Thai Journal of Pharmaceutical Sciences (TJPS)

34th International Annual Meeting in Pharmaceutical Sciences and
2nd CU FPhS - RIKEN CDB Symposium
(IAMPS34 and 2nd CU FPhS - RIKEN CDB)

Antioxidant Activity, Total Phenolic, Total Flavonoid Content and HPTLC Analysis of Morin in *Maclura cochinchinensis* Heartwood Extract

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Keywords: *Maclura cochinchinensis*, antioxidant activity, total phenolic content, total flavonoid content, HPTLC

Introduction

Reactive oxygen species (ROS) are undesirable byproducts of normal cell metabolism that is associated with cell signaling and homeostasis. ROS can be generated endogenously from the mitochondria during cell metabolism. Moreover, they are also produced when exposed to exogenous sources including pollutants, tobacco, toxic substances, drugs and radiation. Overproduction of ROS can potentially damage various cell components (proteins, lipids, and DNA) leading to oxidative stress and impaired cellular function. According to previous evidences, ROS have been implicated in the pathogenesis of various diseases such as aging, cancer, neurodegenerative, other diseases, etc.[1-2] Therefore, the balance of free radicals can help to prevent these diseases. Nowadays, many phytochemicals have been reported as having antioxidant capacity. Among such compounds, a large number of flavonoids can potentially react with wide range of free radicals and provide several health benefits.[3]

Maclura cochinchinensis (Lour.) Corner, a scrambling shrub of the family Moraceae. It is commonly called as Kae Lae in Thai. It is widely distributed in many countries in Asia such as China, Japan, Korea, Taiwan, India, and Thailand.[4-5] According to Thai traditional medicine, *M. cochinchinensis* heartwood is used for treating fever, diarrhea, fainting, abnormality of lymph node, skin infection and diabetes.[4-5] Moreover, *M. cochinchinensis* heartwood is a component of Thai herbal medicine formula for postnatal period included in the National list of essential drugs of Thailand. Many chemical constituents of the heartwood have been reported such as resveratrol, oxyresveratrol, β-sitosterol, quercetin-7-O-β-D-glucoside, kaempferol 3,7-di-O-β-glucopyranoside.[4, 6] Among these compounds, morin (Figure 1) was found predominantly in *M. cochinchinensis* heartwood.[4] Morin possesses several biological activities such as antioxidant[7-8] , anti-inflammatory[8] and antidiabetic.[9-11]

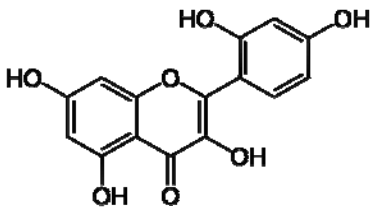

Figure 1. The chemical structure of morin (2′,3,4′,5,7-pentahydroxyflavone)

From previous studies, ethyl acetate and methanolic heartwood extracts of this plant showed anti-herpes simplex activity.[5] The aqueous bark extract showed antibacterial effect against *B. cereus* and *S. aureus*. DPPH radical scavenging activity of aqueous bark extract has been reported with IC_{50} value of 46.32 ± 0.21µg/mL.[12] However, antioxidant profile from the heartwood of *M. cochinchinensis* has not been examined. Therefore, the aim of the present study was to determine the antioxidant activities, total phenolic and total

flavonoid contents of *M. cochinchinensis* heartwood extract. The results obtained from this study will be useful for development of nutraceutical products.

Methods

Plant extraction

The heartwood of *M. cochinchinensis* was dried and ground into coarse powder. Dried heartwood powder (50 g) was boiled with distilled water (1000 mL) for 15 minutes. The extraction was performed three times. The pooled extract was filtered and spray dried. The extract was stored in tight container at 4^{o}C until use.

DPPH radical scavenging assay[13]

DPPH radical was freshly prepared in methanol at the final concentration of 152 µM. Plant extract was dissolved in methanol at varying concentrations. In 96-well plate, 100 µL of extracts were combined with 100 µL of methanolic DPPH solution. Ascorbic acid was used as a positive control and treated under the same condition as sample. The mixtures were allowed to sit at room temperature in the dark for 30 min. The absorbance was measured at 517 nm using microplate reader (Tecan, Switzerland). The experiment was conducted in triplicate. The percentage of scavenging activity was calculated as follows:

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

Where A_c was the absorbance of control solution at 517 nm and A_s was the absorbance of sample solution at 517 nm. The IC_{50} value was expressed as mean ± SD (n=3).

