Original Article

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Antioxidative Compounds from *Aquilaria crassna* Leaf

G. Ray[1], W. Leelamanit[2], P. Sithisarn[1] and W. Jiratchariyakul[1]*
[1] Department of Pharmacognosy, Faculty of Pharmacy, Mahidol University, Bangkok 10400, Thailand.
[2] Department of Biochemistry, Faculty of Pharmacy, Mahidol University, Bangkok 10400, Thailand.

Abstract

The antioxidative activity of *Aquilaria crassna* leaf was investigated through its extracts and isolated compounds. By dissolving the viscous ethanolic extract in a sufficient amount of ethanol and leaving it overnight in the cool place, the precipitate was formed. The filtrate and the precipitate were separately subjected to column chromatography which yielded two compounds. They were compound A from the precipitate and compound B from the filtrate. Compounds A and B were identified using spectroscopic methods as mangiferin and genkwanin. The antioxidative evaluation was performed using 2, 2-diphenyl-1-picrylhydrazyl radical (DPPH) scavenging assay. The result showed that the filtrate, the precipitate, compounds A and B had the IC_{50} values of 32.25 ± 0.48, 15.94 ± 0.16, 15.21 ± 1.20 and 70.05 ± 1.04 µg/mL, respectively. Trolox, the positive control, had the EC_{50} value of 16.81 ± 0.58 µg/mL.

Keyword: *Aquilaria crassna* leaf, Mangiferin, Genkwanin, Antioxidative activity

INTRODUCTION

Aquilaria plants are commonly known as agarwood in European Union, eaglewood in USA, aloeswood in Singapore and kritsana in Thailand. There are 5 *Aquilaria* plants in Thailand, they are *A. crassna* Pierre ex Lecomte., *A. hirta* Ridl., *A. malaccensis* Lamk., *A. rugosa* Kiet & Kessle and *A. subintegra* Ding Hou. *A. crassna* is the most distributed. Only *A. crassna* and *A. malaccensis* can produce fragrant resin embedded in wood (or agar), when they are infected by ascomycetous mould, *Phaeoacremonium parasitica.*[1] Agarwood is wellknown for its distinctive fragrant wood and oil. The volatile oil contains mainly sesquiterpenes, which have sedative effect.[2] It is used in perfumery. The agarwood has been used as one composition in Thai traditional medicine namely Ya-Hom, which is the remedy for fainting[3]. The biological activities of agarwood extract covered cytotoxicity, neuroprotective and inhibition of nitric oxide production. The phytochemicals in the agarwood included 2-(2-phenylethyl) chromenes, lignans, diterpenoids and flavonoid glycosides.[4-9] Our study aimed at the investigation of the plant leaf in the aspect of the isolation of antioxidative compound.

MATERIALS AND METHODS

Plant material

Fresh leaves of *A. crassna* were collected from Nakhon Pathom, Thailand, in October, 2013. The plant was identical to the reference specimen *A. crassna* Pierre ex Lecomte (BKF No. 133637, collected in Trat).The leaves were cleaned and dried in a hot air oven (60 ºC) and ground into coarse powder, yielding 1.2 kg.

Extraction and isolation

The coarse powdered leaf was exhaustively extracted in a Soxhlet apparatus using solvents of increasing polarities. They

***Corresponding author:** Department of Pharmacognosy, Faculty of Pharmacy, Mahidol University, Bangkok 10400, Thailand.
E-mail : weena.jir@mahidol.ac.th

were petroleum ether (25-75 ºC), dichloromethane and 95% ethanol. The solvents were removed under reduced pressure at 50-60 ºC by rotary evaporator. The viscous ethanolic extract was dissolved in ethanol, sonicated in an ultrasonic bath for 30 min and then left in a cool place overnight. The precipitate was formed. It was filtered through Whatman no. 1 filter paper. The dried precipitate had the drug-extract ratio of 150:1 (1 g of precipitate was produced from 150 g of powdered leaves). The dried filtrate had the drug-extract ratio of 24:1(1 g of dried filtrate was produced from 24 g of powdered leaves).

The filtrate and the precipitate were prepared as granules and separately subjected to silica gel columns. The samples were eluted with the gradient mobile phase of dichloromethane and methanol. Compound A was isolated from the precipitate and compound B from the filtrate.

