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*Short Communication*

# Free Radical Scavenging Activity and Total Flavonoid Content of Siamese Neem Tree Leaf Aqueous Extract from Different Locations

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**Abstract** Siamese neem tree (*Azadirachta indica* A. Juss. var. *siamensis* Valeton) is a medicinal plant found in every part of Thailand. Young leaves and flowers of this plant are traditionally consumed as a bitter tonic vegetable. There is a report concerning antioxidant activity of Siamese neem tree leaves including free radical scavenging activity and inhibition of lipid peroxidation in cancer cell line. Moreover, it is found that the leaves of Siamese neem tree contain some antioxidant flavonoids including quercetin and rutin. Therefore, free radical scavenging activity determination by DPPH scavenging assay and total flavonoid content analysis of the leaf aqueous extracts of Siamese neem tree collected from different locations in Thailand were performed in this experiment. It was found that Siamese neem tree leaf extracts contain total flavonoids in a range of 110.0 to 511.4 mg quercetin equivalent per 100 g extract, while $EC_{50}$ values obtained from DPPH method were in a range of 28.85 to 173.77 µg/ml. The free radical scavenging activity and total flavonoid content of the extracts from different regions were significantly different ($p < 0.05$) and samples from the south showed the activity and flavonoid content > the north > the east > the west > the north-east > the central samples. The extract from Songkhla sample promoted both the strongest antioxidant activity ($EC_{50}$, 28.85 µg/ml) and the highest total flavonoid content (511.4 mg quercetin equivalent/ 100 g extract). The results suggested that total flavonoid content in Siamese neem tree leaf extracts correlated with antioxidant activity. 

**Keywords:** *Azadirachta indica*, DPPH, free radical scavenging, Siamese neem tree, total flavonoid

## INTRODUCTION

*Azadirachta indica* A. Juss. var. *siamensis* Valeton or Siamese neem tree is an evergreen tree belonged to Meliaceae family. It is originated in southern Laos, western Cambodia and Thailand where is the largest source of this plant.[1] In Thai traditional medicine, young leaves and young flowers of this plant are used as a tonic and for the treatment of fever and nasal polyposis. The stem bark is used for the treatment of diarrhea and amebic dysentery. Fruits are used as an anthelmintic and for the treatment of haemorrhoids and abnormal urination.[2] Moreover, the young leaves and flowers of this plant are popularly consumed as a bitter tonic vegetable to promote good health.

In our previous reports,[3] the extracts from several parts of Siamese neem tree showed free radical scavenging activity against 1,1-diphenyl-2-picrylhydrazyl (DPPH) and 2,2'-azino-di-(3-ethylbenzthiazoline sulphonate) (ABTS) radicals, especially the leaf, flower and stem bark extracts which showed strong activities. The leaf and flower extracts inhibited lipid peroxidation induced by UV irradiation of bronchogenic cancer cell line (ChaGo K-1). Thin-layer chromatography of the leaf extract of Siamese neem tree showed the spots equivalent to quercetin and rutin flavonoids.[4]

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Siamese neem tree is an interesting plant for future purpose related to its antioxidant activity including medicinal agents and health supplements. Therefore, the aim of this experiment is to investigate free radical scavenging activity of the leaf aqueous extracts of Siamese neem tree collected from different locations in Thailand using DPPH method, and to quantitate total flavonoid content using a spectrophotometric method.

## MATERIALS AND METHODS

### *Materials and Reagents*

Quercetin, rutin, butylated hydroxyl toluene (BHT), DPPH and ascorbic acid were obtained from Sigma (St. Louis, MO, USA). Aluminium chloride was purchased from Merck, Germany. Trolox was obtained from Biomol Research Laboratories, PA, USA. All organic solvents were analytical grade and supplied from Labscan Ltd. (Dublin, Ireland).

