

The antioxidation and antiproliferation activity of flavonoids from *Aquilaria agallocha* and *Aquilaria sinensis*.

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Abstract

Studies have shown that herbal extractions may be effective against various diseases like cancer and cardiovascular disease. In this study, we intended to evaluate the cytotoxicity and antioxidation activity of phytochemical constituents from the leaves of *Aquilaria agallocha* and the stems of *Aquilaria sinensis*. The chemical structures were identified by UV, IR, ESI-MS, ¹H NMR and ¹³C NMR analyses. 5-hydroxy-7, 4'-dimethoxyflavone (1), 5, 3'-dihydroxy-7, 4'-dimethoxyflavone (2), 5-hydroxy-7, 3', 4'-trimethoxyflavone (3), 5, 7, 4'-trihydroxyflavone (4), and 3', 4', 5, 7-tetrahydroxyflavone (5) were isolated and further examined antioxidation and antiproliferation activity by 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging and MTT assay. According these results, 5-hydroxy-7, 3', 4'-trimethoxyflavone (3) had DPPH radical scavenging activity. Further, 5-hydroxy-7, 3', 4'-trimethoxyflavone (3) also showed significant positive anticancer activities (10-100 μM) against human skin cancer cell line A375 cells.

Keywords: *Aquilaria sinensis*, Thymelaeaceae, *Aquilaria agallocha*.

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Introduction

The genus *Aquilaria* (Thymelaeaceae) is widely distributed in Asia. *Aquilaria sinensis* and *Aquilaria agallocha* belongs to Thymelaeaceae [1]. *Aquilaria sinensis* and *Aquilaria agallocha* are famous for the resinous wood and it is called agarwood. Agarwood is a well-known as incense in the oriental region such as in Taiwan, Thailand and China used as a sedative, analgesic, and digestive agent in traditional medicine. Flavonoid glycoside, volatile oil, benzenoid, sesquiterpenes and chromone derivatives were isolated and identified from the seed, leaves or stem of these plants [2-10]. Phytochemical constituents obtained from the agarwood including apigenin-7, 4'-dimethylether, genkwanin, quercetin, kaemperfol and 5, 6, 7, 8-tetrahydro-2-(2-phenylethyl) chromes with different pharmacological activities [8,11,12]. The plant *Aquilaria sinensis* and *Aquilaria agallocha* have several pharmacological activities and shows antioxidant, anti-diabetic, anti-inflammatory, and antibacterial activities [2-6,11-14].

The free radicals are atoms with unpaired electrons. Free radicals can generate from our body by various endogenous systems leading to pathological states. Overproducing free radicals attack lipids, proteins, and DNA and make a lot of human diseases. Oxidative stress is a result of an imbalance between free radical production and antioxidant defenses. Oxidative stress results in many diseases including cancers,

inflammatory diseases, gastric ulcers, hypertension and neurological disorders [15,16]. An antioxidant can protect cell against free radicals. Superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase, and glutathione S-transferase have antioxidant activity and they protect cell from oxidative stress. Antioxidants are found in the diet and natural products. Vitamin E, vitamin C (ascorbic acid), β-carotene and flavonoids are natural anti-oxidants [17-19]. Cancer is the leading cause of death in the world. Chronic inflammation is associated with the risk of developing cancer. Ultraviolet (UV) light can cause chronic inflammation and induce skin tumors. Skin cancer can be divided into melanoma and non-melanoma skin cancer depending on the cell type. There has been a significant increase in worldwide incidence of Non-Melanoma Skin Cancer (NMSC) [20]. However, melanoma skin cancer is much less common, but it is far more dangerous.

In the present study, we reported the isolation, structure identification and biological evaluation of five flavonoids including 5-hydroxy-7, 4'-dimethoxyflavone (1), 5, 3'-dihydroxy-7, 4'-dimethoxyflavone (2), 5-hydroxy-7, 3', 4'-trimethoxyflavone (3), 5, 7, 4'-trihydroxyflavone (4), and 3', 4', 5, 7-tetrahydroxyflavone (5) (Figure 1) from the leaves of *Aquilaria agallocha* and the stems of *Aquilaria sinensis*. The structures of these compounds are shown in Figure 1. Previous studies have indicated that flavonoid possess anti-oxidation

and anti-cancer activity [21]. However, a limited study on anticancer effects of agarwood was conducted. Recently, a study has shown that agarwood essential oil exhibited anticancer activity in breast cancer cell line MCF-7 [22]. In this study, we further isolated novel flavonoid compounds. All of these compounds were further examined the antiproliferation and antioxidation activities.

