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Original Research Article

Potential Biological Activities of Magnoflorine: A Compound from *Aristolochia debilis* Sieb. et Zucc

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Abstract - Magnoflorine, an important compound in *Aristolochia*, was usually used as an anxiolytic chemical. In this study, the magnoflorine was isolated from *Aristolochia* and the biological activities such as antioxidant, α-tyrosinase inhibitory, anti-inflammatory, and anticancer activities were investigated. The magnoflorine showed significant antioxidant activity as a 2,2-diphenyl-2-picryl-hydrazyl (DPPH) free radical scavenger, 50 μg/mL of the magnoflorine scavenged about 70.8% of all the free radicals. And it was good at α-tyrosinase inhibiting, 100 μg/mL of the magnoflorine inhibited 36.5% of the tyrosinase. High dosage of magnoflorine inhibited the inflammation production nitric oxide (NO), and the magnoflorine protected the murine macrophage cells (RAW 264.7) from LPS-induced apoptosis. The cell viability of human colon cancer calls (HT-29) was around 100% when treated with different dose of magnoflorine, it's suggesting that magnoflorine had no anticancer effect.

Key words - Magnoflorine, Antioxidant, α-tyrosinase Inhibitory, Anti-inflammatory, Anticancer

Introduction

Magnoflorine usually used as an anxiolytic chemical, is another important compound in *Aristolochia sp*. It was related to inflammation and cancer as showed to suppress the induction phase of the cellular immune response, and assigned as noncytotoxic to many human cancer cell lines (Mori *et al.*, 1994; Min *et al.*, 2006). It also showed to play a role in high-density lipoprotein (HDL) oxidation (de la Pena *et al.*, 2013) and decreased arterial blood pressure in rabbits and induced hypothermia in mice (Kamal and Tahir, 1991). The antioxidant activity of different structural feature and physico-chemical properties of the magnoflorine have been test in inhibit free radical, peroxidation of dioleoyl phosphatidylcholine, and liposomes (Hung *et al.*, 2007). Magnoflorine also has been reported to have the effect on inhibiting α-glucosidase in *vitro* and in *vivo* (Patel and Mishra, 2012).

Biological activities such as antioxidant, anti-aging, anticancer, anti-anxiety, hypocholesterolemic, anticoagulant, antithrombotic, anti-diabetes, antifungal, antihistaminic, anti-inflammatory, antihistaminic, immunosuppressive, anti-leishmanial, insecticidal, antibacterial, and cytoprotective, which may contribute to the therapeutic effects for various diseases, have been widely investigated (Fujita and Node, 1984; Kosar *et al.*, 2008; Weerakkody *et al.*, 2010; Sharmeen *et al.*, 2012; Hu *et al.*, 2013). The compounds which have antioxidant activity were suggested to play an important role as a health-protection factor. Scientific evidence demonstrated that antioxidants can protect the human body against free radical damage and retard many chronic diseases progress, such as cancer and heart disease (Jacob, 1996; Knight, 1998; Zheng and Wang, 2001). Thus, in this study, the aim was to investigate the antioxidant, α-tyrosinase inhibitory, anti-inflammatory, and anticancer activities of the compound magnoflorine.

Materials and methods

Sample preparation

Magnoflorine was isolated from dried methanol extract of *A. debilis* stems. It's a powder or crystal, has a molecular formula of $C_{20}H_{24}NO_4$ (Fig. 1), and the molecular weight Is 341. The purity of the compound is more than 98% (Fig. S1).

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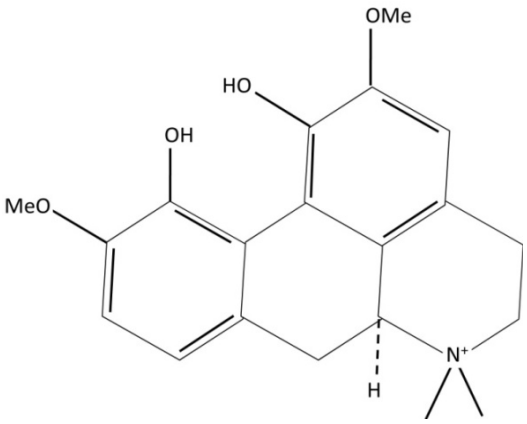

Fig. 1. Structure of magnoflorine.