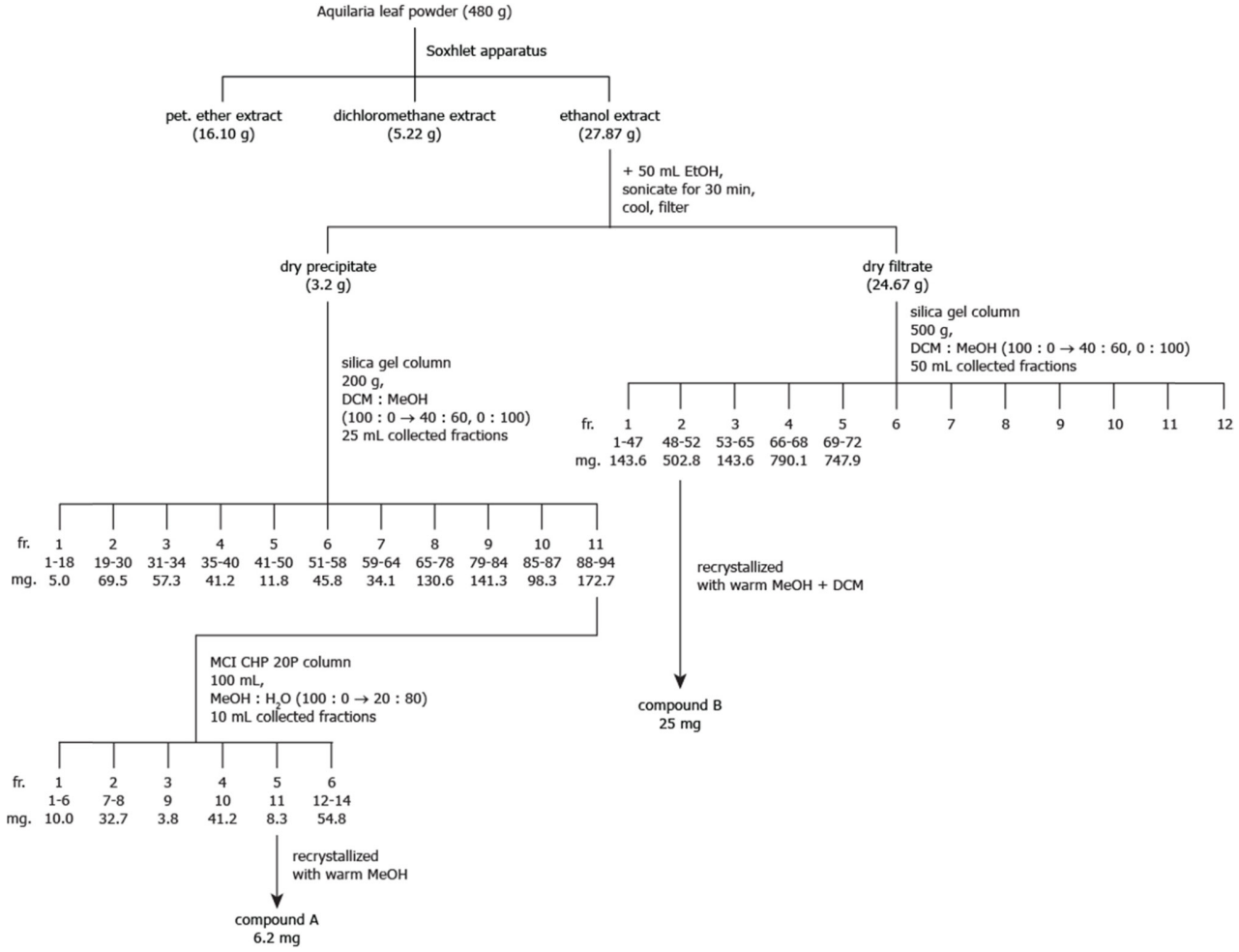

Figure 1. Isolation flowchart of antioxidative compounds from *Aquilaria crassna* leaf
DCM = dichloromethane, MeOH = methanol

Identification

The isolated compounds (A and B) were characterized on thin-layer chromatogram (TLC) under the condition of silica gel GF_{254} pre-coated aluminium sheet as the adsorbent and a solvent system of ethylacetate : formic acid : glacial acetic acid : water (100:11:11:26) as the mobile phase. The melting points were determined on Electrothermal 9100, the UV spectra were recorded on Shimadzu

UV 2600 Spectrophotometer and the FT-IR (liquid film, HATR) on FT-IR Nicolet 6700; these instruments are situated at the Faculty of Pharmacy, Mahidol University, Thailand. The ESI mass spectra and NMR spectra were measured on Bruker microOTOF II spectrometer and Bruker AVANCE 400 spectrometer, respectively, at the Department of Chemistry, Faculty of Science, Ramkhamhaeng University, Thailand.