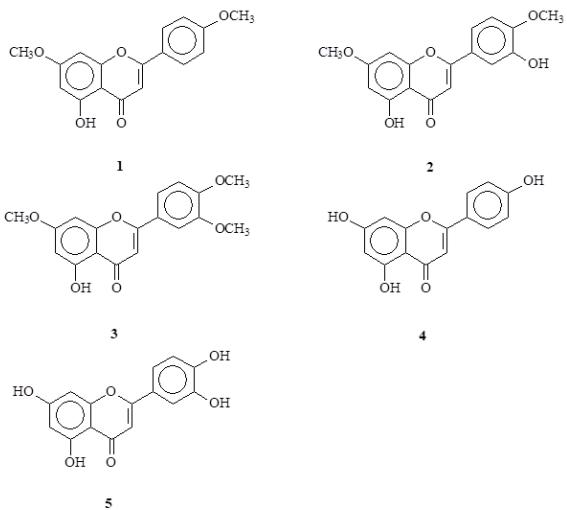


Figure 1. The chemical structure of 5-hydroxy-7, 4'-dimethoxyflavone (1), 5, 3'-dihydroxy-7, 4'-dimethoxyflavone (2), 5-hydroxy-7, 3', 4'-trimethoxyflavone (3), 5, 7, 4-trihydroxyflavone (4) and 3', 4', 5, 7-tetrahydroxyflavone (5).

Materials and Methods

General procedures

UV spectra were obtained on a Jasco UV-240 spectrophotometer in MeCN. IR spectra were measured on a Hitachi 260-30 spectrophotometer (Hitachi, Tokyo, and JP). ¹H-NMR (400/500 MHz) and ¹³C-NMR (100 MHz), HSQC, HMBC, COSY and NOESY spectra were obtained on a Varian (Unity Plus) NMR spectrometer (Varian, CA, USA). For each sample, 128 scans were recorded with the following settings: 0.187 Hz/point; spectra width, 14400 Hz; pulse width, 4.0 μ s; relaxation delay, 2s. Low-resolution ESI-MS spectra were obtained on an API 3000 (Applied Biosystems, CA, USA) and high-resolution ESI-MS spectra on a Bruker Daltonics APEX II 30e spectrometer (Bruker, Bremen, Germany). Silica gel 60 (Merck, 70~230 mesh, 230~400 mesh) was used for column chromatography. Pre-coated silica gel plates (Merck, Kieselgel 60 F-254), 0.20 mm and 0.50 mm, were used for analytical TLC and preparative TLC, respectively, and visualized with 10% H₂SO₄.

Plant material

The stems of *Aquilaria sinensis* were collected from Guansi Township, Hsinchu County, Taiwan in May, 2007. Plant material was identified by Dr. Fu-Yuan Lu (Department of Forestry and Natural Resources College of Agriculture, National Chiayi University, Chiayi, Taiwan). A voucher

specimen (*A. sinensis*) was deposited in the School of Medical and Health Sciences, Fooyin University, Kaohsiung, Taiwan. The specimen of *A. agallocha* was collected from Shanshang District, Tainan City, Taiwan in May, 2008. A voucher specimen was identified by Professor Fu-Yuan Lu (Department of Forestry and Natural Resources College of Agriculture, National Chiayi University) and was deposited in the School of Medical and Health Sciences, Fooyin University, Kaohsiung, Taiwan.

