after the quantitative hydrolysis of trehalose by trehalase (Schulze *et al.*, 1995). In this assay, we found that the intracellular trehalose level in the amentoflavone-treated cells was higher than that of the control cells treated no compound (Fig. 3). This suggests that amentoflavone induces a stress response in fungal cells, which indicates that its antifungal activity is a significant stress to fungal cells.

The cytotoxicity of amentoflavone against human erythrocyte cells was examined by measuring the percentage of hemolysis against human erythrocytes at various concentrations (from 6.25 to 100 μg/mL). Amentoflavone exhibited very low hemolytic activity even at high concentrations while amphotericin B exhibited strong hemolytic activity (Fig. 4). This indicates that amentoflavone show the specific-selective activity on only fungal cells but has almost no hemolytic activity against

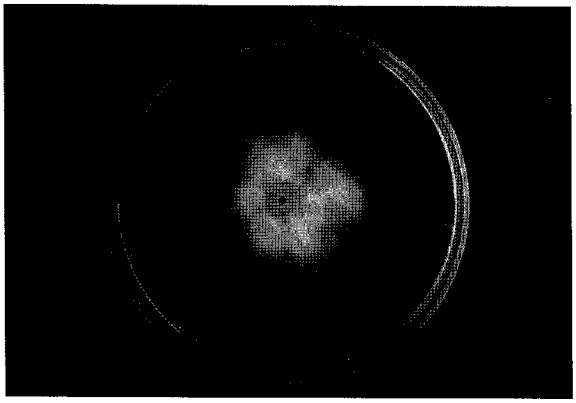

Fig. 3. Agar hole assay for the antifungal effect of amentoflavone against *A. flavus*. The amentoflavone solution (40 μg/100 μl) was located at the center of *A. flavus* mycelium disk on a 1.5% YPD agar plate. The plate was incubated for 3 days at 28°C.

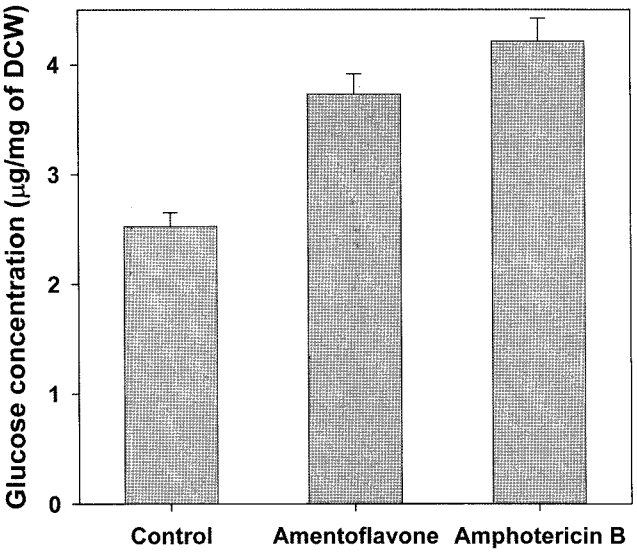

Fig. 5. Trehalose assay after adding amentoflavone and amphotericin B. Subcultured *C. albicans* cells treated with 40 μg of amentoflavone and 5 μg of amphotericin B were incubated at 28°C for 1 h.

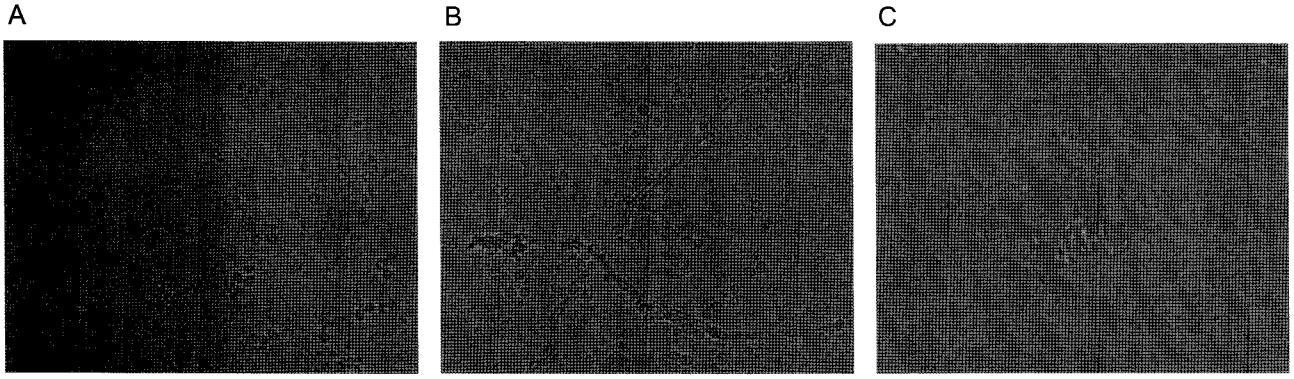

Fig. 4. Effect of amentoflavone on the dimorphic transition in *C. albicans*. The hyphal form was induced by directly supplementing the cultures with 20% fetal bovine serum (FBS). The dimorphic transition in *C. albicans* was investigated from the cultures containing 40 μg of amentoflavone incubated for 48 h at 37°C in YPD media with 20% FBS. A, yeast control; B, with no treated; C, with amentoflavone.

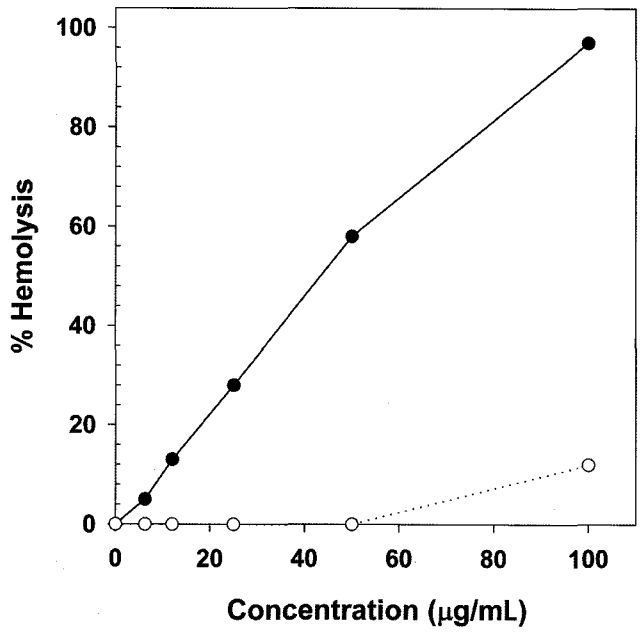

Fig. 6. Hemolytic effect of amentoflavone on human erythrocyte cells. Amentoflavone treated (○), amphotericin B treated (●).

human erythrocytes.

In conclusion, amentoflavone isolated from *Selaginella tamariscina* exhibits a potent antifungal activity against human pathogenic fungi without the hemolytic activity on human erythrocytes. Therefore, amentoflavone is an excellent candidate as an antifungal agent for treating human infectious diseases caused by pathogenic fungi.

ACKNOWLEDGEMENTS

This work was supported by Ministry of Science & Technology (MOST) and the Korea Science and Engineering Foundation (KOSEF) through the Center for Traditional Microorganism Resources (TMR) at Keimyung University.

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