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Antitumor Activity of Flavones Isolated from *Artemisia argyi*

Original Paper

Abstract

The flavones 5,6-dihydroxy-7,3′,4′-trimethoxyflavone (**1**), 5,6,4′-trihydroxy-7,3′-dimethoxyflavone (**2**), 5-hydroxy-3′,4′,6,7-tetramethoxyflavone, 5,7,3′-trihydroxy-6,4′,5′-trimethoxyflavone, ladanein, and hispidulin were isolated from the methanolic extracts of the aerial parts of *Artemisia argyi* and structures of the compounds were elucidated on the basis of their spectral data. These flavones inhibited farnesyl protein transferase with IC_{50} values of 25 – 200 μg/mL. Compound **2** inhibited proliferation of a couple of tumor cell lines and also inhibited neovascularization in a chick chorioallantoic membrane assay. Without loss of body weight of nude mice, compounds **1** and **2** inhibited growth of a colon tumor (SW620) by 44.6% and 14.6%, respectively.

Key words
Artemisia argyi · Compositae · Flavones · Farnesyl protein transferase · Angiogenesis

Abbreviations
FPTase: farnesyl protein transferase
CAM: chick embryo chorioallantoic membrane

Introduction

Artemisia species (Compositae), widespread throughout the world, are important medicinal plants, which are attracting the attention of phytochemists due to their biological and chemical diversity [1]. These species are frequently used for the treatment of diseases such as malaria, hepatitis, cancer, inflammation, and infections by fungi, bacteria, and viruses [1]. Extensive studies of the chemical components of *Artemisia* have led to the identification of many compounds, such as monoterpenes, sesquiterpenes, triterpenes, and flavones from the dry leaves [1]. During investigations on the biologically active compositions of the genus, we have isolated a couple of sesquiterpene lactones from *A. sylvatica* [2], [3]. The interesting biological activities of the isolated compounds led us to further investigate the chemical components of *A. argyi*.

Farnesyl protein transferase (FPTase), a member of the prenyltransferase enzyme family, is a crucial enzyme which participates in the post-translational modification of Ras proteins. When a farnesylation of these proteins is blocked, their oncogenic activity is abolished [4]. Therefore, the identification and synthesis of FPTase inhibitors becomes an active area for the development of antitumor agents and a couple of inhibitors are under clinical testing [5]. We also recently reported a couple of FPTase inhibitors, which were isolated from medicinal plants [2], [3], [6], [7].

In this report, we describe the activity-guided isolation and biological activities of flavones **1 – 6**, which were isolated from the aerial parts of *A. argyi* Levl. et Vant. We have focused on the anticancer effects of the flavones, because flavones also are known to have anticancer properties against a variety of cancer cells [10]. Therefore, we carried out experiments on the growth inhi-

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bition of tumor cell lines, anti-angiogenic activity, and nude-mouse xenografts.

Materials and Methods

Preparation of flavones

The aerial parts of *Artemisia argyi* were collected near Incheon, Korea, in September 2000, and identified by Professor K. Bae, School of Pharmacy, Chungnam National University. A voucher specimen (KRIBB-AP2) is deposited under scientific name at KRIBB, Taejeon Korea. The dried material (2 kg) was extracted with MeOH (2 × 4 L) for 24 h at room temperature. The combined extract was concentrated and the dark residue (85 g) was subjected to silica gel flash chromatography (500 g) and eluted with a gradient of $CHCl_3$-MeOH (from 99 : 1 to 90 : 10, v/v, each 800 mL). Fractions were monitored for FPTase inhibition activity and silica gel TLC ($CHCl_3$-MeOH, 95 : 5). Fractions 3 – 5 eluted with $CHCl_3$ containing gradually increasing amounts of MeOH from 3 to 5 % were collected and concentrated to give a dark yellow solid (350 mg). The solid was dissolved in MeOH and subjected to C18 column chromatography (100 g) with aqueous MeOH (10 to 90 %, v/v, each 200 mL). The two fractions eluted with 60 and 70 % MeOH which showed strong inhibition activity against rat FPTase were collected. Compounds **1** and **2** were isolated through chromatography on a Sephadex LH-20 column (2.5 cm x 100 cm) eluting with MeOH (800 mL) and further purified by recrystallization from acetone-hexane to yield **1** (75 mg) and **2** (35 mg). The other fractions were combined and applied ODS-HPLC (YMCA J'sphere ODS-H80, 4 mm, 250 × 20 mm, 75 % MeOH/H_2O at 340 nm, flow rate 4 mL/min) to yield **3** (3 mg), **4** (12 mg), **5** (8 mg), and **6** (6 mg), respectively.