Extraction and isolation

The stems (2.3 kg) of *Aquilaria sinensis* were chipped and air-dried and extracted repeatedly with MeOH (5 L \times 2) at room temperature. The combined MeOH extracts (58.3 g) were then evaporated and further separated into 5 fractions by column chromatography on silica gel (4.5 kg, 70-230 mesh) with gradients of n-hexane/CH₂Cl₂/acetone/MeOH. Part of fraction 1 (32.2 g) was subjected to silica gel chromatography by eluting with n-hexane-acetone (60:1), enriched with acetone to furnish two further fractions (2-1-2-4). Fraction 2-2 (21.1 g) was further purified on a silica gel column using n-hexane/acetone mixtures to obtain 5-hydroxy-7, 4'-dimethoxyflavone (1) (16.3 mg). Fraction 2-3 (6.2 g) was further purified on a silica gel column using n-hexane/acetone mixtures to yield 5-hydroxy-7, 3', 4'-trimethoxyflavone (3) (5.3 mg). Part of fraction 3 (24.9 g) was subjected to silica gel chromatography by eluting with n-hexane-acetone (40:1), enriched with acetone to furnish two further fractions (3-1-3-4). Fraction 3-2 (10.2 g) was subjected to silica gel chromatography, eluting with CH₂Cl₂-MeOH (100:1), and enriched gradually with MeOH, to obtain two fractions (3-2-1-3-2-2). Fractions 3-2-2 (3.5 g) were subjected to further silica gel column chromatography and purified by preparative TLC (thin layer chromatography) to yield 5, 3'-dihydroxy-7, 4'-dimethoxyflavone (2) (6.1 mg) and 5, 4'-dihydroxy-7, 3'-dimethoxyflavone (5.2 mg). The air-dried branches of *Aquilaria agallocha* (2.5 kg) were extracted with MeOH (50 L \times 6) at room temperature and the MeOH extract (29.2 g) obtained after concentration under reduced pressure. This was chromatographed over silica gel (800 g, 70-230 mesh) using n-hexane/CH₂Cl₂/acetone/MeOH as eluent to produce 5 fractions. These (supporting material) were subjected to repeated Si gel CC and preparative TLC fraction 1 to produce 3 fractions (respectively: fraction 1-1~1-3), fraction 1-1 to afford 5-hydroxy-7, 4'-dimethoxyflavone (1) (12.3 mg); fraction 1-2 to produce 4 fractions (respectively: fraction 1-2-1, 1-2-4), fraction 1-2-4 to afford 5, 3'-dihydroxy-7, 4'-dimethoxyflavone (2) (8.3 mg) and 5-hydroxy-7, 3', 4'-trimethoxyflavone (3) (6.8 mg). Fraction 4 to produce 2 fractions (respectively: fraction 4-1~4-2), fraction 4-1 to afford 5, 4'-dihydroxy-7-methoxyflavone, fraction 4-2 to afford 5, 7, 4'-trihydroxyflavone (4) (4.3 mg); fraction 5 to produce 2 fractions (respectively: fraction 5-1~5-2), fraction 5-2 to afford 3', 4', 5, 7-tetrahydroxyflavone (5) (5.1 mg).

Cell culture

The human skin cancer A375 cell line was derived purchased from the Bioresource Collection and Research Center (BCRC number: 60039, Hsinchu, Taiwan, ROC). The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% Fetal Bovine Serum, 1.5 g/L NaHCO₃, 10 µg/mL penicillin, 10 µg/mL streptomycin, 2 mM L-glutamine and 0.25 µg/mL fungizone at 37°C with 5% CO₂ in a humidified incubator.

Determination of DPPH radical scavenging capacity

DPPH is an abbreviation for an organic chemical compound 2, 2-diphenyl-1-picrylhydrazyl. It is a dark-colored crystalline powder composed of stable free-radical molecules. The mechanism of its radical scavenging activity is the antioxidant transfer of an electron or a hydrogen atom to DPPH. DPPH were obtained from Sigma-Aldrich (GmbH, Sternheim, Germany). Various concentrations of the three compounds were added to 0.1 M of stable DPPH (60 µmole/L) solution. When DPPH reacts with hydrogen-donating antioxidant, it is reduced, resulting in a decrease in absorbance at 517 nm. The analyzed time interval was 10 min per point, up to 30 min by using UV-Vis spectrophotometer (Jasco V-530, Japan Servo Co., Ltd. and Tokyo, Japan). Vitamin C was used as a positive control. Measurements were taken in triplicate. The DPPH• radical scavenging capacity (%) was determined as: 1-((Acontrol-Asample)/Acontrol) × 100.