ABTS assay[14]

The stock solutions were 7.4 mM ABTS solution and 2.6 mM potassium persulfate solution. The working solution was prepared by mixing equal volumes of stock solution and allowed to react for 12-16 hours at room temperature in the dark. After incubation, $ABTS^{\bullet+}$ radical solution was prepared freshly by diluting 1 mL of working solution with 25 mL of methanol to obtain an absorbance of 1.1000 ± 0.0200 units. In 96-well plate, 10 µL of sample or Trolox were mixed with 200 µL of $ABTS^{\bullet+}$ radical solution. After 6 min incubation, the absorbance was taken at 734 nm by microplate reader. The standard regression equation was plotted between the absorbance and the concentration of Trolox at various concentrations. Antioxidant capacity was calculated and expressed as gram Trolox equivalent antioxidant capacity per gram of extract (g TEAC/g extract). All determinations were carried out in triplicate.

Ferric reducing antioxidant power (FRAP) assay[15]

Five hundred microliters of each solution including sample, 0.2 M sodium phosphate buffer (pH 6.6), and 1% potassium ferricyanide solution were mixed before incubation at 50^{o}C for 20 min. Then, 2 mL of 10% trichloroacetic acid was added to the mixture. Five hundred microliters of supernatant was removed, followed by mixing with 500 µL of deionized water and 100 µL of 0.1% (w/v) ferric chloride solution, respectively. Two hundred microliters were transferred to 96-well plate before measuring the absorbance at 700 nm. The assay was done in triplicate. The FRAP value was recorded as mmol $FeSO_4$ equivalent per gram of extract (mmol $FeSO_4$/g extract) based on the standard curve.

*Determination of total phenolic content (*Folin-Ciocalteu method*)*[15]

The plant extract was dissolved in 50% methanol to achieve 0.1 mg/mL. Twenty microliters of sample solution were mixed with 50 µL of 10% Folin-Ciocalteu's reagent (diluted 1:10 with deionized water). The reaction mixtures were allowed to react at room temperature for 3 min then added 80 µL of 7.5% w/v of sodium carbonate solution. After incubation in the dark at room temperature for 2 hours, the absorbance was measured at 765 nm by microplate reader. Sample was replaced by various concentrations of gallic acid to create a standard curve. The assay was performed in triplicate. Total phenolic content was expressed as grams of gallic acid equivalent per gram of extract (g GAE/g extract).

Determination of total flavonoid content[13]

The mixture consisting of 100 µL of plant extract in methanol (50 µg/mL) and 100 µL of 2% w/v aluminium chloride in methanol was incubated for 10 min. The absorbance was read at 415 nm against blank (test sample without aluminium chloride) using microplate reader. Each solution was analyzed in triplicate to

obtain the mean absorbance values. Standard quercetin was diluted serially to the range of 6.25 to 100 µg/mL and treated in the same procedure as sample to create a standard curve. Total flavonoid content was calculated against the quercetin calibration curve and expressed in terms of grams of quercetin equivalent per gram of extract (g QE/g extract).

HPTLC analysis

The HPTLC separation was performed on silica gel HPTLC plates F_{254} (20x10 cm with 0.2 mm thickness; Merck, Germany). Samples and standard solution were spotted in the form of bands of 7 mm long, 10 mm from the bottom edge of plate with constant application rate (100 nL/s) by Linomat 5 automatic sample applicator (CAMAG, Switzerland). Different volumes (1.5-3.5 µL) of morin standard solutions (200 µg/mL) corresponding to morin 300-700 ng/band were applied on the HPTLC plate. The plate was developed in CAMAG twin trough chamber, presaturated with a mobile phase consisting of toluene: ethyl acetate: formic acid (36: 12: 7, v/v/v) at room temperature. The plate was run to the distance of 80 mm, and scanned at 410 nm using TLC scanner 3 (CAMAG, Switzerland). The Rf values of morin reference standard and sample were determined. The results were analyzed by WinCATs software. The regression equation of morin was obtained from plotting between the peak areas and the amount of morin. Morin content in the *M. cochinchinensis* heartwood extract was calculated and expressed in terms of %w/w.

