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Original Article

Extraction method for high free radical scavenging activity of Siamese neem tree flowers

Worarat Chaisawangwong and Wandee Gritsanapan*

Department of Pharmacognosy, Faculty of Pharmacy,
Mahidol University, Ratchatewi, Bangkok, 10400 Thailand.

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Abstract

Siamese neem tree (*Azadirachta indica* A. Juss. var. *siamensis* Valeton) is a medicinal plant found in Thailand. Young leaves and young flowers of this plant are commonly consumed as a bitter tonic vegetable. The flowers are also used for treatment of fever. The flower extract has been reported to exhibit *in vitro* free radical scavenging activity and can inhibit lipid peroxidation of bronchogenic cancer cell line. Active compounds in the flowers are flavonoids such as rutin and quercetin. The content of these compounds in the crude extract depends on the method of extraction. Therefore, the appropriate extraction method promoting high yield of total flavonoids and high free radical scavenging activity was investigated in this study. Six different extraction methods, i.e. maceration, percolation, decoction, soxhlet extraction, ultrasonic extraction (UE), and microwave assisted extraction (MA) were carried out for extracting dried powder of Siamese neem tree young flowers. The solvent used for maceration, percolation, and soxhlet extraction was 50% ethanol, while distilled water was used for decoction and MA, and both solvents were used for UE. The content of crude extract, free radical scavenging activity, and total flavonoids content of each extract were investigated and compared. Comparing the various extraction methods, decoction provided an extract containing a high amount of total flavonoids (17.54 mgRE/g extract) and promoting the highest scavenging activity at EC_{50} 11.36 μg/ml. Decoction is also simple, cheap, and convenient and could be used in developing countries. Thus, it should be the recommended extraction method for the flowers of Siamese neem tree for further development of antioxidant pharmaceutical preparations.

Keywords: antioxidant, Siamese neem flower, *Azadirachta indica* var. *siamensis*, flavonoid, extraction method

1. Introduction

In recent years, increasing attention has been paid to the role of diet for human health. Several epidemiological studies have indicated that a high intake of plant products is associated with a reduced risk of a number of chronic diseases, such as atherosclerosis and cancer (Temple, 2000; Hashimoto *et al.*, 2002, Gosslau and Chen, 2004). These beneficial effects have been partly attributed to the compounds, which possess antioxidant activity. The major antioxidants in vegetables are vitamin C and E, carotenoids, and phenolic compounds, especially flavonoids. These antioxidants scavenge free radicals and inhibit the chain initiation or break the chain propagation (Podsedek, 2007).

Siamese neem tree (*Azadirachta indica* A. Juss var. *siamensis* Valeton), which belongs to the Meliaceae family, has been found in Southeast Asian countries such as Laos, Myanmar, Cambodia and also Thailand (Sombatsiri *et al.*, 1995). In Thailand, the young leaves and young flowers of this plant are commonly consumed as a vegetable for bitter tonic. The flowers were also traditionally used as an element tonic for the treatment of fever and nasal polyposis (Clayton

*Corresponding author.
Email address: pywgs@mahidol.ac.th

et al., 1996). There are a small number of publications concerning biological activities of Siamese neem tree. One of those showed that the extracts from several parts of the Siamese neem tree, especially flowers and leaves, promoted high free radical scavenging activity against 1,1'-diphenyl-2-picrylhydrazyl (DPPH) and 2,2'-azinodi-(3-ethylbenzthiazoline sulphonate). The flowers decrease the activity of phase I enzymes, cytochrome P450, aniline hydroxylase (ANH), and aminopyrone-N-demethylase (AMD), but increase the activity of the phase II enzyme glutathione-S-transferase (GST) (Kusamran *et al.*, 1998). Active compounds in the flowers are flavonoids, such as rutin and quercetin (Sithisarn *et al.*, 2005). The content of active compounds in the crude extract depends on the solvent, method of extraction, extraction time, and temperature. Therefore, the aim of this study was to find the appropriate extraction method to gain a high yield of total flavonoids and to gain a high free radical scavenging activity of Siamese neem flowers for further standardization of the extract for pharmaceutical purposes.

