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# DEVELOPMENT OF A NEW ANALYTICAL METHOD FOR DETERMINATION OF ASIATICOSIDE CONTENT IN *CENTELLA ASIATICA*

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**KEYWORDS:** *Centella asiatica* L., Apiaceae, Asiaticoside, TLC-Densitometry, 2-naphthol

## INTRODUCTION

Asiaticoside is a major active compound in *Centella asiatica* L. (Apiaceae) [1,2]. It has many pharmacological activities, such as wound healing[3], anti-inflamatory, memory enhancing, immunomodulatory activity, etc.[1,4,5] It is an ursane-type triterpene glycoside which has weak UV absorption (≤200 nm) due to lacking of a chromophore in its structure.[4,5](Figure1) Various analytical methods have been developed for determination of asiaticoside content in raw materials or finished products.[6,7,8] Presently, HPLC is the only standard analytical method used for the analysis but it is not so efficient owing to the weak UV absorption of asiaticoside.

In this study, derivatization of asiaticoside with 2-naphthol directly on TLC silica gel plate was developed for detection and determination of the triterpenoid glycosides in *C. asiatica* crude extracts using the UV–visible range. The developed densitometric TLC appeared to be simple, accurate, precise and fast. Eighteen samples could be performed simultaneously in a single run per plate within 15 minutes.

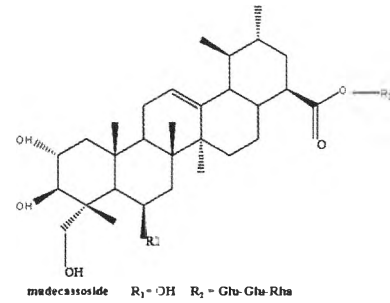


**Figure1**: The structures of triterpenoics in *C. asiatica*

## MATERIALS AND METHODS

### Plant Material

Samples of *C. asiatica* with commercial maturity (2-4 months) was purchased from a garden in Nonthaburi province.

### Preparation of sample Solutions

Crude extracts of *C. asiatica* were prepared from the plant materials under reflux with 80% methanol and partially purified using liquid-liquid extraction by dichloromethane and butanol, precipitated by ethyl acetate to powder. A sample solution (1 mg/ml) was prepared by dissolving asiaticoside (10 mg) in methanol (1ml) then pipetting 0.1 ml and diluted to 1 ml with methanol.

### Preparation of Standard Solutions

Asiaticoside standard was prepared from the aerial part of *C. asiatica* . In brief, the dried powdered leaves of *C. asiatica* (600g) were sonicated with 80% ethanol. The compounds were extracted by liquid-liquid extraction using dichloromethane and butanol. Ethyl acetate was added into butanol part to salting out the active compounds.Then , recrystallization of the precipitate using ethanol to give pure asiaticoside. Identification and purity of the compound was performed using a silica TLC plate and solvent system of chloroform : methanol : DI water (30:15:1.2) and HPLC using the conditions: C-18 column (300x4.60 mm); 20 ul injection of sample solution; isocratic elution phosphate buffer: acetonitril (8: 2); flow rate 0.1 ml/min; retention time of asiaticoside 2 min.

A standard solution with an accurate concentration of 1 mg/ml was prepared by dissolving 10mg asiaticoside in 1 ml methanol, then pipetting 0.1 ml to mix with 0.9 ml methanol. From this solution, various standard solutions (0.50, 0.45, 0.40, 0.35, 0.30, 0.25, 0.20, 0.15, 0.10 and 0.05 mg/ml) were prepared by the same method of dilution.

