



Research Paper

HPLC SEPARATION AND ISOLATION OF ASIATICOSIDE FROM *CENTELLA ASIATICA* AND ITS BIOTRANSFORMATION BY *A. NIGER*

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The study was carried out to develop a rapid and accurate HPLC method to isolate the major bioactive phytochemical asiaticoside in *Centella asiatica* and to transform this metabolite using microorganism. Asiaticoside was separated using an optimized HPLC method using Phenomenex, Luna, 5 μ m (C18) (150 mm x 4.6 mm) eluted with Water PH3: Acetonitrile (50:50 V/V). The reported method is easy, fast and accurate, and produced highly pure, resolved isolate with high concentration. Asiaticoside was biologically transformed by *Aspergillus niger*, and the prescreening results on TLC and 1D and 2D IR showed that the transformed product has at least two byproducts of the ancestor compound. In addition to that the preliminary screening showed that the transformed product showed very high cytotoxicity against HaCat Cells in comparison to the original compound.

Keywords: *Centella asiatica*, Asiaticoside, Biotransformation, Biocatalysis, HPLC, *Aspergillus niger*, 2D IR, HaCat Cells, Cytotoxicity

INTRODUCTION

Centella asiatica or “gotu kola” has been used since long time ago as an ethno-pharmacological plant and supposed to be a potent medicinal plant for its various pharmacological effects. Many studies described the noteworthy protective effect of *C. asiatica* against many diseases, such as skin problems, wound healing, nerves and brain cells stimulating. That is why in India they called it as “Brain food or the Brain tonic”. Recently in 2013, we already reviewed the most studies that have been conducted on the potent bioactivities

of *C. asiatica*. Most of the studies linked *C. asiatica* activities to its triterpenoid content of the various physiological effects. Asiaticoside, assumed to be the greatest significant component in *C. asiatica*, which reported to have many bioactivities such as wound healing, burn wound repair, lung injury of mice varicose vein, anti-tumor, anti-oxidant, anti-tuberculosis and other activities (Alfarra and Omar, 2013b).

Previous studies reported the difficulties to separate asiaticoside and other derivatives from *C. asiatica* by HPLC (Schaneberg *et al.*, 2003;

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Zheng and Lu, 2011). Zheng and Lu (2011) reported many other attempts to separate asiaticosides and to quantify it precisely using HPLC, but the reported efforts could not give the wished results, thus he and his group worked to develop a method to separate them. In this communication we report original optimized approach to separate asiaticoside and to quantify it precisely. Which could be simpler, reproducible and faster to achieve this goal. In addition to that we report the microbial transformation of isolated asiaticoside by *A. niger*.

MATERIALS AND METHODS

Plant Material and Preparation of Extract

Centella asiatica (pegaga) whole plant was purchased from the local market in Kuala Lumpur, Malaysia, washed carefully, and leaves were separated from the other parts, then dried under shade for 14 days, after that it powdered using a common house grinder to give 1890 g net weight.

Powder was divided into (300 g) then put in a cellulose thimble, after that it was exposed to 4 L of n-Hexane, 4 L of DCM and 4 L of Methanol for 12 h each in that order, using Soxhelt apparatus at 50-60°C. The Methanolic extract was concentrated at the 40°C under reduced pressure using a rotary evaporator to give (300 g) total extract residue.

Fractionation, Chromatography and Compounds Isolation

The (150 g) residue was portioned in an Ethyl acetate (AcOEt) -water mixture (1:1) ratio. (100 g) EtOAc extract was mixed with silica gel to make a slurry, divided to sections and then were subjected to column chromatography [silica gel (mesh 70-230, 250 g), column, 5 x 50 cm] eluted with 2 L eluent of an increasing polarity started

from 100% CHCl₃ to 100% MeOH, the fractions were 100 mL with 5 mL/min flow rate. After that fractions were checked on TLC with mobile phase (CHCl₃/CH₃OH/H₂O 10/3/0.2 respectively) and vanillin reagent was used to detect the triterpenoids contents and the interested phytochemicals, fractions 5-10 (85%-75% CHCl₃/MeOH) showed to have the most interesting compound and triterpenoids content, they combined together then concentrated at 40°C under vacuum pressure to give 30 g fraction, which again subjected to another column chromatography with the same conditions mentioned above, with eluent volume 1 L and fraction size 50 mL each fractions 5-10 again gave the same interested compound—asiaticoside—in addition to other compounds shown on TLC. They merged together to give 5 g residue.

