DETECTING CENTELLOSIDES IN PEGAGA EXTRACT VIA HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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Abstract

A chemical investigation of pegaga or *Centella asiatica* (L.) Urb. was performed via the Reversed Phased-High Performance Liquid Chromatography (RP-HPLC). The mobile phase was modified from the Malaysian Herbal Monograph. Instead of utilizing a non-acidified mixture of water and acetonitrile, this work involved an aqueous solution of formic acid (0.3%), with similar organic solvent. The chromatograms were recorded, and four major peaks were observed in the stepwise gradient. These signals represent four individual constituents, suggestive of the pentacyclic triterpenoids, including madecassoside (1), asiaticoside (2), madecassic acid (3), and asiatic acid (4). More experiments should be carried out to understand the elution of the pegaga's compounds. It is hoped that all the plant parts could be sustainably participated in the chromatographic analysis, for the pharmaceutical research and development of the metabolites.

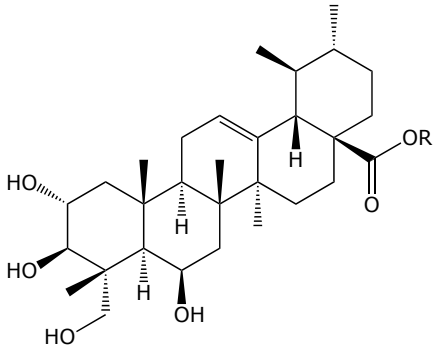
Keyword: *Centella asiatica*, centellosides, RP-HPLC-DAD, stepwise gradient

Introduction

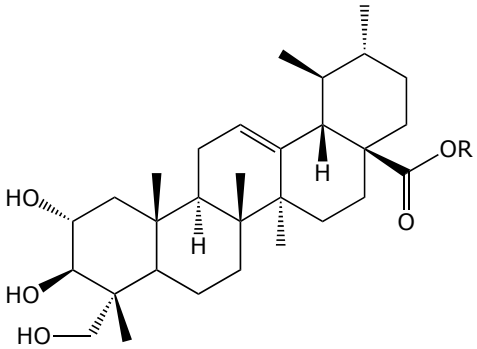
Pegaga or *Centella asiatica* (L.) (**Figure 1**) is classified in the Apiaceae family. It is known as Indian pennywort or gotu kola (Ahmad *et al*., 2016), and has become a pharmaceutically important medicinal plant (Arpita & Navneeta, 2017). From an ethnobotanical survey, *C. asiatica* was used as the traditional salad (or ulam) in Kota Belud, Sabah, Malaysia. It is believed to cure memory loss and as anti-aging herbal medicine (Awang-Kanak *et al*., 2018). *C. asiatica* has been subjected to quite extensive experimental and clinical investigations (Tan, 2017). The important therapeutic secondary metabolites known as centellosides, including compound (1) – (4), are shown in **Figure 2** (James *et al*., 2009; Gallego *et al*., 2014). The separation of pharmacologically relevant triterpenes (**Figure 2**) was achieved by high performance liquid chromatography (HPLC), employing the reversed-phase (RP) system of acetonitrile/water on RP C-18 columns at a detection wavelength of 205 nm (Güntherm & Wagner, 1996).

The quantification of these targeted bioactive centellosides was later carried out by using Ultra-High-Performance Liquid Chromatography (UHPLC) (Sabaragamuwa *et al*., 2022) and High-Performance Thin Layer Chromatography (HPTLC) (Kunjumon *et al*., 2022). The emphasis was to standardize all four compounds as the biomarkers in *C. asiatica* raw drugs, pharmaceutical and cosmetic products. In this study, the chromatograms from a High-Performance Liquid Chromatography with Diode Array Detector (HPLC-DAD) will be presented. It is hoped that the HPLC chromatographic protocol could be a common tool to identify the major triterpenes of *C. asiatica* extract.