### TLC-Densitometric analysis

A CAMAG TLC system (Linomat, Switzerland) equipped with an automatic TLC sampler, a TLC scanner and a CATS software, was used. The crude extract of *C. asiatica* samples and asiatocoside standard solutions were spotted onto a pre-coated siliga gel (Siliga gel 60 F 254, 0.25 mm thickness). The TLC plate was then developed using the solvent system of chloroform : methanol : DI water (30:15:1.2) with 9 cm. height of solvent front. The plate was dried, dipped into 2-naphthol sulfuric acid reagent[9] then

heated with TLC plate heater at 120°C for 5 minutes for complete derivatization. After that, the TLC plate was scanned using the wavelength of 530nm. (Figure2,3)

**Method Validation**

The TLC densitometric analytical method was validated with respect to linearity, accuracy and precision.

Linearity was determined over the range of 100-1000 ng/spot. Standard asiaticoside solutions with varied concentrations were loaded (2.0 ul each) on to the TLC plate to give spots containing asiaticoside from 100 to 1000 ng/spot. A plot of average area under curve (AUC) versus concentration (ng/spot) was obtainedas standard curve. Linearity was expressed as the correlation coefficient ($r^2$). Accuracy was determined using the addition method. In practice, two samples of asiaticoside solutions were loaded (2.0 ul each) on to the TLC plate, followed by additional loading of 1 ul 100ug/ml. standard asiaticoside solutions on to the sample spot to give the spots containing additional asiaticoside (100ng/spot). After running, the AUC values were used to calculate the asiaticoside content and % recovery.

Precision was evaluated in terms of repeatability and reproducibility, each expressed as the standard deviation (SD) and the percent coefficient of variation (% CV) values. The sample solution was applied as a band onto the TLC plate. The AUC was recorded and asiaticoside content was determined. Data for the study of repeatability were obtained from 9 separate bands on the same plate, whereas those for reproducibility study were from two 2 plates (9 bands each) taken on 3 different days. The SD and % CV values were calculated for each experiment.

**Comparison with HPLC**

Comparison with HPLC was carried out by determining the asiaticoside content in unknown sample solutions using both HPLC technique and newly developed method then compare the results from both techniques. Two samples of unknown concentration asiaticoside solutions coding E1 and G1 were prepared and determine the concentration by HPLC technique. Chromatographic separation was performed with a reversed phase C18 as shown before. After that, repeat the determination again using TLC- densitometric method as previous then calculated the asiaticoside content and compared the result with the results taken by HPLC methods.

**Simultaneous determination of Asiaticoside and Madecassoside**

Comparison of asiaticoside and madecassoside was carried out by ploting the calibration curve of the two compounds. Six concentrations of standard madecassoside 0.50, 0.40, 0.30, 0.20, 0.10 and 0.05mg/ml of the two compounds were prepared and loaded (2.0 ul each) on to the TLC plate to give spots containing asiaticoside and madecassoside (100, 200, 400, 600, 800 and 1000 ng/spot). A plot of average area under curve (AUC) versus concentration (ng/spot) was obtained.

**Sample preparation of *C. asiatica* Products for TLC analysis**

Three brands of *C. asiatica* capsules, Four brands of *C. asiatica* infusions were purchased from drugstores and 1 sample of dry aerial part of *C. asiatica* was collected from its natural habitat. Crude extracts of these products and plant natural of *C. asiatica* were prepared from 500mg of the plant materials with 10ml. of 80% methanol at 60C for 15 mins under sonication. The extracts were centrifused and the supernatant part was analysed. Loaded 3.0 ul each on to the TLC plate and determine for the asiaticoside content.

## RESULTS

**Method validation**

The developed TLC-densitometric system showed its linear calibration curve of asiaticoside in the concentration range of 100-1000 ng/ spot. The graph was characterized by the equation: $y = 9.7171\ x + 916.42$ (r 2 = 0.9984), where $y$ is AUC and $x$ is concentration of asiaticoside (ng/spot).In term of accuracy, this was indicated by the percentage recovery values which was found to be in range of 98.18-104.3% .The precision was expressed as the % CV, which was 1.15- 1.9 %.

