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Evaluation of antioxidant activities and HPTLC analysis of *Lysiphyllum strychnifolium* (Craib) A. Schmitz leaf extract

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Introduction

A balance between free radicals and antioxidants is necessary for proper physiological function. Free radicals are generated in living organisms by a variety of endogenous systems, and after exposure to different chemical and physical factors (e.g., UV radiation, pollutants, drugs, pesticides, food additives).[1] Oxidative stress leads to a disturbance in the balance of free radicals and antioxidant defenses in living organisms. The harmful action of free radicals can be blocked by antioxidant substances, which scavenge the free radicals and detoxify the organism. The reactive species are implicated in mediating various pathological processes in human including aging, asthma, atherosclerosis, diabetes, cancer, Parkinson's and Alzheimer's diseases.[2-3] Several plant extracts and different classes of phytochemicals have been shown to have antioxidant activity. The search for newer natural antioxidants, especially of plant origin, has been increasing ever since. Plants have been a constant source of drugs and, recently, much emphasis has been placed on finding new therapeutic agents from medicinal plants.

L. strychnifolium (Craib) A. Schmitz (Leguminosae) is commonly known as Khayan or Yanang Dang in Thai. Its climbing stem with tendrils is 5 m long. The leaves are simple, alternate, obovate shape 4-5 cm with 3-5 secondary nerves. The inflorescence is red color with the length of 15-100 cm. The calyx is 1 cm long in cup-shaped. Its ovary is pubescent with the length 0.7 cm. It used to be classified as a *Bauhinia* species, but molecular phylogenic analysis proves that it belongs to the genus *Lysiphyllium.*[4] Several chemical constituents have been reported such as quercetin, 3,5,7,3′,5′-pentahydroxy-flavanonol-3-O-α-L-rhamnopyranoside, 3,5,7-trihydroxychromone-3-O-α-L-rhamnopyranoside, β-sitosterol, stigmasterol and gallic acid. These compounds possess several biological activities such as antioxidant, anticancer and antidiabetic.[5]

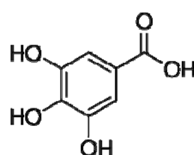

Figure 1. The chemical structure of Gallic acid

In Thai traditional medicine, *L. strychnifolium* is one of Thai longevity medicinal plant. It is widely used for detoxification of the body. The stem and root have been used to treat cancer, fever, alcoholic intoxication and allergy. Dried stems and leaves of *L. strychnifolium* are prepared as herbal tea in order to use for detoxification and promoting health. Brewing of its dried stems and leaves is used as health-promoting herbal tea and hydroalcoholic extracts are prescribed by Thai traditional doctors[6-7].

The aim of this study to evaluate its antioxidant potential including DPPH radical scavenging, ferric reducing power (FRAP) and ABTS radical scavenging assays. Total phenolic and total flavonoid contents were also determined.

Materials and Methods

Plant extraction

L. strychnifolium leaves were dried at 50°C, then ground into fine powder. Dried, powdered leaves (100 g) were extracted in boiling water (1 L) for 15 min three times then filtered. The filtrates were pooled and initially concentrated by rotary evaporator. The water extract was lyophilized, then stored in a sealed container at -20°C until further used.

DPPH free radical scavenging assay[8]

DPPH radical was freshly prepared in methanol at the final concentration of 152 µM. Each plant extract was diluted in methanol at varying concentrations. In 96-well plate, 100 µL of each extract was added to each well, followed by 100 µL of methanolic DPPH solution. Ascorbic acid was used as a positive standard and treated under the same condition as samples. The mixtures were allowed to stand at room temperature in the dark for 30 minutes. The absorbance was recorded at 517 nm using microplate reader (Infinite® M200, Tecan). Each experiment was performed in triplicate. The percentage of scavenging activity and half inhibitory concentration (IC_{50}) were calculated.

$$\% \text{ inhibition} = [(A_c - A_s)/A_c] \times 100$$

Whereas A_c was the absorbance of DPPH solution without plant extract, A_s was the absorbance of plant extract

ABTS free radical scavenging assay[9]

The stock solutions included 7 mM ABTS solution and 2.45 mM potassium persulfate solution. The working solution was prepared by mixing the two stock solutions in equal quantities and allowed them to react for 12-16 hours in the dark at room temperature. The solution was then diluted by mixing 1 mL ABTS solution with 25 mL of methanol to obtain an absorbance of 1.100 ± 0.020 units at 734 nm using a microplate reader. Fresh ABTS solution was prepared for each assay. 10 µL of samples were mixed with 200 µL of $ABTS^{\bullet+}$ radical cation solution in 96-well plates. Then the absorbance was taken at 734 nm after 6 minutes. All determinations were carried out in triplicate. Trolox was used for the standard curve. Results were expressed in mg Trolox equivalent antioxidant capacity (TEAC)/g extract.