Figure 1 *Centella asiatica* (pegaga) leaves



(1) Madecassoside,
R = Rha-Glc-Glc (MAD)
(3) Madecassic acid, R = H (MDA)



(2) Asiaticoside, R = Rha-Glc-Glc (ASI)

(4) Asiatic acid (ASA)

Figure 2 The structures of chemical compounds from *C. asiatica* (Gallego *et al*., 2014)

Materials and Methods

The leaves of *C. asiatica* were obtained from the local market in Puncak Alam, Selangor Darul Ehsan, Malaysia. The samples were identified and deposited in the Faculty of Pharmacy, UiTM Puncak Alam, Selangor Darul Ehsan (voucher specimen no. PH-NAS01). The plant materials were chopped and crushed by using a mortar and pastel, to increase the surface area. The samples were dried, powdered, and sonicated in methanol, prior to the liquid chromatography (Sabaragamuwa *et al*., 2022). The extract was filtered through a 0.45 µm Millipore filter, into the vials. The profiling of the extract was performed by using the Reverse-Phase High-Performance Liquid Chromatography (Agilent 1200 RP-HPLC) (Globinmed, 2022). About 10 µl of the sample was injected for the HPLC, which was run through a column (Thermo C-18; 250 mm × 4.6 mm and 5 µm of pore size) as a stationary phase. Two approaches were employed to compare the extract profiles, between a rapid and a non-isocratic, stepwise gradient (**Table 1** and **Figure 3**) elutions in RP-HPLC methods. The mobile phase consisted of various composition of 0.3% formic acid in water and acetonitrile, with a flow rate of 1 ml/min. The data were recorded via dual wavelengths (210 and 360 nm) through diode array detector (DAD) and the column temperature was maintained at 36°C throughout the experiment. The running time was set at 30 and 50 minutes, for the rapid and stepwise gradient modes, respectively. The data acquisition was performed by using ChemStation software.

Table 1 The solvents for the stepwise gradient elution

Time (minutes)	Acetonitrile (%)	0.3% Formic Acid in Water (%)
0	10	90
5	10	90
20	40	60
22	40	60
30	70	30
32	70	30
40	90	10
45	90	10
47	10	90
50	10	90

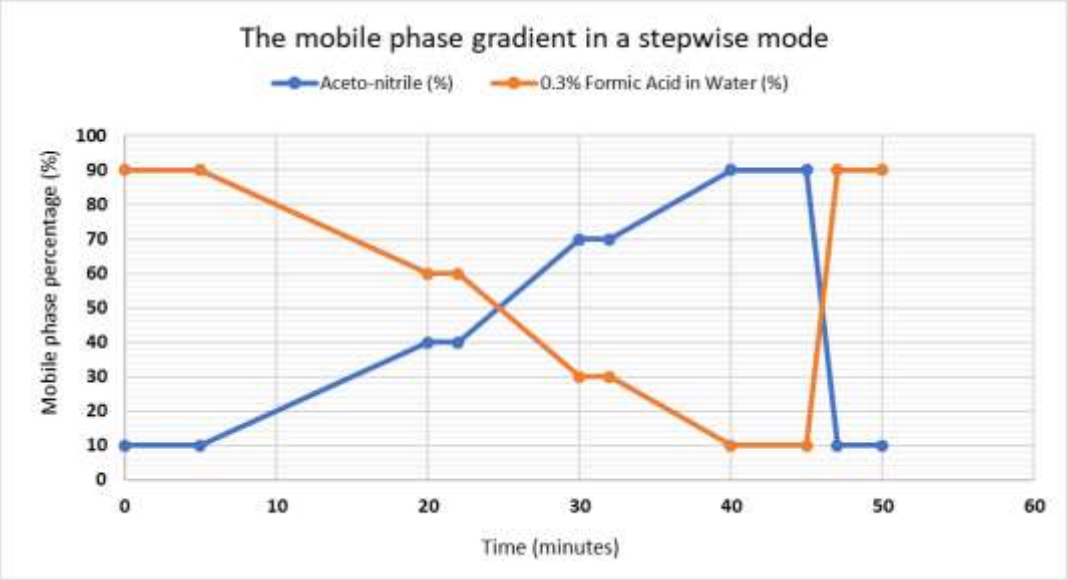


Figure 3 The composition of mobile phase for the HPLC run (stepwise gradient mode)

Results and Discussion

The chromatogram of the methanol extract in a fast gradient was shown in **Figure 4**. Significant peaks of compounds (1) – (4) can be referred at 9.547, 9.714, 11.810, and 12.612 minutes, respectively. The fourth signal or compound 4 could be the major constituent of this sample, based on the highest peak intensity. Meanwhile, **Figure 5** showed the chromatogram in a stepwise gradient mode. There were differences in the retention times (t_R) for the peaks that emerged after t_R = 9.5 minutes, in the fast method. In comparison, these four obvious, isolated signals were later found at retention time (t_R); 17.371, 18.635, 28.736, and 32.310 min, consecutively for compound (1) - (4), when the stepwise gradient mode was applied (**Figure 4**). Better resolution and base line separation were achieved for the two, twinning peak of compound 1 and 2. As projected, compound 4 could still be the main component of the sample, due to its highest absorbance value.

