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PURIFICATION AND CHARACTERIZATION OF CHEMICAL MARKER COMPOUND FROM *AZADIRACHTA INDICA* VAR. *SIAMENSIS* FLOWERS

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KEYWORDS: *Azadirachta indica*, Sadao, Siamese neem tree, DPPH, HPLC

INTRODUCTION

Azadirachta indica A. Juss. var. *siamensis* Valeton or Siamese neem tree is perennial plant in the Meliaceae family. This plant is widespread in southern Laos, Cambodia and Thailand (**1**). In Thailand, the flower of the neem tree which is called "dok sadao" is commonly consumed as a vegetable. Besides that, it is also recognized to have some medicinal activities (**2**) especially for the treatment of some pathological conditions related to oxidative disorders, such as inflammation and skin diseases (**3, 4**). We are interested in phytochemical isolation of marker compound for qualitative and quantitative control of its flower alcoholic extract. We also compared the antioxidant activity of neem tree flower with five kinds of stigmas which is called "Gaysorn Tang Ha", using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) scavenging assay. Gaysorn Tang Ha is composed of the stigmas of *Jasminum sambac* (L.) Ait., *Mimusops elengi* L., *Mesua ferrea* L., *Mammea siamensis* Kosterm., and *Nelumbo nucifera* Gaertn.

MATERIALS AND METHODS

Instrument and reagents

Reagents and solvents were reagent grade and used without further purification. TLC was performed on silica gel GF_{254} (Merck). For column chromatography, silica gel (Merck 230-400 mesh) was used. NMR spectra were recorded with a Bruker Avance (^{1}H, 300 MHz) spectrometer. Chemical shifts are reported in ppm, and coupling constants are reported in Hz. All NMR spectra were obtained in deuterated chloroform ($CDCl_3$) and referenced to the residual solvent peak. Mass spectra were obtained on a Thermo Finigan Polaris Q spectrometer. α-Tocopherol and 1,1-diphenyl-2-picrylhydrazyl (DPPH) were purchased from Sigma.

Plant materials

Flowers of Siamese neem tree (*Azadirachta indica* var. siamensis) were collected in April 2012 from Prachuapkhirikhan and Saraburi provinces, Thailand. The flowers were identified by comparison with the specimens at the Forrest Herbarium, Department of National Park, Wildlife and Plant Conservation, Ministry of Natural Resources and Environment, Bangkok. The voucher specimens of *A. indica* var. siamensis was deposited at the Faculty of Oriental Medicine, Rangsit University, Pathumthani, Thailand. Samples were dried in hot air oven at 55 °C for 8 hours and then powdered.

Preparation of crude extracts

The dried, powdered flowers (250 g) were extracted with 95% ethanol (3 liters) at room temperature for 7 days. The extract was filtered with Whatman filter paper No.1 and then evaporated under reduced pressure with rotary evaporator to obtain 15 g of crude, dark green extract.

Extraction and isolation

The crude extract was partitioned with dichloromethane (200 ml) and water (200 ml). After removal of the solvent, the dichloromethane layer gave 7.8 g of extract and the aqueous layer gave 0.98 g of extract. The dichloromethane extract was dissolved in dichloromethane, and then mixed with 80 g of Silica gel 60 and loaded onto a silica gel column (20 cm x 2.0 cm). The column was eluted with a gradient of dichloromethane : methanol (1:0 → 98:2) and seven fractions were collected.

Identification of marker compound in the crude extract by HPLC

Dried, powdered *A. indica* var. *siamensis* flowers (30.0 g) were accurately weighed and transferred to a 100 mL volumetric flask. Methanol (50 mL) was added and the flask was immersed in an ultrasonic bath and sonicated for 30 min. It was filtered through Whatman filter paper No. 1 and concentrated using rotary evaporator. The dried extract (0.8 g) was dissolved in methanol, transferred to a 100 mL volumetric flask, and adjusted to volume with methanol. The solution was filtered through a membrane filter (0.20 μm) and 20 μL of the filtrate was injected into the HPLC system (Shimadzu SPD-10AD Shimadzu LC-10AD Pump and a diode array detector SPD-10A with detection wavelength at 292 nm). The separation was performed on Inertsil ODS-3 (5 μm, 250 x 4.6 mm) column; flow rate was 0.8 mL/min and the solvent system was a gradient elution of 1% acetic acid in water and CH_3CN ranging from 90:10, 60:40, 40:60, 20:80, 0:100, at 0, 15, 30, 40, and 60 min, respectively. For the identification of marker in the flower extract, stock standard solution was prepared by dissolving 10.0 mg of sophoraflavanone in 100 mL of methanol (Figure 2).