High Performance Liquid Chromatography (HPLC) Analysis

Triterpene standard (asiaticoside) stock was made using 5 mg (Sigma, Malaysia) dissolved in 10 mL HPLC grade methanol and then diluted further to obtain concentrations of 50, 100 and 150 ug/ml, respectively. HPLC analysis carried out with PerkinElmer, Flexar, PDA plus Detector connected to computer with Chromera software. The column used was brand new product of Phenomenex, Luna, 5 µm (C18), (150 mm x 4.6 mm). 1.361 g of KH₂PO₄ was dissolved in 1 L of d.d H₂O adjusted to the PH 3 using orthophosphoric acid and then used to prepare 50/50 Acetonitrile/H₂O mobile phase. 30 mg sample of the residue were dissolved in 3 mL CHCl₃/CH₃OH 85/15 to make final concentration 1 mg/mL. The sample and the standard were filtered using a 0.45 µm nylon membrane syringes filter. The detection wavelength was set at 205 nm, injection volume was 1 µL with three injections

being performed for each sample and standard, 1 mL/min flow rate and the column was maintained at 28°C with pressure in range 980-1000 Psi. The concentration of asiaticoside in the sample was estimated from the peak areas that were constructed by Chromera application. Concentrations of standard asiaticoside sample purchased from Sigma were used as 50, 100, 150 ug/mL, and the calibration curve was created by Micorsoft excel software to give the regression equation

$$y = 1185.2x + 756.83; R^2 = 0.99441$$

Figure 3 shows the standard curve of asiaticoside.

Biotransformation of Asiaticoside by *A. Niger*

Preparation for Preliminary Screening and Microbial Transformation

A pure fungal culture of *A. niger* (ATCC 16404) was obtained from Microbiology Laboratory, University Malaysia Pahang. *A. Niger* was culture was streaked and cultivated on Potato Dextrose Agar slant (PDA) (Difco) at 30°C for a week and then stock cultures of *A. niger* stored on slant PDA agar at 4°C for further use.

All preliminary screening and time course experiments were performed in 250 mL conical flasks containing 100 mL Sabouraud Dextrose Broth (SDB) media. Media contained peptone (2.5 g), yeast extract (2.5 g), KH_2PO_4 (1 g), MgSO_4 (0.5) in 1 L deionized distilled H_2O ; media was sterilized at 121°C for 15 min. *A. Niger* was cultured on a petri dish for 48 h to get well developed mycelia. The mycelia were then carefully scratched and suspended in 1 mL media using a sterile loop then aseptically transferred to a 250 mL conical flask containing 100 mL fresh media and subsequently incubated with shaking

in the incubator shaker operating at 120 rpm at 30°C for another 48 h. Then, mycelia suspensions were dispersed among 14 flasks containing sterile broth medium (100 mL each) and incubated for 24 h using same conditions mentioned above using the same method we already published in other work with a slight modifications (Nor Hazwani *et al.*, 2013). The substrate 140 mg asiaticoside was dissolved in 5 mL Dimethyl Sulfoxide (DMSO) to give final concentration 0.1 mg/mL in each flask, then was added to 7 conical flasks of the 24 h old culture and to another 7 conical flasks contains only sterile medium without microorganism (substrate control), and 7 flasks consist of the sterile media with the microorganisms (culture control). All the 21 flasks were incubated at the same conditions mentioned above, the extraction was performed after 2, 4, 6, 8, 10, 12 and 14 days for the time course and preliminary screening experiments.