Cell lines and cell culture

Embryonic kidney cell (HEK293), murine macrophage cell (RAW 264.7) and human colon cancer cell (HT-29) lines were purchased from the Korean Cell Line Bank (Seoul, Korea). HEK293 cells were grown in DMEM; RAW 264.7 and HT-29 cells were grown in RPMI 1640, respectively, supplemented with10% fetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin. Cells were cultured in a humidified atmosphere and incubated at 37°C in 5% CO_2.

Determination of DPPH free radical scavenging activity

DPPH free radical scavenging activity was evaluated according to Zhang *et al.* (2009) with some modifications. Briefly, the sample was vortex-mixed with a 50 μL of 0.1 mM DPPH solution. After incubated at room temperature for 30 min, the absorbance was measured at 515 nm against a blank. The scavenging ratio by each sample extract was calculated using the following equation:

DPPH scavenging activity (%) = $[A_0 - (A_1 - A_s)] / A_0 \times 100$

Where A_0, A_1, A_s were the absorbance of the control, sample and blank.

The scavenging ratio by pure compound was calculated using the following equation:

DPPH scavenging activity (%) = $(A_0 - A_1) / A_0 \times 100$

Where A_0, A_1 were the absorbance of the control and test sample. In this experiment, we used α-tocopherol as the positive control.

The IC_{50} values were calculated from the graph of DPPH scavenging activity against extract concentration.

α -tyrosinase inhibitory activity assay

The tyrosinase inhibition assay was determined according to the method described by Alam *et al.* (2010), by using an ELISA plate reader. Briefly, 40 μL *A. debilis*, 40 μL of 125 U/L tyrosinase enzyme, and 80 μL of 67 nM phosphate-buffered saline (PBS, pH 6.8) were mixed and pre-incubated at 37°C for 5 min. To the substrate was added 40 μL of 25 nM L-DOPA. Then, the tyrosinase activity was tested by measuring the absorbance at 492 nm. The inhibition of the enzyme activity was calculated as follows:

Percent inhibition (%) = $[A_{control}-(A_{sample}-A_{blank})]/A_{control}\times100$.

L-ascorbic acid was used as a positive control.

Measurement NO inhibitory activity in RAW 264.7 cells

Exponentially growing RAW 264.7 cells were plated at a density of 1×10^5 cells/well in 96-well microplates in 200 μL of culture medium and allowed to adhere for 24 h before treatment. Then, cells were divided into 2 groups, stimulated with/without 2 μg/mL of LPS, in the presence or absence of various concentration of the compound for 24 h. Aliquots of 100 μL of cell culture medium were mixed with 50 μL of 1% sulfanilamide (in 5% phosphoric acid) and 50 μL of 0.1% naphthyl-ethylenediamine dihydrochloride.

MTT assay for cell viability

HEK293, RAW 264.7 and HT-29 cells were seeded in 96-well plates (1×10^5 cells/well) for 24 h, respectively. Then, RAW 264.7 cells were treated with LPS (2 μg/mL) in the presence of 25, 50, 100, 200, and 400 μg/mL of the compound (with 0.1% DMSO) for 24 h. HEK293 and HT-29 cells were pretreated with 25, 50, 100, 200, and 400 μg/mL of the compound (with 0.1% DMSO) for 24 h. 0.1% DMSO was used as a blank. After incubation, the cell viability was measured by an MTT assay. MTT is a pale yellow substrate that was reduced by living cells to yield a dark blue formazan product. After reacting for 4 h, the supernatant was removed and the formazan crystals were dissolved in DMSO. Absorbance was measured at 550 nm.

Statistical analysis

All experiments were conducted in independent triplicate (n = 3) and data were expressed as mean ± SD. Statistical

significance was evaluated using an analysis of variance with SPSS 16.0 (SPSS Inc., Chicago, IL, USA).

Result

Antioxidant activity of magnoflorine

The antioxidant activity of magnoflorine has been tested by a DPPH free radical instrument. DPPH, which has a deep violet color, can show a strong absorption band around 517 nm. 12.5, 25, 50, 100, and 200 μg/mL of the magnoflorine (Fig. 1) were used, and the scavenging activity was showed in a dose-dependent manner (Fig. 2). 50 μg/mL of the magnoflorine scavenged about 70.8% of all the free radicals. The IC_{50} value of the magnoflorine was 31.7 μg/mL. Compared to positive control (α-tocopherol), which IC_{50} value was 11.5 μg/mL (Fig. S2), the free radical scavenging activity of magnoflorine was significantly.