Cell viability assay-MTT assay

The effects of compounds on cell growth were according to the MTT assay procedures. MTT were obtained from Sigma-Aldrich. MTT form impermeable crystals of a dark-blue formazan, thus resulting in accumulation within healthy cells. Briefly, cells were seeded in 96-well microplates at a density of 1 × 10⁴ cells/well. The testing samples were dissolved in sterile DMSO to treat a working concentration. Each concentration was added to a microplate in three replicates and incubated under the same conditions as above for 24 h. After 24 h of incubation, the medium was replaced with 100 µL of fresh medium including 0.5 mg/mL MTT.

The microplate was cultured in a 37°C incubator filled with 5% CO₂ for 2 h. Each precipitate was dissolved in 100 µL of DMSO to dissolve the purple formazan crystals. After the dishes were gently shaken for 10 min in the dark to ensure maximal dissolution of formazan crystals, the absorbance (A) values of the supernatant were measured at 595 nm (UV_{vis}, BioTek, Winooski, VT). Cell growth was calculated as: (Asample-Ablank)/(Acontrol-Ablank) × 100%. All experiments were repeated at least 3 times.

Statistical analysis

All experiments were carried out at three times and at least triplicate. The results were expressed as the average of the mean values ± standard deviation (SD).

Analysis of the data was done with SigmaPlot software (Version 8.0, SPSS Scientific, and Chicago, IL, USA) and SigmaStat (Version 2.03, SPSS Scientific) run on an IBM-compatible computer.

Results

DPPH radical scavenging activity

DPPH has been widely used for free radical-scavenging activity of antioxidant compounds such as phenolic compounds or crude extracts of plants. DPPH radical was scavenged by antioxidants through the donation of hydrogen. In the present study, all compounds were flavonoids. 5-hydroxy-7, 3', 4'-trimethoxyflavone (3) was found to be effective scavengers against DPPH radical at the concentration of 10 mM compared with vitamin C. Vitamin C is as a positive control in this study. As shown in Table 1, 5-hydroxy-7, 3', 4'-trimethoxyflavone (3) had 20.68 ± 5.50% activity at the concentration of 10 mM.

Table 1. DPPH radical scavenging activity (%) of various compounds from *Aquilaria agallocha* and *Aquilaria sinensis*.

DPPH radical scavenging activity (%)	
5-hydroxy-7, 4'-dimethoxyflavone	5.23 ± 0.11
5, 3'-dihydroxy-7, 4'-dimethoxyflavone	4.38 ± 0.33
5-hydroxy-7, 3', 4'-trimethoxyflavone	20.68 ± 5.50
5, 7, 4-trihydroxyflavone	3.74 ± 0.08
3', 4', 5, 7-tetrahydroxyflavone	6.22 ± 1.21
Vitamin C	100.00 ± 9.87

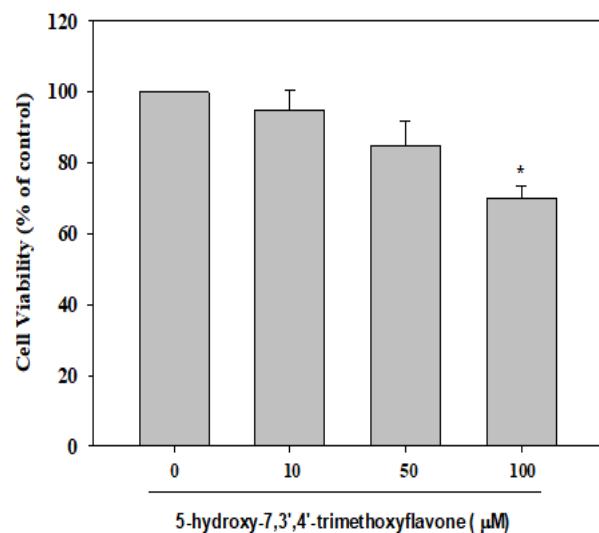


Figure 2. Anti-proliferative effects of 5-hydroxy-7, 3', 4'-trimethoxyflavone in A375 cells. Cell growth was determined by MTT assay after incubation with 10, 50, 100 µM of 5-hydroxy-7, 3', 4'-trimethoxyflavone. Results are expressed as the percent of the cell proliferation of the vehicle control at 24 h. Values are expressed as means ± S.D. of n=3; *p<0.05 versus vehicle control group.

