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Biological Activities and Stability of a Standardized Pentacyclic Triterpene Enriched *Centella asiatica* Extract

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Abstract – Pentacyclic triterpenes, mainly, asiatic acid, madecassic acid, asiaticoside, and madecassoside are the active constituents of *Centella asiatica*. A pentacyclic triterpene enriched *C. asiatica* extract (PRE) was prepared and standardized to contain a total pentacyclic triterpenes not less than 65% w/w. This work was focused on determination of antiinflammatory, antioxidant, and tyrosinase inhibitory activities of PRE and its stability. The PRE exhibited a satisfactory nitric oxide inhibitory effect, with an IC_{50} value of 64.6 μg/mL. In addition, the PRE inhibited tyrosinase enzyme activity with an IC_{50} value of 104.8 μg/mL. In contrast, the PRE possessed only weak antioxidant activity. The PRE was stable over a period of four months when stored as a dried powder but only in a well-closed container protected from light at 4 ºC. An aqueous alcoholic solution of the PRE was stable at pH values of 5.8 and 7.0, but was not stable at a pH of 8.2. Preparations of the PRE in an aqueous solution should be performed in acidic or neutral conditions.
Keywords – *Centella asiatica*, Pentacyclic triterpenes, Antiinflammatory, Antioxidant, Tyrosinase inhibition, Stability

Introduction

Centella asiatica is one of a well known medicinal plant in Thailand and other Asian countries that possess a wide range of pharmacological properties. Pentacyclic triterpenes, mainly, asiatic acid, madecassic acid, asiaticoside, and madecassoside (Fig. 1) are the active constituents with various pharmacological properties that include wound healing,[1,2] antiinflammatory,[3,4] anti-ulcer,[5] antibacterial,[6] and anticancer[7] effects.

The method for preparation of the pentacyclic triterpenene enriched *C. asiatica* extract (PRE) has been previously described. In a recent study it was shown that a standardized PRE that contained total pentacyclic triterpene content of not less than 65% w/w possessed satisfactory antibacterial activity against *Streptococcus* spp., with a potency stronger than asiaticoside and madecassoside, and almost equal to that of madecassic acid.[6] In this study, the antiinflammatory, antioxidant, and tyrosinase inhibitory activities of the PRE and the four pentacyclic triterpenes, asiatic acid, madecassic acid, asiaticoside, and madecassoside were evaluated. The stability of the PRE was also studied in order to obtain useful information for future studies on the development of herbal medicines from the extract.

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Experimental

Plant material – Aerial parts of *C. asiatica* were collected from Nakornsrithammarat province, Thailand. A voucher specimen (specimen no. SKP 199 03 01 01) was authenticated by Associate Professor Pharkphoom Panichayupakaranant, and has been deposited at the herbarium of the Faculty of Pharmaceutical Sciences, Prince of Songkla University, Thailand. The plant material was washed and dried at 50 ºC for 24 h in a hot air oven, and reduced to powder using a grinder and a sieve no. 45.

Preparation of PRE – The PRE was prepared by the method as previously described.[6] Briefly, the dried powder of *C. asiatica* was extracted by microwave assisted

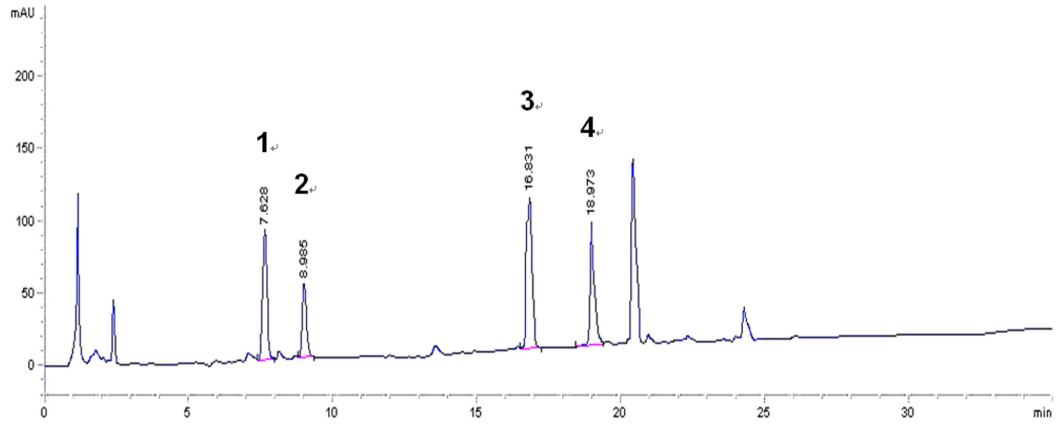


Fig. 1. HPLC chromatogram of the standardized PRE. Madecassoside (1); asiaticoside (2); madecassic acid (3); asiatic acid (4).