Extraction of Transformed Products

The culture media and mycelium were filtered using vacuum filtration and the washed three times using 100 mL EtOAc for and the organic phase was collected and extracted 3 times by EtOAc (100 mL each time). The organic layer was dried over anhydrous Na_2SO_4 , after that the solvent was evaporated under reduced pressure to give dark brown crude. The same method was used for the control group. The extracts from both experimental and control groups were checked under TLC to confirm the presence of transformed product. The same procedure was applied to all time course and screening experiments until the end of the 14 days. For preparative scale experiments, 520 mg of asiaticoside was dissolved in 16 mL dimethyl sulfoxide (DMSO) and distributed among 52 conical flasks containing 100 mL media and 24 h

old culture with final concentration (0.1 mg/mL in each flask) and continuously shaken for 8 days using a rotary shaker (120 rpm) at 30°C, in addition to the substrate control flask and the culture control flask. Product extraction of the preparative scale experiments was done similarly as mentioned above with the prescreening experiments and to afford around 80 mg of dark brown residue analyzed by TLC and 1D and 2DIR.

The emerging molecular spectroscopy 2DIR is a technique became widely used in biomedical and herbal medicine sciences to characterize their molecular structure, give information about the herbs contents in heterogeneous samples.

To prepare the sample for 2DIR small amount of each sample was grinded using a pestle and mortar and mixed with proper amounts of KBr to reduce its water contents. After that the fine mixture of sample/KBr powder was placed in the die set to make the pellet and a pressure of 10 ton was applied for 20 s and then removed to place pellet in the 2DIR set then fixed to instrument after the background reading, sample scanning was started with temperature 40, 50, 60 until 120°C after and then the results were processed using 2DIR software to get the results in Figure 4.

RESULTS AND DISCUSSION

A broad review of the literatures showed that *C. asiatica* is used widely in the traditional medicine (Alfarra and Omar, 2013b).

HPLC now a day considered a great broadly used technique of quantitative analysis in the pharmaceutical business and in medicinal investigation laboratories. There are different but small number of HPLC methods was reported and few of them were explained with details. It

can be said most of the reported methods show major disadvantages, such as the very long separation time, the compounds are not on the base line. Some of the reported methods were used in our experiment to separate and analyze asiaticoside in *C. asiatica* fractions, however none of them gave the best result for our study. Therefore, to achieve finest separation of the asiaticoside from *C. asiatica*, some experimental parameters such as mobile phase strength, its acidity, composition and flow-rate were taken into consideration. Isocratic HPLC was used in this method and it gave the finest separation of asiaticoside (Figure 2) and its microbial transformation products. We noted that using percentages of acid in the preparation of mobile phase was not very satisfactory to get the accurate pH and good results; therefore we used to adjust the pH of the salted H₂O by diluted orthophosphoric acid to pH range 2.7-3.

Figure 1: The Chromatogram of the Asiaticoside Standard

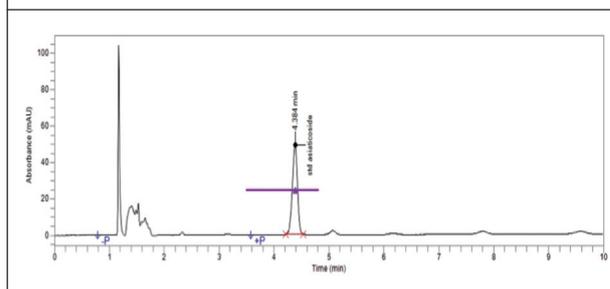
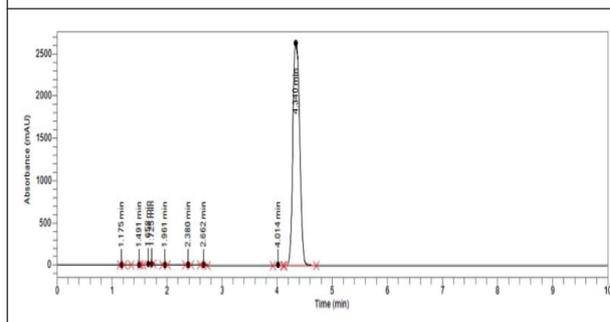