Determination of antioxidative activity

DPPH (2, 2-diphenyl-1-picrylhydrazyl) free-radical scavenging assay was applied to determine the antioxidative activity of the samples. DPPH is an organic nitrogen radical with purple color. It is reduced by antioxidants to form pale yellow hydrazine. The samples were prepared at the concentration of 1 mg/mL in MeOH (for the filtrate and the precipitate) and in DMSO (for compounds A and B). They were diluted to various concentrations in a 96-well microplate. Volumes of 100 mL of the samples along with 100 mL of 0.2 mM DPPH solution were added to the individual wells, giving final volume of 200 mL. The plate was wrapped in aluminium foil and incubated for 40 min. The optical density of the solutions was read by microplate reader at the wavelength 517 nm. The experiment was performed triplicately and averaged. Trolox was used as a positive control. The percent inhibition (IC_{50}) was calculated using the following equation :

$$\% \text{ inhibition} = [A0 - As/ A0] \times 100$$

A0 = absorbance of control

As = absorbance of sample

Statistical analysis

All values were exposed as mean ± standard deviation (n = 3). Statistical differences of the samples were determined by one-way analysis of variance (one-way ANOVA) at the 5% level of significance. The samples that had *p*-value less than 0.5 were considered to have a statistical difference.

RESULTS AND DISCUSSION

A. crassna leaf produced the highest yield of ethanol extract (5.89%) in comparison to petroleum ether extract and dichloromethane extract which had the yields of 3.35 and 1.10%, respectively. The insoluble part (the precipitate) of the ethanol extract had a yield of 0.67% and the soluble part (the filtrate) 5.12% (Figure 1.)

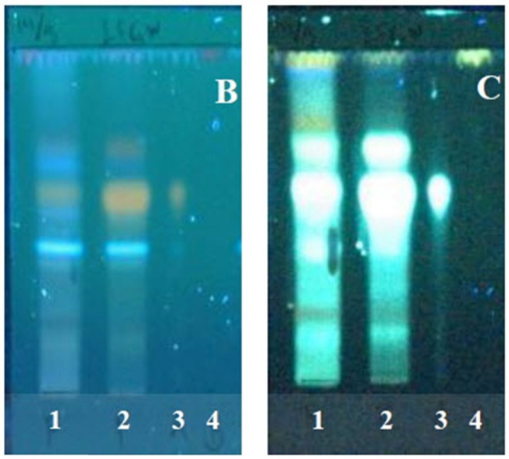

Figure 2. Thin-layer chromatogram of *Aquilaria crassna* leaf

TLC condition	see Materials and methods
Detection	B : under UV 366 nm C : with NP/PEG reagent under 366 nm (NP = natural product reagent; 1% diphenyl-boryloxyethylamine in methanol, PEG = 5% polyethylene glycol-4000 in ethanol)
Sample	Track 1 : the filtrate in ethanol (1 mg/mL), 20 µL Track 2 : the precipitate in ethanol (1 mg/mL), 20 µL Track 3 : compound A (0.5 mg/mL), 5 µL Track 4 : compound B (0.5 mg/mL), 5 µL

The filtrate and the precipitate contained compound A. Only the filtrate had compound B (Fig. 2). The chemical structures of compounds A and B were elucidated using spectroscopic methods as mangiferin and genkwanin, respectively (Fig. 3).

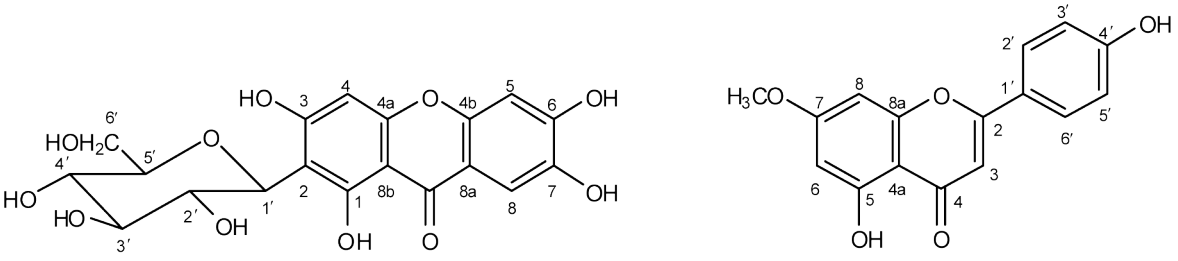

Figure 3. Chemical structures of compounds A and B (M_R = relative molecular mass)