2. Materials and Methods

2.1 Plant materials

Fresh young flowers of Siamese neem were collected from Kanchanaburi Province in January 2007. The sample was identified by comparison with specimens at the Forest Herbarium, Department of National Park, Wildlife and Plant Conservation, Ministry of Natural Resources and Environment, Bangkok. The voucher specimen (WAI0107) was deposited at Department of Pharmacognosy, Faculty of Pharmacy, Mahidol University. The flowers were dried in a hot air oven at 55°C for 24 hours, then moderately powdered and passed through a sieve (20 meshs).

2.2 Chemicals and reagents

Quercetin, rutin, and DPPH were obtained from Sigma-Aldrich Chemical Company (St. Louis, MO, USA). All chemicals and reagents used were analytical grade, except ethanol, which was the commercial grade obtained from the Excise Department, Bangkok.

2.3 Extraction methods

2.3.1 Maceration

The powder of Siamese neem young flowers was macerated with 50% ethanol (1:20, w/v) at room temperature for 4 days and filtered through a Whatman no.1 filter paper. Other portions of the solvent were added to the marc and the extraction was repeated until the last extract was colorless. The extracts were combined and concentrated under reduced pressure at 45°C using a rotary vacuum evaporator. The crude extract was then evaporated on a boiling water bath until a constant weight was obtained to afford the maceration extract.

2.3.2 Percolation

The powder of Siamese neem young flowers was percolated with 50% ethanol (1:20, w/v) at room temperature (flow rate 1 ml/min). Other portions of the solvent were added and the extraction was repeated until the last extract was colorless. The combined extract was filtered and the filtrate was concentrated and evaporated under the same condition as described before to afford the percolation extract.

2.3.3 Soxhlet extraction

The powder of Siamese neem young flowers was extracted with 50% ethanol using a soxhlet apparatus (60-80°C; 1:50, w/v) until the last extract was colorless. The combined extract was filtered and the filtrate was concentrated and evaporated under the same condition as described before to afford the soxhlet extract.

2.3.4 Ultrasonic extraction (UE)

The powder of Siamese neem young flowers was separately extracted by sonication with 50% ethanol and distilled water (1:20, w/v) for 30 min each and then filtered. Other portions of the solvent were added to the marc at the same portion and the extraction was repeated until the last extract was colorless. The combined extract was filtered and the filtrate was concentrated and evaporated under the same condition as described before to afford the 50% and aqueous ultrasonic extracts.

2.3.5 Decoction

The powder of Siamese neem young flower was extracted by boiling with distilled water (1:20, w/v) for 6 hrs and then filtered. Other portions of the distilled water were added to the marc and the extraction was repeated until the last extract was colorless. The combined extract was filtered and the filtrate was evaporated on a boiling water bath until constant weight was obtained to afford the decoction extract.

2.3.6 Microwave assisted extraction (MA)

A commercial household microwave oven (Panasonic Model NN-MX21WF) was used for extraction. The microwave oven was operated at 2450 Hz single-phase output of 800 W. A portion of the sample (10.0 g) was placed in a 600 ml flask, followed by the addition of 200 ml of distilled water. The irradiation cycle was as following: 3 min preheated (temperature 70°C), 1 min power-on, followed by 2 min power-off to hold the temperature at 70-85°C, the extraction process was then repeated 8 cycles. After finishing the extraction, the flask was allowed to cool to room temperature, then the mixture was filtered through a Whatman no. 1 filter paper. Other portions of the solvent were added

to the marc and the extraction was repeated until the last extract was colorless. The combined extract was then evaporated on a boiling water bath until a constant weight was obtained to afford the microwave extract.

2.4 Thin-layer chromatographic fingerprints

The extract of Siamese neem flower prepared by each extraction method was analyzed using thin-layer chromatography (TLC). The TLC was performed on TLC precoated silica gel G60 F_{254} plate 10×10 cm (Merck, Germany) using a homogenous solvent system comprising ethylacetate: dichloromethane: formic acid: acetic acid: water at 100: 25: 10:10:11 as the mobile phase. The TLC plate was detected using a CAMAG viewing box UV detector (CAMAG, Muttenz, Switzerland) and a natural product polyethylene glycol (NP-PEG) spraying reagent under UV 366 nm was used for detecting the flavonoids (Figure 1).