**Comparison with HPLC**

The results of asiaticoside content in two unknown concentration asiaticoside solution,E1 and G1, using the developed TLC-densitometric method were in range of 98.572%-101.264% which is comparable with the results from HPLC which is the gold standard technique.

**Simultaneous determination of Asiaticoside and Madecassoside**

The calibration curves of asiaticoside and madecasssoside showed the same pattern in the range 100ng-1000ng with $r^2 \geq 0.99$ (asiaticoside = 0.9931, madecassoside = 0.9907). (Figure 4) These results suggested the possibility of using the TLC densitometric method to determine both compounds at the same time in *C. asiatica*.

**Analysis of asiaticoside in commercial products**

This newly TLC densitometric method was then used to evaluate the quality of *C. asiatica* product samples in various brand and dosage form, and the results are shown in table 1. It can be seen that the commercial product samples contained a wide range of asiaticoside content(0.16%-0.60% w/w) which lower than the sample from the natural source (0.68% w/w). Apaiphubeth's product has the lowest asiaticoside content in both dosage forms (0.21% and 0.17%w/w).

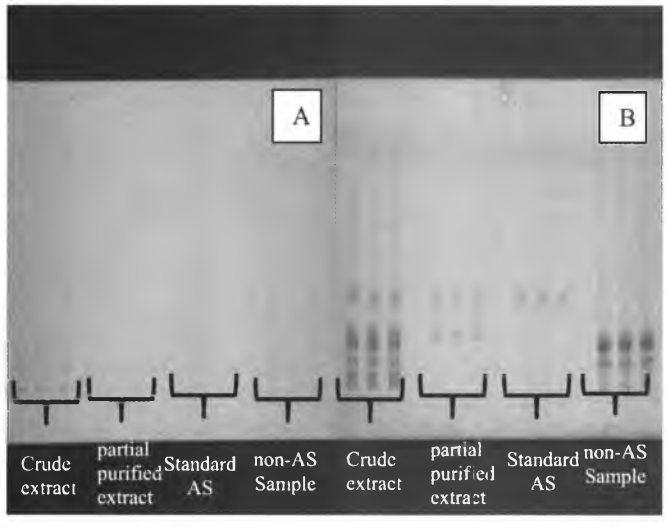


**Figure 2**: TLC-patterns of some *C. asiatica* crude extracts observed under visible light before (A) and after (B) derivatization with 2-naphthol acid reagent.

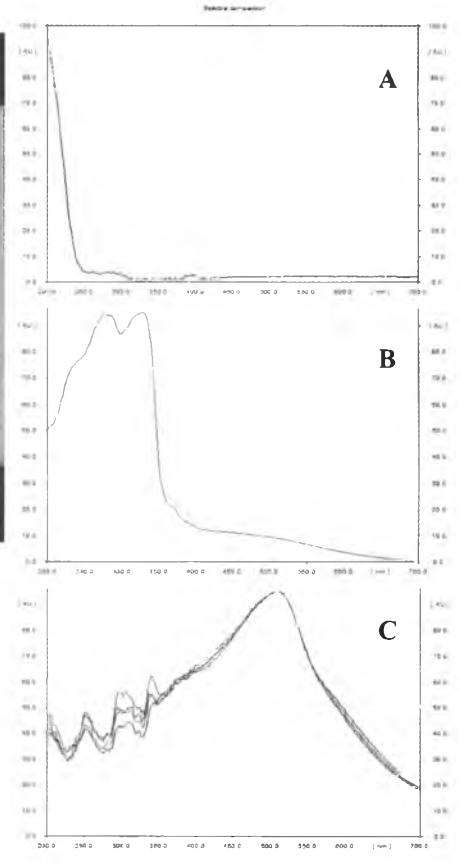


**Figure 3** (right): Absorption spectra obtained from TLC -densitometric scan of asiaticoside (A), 2-naphthol (B) and derivative asiatocoside after treated with 2-naphthol acid reagent (C).