Free radical scavenging activity

The antioxidant activity of six flower extracts on the scavenging activity of the stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical was assayed. The decrease in absorbance was determined at 520 nm. The stock solutions of *A. indica* var. *siamensis*, *M. siamensis, M. ferrea, J. sambac*, *M. elengi* and *N. nucifera* extracts were diluted into 4 concentrations (75, 100, 150 and 200 μg/mL). An aliquot of the extract (2.5 mL) at the concentration of 0.1 mg/mL was added to 2.5 ml of 0.06 mM DPPH solution. The mixture was mixed and left to stand for 5 min at room temperature. In this experiment, 0.05 mg/mL of α-tocopherol (Vitamin E) was used as the positive control. The absorbance of the resulting solution was measured by UV-Visible spectrophotometer at 520 nm and the percent inhibition activity was calculated.

RESULTS AND DISCUSSION

Fraction 7 of the dichloromethane extract of *A. indica* var. *siamensis* flowers was further purified by column chromatography and preparative TLC to give sophoraflavanone B (**5**). The aqueous layer (0.975 g) was subjected to RP-18 column chromatography, eluted with methanol:water (40:60 to 1:0) to obtain five fractions. The selected fraction was further purified by preparative TLC to yield two compounds which were identified as rutin (**6**) and quercetin (**6**) (Figure 1). All compounds were elucidated by spectroscopic methods and the data were compared with the literatures.

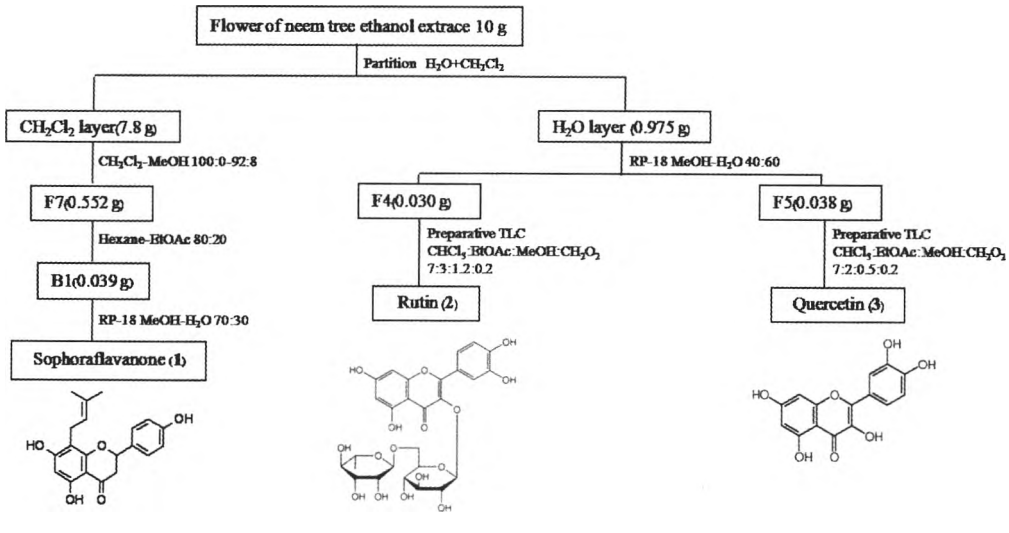

Figure 1: Isolation of compounds 1-3 from the dried flowers of *Azadirachta indica* var. *siamensis*

For the identification of marker compound in the crude extract by HPLC, sophoraflavanone B was used as a marker in this extract. This compound has an inhibitory effect on copper-induced protein oxidative modification (**7**). Sophoraflavanone B, showing a retention time of 37.0 min (t_R) with good resolution, appeared as one of the major compounds in the extract (Figure 2).