Ferric reducing antioxidant power (FRAP) assay[10]

Sample solution (500 µL of 1 mg/mL) was mixed with 500 µL of potassium phosphate buffer (0.2 M, pH 6.6) and 500 µL of 1% w/v potassium ferricyanide solution. The mixture was incubated at 50 °C for 20 min. Then, 2 mL of trichloroacetic acid was added to stop the reaction. Then, 100 µL of supernatant was mixed with 100 µL of deionized water before the addition of 20 µL of 0.1% w/v ferric chloride solution. The procedure was carried out in triplicate and allowed to stand for 30 min before measuring the absorbance at 700 nm using microplate reader. Ferrous sulfate was used for the standard curve. The absorbance was expressed as mmol ferrous sulfate equivalent /g extract. Standard curve of six concentrations of $FeSO_4$ (0.0156, 0.03125, 0.0625, 0.125, 0.25, 0.5 mM) was prepared using distilled water as solvent. 500 µL of $FeSO_4$ solution, 100 µL of 1% w/v potassium ferricyanide, and 400 µL of deionized water were mixed together. Then, the absorbance of the mixtures was measured at 700 nm. The standard curve was obtained from plotting between concentrations of $FeSO_4$ and the absorbances.

Determination of total phenolic content

Total phenolic content was determined using Folin-Ciocalteu method with some modifications.[11] The extract was dissolved in methanol at varying concentrations. 20 μL of sample solution and 50 μL of

Folin-Ciocalteu's reagent (diluted 1:10 with deionized water) were mixed in 96-well plate for 3 minutes, then 80 μL of 75% w/v sodium carbonate was added. After incubation for 2 hours in the dark at room temperature, the absorbance was measured at 765 nm. The assay was performed in triplicate. Gallic acid was used for the standard curve. The total phenolic content in extract was expressed in terms of gallic acid equivalent per gram of extract (mg of GAE/g of extract).

Determination of total flavonoid content[12]

The assay for total flavonoid content was conducted by mixing 100 μL of plant extract in methanol (1 mg/mL) with 100 μL of 2% w/v aluminium chloride in methanol. The sample was incubated in 96-well plate for 10 minutes at room temperature. The absorbance was recorded using microplate reader at 415 nm. The analysis was done in triplicate and the mean value of absorbance was obtained. Standard solutions of quercetin was used to construct the calibration curve. The content of flavonoids in extract was expressed in term of quercetin equivalent per gram of extract (mg of QE/g of extract).

HPTLC analysis[13]

HPTLC chromatogram was performed on 20 x 10 cm aluminum plate coated with 0.2 mm layers of silica gel 60 F_{254}. The samples and standard were applied on the plates as 6 mm width bands, positioned 15 mm from lower edge of the plate using Linomat V applicator with a nitrogen flow providing delivery from the syringe at a speed of 150 nL/s. The mobile phase consisted of toluene-ethyl acetate-formic acid (5:4:1 v/v/v). The plates were developed with a distance of 80 mm from lower edge and scanned at wavelength of 254 nm with TLC scanner3. Slit dimension was 6.00 x 0.45 mm and scanning speed was 20 nm/s. These parameters were maintained for all quantitative analysis.

Results

Determination of anitioxidant activity, total phenolic content, and total flavonoid content

The yield of *L. strychnifolium* leaf extract from decoction method was 23.81 %w/w. The results of antioxidant assays were shown in Table 1. The IC_{50} value of extract determined by DPPH was 10.97 ± 0.52 μg/mL while ascorbic acid showed the IC_{50} value of 3.32 ± 0.01 μg/mL. The ABTS and FRAP assays showed the value of 559.11 ± 23.79 mg TEAC/g extract and 1.1568 ± 0.07 mmol $FeSO_4$/g extract, respectively. The contents of phenolic compounds and flavonoids were 197.82 ± 5.78 mg GAE/g extract and 32.22 ± 1.23 mg QE/g extract, respectively.