extraction (MAE). The optimal conditions for the MAE were: extraction with absolute ethanol, a microwave irradiation power of 600 W, at 75 ºC, with four irradiation cycles, and four extraction times. The pooled extracts were then dried *in vacuo* and dissolved in 25% v/v ethanol. After filtering through cotton wool, the solution was loaded into a macroporous resin (Diaion® HP-20) column, and eluted with 25% v/v ethanol. The residue from the filtering step was dissolved in 50% v/v ethanol, and the solution was then loaded into the same column, and eluted with 50% v/v ethanol. The process was repeated by altering the eluent as follows; 75% v/v ethanol, ethanol, and ethyl acetate, respectively. The obtained pentacyclic triterpenes enriched fractions were pooled and evaporated to dryness *in vaccuo* and then subjected to decolorization using activated charcoal. The extract was then evaporated to dryness *in vaccuo* to obtain the PRE.

HPLC quantitative analysis of the pentacyclic triterpenes – A 5 mg sample of PRE was accurately weighed and dissolved in methanol, and the volume adjusted to 5 mL. This solution was filtered through a 0.45 µm membrane filter and the pentacyclic triterpenes content was determined by the standard HPLC method previously described.[6]

Assay for NO inhibitory effect using RAW264.7 cells – The inhibitory effect on NO production by murine macrophage-like RAW264.7 cells was evaluated using the method previously described.[8]

DPPH radical scavenging assay – The 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity was evaluated using the method previously described.[9]

Assay for non-enzymatic lipid peroxidation in liposomes – Assay for non-enzymatic lipid peroxidation in liposomes was carried out using the method described by Burits and Bucar (2000).[10]

***In vitro* assay for tyrosinase inhibition** – Tyrosinase inhibitory activity was evaluated using the method described by Rangkadilok et al. (2007).[11]

Stability evaluation – Stability evaluations, including effect of light, temperature, pH, and accelerated conditions on the stability of the PRE were performed using the method previously described.[12] An aliquot of each sample was taken at 0, 1, 2, 4, 8, 12, and 16 weeks and subjected to quantitative analysis of the four pentacyclic triterpenes using HPLC. The experiments were performed in triplicate.

Statistics – Values were expressed as means ± S.D. Statistical significance ($p < 0.01$) was calculated by one-way analysis of variance (ANOVA), followed by Tukey's test.

Result and Discussion

The PRE used in this study was standardized by the HPLC method to obtain a total pentacyclic triterpene content of not less than 65% w/w. The content of asiatic acid, madecassic acid, asiaticoside, and madecassoside in the PRE were 10.1, 16.7, 17.4, and 22.0% w/w, respectively (Fig. 1). The total pentacyclic triterpene content of the PRE was 66.2% w/w. Evaluation of the antiinflammatory activity of PRE and the four pentacyclic triterpenes *via* its inhibitory effect against nitric oxide (NO) production by RAW264.7 cells showed that PRE possessed satisfactory antiinflammatory activity with an IC_{50} value of 64.6 µg/mL. The antiinflammatory activity of PRE was higher than those for asiaticoside, madecassoside and madecassic acid separately, but lower than that for asiatic acid (Table 1). Asiatic acid was therefore the major active compound that contributed to the inhibitory effect of PRE on NO production. In addition, it has been previously reported that asiatic acid and madecassic acid were more potent

Table 1. Biological activities of the PRE and four pure pentacyclic triterpenes from *C. asiatica*

Samples	IC_{50} μg/mL (μM)			
	NO inhibition	DPPH	Lipid peroxidation	Tyrosinase inhibition
PRE	64.6	348.3	n.a.[#]	104.8
Asiatic acid	12.4 (25.4)	n.a.[*]	n.a.[#]	n.a.[#]
Madecassic acid	n.a.[+]	n.a.[*]	n.a.[#]	n.a.[#]
Asiaticoside	n.a.[+]	n.a.[*]	n.a.[#]	n.a.[#]
madecassoside	n.a.[+]	n.a.[*]	n.a.[#]	n.a.[#]
Aspirin	12.9 (71.7)	–	–	–
CAPE	1.6 (5.6)	–	–	–
BHT	–	60.9 (276.3)	–	–
Qurcetin	–	1.7 (5.6)	1.7 (5.6)	–
Kojic acid	–	–	–	5.0 (35.2)