Anti-proliferative properties of compounds 3 on A375 cells

The MTT assay is a colorimetric assay used to measure cell viability. The MTT assay was used to investigate the anti-proliferation activity of tested compounds in A375 cells after 24 h treatment. The cells were treated with 5-hydroxy-7, 3', 4'-trimethoxyflavone (3) at different concentrations (10, 50 and 100 μM). The compound had significant inhibition at the concentration of 100 μM in A375 cells. 5-hydroxy-7, 3', 4'-trimethoxyflavone (3) had $68.00 \pm 1.33\%$ of cell survival rate (Figure 2).

Discussion

Oxidative stress can contribute to many diseases including inflammatory, cancers and aging. Reactive oxygen, nitrogen species and their metabolites play an important role in carcinogenesis. ROS can break DNA structure and DNA-protein cross link. An antioxidant can donate an electron to the free radical or remove the ROS/reactive nitrogen species. Synthetic and natural food antioxidants are usually used in food industry and medicine. Studies have shown that consumption of high antioxidant rich food and medical plants is useful for the prevented diseases. Many antioxidant compounds extracted from plant have been identified as free radical scavengers. Polyphenolic phytochemicals are bioactive secondary metabolites from a variety of plants. Flavonols, flavones, isoflavones, flavanones, procyandins, and anthocyanins belong to flavonoids. Flavonoids are a group of nature compounds with phenolic structure and common characteristics C6-C3-C6 skeleton consisting of two benzene rings. Flavonoids possess anti-oxidative effects as free radical scavengers and metal ion chelators properties associated with the phenolic hydroxyl groups attached to ring structures. Intensity of the antioxidant activity of a flavonoid strongly depends on its chemical structure. Flavonoids possess anti-oxidation, anti-cancer, and anti-inflammatory activities [23]. Many *in vitro* studies have shown flavonoids have chemoprevention and chemotherapy activities in many cancer types including breast, prostate, pancreas and colon cancer [24]. Melanoma is a highly aggressive skin cancer and often resistant to cancer therapy. Pigmentation is a complex biochemical process involving different signal pathways and enzymes.

In this study, we isolated five compounds. Among them, luteolin (3', 4', 5, 7-tetrahydroxyflavone) is a well known flavonoid. Previous study has indicated that luteolin inhibited melanin synthesis in B16 melanoma cells [25]. 2, 2-diphenyl-1-picrylhydrazyl radical (DPPH assay), ferric reducing antioxidant power (FRAP assay) and ABTS assay are *in vitro* anti-oxidation assays. Studies have shown that EGCG, catechins, luteolin, baicalein and kaempferol are flavonoids with anti-oxidation activity. These compounds display anti-melanoma effect *via* inhibiting cell proliferation, invasion and inducing apoptosis with multiple pharmacological mechanisms [25-32]. Flavonoids exhibit anti-proliferative and apoptotic effects *via* HGF/SF-Met signaling, PI3K-AKT pathway,

MAPK pathway or cell cycle regulation in melanogenesis. A study has shown that luteolin inhibited xanthine oxidase activity and reduced cellular ROS levels in B16 cells [33]. Taken these the results of previous studies together, the phenolic compounds may display anti-oxidative actions through different mechanisms inhibiting cell proliferation.

Aquilaria agallocha and *Aquilaria sinensis* are used to treat inflammation in folk medicine. The purpose of the study was to extract five new compounds of flavonoid and examine the antioxidation and antiproliferation activity. It was observed that 5-hydroxy-7, 3', 4'-trimethoxyflavone (3) was shown to scavenge directly the stable DPPH radical scavenging and antiproliferation activities. These findings indicate that 5-hydroxy-7, 3', 4'-trimethoxyflavone (3) may be useful in as free radical scavengers for preventing diseases.

Conclusions

Based on this study it can be concluded that 5-hydroxy-7, 3', 4'-trimethoxyflavone was found to be more sensitive to human skin cancer cell line A375 cell line with anti-oxidation. The detail pharmacological study and *in vivo* study need to be conducted in the future.

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