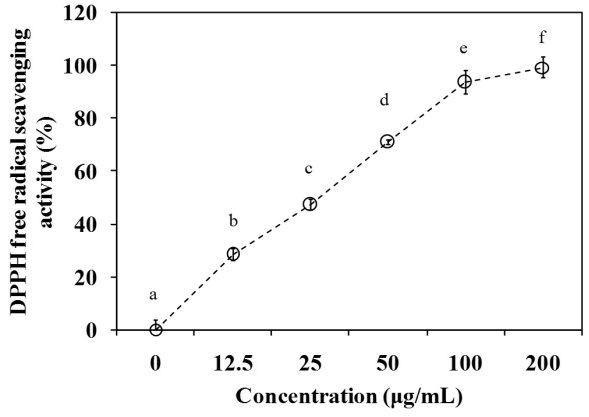

Fig. 2. DPPH free radical scavenging activity of magnoflorine. The scavenging activity was expressed in the present of 12.5, 25, 50 100, and 200 μg/mL magnoflorine.

α -tyrosinase inhibitory effect of magnoflorine

In this study, the tyrosinase inhibition activity was determined by using L-DOPA as the substrate. The positive control, L-ascorbic acid inhibited the α-tyrosinase in a dose-dependent manner, and 100 μg/mL of the positive control showed 100% inhibitory effect (Fig. 3). The activity of magnoflorine was

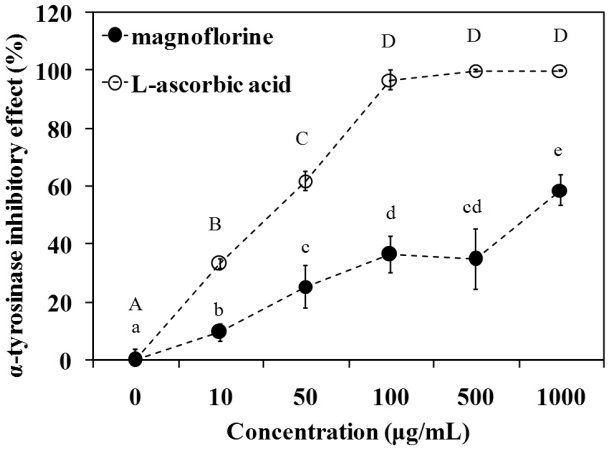

Fig. 3. α-tyrosinase inhibitory effect of magnoflorine. 125 U/L tyrosinase enzymes were used as the target. The inhibitory effect was expressed in the presence of 10, 50, 100, 500, and 1000 μg/mL magnoflorine. L-ascorbic acid was used as a positive control.

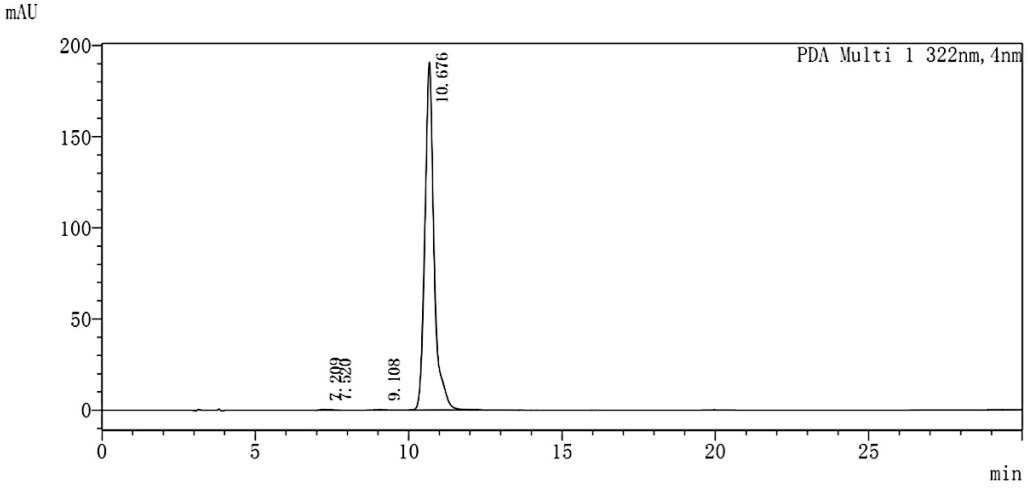

Fig. S1. HPLC chromatograms result.