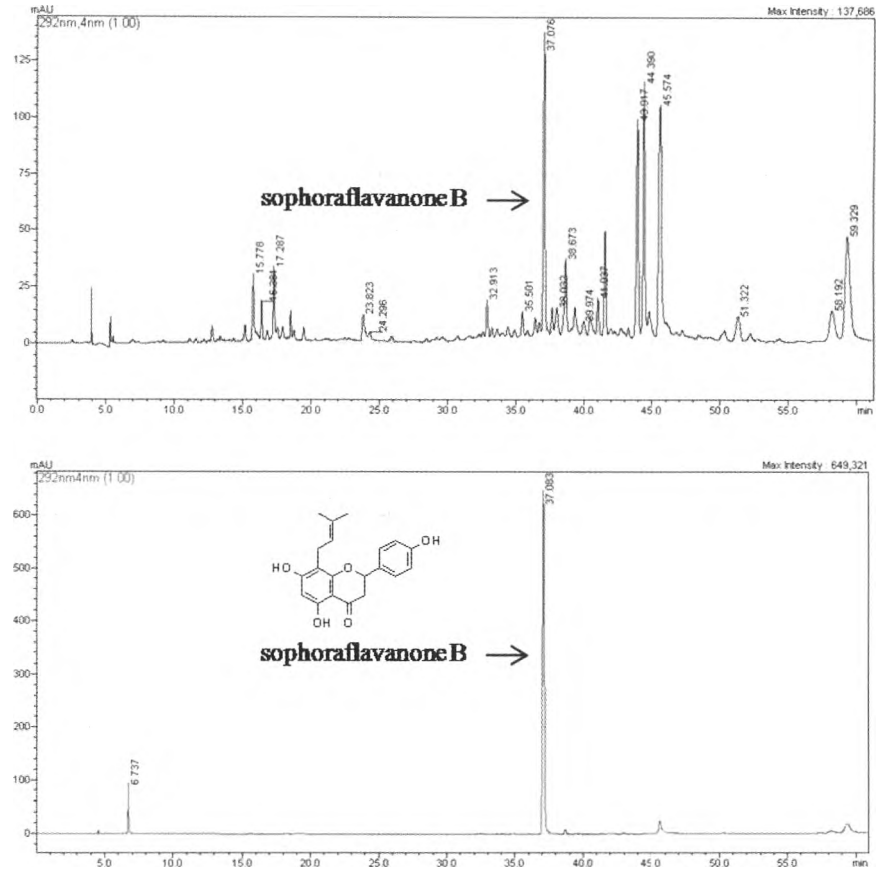

Figure 2. Chromatogram of flower of *Azadirachta indica* var. *siamensis* extract (above) and standard sophoraflavanone B (below)

This study also evaluated the radical scavenging activity of crude extract from *Azadirachta indica* var. *siamensis* flowers, together with five kinds of stigmas called "Gaysorn Tang Ha". Gaysorn Tang Ha is composed of the stigmas of *Jasminum sambac* (L.) Ait., *Mimusops elengi* L., *Mesua ferrea* L., *Mammea siamensis* Kosterm. and *Nelumbo nucifera* Gaertn. After performing the DPPH assay, the 50% scavenging activity (IC_{50}) of each sample was calculated and plotted in bar graph (Figure 3). The highest antioxidant activity belonged to *Mimusops elengi* L., while *Azadirachta indica* var. *siamensis* came in the second place. *Nelumbo nucifera* extract showed the lowest radical scavenging activity.

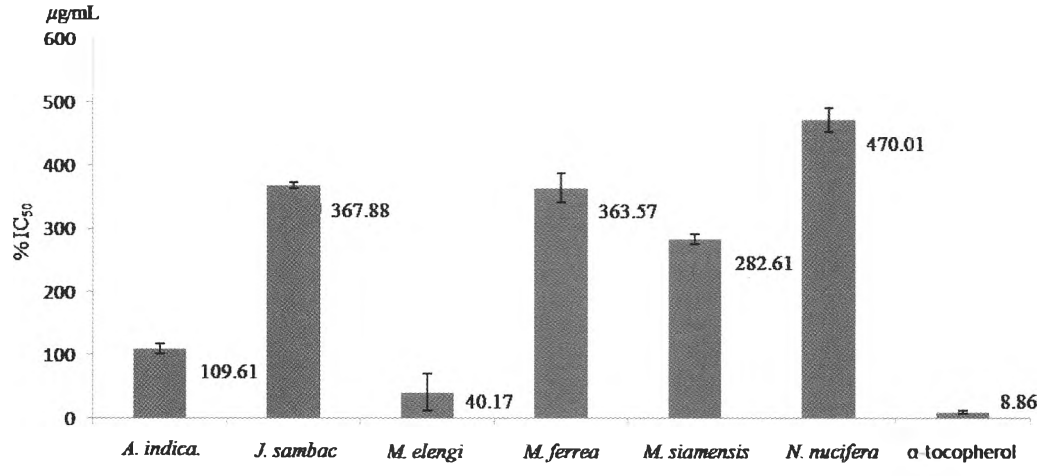

Figure 3. DPPH radical-scavenging activities of ethanol extracts of *A. indica* var. *siamensis* and "Gaysorn Tang Ha"

CONCLUSION

From our study, three flavonoids, sophoraflavanone B, rutin, and quercetin, were isolated from the flowers of *Azadirachta indica* var. *siamensis*. Sophoraflavanone B is a major compound in the extract and has inhibitory effect on copper-induced protein oxidative modification. These two properties make it a good marker for quality control of the extract by HPLC. For the radical scavenging activity, the crude extract from *A. indica* var. *siamensis* flowers showed a moderate antioxidant activity.

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