Table 1 Antioxidant activity, total phenolic and total flavonoid content of *L. strychnifolium* leaf extract.

Sample / Assay	Aqueous *L. strychnifolium* leaf extract	Ascorbic acid
Antioxidant activity*		
-DPPH (IC_{50} μg/mL)	10.97 ± 0.52	3.32 ± 0.01
-ABTS (mg TEAC/g extract)	559.11 ± 23.79	-
-FRAP (mmol $FeSO_4$/g extract)	1.16 ± 0.07	-
Total phenolic content* (mg GAE/g extract)	197.82 ± 5.78	-
Total flavonoid content* (mg QE/g extract)	32.22 ± 1.23	-

* Each value was expressed in terms of mean ± SD (n=3), TEAC= trolox equivalent antioxidant capacity, GAE = gallic acid equivalent , QE= quercetin equivalent

HPTLC analysis

The specificity of gallic acid in *L. strychnifolium* leaf extract (R_f = 0.41) was confirmed by overlaying UV spectra of gallic acid reference standard and sample (Figure 2A). Densitograms of gallic acid reference standard and *L. strychnifolium* leaf extract were shown in Figures 2B and 2C, respectively. The calibration curve of gallic acid appears to be linear over the range of 200 to 1,200 ng/spot. The correlation coefficient (r^2) value was over 0.997. The content of gallic acid in *L. strychnifolium* leaf extract was found to be 3.54% ± 0.08 %w/w.

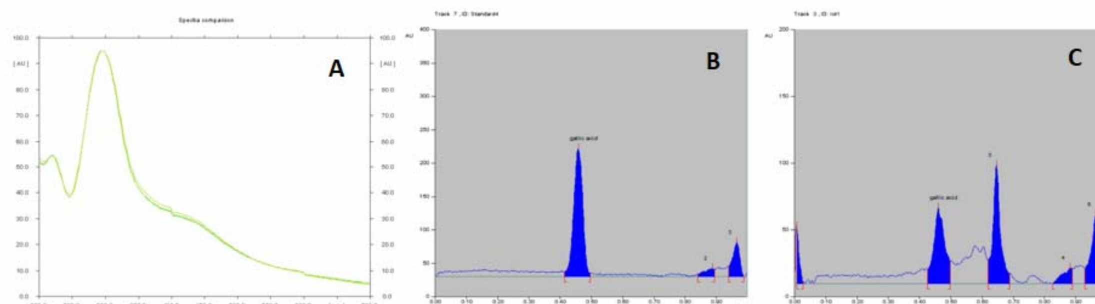

Figure 2. A) Overlaid UV spectra (scanning from 200 to 700 nm) of HPTLC of gallic acid reference standard and sample, B) Densitogram of gallic acid reference standard (R_f = 0.41), C) Densitogram of aqueous *L. Strychnifolium* leaf extract**.**

Discussion

The aqueous extract of *L. strychnifolium* leaves displayed strong antioxidant activities. The decoction method was environmentally benign, low cost and closely similar to its traditional way of use. From the results of HPTLC analysis, gallic acid appeared to be a major compound which could be responsible for its antioxidant effect. Structurally, gallic acid has phenolic groups that serve as a source of readily available hydrogen atoms such that the subsequent radicals produced can be delocalized over the phenolic structure.[14-15] The interest in these phenolic acids is due to its pharmacological activity as radical scavengers.[16-17] They been demonstrated to have preventive and therapeutic effects in many diseases.

Conclusion

L. strychnifolium leaf extract obviously contained high amount of total phenolic compound and exhibited potent antioxidant activity. The high antioxidant activity of *L. strychnifolium* leaf extract may relate to the amount of gallic acid in the extract. Therefore, *L. strychnifolium* leaf extract is a suitable natural antioxidant source which could be further applied in nutraceutical and pharmaceutical production. Further investigation of *in vivo* antioxidant activities and other pharmacological activities is needed.

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