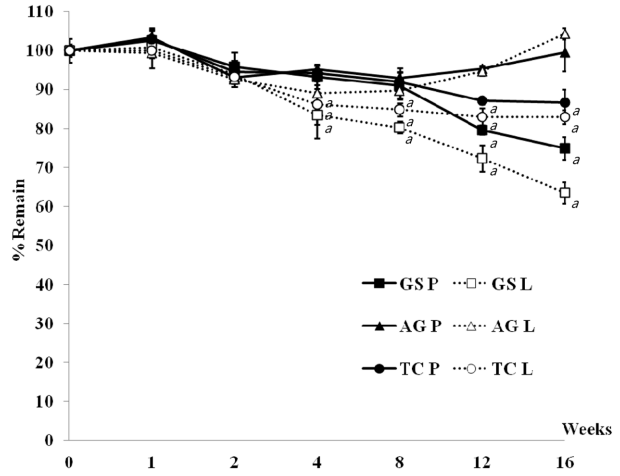
– = not performed
n.a.[+] = not active at concentration of 100 μg/mL
n.a.[*] = not active at concentration of 400 μg/mL
n.a.[#] = not active at concentration of 1000 μg/mL

inhibitors of LPS-induced NO than asiaticoside and madecassoside. The antiinflammatory properties of asiatic acid and madecassic acid might result from the inhibition of iNOS, COX-2, IL-6, IL-1 beta, and TNF-alpha expressions through the down-regulation of NF-kappa B activation in RAW 264.7 cells.[3,4]

Determination of the antioxidant activity of the PRE and the four pentacyclic triterpenes using the DPPH radical scavenging assay showed that PRE possessed weak antioxidant activity, with an IC_{50} value 348.29 μg/mL. In contrast, none of the pure pentacyclic triterpenes showed antioxidant activity at concentrations of up to 400 μg/mL (Table 1). Moreover, neither PRE nor the pure pentacyclic triterpenes showed antioxidant activity *via* the assay for non-enzymatic lipid peroxidation in liposomes. However, it has been reported that an aqueous extract of *C. asiatica* leaves did possess satisfactory antioxidant activity, with an IC_{50} value of 31.25 μg/mL.[13] This shows that *C. asiatica* pentacyclic triterpenes do not have significant antioxidant activity. It is possible that the antioxidant activity of the aqueous extract of *C. asiatica* was due to the presence of phenolic compounds or flavonoids, including gallic acid, naringin, chlorogenic acid, catechin, rutin, rosmarinic acid and quercetin.[14] Also, two flavonoids from *C. asiatica* named castilliferol and castillicetin did exhibit good antioxidant activity (*via* DPPH radical scavenging assay) with IC_{50} values of 23.10 and 13.31 μg/mL, respectively.[15]

Evaluation of the tyrosinase inhibitory activity of PRE and the four pentacyclic triterpenes showed that PRE exhibited tyrosinase inhibitory activity, with an IC_{50} value of 104.8 μg/mL. In contrast, neither of the pure pentacyclic triterpenes showed tyrosinase inhibitory activity at concentrations of up to 1000 μg/mL (Table 1). This implies that *C. asiatica* pentacyclic triterpenes do not have tyrosinase inhibitory activity so other compounds in PRE must be involved. The tyrosinase inhibitory activity of PRE should be contributed by flavonols, e.g. quercetin.[16] The result from this study supports a conclusion that the chemical complexity of the extract provides a wider range of pharmacological activities than any of the isolated pure compounds.

The extracts were kept in well-closed containers and stored either under fluorescent light or protected from light, at room temperature for a period of four months. When the PRE was kept exposed to light it changed its color from off-white to pale yellow, while the PRE kept in the dark in well closed containers did not change. The total pentacyclic triterpene content of the PRE that was kept exposed to light decreased significantly by 14% compared to a loss of 6% in the dark after four weeks of storage, while after 16 weeks of storage in the light the loss was 17% while in the dark it was only 13% (Fig. 2). The results also demonstrated that the aglycone form of the pentacyclic triterpenes was more stable than the glycoside form. The content of the aglycone (asiatic acid and madecassic acid) in the PRE kept either exposed to light or in the dark did not decrease through the period of four months. Instability of the PRE in both conditions was therefore due to decomposition of the glycosides (asiaticoside and madecassoside). These results indicated that the PRE should be kept in a well-closed container,

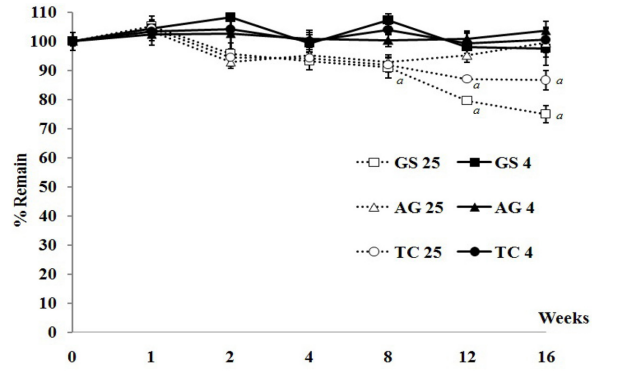


a = Significance at $p < 0.01$ compared with the content at initial time.