Compound **3** was also prepared from methylation of **1** with diazomethane. In brief, 20 mg of **1** was dissolved in 100 mL of methanol and an excess of diazomethame in ethyl ether was added. The reaction mixture was stirred for 1 h at room temperature, and then the solution was concentrated under reduced pressure. The residue was subjected to silica gel column chromatography to give a compound **3** (19 mg, 91 % yield). Isolated compounds were identified by comparison of their spectral data with reported values.

5,6-Dihydroxy-7,3′,4′-trimethoxyflavone **(1)**: Pale yellow powder; m. p. 244 – 247 °C; HREIMS: *m/z* $[M + H]^+$ = 344.0881; calcd. for $C_{18}H_{16}O_7$: 344.0895 [9].

5,6,4′-Trihydroxy-7,3′-dimethoxyflavone **(2)**: Pale yellow powder; m. p. 268 – 270 °C; HREIMS: *m/z* $[M + H]^+$ = 330.0812; calcd. for $C_{17}H_{14}O_7$: 330.0392 [9], [10].

5-Hydroxy-6,7,3′,4′-tetramethoxyflavone (**3**): Pale yellow powder; m. p. 242 – 245 °C; HREIMS: *m/z* $[M + H]^+$ = 359.1131, calcd. for $C_{19}H_{19}O_7$: 359.1130 [9].

5,7,3′-Trihydroxy-6,4′,5′-trimethoxyflavone **(4)**: Pale yellow powder; m. p. 252 – 256 °C; $C_{18}H_{16}O_8$ [11].

Ladanein **(5)**: Pale yellow powder; m. p. 213 – 216 °C; $C_{17}H_{14}O_6$ [12].

Hispidulin **(6)**: Pale yellow powder; m. p. 287 – 289 °C; $C_{16}H_{12}O_6$ [13].

In vitro activities of the flavones

FPTase assays were done using a scintillation proximity assay (SPA) kit, which was provided by Amersham Bioscience (code TRKQ 7010), following the protocol described by the manufacturer except that a biotinylated substrate peptide containing the Ki-Ras carboxyl-terminal sequence was used. The C-terminal peptide of Ki-Ras (Biotin-KKKSKTKCVIM) was synthesized by solid-phase peptide synthesis. FPTase activity was determined by measuring transfer of [^3H]-farnesyl from [^3H]-farnesyl pyrophosphate to Biotin-KKKSKTKCVIM. FPTase was partially purified from rat brain homogenates by sequential ammonium sulfate fraction and Q-sepharose column chromatography [7]. 2-Hydroxycinnamaldehyde was used as a positive control [6].

In vitro cytotoxicity assay

Human tumor cell lines used in the experiment were SW620 (colon), A549 (lung), PC-3 (prostate), LOX-IMVI (melanoma), and HCT15 (colon) obtained from the National Cancer Institute (NCI), NIH, USA. All of the cell lines were maintained in RPMI 1640 (Gibco/BRL) supplemented with 10 % heat-inactivated FBS (Gibco/BRL) under a humidified atmosphere of 5 % CO_2 in an incubator at 37 °C. *In vitro* growth inhibition by the flavones against human cancer cell lines was evaluated by the sulforhodamine B (SBR) assay [14]. In brief, cells were divided into 96-well plates and preincubated on the plates for 24 hours. The compounds were added to the wells and incubated for 48 hours. At the termination of the incubation, the culture medium in each well was removed, and cells were fixed with cold 10 % tricholoroacetic acid. 0.4 % SRB solution in 1 % acetic acid was added to each well. Optical density was measured in microtiter plate reader at 540 nm.

Antiangiogenic activity

The antiangiogenic activity was examined in a chick chorioallantoic membrane (CAM) assay system, according to a modification of the method described previously [15]. In brief, fertilized eggs were incubated at 37 °C for 3 days. A test sample dissolved in ethanol was placed on a thermanox coverslip and dried. The coverslip coated with a test sample was placed on the surface of 4 day-old CAM. After 5 days, 1 mL of intralipose (fat emulsion) was injected into the chorioallantois and the antiangiogenic activity was determined by measuring an avascular zone in the CAM.