| Product brand | Dosage form | Averrage AS content %w/w |
|---|---|---|
| Uthaiprasit | Capsules | 0.37% |
| Aphaiphubeth | Capsules | 0.21% |
| Thanyaporn | Capsules | 0.33% |
| Pathom asok | Infusions | 0.45% |
| Thanyaporn | Infusions | 0.60% |
| Aphaiphubeth | Infusions | 0.17% |
| Lampang-RuK | Infusions | 0.60% |
| Dry leave | | 0.68% |

**Table 1**: the asiaticoside content in commercial products

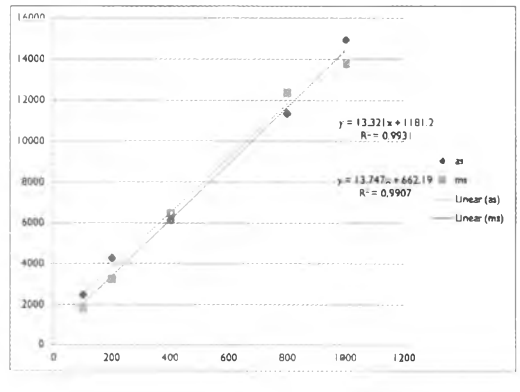


**Figure 4**: The comparison between the calibration curves of asiaticoside and madecassoside derivatized with the 2-naphtholsulfuric acid by AUC of absorbance measurement at 530 nm

## DISCUSSION

We have shown here a simple, accurate, method of TLC- densitometric analysis of asiaticoside. This newly developed method consists of 2 steps. First, asisticoside present the crude extracts of *C. asiatica* is separated from other components on a siliga gel plate by a normal thin layer chromatography. Second, the band of asiaticoside on the plate is treated with 2-naphthol acid reagent to add the chromophores of 2-naphthol to its structure which can be quantitated directly from its TLC-densitometry chromatogram (λ530). It is thought that the molecule of 2-naphthol reacts with the sugar molecule of asiaticoside. The predicted mechanism of this reaction has 3 steps. First, the strong acid (sulfuric acid in the reagent) cause the hydrolysis reaction of glycosidic bond of asisticoside and cleave each sugar molecule apart. Second step is monosaccharides are dehydrated forming furfuryl derivates by conc. $H_2SO_4$ and attacked by 2-naphthol as a last step. 2-naphthol acts as a nucleophile and added to the positive polarized carbon of the aldehyde group of the furfural. After an intramolecular proton migration water is eliminated and a C-C double bond is formed. The method showed good sensitivity and selectivity and appears to be comparable to the UV-HPLC method witch is standard method.

The developed TLC densitometry was use to evaluate the asiaticoside content in various commercial products which showed variable asiaticoside content. This might be affected by many factors such as storage environment, source and origins, production process, time of harvesting, etc.[10] This newly analytical method should be useful for the examination the active compounds content of *C. asiatica* products on the drug market. The information gathered this way could be beneficial to the customers as the dose of the active compounds could be adjusted so that maximal therapeutic efficacy is achieved and lead to greater cost-benefits ratio of using herbal products.

## CONCLUSION

A new technique of TLC-densitometry has been developed for the determination of asiaticoside in the crude extracts of *C. asiatica* and its commercial products. In practice, the crude extracts are prepared from the plant materials under reflux with 80% methanol and patially purified by dichloromethane and butanol. Analysis is performed on a silica gel 60 $F_{254}$ TLC plate (20x10cm.) with chloroform/methanol/water system as the mobile phase. Densitometric analysis is performed at 530 nm after post-chromatographic derivatization with 2-naphthol sulfuric acid reagent (brownish band for glycoside) [1]. This new method showed good sensitivity and selectivity. The linear range for the analysis of asiaticoside was 100-1000 ng/band ($r^2 \geq 0.99$) with good precision and accuracy (1.15- 1.9 %RSD, 98.18-104.3%).

## ACKNOWLEDGMENTS

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