Figure 2: The Chromatogram of the Extracted Fraction of *C. asiatica*



The HPLC analysis of fractions from *C. asiatica* showed asiaticoside peak at retention time (Rt) 4.342 min (Figure 2), which matched the same Rt of the authentic compound (Figure 1). Figure 1 display chromatogram of standard asiaticoside and Figure 2 showed chromatogram of CHCl₃/CH₃OH80/20 fraction of *C. asiatica*.

The concentration of the was calculated by using the regression $Y = 1185.2x + 756.83$ where Y was the peak area and X is the Concentration, accordingly the concentration was

$$708658 = 1185.2x + 756.83 \text{ and then } X \text{ will } = (708658 - 756.83)/1185.2 = 0.597 \text{ mg/ml}$$

It can be said that this method we described above is simple and rapid in its application, that can be used in to isolate asiaticoside from *C. asiatica* easily, purely, and faster.

Microbial transformation has been extensively used, to create new and useful metabolites of almost all classes of terpenes, steroids and herbal

extracts such as tea extracts as a substitute of chemical synthesis for preparation of pharmacologically active compounds. Biotransformation can be some times the only predictable technique to yield specific compounds, such as the hydroxylation of the non activated carbon atoms (Omar *et al.*, 2012; and Alfarra and Omar, 2013a).

Time course screening biotransformation of the isolated asiaticoside showed that the used *A. niger* strain converted the substrate in the time period of 8-14 days, but the best result shown after 8 days (Figure 4) shows the transformed products of asiaticoside on TLC and (Figure 5) represent the 2D IR of asiticoside and the transformed product which extracted from microorganism metabolites, from the figure it can be easily noted that there are major changes on the original phytochemical resulted from microbial transformation, figures show clear differences between the peak of asiaticoside before biotransformation and the peaks that resulted from the biotransformation.