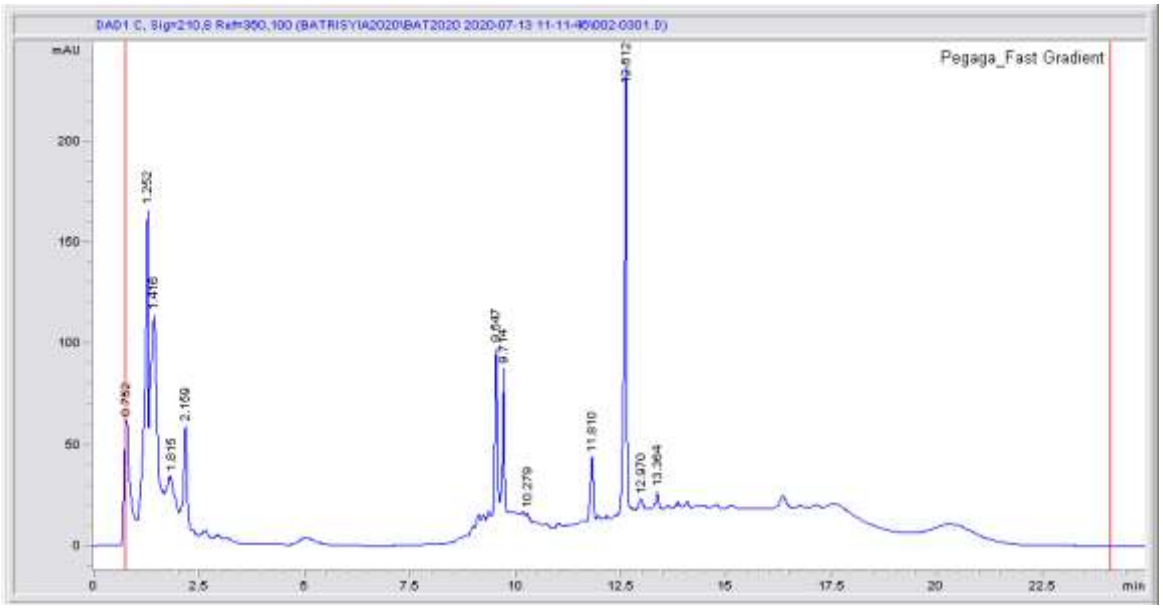


Figure 4 The chromatogram of methanol extract of *C. asiatica* (fast gradient)