Mangiferin[10] (compound A) : It was yellow powder with a melting point of 271 ºC. On TLC it had the hR_f value of 65 (Fig. 2). The UV spectrum showed the maximum absorption in methanol, λ_{max}^{MeOH}, at 365, 316 and 257 nm. IR (HATR-FTIR, $\upsilon_{CH_2Cl_2}^{cm^{-1}}$) 3419, 1647, 1621; ESI-MS, negative ion mode, m/z = 421.3 $[M\text{-}H]^-$; ^{1}H-NMR (400 MHz, DMSO-d6), δ 7.35 (1H, *s*, H-8), 6.82 (1H, *s*, H-5), 6.35 (1H, *s*, H-4), 4.57 (1H, *d*, *J* = 9.8, H-1′), 4.03 (1H, m, H-2′), 3.67 (1H, *d*, *J* = 11.3, H-6′); ^{13}C-NMR (100 MHz, DMSO-d6), δ 179.1 (C-9), 163.9 (C-3), 161.8 (C-1), 156.2 (C-4a), 154.2 (C-6), 150.8 (C-4b), 143.8 (C-7), 111.7 (C-8a), 108.0 (C-2), 107.6 (C-8), 102.6 (C-5), 101.3 (C-8b), 93.3 (C-4), 81.6 (C-5′), 79.0 (C-3′), 73.1 (C-1′), 70.6 (C-4′), 70.3 (C-2′), 61.5 (C-6′)

Genkwanin[11] (compound B) : It was yellow powder with a melting point of 288 ºC. It had the hR_f value of 44 on TLC using silica gel as an adsorbent and dichloromethane : methanol (95:5) as solvent system. The UV spectrum showed the maximum absorption in methanol, λ_{max}^{MeOH}, at 366, 268 and 239 nm. IR (HATR-FTIR, $\upsilon_{CH_2Cl_2}^{cm^{-1}}$) 3279, 1604, 2973; ESI-MS, negative ion mode, m/z = 283.4 $[M\text{-}H]^-$; ^{1}H-NMR (400 MHz, DMSO-d6), δ 12.98 (1H, *s*, OH-5), 10.41 (1H, br, OH-4′), 7.93 (2H, *d*, *J* = 8.2, H-2′, H-6′), 6.92 (2H, *d*, *J* = 8.2, H-3′, H-5′), 6.82 (1H, *s*, H-3), 6.74 (1H, br*s*, H-6), 3.85 (3H, *s*, OCH_3-7′); ^{13}C-NMR (100 MHz, DMSO-d6), δ 181.9 (C-4), 165.1 (C-7), 164.0 (C-2), 161.3 (C-4′), 161.2 (C-8a), 157.2 (C-5), 128.5 (C-6′), 128.3 (C-2′), 121.0 (C-1′), 115.9 (C-3′, C-5′), 104.6 (C-4a), 103.0 (C-3), 97.9 (C-6), 92.6 (C-8), 56.0 (OCH_3-7).

The free radical scavenging activity of the extracts and the isolated compounds using DPPH radical produced the IC_{50} values as shown in Table 1.

Table 1. IC_{50} values of *A. crassna* leaf using DPPH free radical scavenging activity assay.

Sample	IC_{50} values (μg/mL)
the filtrate	32.25 ± 0.48
the precipitate	15.94 ± 0.16
compound A	15.21 ± 1.20
compound B	70.05 ± 1.04
trolox (positive control)	16.81 ± 0.58

We used the non-polar solvents to remove the fatty and the coloring substances from the plant leaf. The ethanol extract contained mostly the polar substances. The dry ethanolic extract was dissolved in a sufficient amount of ethanol and left it in a cool place. We easily obtained the precipitate of which some coexisting substances were removed. This precipitate possessed twofold antioxidative activity when compared with the filtrate. The antioxidative substance in the precipitate was mangiferin (compound A), which was the main component, and more active than trolox, the positive control. Mangiferin was previously reported as antiinflamatory, antihepatotoxic, antiviral agents and central nervous stimulant.[12] This finding supported the traditional use of *Aquilaria* leaf as anti-inflammation and hepatoprotective.[13]

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