Figure 3: The Standard Curve of Asiaticoside

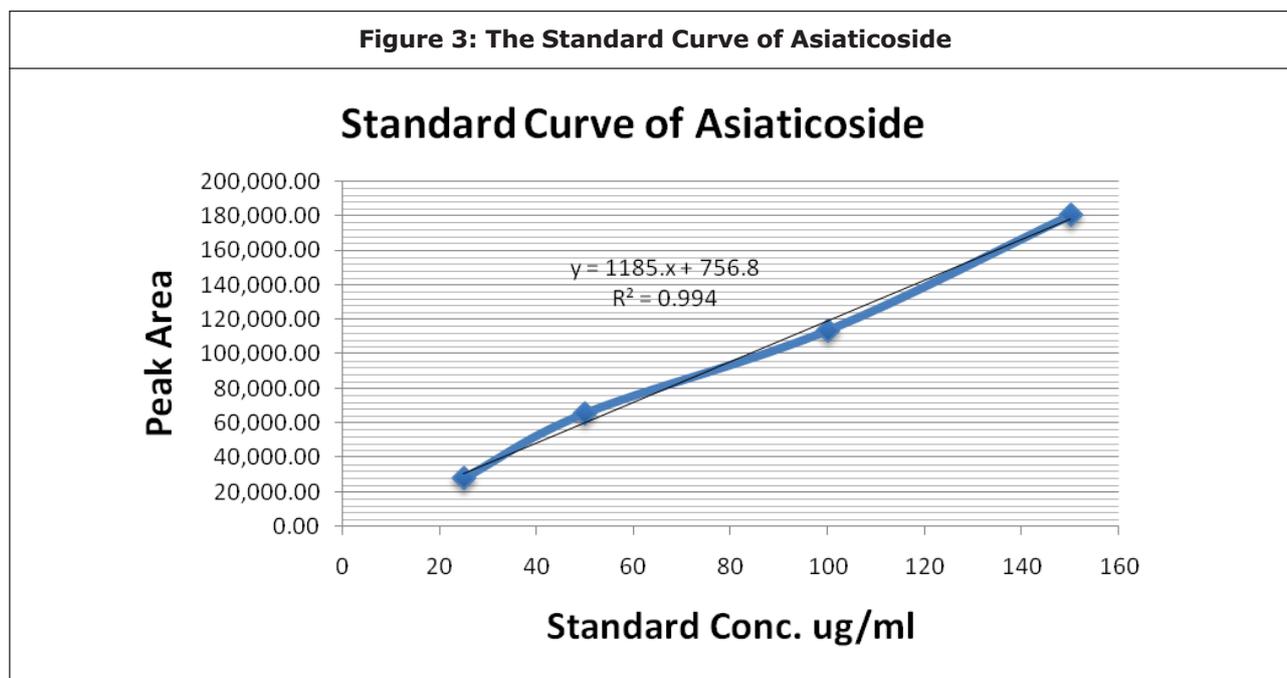


Figure 4: The Results of the Microbial Transformation of Asiaticoside by *A. niger*, where Sub A.S is Substrate of Asiaticoside, T1 is Positive Control, T2 is Negative control, T3, T4 and T5 are the First 5 Days of Metabolism, T6 and T7 is the day 8 and day 11 of Biotransformation Experiment

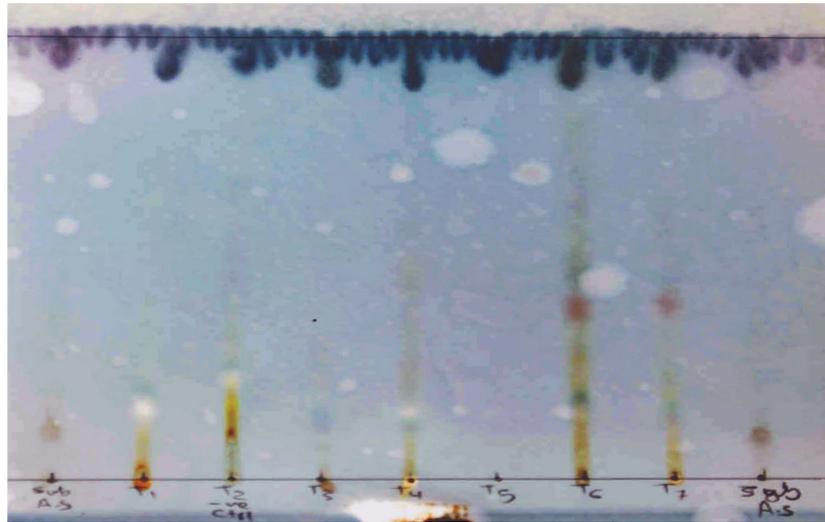
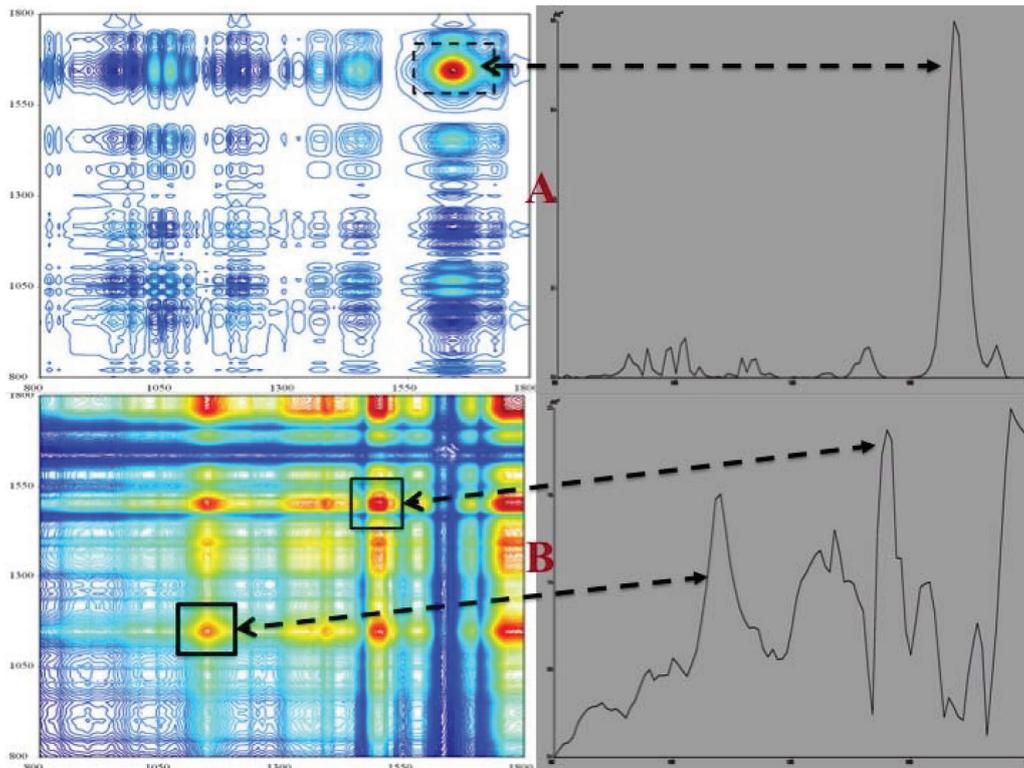