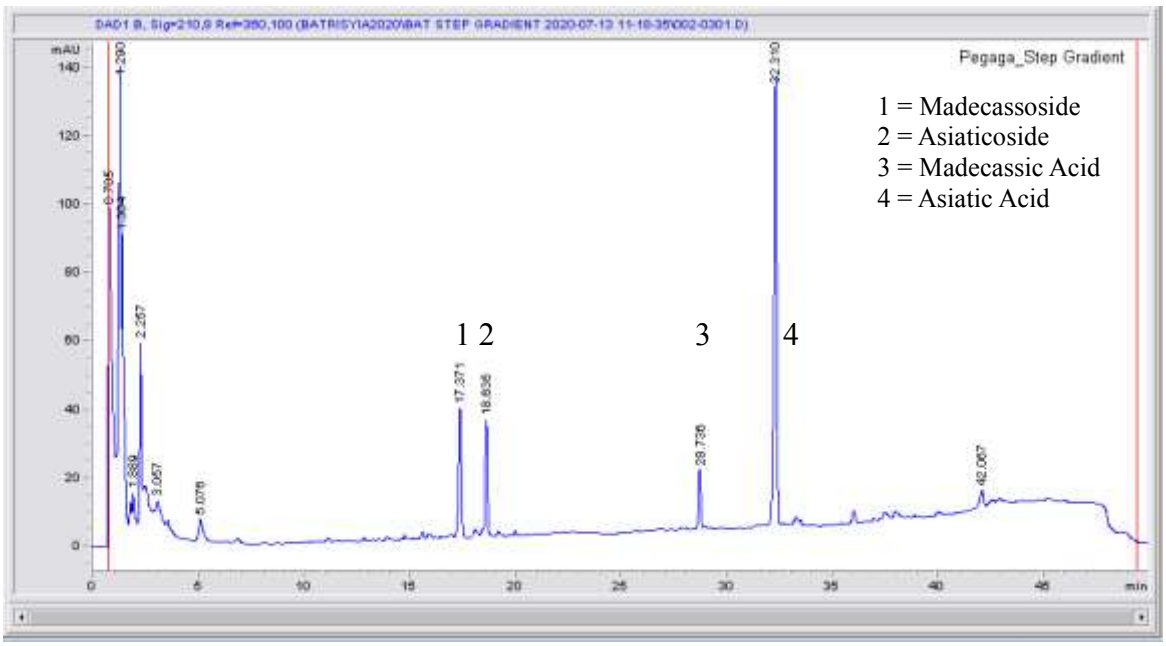


Figure 5 The chromatogram of methanol extract of *C. asiatica* (stepwise gradient)

The chromatographic baseline drift was experienced during the gradient mode, as commented by Sabaragamuwa *et al* (2022). Based on the chromatographic shifts on the peaks in the slow gradient program (Table 2), it was suggested that the order of the eluted compounds would begin with madecassoside (1), followed by asiaticoside (2), madecassic acid (3), and asiatic acid (4) (Globinmed, 2022). The twinning pattern (t_R = 17.371, 18.635) would be for the triterpenoid saponins, not for madecassic and asiatic acids. Those acid molecules interacted longer with the RP column, than their respective glucosides (Ahmad *et al*., 2016). According to the online monograph (Globinmed, 2022), the RP-HPLC condition was set for an ethanol extract of *C. asiatica*. The mobile phase consisted of a non-acidified mixture of water and acetonitrile (Ahmad *et al*., 2016). The peak for madecassic acid (3) was selected as reference. It was compared with the other three compounds. According to the recorded data of the RRT, or relative retention time for the centellosides, similar estimations were performed. Owing to

observations upon the chromatogram peaks in the experimental data of the stepwise gradient (**Table 2**), similar rule was applied to the unknown peak 1-4. The peak elution or the chromato-graphic behavior would be in the same order, as seen in the pattern or peak height. The RRT was comparable among the yet, non-elucidated compounds (**Table 3**).

Table 2 The retention times (minutes) for the peaks of interest, in both fast and stepwise gradient modes

Peak No.	Fast Gradient	Stepwise Gradient
1	9.547	17.371
2	9.714	18.635
3	11.810	28.736
4	12.612	32.310

Table 3 The relative retention times (RRT) for the peaks of interest in the stepwise gradient mode, compared to the Malaysian Herbal Monograph

Peak No.	RRT according to guideline	Experimental RRT	Suggested Compound
1	0.67	0.60	Madecassoside
2	0.69	0.58	Asiaticoside
3	1.00	1.00	Madecassic Acid
4	1.17	1.12	Asiatic Acid

Conclusion

This study reports on the primary chromatographic data of *C. asiatica* with a substantial hypothesis on the elution order or signal character of the centellosides. In conclusion, this sample contains centellosides and they were detected using via RP-HPLC-DAD. The chromatographic procedure could be a routine analysis of the pegaga content, hence, the terpenoid glycosides could present in the research specimen. Such monitoring of common secondary metabolites would indicate the herbal quality. This information is useful to the plant analyst in sustainable cultivation, harvest, and production of this healthy herb.

Ethics Statement

The research does not require research ethics approval.

Authors Contribution

Writing – Original draft preparation, Abdul Wahab, I.; sample identification - Mohsin, H. F.; Methodology - Mohsin, H. F. and K. J. Jalani.

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Conflict of interests

The authors declare that there is no conflict of interest concerning the publication of this paper.

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