In vivo activities of the flavones

For the evaluation of *in vivo* antitumor activity, SW620 human colon adenocarcinoma cells (3×10^7 cells/mL) were implanted subcutaneously into the right flank of nude mice on day 0. Compounds were dissolved in 0.5 % tween 80 and were intraperitoneously administered at a concentration of 60 mg/kg per day for 22 days. Adriamycin was used as a reference compound at a dosage of 2 mg/kg. Test substances were administrated in a volume of 0.2 mL per 20 g body weight of animals. On day 22, the mice were sacrificed and tumor volumes were estimated [length (mm) × width (mm) × height (mm)/2]. To determine the toxicity of the compounds, the body weight of tumor-bearing animals was measured. Animal experiments were performed with per-

mission according to the "Institutional Guidelines of Animal Experiments" of Korea Research Institute of Bioscience & Biotechnology.

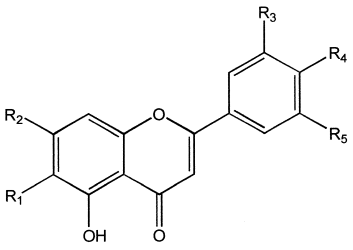
Results and Discussion

During the course of screening medicinal plant extracts for the FPTase inhibitors, a methanolic extract of the aerial parts of *Artemisia argyi* exhibited a strong inhibition activity against an FPTase. Compounds **1 – 6** (Fig. **1**) were isolated from the methanolic extract of the aerial parts of *A. argyi* through activity-guided isolation and purification. They were identified as flavones such as 5,6-dihydroxy-7,3′,4′-trimethoxyflavone (**1**), 5,6,4′-trihydroxy-7,3′-dimethoxyflavone (**2**), 5-hydroxy-3′,4′,6,7-tetramethoxyflavone (**3**), 5,7,3′-trihydroxy-6,4′,5′-trimethoxyflavone (**4**), ladanein (**5**), hispidulin (**6**) by comparison with reported spectral data. Flavones exhibit a wide range of biological activities including antimutagenic, antitumoric and anti-inflammatory activities [8], [16], [17]. And also flavones are well known pharmaceutical constituents from the *Artemisia* genus [18].

The structure of compound **1** was determined by the extensive analysis of NMR and mass spectral data. Analysis of the HREIMS ($[M]^+$: *m/z* = 344.0881, calcd. 344.0895 for **1**) and ^{13}C NMR spectrum of **1** led to a molecular formula $C_{18}H_{16}O_7$, which was identified as 5,6-dihydroxy-7,3′,4′-trimethoxyflavone by comparison with reported spectral data [11]. However, the methoxy groups of the flavones were not completely assigned by NMR and, therefore, compound **3** was prepared from the reaction of **1** with CH_2N_2. The four methoxy groups were assigned on the basis of the NOSEY spectrum, and the strong correlation between CH_3 groups and oxygen-bearing carbons (Fig. **2**).

In vitro activities of the flavones

These flavones inhibited the activity of FPTase by 62 (**1**), 84 (**2**), 35 (**3**), 65 (**4**), 63 (**5**) and 75 (**6**)%, respectively, at a concentration of 100 μg/mL. As shown in Fig. **3**, compounds **1** and **2** inhibited FPTase in dose-dependent manner and the IC_{50} values of the compounds were 25 and 63 μg/mL, respectively.



Compounds	R^1	R^2	R^3	R^4	R^5
1	OH	OCH_3	OCH_3	OCH_3	H
2	OH	OCH_3	OCH_3	OH	H
3	OCH_3	OCH_3	OCH_3	OCH_3	H
4	OCH_3	OH	OH	OCH_3	OCH_3
5	OH	OCH_3	H	OCH_3	H
6	OCH_3	OH	H	OH	H

Fig. **1** Structures of isolated flavones **1 – 6**.

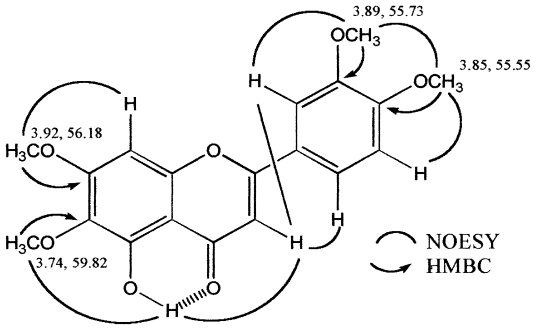


Fig. **2** NOESY and HMBC correlations of **3**.