2.5 Determination of scavenging activity by DPPH method

The scavenging activity of the extracts obtained from several extraction methods and standards quercetin and rutin were determined based on the radical scavenging ability in reacting with stable DPPH free radicals (Yamasaki *et al.*, 1994). A total of 750 ml extract (50 to 1000 mg/ml) or of the standards quercetin and rutin, where absolute ethanol was used as a blank, was added to 750 ml of DPPH in absolute ethanol solution (152 mM) using a 1.5 ml Eppendorf tube. After incubation at 37°C for 20 minutes, the UV-absorbance of each solution was determined at 520 nm (Shimadzu UV spectrophotometer), where were methanol without a sample was used as a blank. The corresponding blank readings were also taken and % inhibition was then calculated as follows:

$$\% \text{ Inhibition} = \frac{(A_{blank} - A_{extract}) \times 100}{A_{blank}}.$$

The EC_{50} value, which is the concentration of the sample required for 50% inhibition of DPPH free radicals, was determined from the curve between % inhibition and concentration. Each sample was done in triplicate. The average of the three EC_{50} values was then calculated.

2.6 Total flavonoid content

The Total flavonoid content was determined as described by Meda *et al.* (2005). Five millilitres of 2% aluminium chloride ($AlCl_3$) in methanol were mixed with the same volume of the sample solution. Absorption readings at 415 nm were taken after 10 min against a blank consisting of 5 ml sample solution and 5 ml of methanol without $AlCl_3$. The total flavonoid content was determined using a standard curve of rutin (10-100 mg/ml). The mean of three readings was used and expressed as mg of rutin equivalents (RE)/100 g of the extract.

2.7 Statistical analysis

The results were expressed as mean ± standard deviation (SD) (n=3). The data were statistically analyzed using one way ANOVA with least square difference (LSD) using SPSS 13 software. The statistical probability (*p*-value) less than 0.05 indicated a statistical difference between groups.

3. Results and Discussion

In a previous study (Sithisarn *et al.*, 2006), a comparison of solvents used for extraction of the leaves of Siamese neem tree, using 20%, 50%, 80%, 95% ethanol by maceration and 50%, 80%, 95% ethanol by percolation, was done. It was found that 50% ethanol affords the extracts with high free radical scavenging activity when prepared by maceration (EC_{50}=201.45 μg/ml) and percolation (EC_{50}=122.01 μg/ml), while the EC_{50s} of 20%, 80%, 95% ethanol extracts from maceration were 265.05, 217.08, and 265.18 μg/ml, respectively and the EC_{50} of 80% and 95% ethanol extracts from percolation were 236.86 and 169.88 μg/ml, respectively. Therefore ethanol 50% (v/v) was used as a solvent for maceration, percolation, soxhlet extraction, and UE in this study, while distilled water was used for UE, decoction, and MA. Ethanol 50% (v/v) was not used in decoction and MA due to the loss of ethanol from high temperature under decoction condition and to avoid unexpected explosions under MA condition. Distilled water was not used in maceration and percolation because of microbial growth contamination during the processes, which took a relatively long time. TLC-fingerprints of the extracts prepared by different extraction methods showed the same pattern with rutin as the major and quercetin as the minor component (Figure 1). The main spot

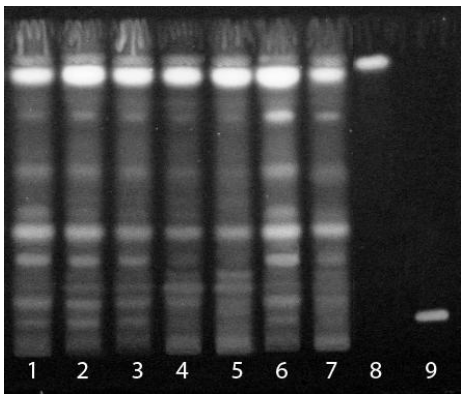

Figure 1. Thin-layer chromatograms of Siamese neem flower extract prepared by different methods of extraction. 1 = Maceration, 2 = Percolation, 3 = Soxhlet extraction, 4 = Ultrasonic extraction with 50% ethanol, 5 = Ultrasonic extraction with distilled water, 6 = Decoction, 7 = Microwave assisted extraction, 8 = Quercetin, 9 = Rutin. Absorbent: Silica gel G60 F_{254}, Solvent system: ethylacetate: dichloromethane: formic acid: acetic acid: water 100: 25: 10: 10: 11. Detection: NP/PEG spraying reagent, under UV 366 nm