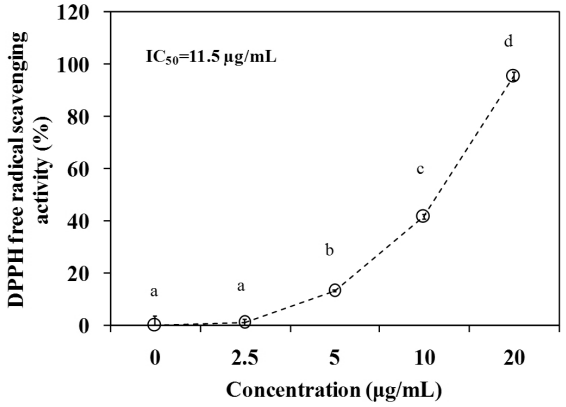

Fig. S2. DPPH free radical scavenging activity of α-Tocopherol. The scavenging activity was expressed in the present of 2.5, 5 10, and 20 μg/mL α-Tocopherol. The IC_{50} value was 11.5 μg/mL.

weaker than L-ascorbic. 100 μg/mL of the magnoflorine inhibited 36.5% of the tyrosinase.

Anti-inflammatory activity of magnoflorine

The effect of magnoflorine on NO inhibition was determined by treating the RAW264.7 cells with it in the presence or absence of LPS stimulation. The LPS produced significant levels of NO in conditioned medium (Fig. 4A). However, the magnoflorine didn't change the level of NO production induced by LPS in RAW cells. The cytotoxicity of the magnoflorine was determined by an MTT assay in the incubated RAW 264.7 cells with different dosages of magnoflorine for 24 h. Cell viability did not seem to be affected for magnoflorine (Fig. 4B). On the contrary, the magnoflorine protected the RAW 264.7 cells from LPS-induced apoptosis. 49.05% of cells were viable after stimulation with LPS for 24 h. However, the viability of the RAW cells increased to 53.5 and 58.7% after treatment with 200 and 400 μg/mL of magnoflorine, respectively.

Cell viability of magnoflorine-stimulated HT-29 and HEK293 cells

The overdose of *Aristolochia* plant may have serious side-effect on renal. In the study of magnoflorine toxicity, we also used the HEK293 as the target. By an MTT assay, the result showed that the magnoflorine have no toxicity on HEK293 cells. Even the dose of the magnoflorine was 400 μg/mL, the cell viability of HEK293 preserved 100% (Fig. 5).

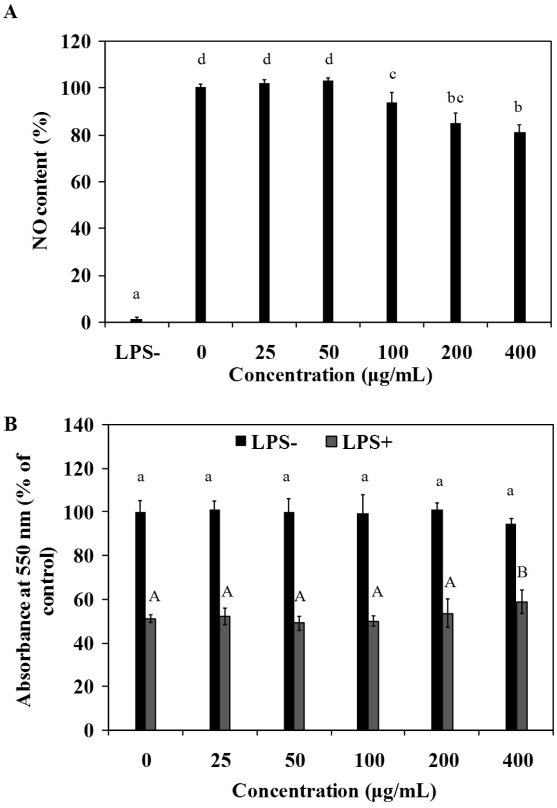

Fig. 4. NO scavenging activity and cytotoxicity of magnoflorine on LPS-stimulated RAW 264.7 cells. A, RAW 264.7 cells were treated with LPS (2 μg/mL) in the presence of 25, 50, 100, 200, and 400 μg/mL magnoflorine for 24 h. B, Cytotoxicity of magnoflorine on LPS-stimulated RAW 264.7 cells.