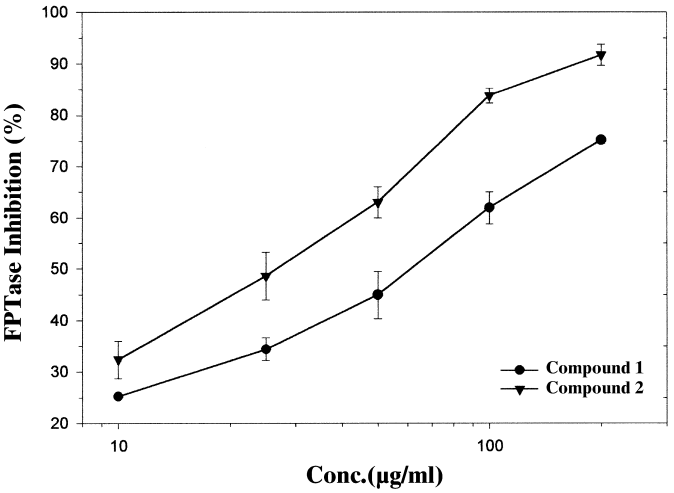


Fig. **3** Dose-dependent inhibition of flavones on the FPTase. The data are expressed as means SD (bars). 2-Hydroxycinnamaldehyde with the IC_{50} values of 18 μg/mL was used as a positive control [6].

Hispidulin (4′,5,7-trihydroxy-6-methoxyflavone, **6**) is well-known for its cytotoxic properties and it was reported that it strongly inhibited the growth of ZR-75 – 1 cells (hormone-dependent human breast cancer cell line) with GI_{50} value of 1.2 μg/mL [19]. In Table **1**, the growth inhibitory activities of the compounds **1** and **2** against different human tumor cell lines are

Table **1** Growth inhibition by compounds **1** and **2** against human cancer cell lines[a]

Compounds \ Cell lines[b]	SW620	A549	PC-3	LOX-IMVI	HCT 15
Compound (**1**)	9.5 ± 1	19.3 ± 2	13.4 ± 1	4.9 ± 0.5	8.8 ± 0.6
Compound (**2**)	5.0 ± 0.4	11 ± 3	3.4 ± 0.3	2.6 ± .0.3	4.1 ± 0.3
Adriamycin[c]	0.34	0.21	0.39	0.12	0.84

[a] Results are expressed as GI_{50} values (μM) and experiments were performed at least three times with consistent and repeatable results.

[b] A549, human lung cancer; PC-3, human prostate cancer; LOX-IMVI, human melanoma cancer; HCT-15, human colon cancer.

[c] Adriamycin as positive control.