Twelve samples of the leaves of Siamese neem tree (*Azadirachta indica* A. Juss. var. *siamensis* Valeton) were collected from 12 provinces, i.e. Ratchaburi, Roi Et, Nakhon Pathom, Maha Sarakham, Ang Thong, Chanthaburi, Lampang, Lop Buri, Prachin Buri, Nakhon Si Thammarat, Songkhla and Phetchabun, Thailand, during April to May 2004. The samples were identified by Mrs. Vachalee Prachasaisoradej, an Agricultural Scientist, Plant Variety Protection Division, Research Unit of Princess Sirindthorn Plant Herbarium, Bangkok, Thailand. The Voucher specimens were deposited at the same place (BK63512 Siamese neem tree). Samples were cleaned, dried in a hot air oven (55$^{o}$C) for six hours then powdered and passed through a sieve with mesh number 20.

### *Apparatus and Equipment*

A Shimadzu U160 ultraviolet spectrophotometer (4973C SPEC, Shimadzu, Kyoto, Japan) was used for antioxidation activity determination by DPPH method and for total flavonoid content determination.

### *Preparation of the Extracts*

Dried powder of leaf samples were separately boiled with distilled water for 6-8 hours (plant : water = 1:20, w/v) then filtered. The filtrate was evaporated to dryness on a boiling water bath to yield dried leaf aqueous extracts.

### *Free Radical Scavenging Activity Determination by DPPH Method*

Antioxidant activity of the extracts of Siamese neem tree leaves from several locations and standard solutions (BHT, vitamin C, trolox, quercetin and rutin) were determined based on the radical scavenging ability in reacting with a stable DPPH free radical.[5] The amount of 750 µl of the extract (at concentrations of 50 to 1000 µg/ml) or a standard was added to an eppendorf tube (1.5 ml) containing 750 µl of DPPH in absolute ethanol solution (152 µM). After incubation at 37$^{o}$C for 20 minutes, the absorbance of each solution was determined at 520 nm using an ultraviolet spectrophotometer. The corresponding blank readings were also taken and % inhibition was then calculated as follows:

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

A = absorbance

$EC_{50}$ value, the concentration of sample required for 50% inhibition of DPPH free radical, was determined from the plot between % inhibition and concentration. Each sample was done in triplicate. The average of $EC_{50}$ values was then calculated.

### *TLC Fingerprint of the Extract*

Thin layer chromatography of the leaf aqueous extract of Siamese neem tree (from Ratchaburi) was performed on TLC pre-coated silica gel 60 $GF_{254}$ plate 10 x 10 cm (Merck, Germany) using chloroform : ethyl acetate : methanol : formic acid (7:2:1:1) as a mobile phase. TLC plate was detected using Camag viewing box UV detector (Camag, Muttenz, Switzerland) and natural product-polyethylene glycol (NP-PEG) spraying reagent under UV 366 nm for detecting of flavonoids.

### *Total Flavonoid Content Determination*

Total flavonoid content was determined using the method adapted by Meda *et al.*[6] Briefly, 5 ml of 2% aluminium chloride ($AlCl_3$) in

methanol was mixed with the same volume of Siamese neem tree leaf extracts (0.02 mg/ml). Absorption readings at 415 nm were taken after 10 minutes against a blank sample without $AlCl_3$. The total flavonoid content was determined using a standard curve of quercetin (0.01-0.1 mg/ml). The mean of three readings was used and expressed as mg quercetin equivalent (QE)/100 g extract.