GS = Glycoside form; AG = Aglycone form; TC = Total content of pentacyclic triterpenes

P = Protected from light; L = Exposed to light

Fig. 2. Effect of light on the stability of the PRE.

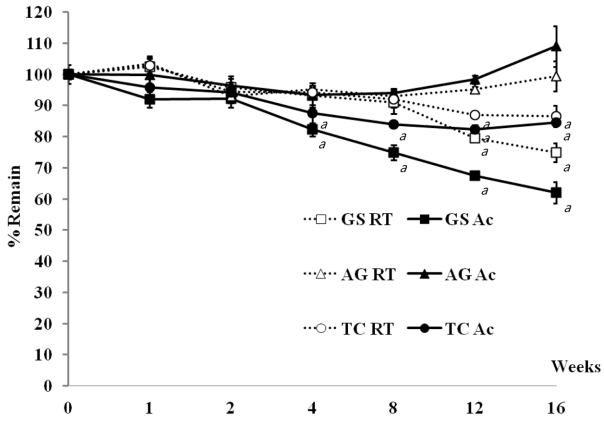


a = Significance at $p < 0.01$ compared with the content at initial time.

GS = Glycoside form; AG = Aglycone form; TC = Total content of pentacyclic triterpenes

25 = at 25°C; 4 = at 4°C

Fig. 3. Effect of temperature on the stability of the PRE.

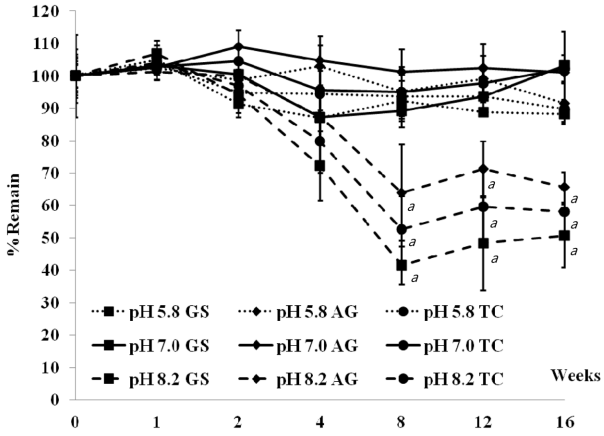


a = Significance at $p < 0.01$ compared with the content at initial time.

GS = Glycoside form; AG = Aglycone form; TC = Total content of pentacyclic triterpenes

RT = Room temperature; Ac = Accelerted conditions

Fig. 4. Effect of accelerated conditions on the stability of PRE.



a = Significance at $p < 0.01$ compared with the content at initial time.

GS = Glycoside form; AG = Aglycone form; TC = Total content of pentacyclic triterpenes

Fig. 5. Effect of pH on the stability of PRE.

protected from light.

The effect of temperature on the stability of the PRE was examined under two temperatures, 4 ºC and 25 ºC, both protected from light. The physical appearance of the extracts at both tested temperatures did not change through the four-month period. However, the total pentacyclic triterpene content of the PRE was stable over a period of four months only when stored in a well-closed container protected from light at 4 ± 2 ºC. (Fig. 3). The PRE should therefore be kept in a well-closed container, protected from light at 4 ± 2 ºC.

The accelerated stability test of the PRE was carried out using a stability chamber at 45 ºC and 75% RH. The result indicated that the physical appearances of the extracts did not change even stored under accelerated conditions over a period of four months. Unfortunately, the total content of pentacyclic triterpenes was significantly decreased from 67.28 to 56.85% w/w after four weeks of storage (Fig. 4). This instability of the PRE in the accelerated conditions was most likely due to decomposition of the glycosides (asiaticoside and madecassoside) at the higher temperature.

The evaluation of the acid-base stability of the PRE in the solution was determined at the three different pH values of 5.8,7.0, and 8.2. The total pentacyclic triterpene content of the PRE in the solution at pH 5.8, pH 7.0 did not significantly decrease through the period of four

months (Fig. 5). In contrast, the total pentacyclic triterpene content of the PRE in the solution at pH 8.2 did significantly decrease after 8 weeks of storage. This indicates that the pentacyclic triterpenes are not stable under aqueous alcoholic alkaline conditions. Thus, preparation of the PRE in an aqueous solution should be performed in acidic or neutral conditions.

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