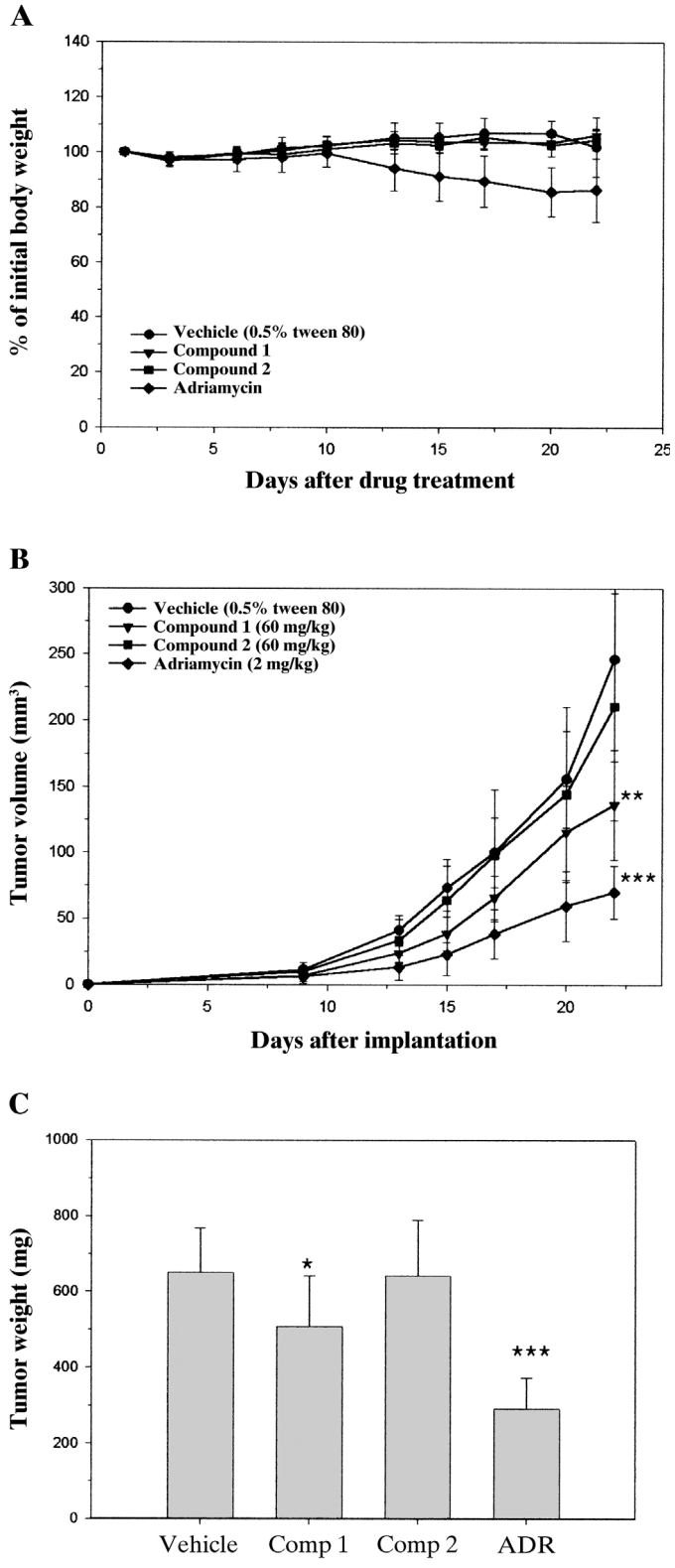


Fig. 4 Effects of compounds **1** and **2** on tumor growth. **A**: Body weight, **B**: tumor volume, **C**: tumor weight. Adriamycin is a positvive control. Vehicle is polyethylene glycol. ADR (adriamycin) used as a positive control. Statistical significance between the control and treatment groups was evaluated using Student's t test (* $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$).

summarized. So far antitumor effects against tumor cell lines of these compounds have not been reported. Compounds **1** and **2** showed the most potent activities against the melanoma cell line (LOX-IMVI) with GI_{50} = 4.9 and 2.6 μM, respectively. Compound **2** displayed more potent activity in several cancer cell lines. We also examined the antitumor effects on breast cancer cell lines such as MDA-MB-231, MCF7 and T47D cells, however, the isolated compounds **1 – 6** did not show any growth inhibitory activity against these tumor cell lines at 20 μg/mL.

Fotsis and coauthors reported several isoflavones, including genistein, which were effective inhibitors of endothelial cell proliferation and *in vitro* angiogenesis [20]. Therefore, we investigated the antiangiogenic activity of compounds in the chicken embryo chorioallantoic membrane (CAM) assay. Compound **2** induced 55% inhibition of angiogenesis at a dose of 10 μg, but the other compounds did not inhibited angiogenesis at that concentration.

In vivo activities of the flavones

It is well known that a high incidence of *ras*-muation and activation is found in colon cancers (50%). Therefore, the antitumor effect of compounds **1** and **2**, which are the major components of the aerial parts of *A. argyi*, were evaluated with SW620 human colon cancer cells in a human tumor xenograft model in nude mice. The compound in 0.5% tween 80 was intraperitoneously administered at a dose of 60 mg/kg for 22 days. As shown in Fig. **4**, the growth of tumors was moderately inhibited by the compounds. Flavones **1** and **2** reduced the tumor volumes 44.6 and 14.6%, respectively, at the final day. Fortunately, a loss of body weight was not observed in nude mice administered with compounds **1** and **2** at the dose of 60 mg/day.

Although further studies with cell lines and *in vivo* are needed to determine the efficacy of these flavones as antitumor agents and also to clarify the mechanism of the antitumor activities of flavones **1** and **2**, the present results suggest that such flavones could be good candidates as antitumor agents.

Acknowledgements

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