Results

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

The yield of *M. cochinchinensis* heartwood extract from decoction method was 18.59 ± 0.97 %w/w. The results from various antioxidant assays were shown in Table 1. The IC_{50} value of the extract determined by DPPH was 5.07 ± 0.29 µg/mL while ascorbic acid showed the IC_{50} value of 3.28 ± 0.03 µg/mL. From the ABTS and FRAP assay, *M. cochinchinensis* heartwood extract expressed the values of 1.27 ± 0.01 g TEAC/g extract, and 7.60 ± 0.93 mmol $FeSO_4$/g extract, respectively. The amounts of phenolic and flavonoid compounds were 1.90 ± 0.01 g GAE/g extract and 0.34 ± 0.01 g QE/g extract, respectively.

Table 1. antioxidant activity, total phenolic content, and total flavonoid content of aqueous *M. cochinchinensis* heartwood extract.

Sample / Assay	Aqueous *M. cochinchinensis* heartwood extract	Ascorbic acid
Antioxidant activity*		
- DPPH (IC_{50}, µg/mL)	5.07 ± 0.29	3.28 ± 0.03
- ABTS (g TEAC/g extract)	1.27 ± 0.01	-
- FRAP (mmol $FeSO_4$/g extract)	7.60 ± 0.93	-
Total phenolic content* (g GAE/g extract)	1.90 ± 0.01	-
Total flavonoid content* (g QE/g extract)	0.34 ± 0.01	-

*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 morin in *M. cochinchinensis* heartwood extract was confirmed by overlaying UV spectra between morin reference standard and sample (Figure 2A). Densitograms of morin reference standard and aqueous *M. cochinchinensis* heartwood extract were shown in Figures 2B and 2C, respectively. The calibration curve of morin appears to be linear over the range of 300 to 700 ng/spot. The correlation coefficient (r^2) value was over 0.995. The content of morin in aqueous *M. cochinchinensis* heartwood extract was found to be 12.79 ± 0.21 %w/w.

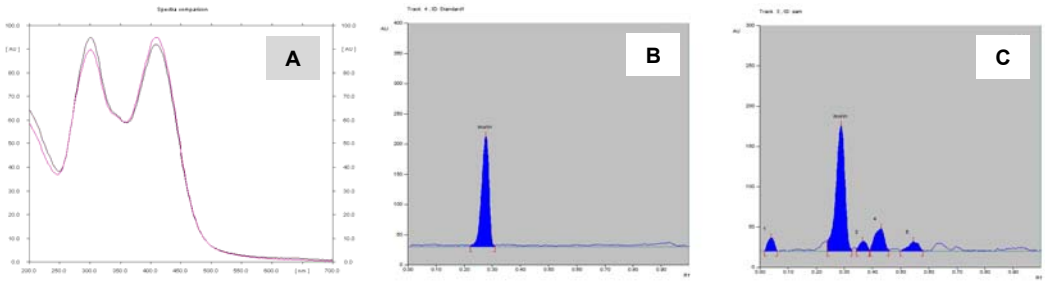

Figure 2. A) overlay UV spectra (from 200 to 700 nm) of morin reference standard and sample, B) densitogram of morin reference standard (R_f = 0.29 ± 0.01), C) Densitogram of aqueous *M. cochinchinensis* heartwood extract.

Discussion

According to *in vitro* antioxidant assays, aqueous *M. cochinchinensis* heartwood extract displayed strong antioxidant activities. Morin, a major compound in *M. cochinchinensis* heartwood extract, could be responsible for its antioxidant effect.[7] There were some studies indicating the antioxidant effects of morin.[3, 18] The presence of hydroxyl groups at positions 3, 2′, 4′, and double bond at position C2-C3 in morin molecule were related to its potent antioxidant activities.[3, 18] The results of antioxidant activities obtained from the present study support traditional uses of *M. cochinchinensis* and may provide essential information for the utilization of this plant as a source of low-cost natural antioxidants or nutraceutical products.

Conclusion

The present study reported the evaluation of *in vitro* antioxidant activities of *M. cochinchinensis* heartwood extract. The extract exhibited strong antioxidant activities in all tested assays. HPTLC analysis showed the presence of morin which is the major active compound. Therefore, *M. cochinchinensis* heartwood extract has the potential to be developed as antioxidant nutraceutical products. Determination of related biological activities and toxicity test on the extract should be conducted in the future.

Acknowledgements

The authors would like to thank Faculty of Pharmacy, Mahidol University for supporting necessary equipment and facilities.

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