Figure 5: 2DIR Spectrums, Where A is the Auto Peak Pectrum (right) and 2DIR Synchronous Spectrum (left) of Asiaticoside, and B is the Auto Peak Spectrum (right) and the 2DIR Synchronous Spectrum (left) of Transformed Product of Asiaticoside, Figures Show Clear Differences Between the Peak of Asiaticoside Before Biotransformation and the Peaks that Resulted from the Biotransformation



The preliminary screening of the transformed product of asiaticoside showed a very cytotoxicity against HaCat cell lines even in the low concentrations with comparison to the original compound, this observations might lead to further research to test the product effects on some other cancer cell lines.

CONCLUSION

Herbs and its medicinal ingredients extraction and isolation are not easy and needs much time to adjust the possible method to separate the interested compounds. Most of the research results reported that *C. asiatica* activities are related to the triterpenoid content. Other studies reported that its activities belong mainly to the presence of asiaticoside as it is found in majority. We here tried to take the advantage of HPLC to standardize asiaticoside the major active compound of *C. asiatica* and it transformed the products. Our original optimized method provided an excellent resolution and separation of asiaticoside from other constituents of *C. asiatica*. This reported HPLC method in comparison to the other reported methods in Chang and Wang (2011) Inamdar *et al.*, (1996), Jain and Agrawal (2008), Kwon *et al.* (2011), Schaneberg *et al.* (2003), Verma *et al.* (1999), Zheng and Lu (2011) supposed to be rapid, modest and accurate to isolate pure asiaticoside from *C. asiatica*—using fraction collector that can be attached to the HPLC instrument—and can be used to quantify asiaticoside in *C. asiatica* fractions, though, the reported results in this study showed very good and novel outcome working on developing other methods to separate this compound and it bio transformed products without using acidic mobile to avoid the effects of acids on the isolated compounds specially the saponins. The

cytotoxicity of the isolated compound and its biocatalysed product were tested on HaCat cell lines, and it was observed that the transformed product showed a sever cytotoxicity on the cells even with very low concentrations which might lead to test its toxicity on other cancer cell lines. However more bioactivity tests still ongoing, products will be tested on the wound healing of human cell lines, then a comparison will be done to study which has more effect the original isolated compound or its microbial transformation product. Further conclusion and recommendation on that will be reported.

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