## RESULTS AND DISCUSSION

Yields (% w/w), extraction ratio (powder : water extract), free radical scavenging activity and total flavonoid content of the leaf aqueous extracts of Siamese neem tree collected from different locations in Thailand were shown in Table 1. Thin layer chromatogram of the leaf aqueous extract of Siamese neem tree showed a spot with Rf value equivalent to quercetin. Thus, total flavonoid content in all extracts was determined based on quercetin equivalent. The extracts exhibited antioxidant activity with $EC_{50}$ values from 28.85 to 173.77 µg/ml. According to Cervantes-Cervantes,[7] five extracts showed high antioxidant activity, while three samples showed moderate activity and the other four samples showed low activity. Average $EC_{50}$ value of Siamese neem tree leaf extracts from 12 locations is 78.12 µg/ml which is about 23 times more than $EC_{50}$ values of standards vitamin C and trolox ($EC_{50}$ = 3.43 and 3.38 µg/ml, respectively) and was considered as moderate antioxidant activity. There was a report of antioxidant activity tested by DPPH scavenging assay showed that tea leaves (*Camellia sinensis*) from Japan, Mynmar and Thailand exhibited very low $EC_{50}$ values (2.80, 2.94 and 3.07 µg/ml, respectively).[11] Moreover, DPPH scavenging activities of some Thai Medicinal plant extracts were reported with $EC_{50}$ values in a range from 1.11 µg/ml (*Polygonum odoratum* leaves) to 472.76 µg/ml (*Acacia pennata* leaves),[12] while green tea *(Camellia sinensis*) and safflower tea (*Carthamus tinctorius*) in Thailand showed $EC_{50}$ values of 7.05 and 129.83 µg/ml, respectively.[13]

Total flavonoid content of the extracts was in the range from 110.0 to 511.4 mg calculated as QE/100 g extract. The average total flavonoid content was 312.3 mg QE/100 g extract or 1,061 mg QE/1 kg dried leaves. There was a report of flavonoid content in edible plants[8] indicating that some edible plants in Malaysia contain total flavonoids in the range of 0 (not detectable) such as sweet potato shoot (*Ipomoea batatas*), winged bean (*Psophocarpus tetragonolobus*), sting bean (*Vigna sinensis*) and oyster mushroom (*Pleurotus sajor-caju*) to 2,720.5 mg/kg (dried weight) in onion leaves (*Allium fistulosum*). Moreover, Hertog *et al.*[9] studied the flavonoid content in 28 vegetables and 9 fruits and suggested that quercetin levels in the edible parts of most vegetables were generally below 10 mg/kg, while Howard *et al.*[10] reported that spinach contains high total flavonoid content which was 100 mg/kg. Therefore, Siamese neem leaves should be considered to contain moderate to quite-high total flavonoid content, calculated as quercetin equivalent.

Comparison of antioxidant activity and total flavonoid content of the extracts from different regions, each sample was significantly different ($p < 0.05$). Samples from the south showed antioxidant activity and total flavonoid content > the north > the east > the west > the north-east > the central. The extract from Songkhla sample showed the strongest antioxidant activity ($EC_{50}$, 28.85 µg/ml) and the highest total flavonoid content (511.4 mg QE/100 g extract). The wide ranges of the $EC_{50}$ values and flavonoid content of Siamese neem tree leaf extracts could be due to many factors including age of plant, locations, altitude, climate, temperature and variation of plant variety which might affect biosynthesis of plant flavonoids. From the present results, the extracts of samples from some locations, i.e. Ratchaburi, Chantaburi, Phetchabun, Nakhon Si Thammarat and Songkhla exhibited high antioxidant activity. However, the extracts of samples from Ratchaburi, Chantaburi and Nakhon Si Thammarat exhibited high antioxidant activity but moderate total flavonoid content. This suggested that other chemical components such as ascorbic acid, ß-carotene

**Table 1.** Yield, free radical scavenging activity and total flavonoid content of the leaf aqueous extracts of Siamese neem tree from different locations in Thailand