Table 1. Extraction time, amount of solvent, yield, extract ratio, total flavonoid content, and free radical scavenging activity of the extracts

Method	Solvent	Sample: solvent (g:ml)	Extraction time (hrs)	Amount of solvent (ml)	Yield of crude extract (% dry weight)***	Extract ratio	Total flavonoid content (mg RE/g extract)***	EC_{50} (μg/ml)***
Maceration	50%Ethanol	1:20	840	1,000	43.50±1.24[b]	2.29 : 1	10.68±1.28[d]	50.16±1.32[g]
Percolation	50%Ethanol	1:20	72	4,320	50.21±2.56[a]	1.99 : 1	14.68±1.86[c]	39.14±0.67[f]
Soxhlet	50%Ethanol	1:50	48	1,000	40.47±1.05[b]	2.47 : 1	22.30±1.10[a]	25.74±0.37[c]
UE*	50%Ethanol	1:20	30	1,000	42.33±0.82[b]	2.36 : 1	8.56±0.49[d]	33.90±1.71[e]
	Distilled water	1:20	36	1,200	38.18±1.87[b]	2.62 : 1	14.14±0.68[c]	18.00±0.31[b]
Decoction	Distilled water	1:20	30	1,000	45.32±0.98[b]	2.20 : 1	17.54±1.67[b]	11.36±0.52[a]
MA**	Distilled water	1:20	0.9	1,200	37.81±2.12[b]	2.64 : 1	9.73±2.23[d]	27.85±1.40[d]
Quercetin								2.34±0.14
Rutin								26.00±0.50

* Ultrasonic extraction **Microwave assisted extraction

***Different letters in each column indicate a significant difference at $p < 0.05$ in one-way ANOVA

seen under quercetin was found to be a combination of few minor unidentified flavonoids. TLC chromatograms of the decoction extract showed bigger spots of flavonoids than the extracts from other extraction methods indicating a higher flavonoid content. Compared to the TLC chromatograms of the leaves extract, they also showed spots corresponding to quercetin and rutin flavonoids exhibiting antioxidant activities (Sithisarn *et al.*, 2006). Yields, extract ratio (powder: 1 g extract), free radical scavenging activity, and total flavonoid content of Siamese neem flower extracted by different methods are shown in Table 1. Percolation with 50% ethanol provided the highest yield of crude extract (50.21% dry weight) but medium free radical scavenging activity (EC_{50}= 39.14 μg/ml). Soxhlet extraction gave the highest total flavonoid content at 22.30 mg RE/g extract, but also showed medium free radical scavenging activity (EC_{50}= 25.74 μg/ml). The most active free radical scavenging activity extract (EC_{50} = 11.36 μg/ml) was obtained from the decoction method, which gave a high yield of crude extract (45.32% dry weight) but a medium yield of total flavonoids (17.54 mg RE/g extract). The decoction method affording the highest scavenging effect of the flower extract confirms the previous report of Siamese neem tree leaf extracts (Sithisarn *et al.*, 2006). These results also suggest that the active antioxidant components in the flowers of Siamese neem tree were better extracted by hot water than by 50% ethanol. Comparing three extraction methods using hot water as a solvent, decoction gave an extract that was promoting higher free radical scavenging activity than UE and MA. MA took the shortest time but gave the lowest yield of the crude extract and low content of total flavonoids. Compared with the other methods, decoction is simple, convenient and carried low cost in terms of reagent and instrumentation. As it is economical, the decoction method can be used especially in developing countries.

4. Conclusions

The decoction method gave a high yield of crude extract and a high content of total flavonoids. The decoction extract promoted the highest antioxidant activity against the DPPH radicals. Thin layer chromatograms of the flower extracts showed spots of rutin and quercetin flavonoids as previous reported in the young leaf extract (Sithisarn *et al.*, 2006), but rutin was the major component. Decoction is here the recommended extraction method for the high antioxidant activity of Siamese neem flowers. This finding will be useful for further developments of the extracts for antioxidant pharmaceutical production and commercial use.

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