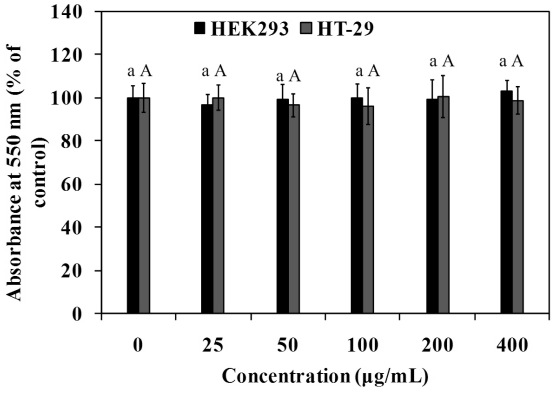

Fig. 5. Cytotoxicity effect of magnoflorine. Cell viability expressed the cytotoxicity of magnoflorine on human embryonic kidney cells (HEK293) and human colon cancer cells (HT-29) cells by an MTT assay.

Similarly, magnoflorine showed no cytotoxicity to the HT-29 cells. The cell viability of HT-29 was around 100% when

treated with different dose of magnoflorine. That means, the magnoflorine has no anticancer effect on HT-29 cell line.

Discussion

Magnoflorine, a powder or crystal was isolated from *Aristolochia debilis*. Biological activities such as antioxidant, anti-α-glucosidase, α-tyrosinase inhibitory, anti-inflammatory, and anticancer activities were investigated. The magnoflorine showed significant antioxidant activity as a DPPH free radical scavenger. Hung *et al.* (2007) also have reported that, magnoflorine played a role in protecting high-density lipoprotein (HDL) under oxidative stress. Different to the report by Patel and Mishra (2012), which indicated that the magnoflorine from *Tinospora cordifolia* stem inhibited α -glucosidase ability, the α-glucosidase inhibitory in this study was weak by a *in vitro* test (data not shown).

Tyrosinase is a rate-limiting, essential enzyme in the biosynthesis of the pigment melanin in the plants, micro-organisms, and mammalian cells (Shimizua *et al.*, 2001). In the human melanogenesis, tyrosinase plays a key role in catalyzing the hydroxylation of monophenols (tyrosine) to *o*-diphenols and their subsequent oxidation to *o*-quinones (Husni *et al.*, 2011). Furthermore, tyrosinase was reported to be related to cancer and some neurodegenerative diseases (Schurink *et al.*, 2007). And the tyrosinase inhibitors have been studied for the cosmetics and pharmaceutical applications. In this study, the magnoflorine showed considerable α-tyrosinase inhibitory effect. Thus, magnoflorine may contain potential skin-whitening agents that inhibit melanogenesis in cosmetics.

In inflammation, NO is an important production. In LPS-stimulated RAW 264.7 cells, the NO content was increased acutely; however, the magnoflorine didn't change the NO content significantly. And the protective effect of magnoflorine on LPS-induced apoptosis on RAW 264.7 cells also was not very high. But, when the dosage was high, the magnoflorine showed considerable anti-inflammatory activity. Thus, the high dosage of magnoflorine may have positive effect in treating or preventing inflammation-relate diseases.

The overdose of *Aristolochia* plant may have serious side-effects on renal. For testing the toxicity of *A. debilis*, we firstly measured the cytotoxicity on HEK293 cells. Thus, in the study of magnoflorine toxicity, we also used the HEK293 cells as the target. The magnoflorine showed no toxicity on HEK293 cells even at high dosage. However, the magnoflorine also had no toxicity on HT-29 cells. That means, the magnoflorine had no anticancer effect on HT-29 cell line. It was different to the report of Stévigny *et al.* (2005), that S-magnoflorine showed selective cytotoxicities against the murine leukemia (P388) cell line. The *A. debilis* extract could inhibit HT-29 cell proliferation significantly. The magnoflorine was not the active compound on *Aristolochia* to have anti-proliferation effect (Li and Wang, 2013).

In summary, the magnoflorine showed significant antioxidant activity as a DPPH free radical scavenger, and it was good at α-tyrosinase inhibiting. And the high dosage of magnoflorine showed considerable anti-inflammatory effect. But it had no anti-proliferative effect on HT-29 cell lines. All the result suggested that the magnoflorine may be used as an antioxidant compound in preventing oxidative relate diseases.

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