| Location | Region | Yield (% w/w) | Extraction ratio | DPPH method $EC_{50}$ (µg / ml) | Antioxidant activity[a] | Total flavonoid content | |
|---|---|---|---|---|---|---|---|
| | | | | | | mg QE / 100 g extract | mg QE / 100 g dried leaf |
| Ang Thong | Central | 27.71 ± 1.54 | 3.6 : 1 | 173.77 ± 4.74 | Low | 158.4 ± 12.1 | 43.90 ± 4.97 |
| Lop Buri | Central | 32.90 ± 0.98 | 3.0 : 1 | 129.36 ± 2.43 | Low | 110.0 ± 15.9 | 36.20 ± 1.09 |
| Nakhon Pathom | West | 20.98 ± 2.01 | 4.8 : 1 | 145.44 ± 8.24 | Low | 345.8 ± 12.6 | 72.54 ± 4.92 |
| Ratchaburi | West | 38.88 ± 1.23 | 2.6 : 1 | 31.41 ± 0.25 | High | 289.1 ± 5.31 | 112.4 ± 2.65 |
| Chantaburi | East | 33.52 ± 0.55 | 3.0 : 1 | 41.26 ± 0.55 | High | 283.5 ± 16.3 | 95.02 ± 6.36 |
| Prachin Buri | East | 40.12 ± 2.41 | 2.5 : 1 | 91.97 ± 5.11 | Moderate | 414.4 ± 3.37 | 166.3 ± 3.62 |
| Lampang | North | 38.41 ± 0.66 | 2.6 : 1 | 84.19 ± 1.06 | Moderate | 224.1 ± 14.5 | 86.08 ± 5.65 |
| Phetchabun | North | 48.15 ± 1.42 | 2.1 : 1 | 30.33 ± 2.47 | High | 493.2 ± 3.91 | 211.5 ± 3.92 |
| Maha Sarakham | North-east | 17.38 ± 1.16 | 5.7 : 1 | 81.13 ± 1.90 | Moderate | 477.3 ± 5.83 | 82.96 ± 2.27 |
| Roi Et | North-east | 25.11 ± 2.07 | 4.0 : 1 | 125.21 ± 0.83 | Low | 147.4 ± 3.71 | 37.02 ± 5.94 |
| Nakhorn Si Thammarat | South | 47.97 ± 0.78 | 2.1 : 1 | 37.80 ± 3.45 | High | 293.5 ± 8.56 | 140.8 ± 7.97 |
| Songkhla | South | 37.00 ± 0.96 | 2.7 : 1 | 28.85 ± 3.82 | High | 511.4 ± 6.85 | 189.2 ± 7.24 |
| **Average** | | **34.01 ± 9.77** | **3.2 : 1 (3-4 : 1)** | **78.12 ± 52.15** | **Moderate** | **312.3 ±138** | **106.1 ± 59.2** |
| Standard BHT | | | | 14.87 ± 3.66 | High | | |
| Standard vitamin C | | | | 3.43 ± 0.06 | High | | |
| Standard trolox | | | | 3.38 ± 0.02 | High | | |
| Standard quercetin | | | | 2.29 ± 0.06 | High | | |
| Standard rutin | | | | 34.67 ± 4.72 | High | | |

[a] from reference 7
BHT = butylated hydroxyl toluene, DPPH = 1,1-diphenyl-2-picrylhydrazyl, QE = quercetin equivalent

or other phenolic compounds in the extracts could also act as antioxidants. Consideration that Siamese neem tree could be consumed as a vegetable in a high quantity in each meal, it is interesting to study this plant in more details about the antioxidant activity and chemical components.

## CONCLUSION

The leaf aqueous extracts of Siamese neem tree contained some flavonoids and showed moderate-high free radical scavenging activity when tested by DPPH scavenging method. The samples collected from the south exhibited the highest antioxidant activity and total flavonoid content while those from the central area presented the lowest activity and total flavonoid content. The average $EC_{50}$ value of Siamese neem tree leaf extract showed a moderate level of free radical scavenging activity while average total flavonoid content was quite high comparing to previous reports of other plants. From the results, it is suggested that total flavonoid content in almost Siamese neem tree leaf extracts correlate to their antioxidant activities. The ranges of flavonoid content and antioxidant activity will be useful for standardization of Siamese neem tree leaf extracts for further pharmaceutical productions. Further works should include the separation and identification of active components and determination of antioxidant activities by other methods.

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