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An improved HPLC method for quantitative determination of six triterpenes in *Centella asiatica* extracts and commercial products

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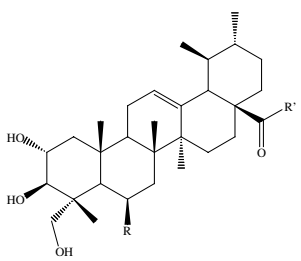
An improved HPLC qualitative and quantitative method of six triterpenes (asiaticoside, madecassoside, asiatic acid, madecassic acid, terminolic acid, and asiaticoside-B) in *Centella asiatica* (raw plant material and preparations) is described in this paper. After 50 minutes the six active triterpenes were separated and detected in the methanolic extract at a limit of 0.01 μg/ml. The method uses a Phenomenex Aqua 5μ C18 (200 Å) column as the stationary phase, a gradient mobile phase of water (0.1% TFA), acetonitrile (0.1% TFA), and methyl tert-butyl ether (0.1% TFA), and UV detection at 206 nm. The correlation coefficients for the calibration curves and the recovery rates ranged from 0.995 to 0.999 and from 98.39% to 100.02%, respectively. The qualitative and quantitative results are discussed.

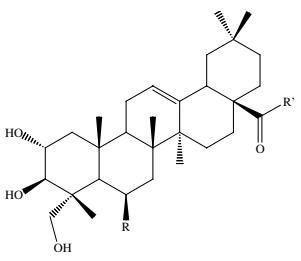
1. Introduction

Preparations from *Centella asiatica* (L.) Urban. (Umbelliferae) (also *Hydrocotyle asiatica*), commonly named Gotu Kola have been used for centuries by many to treat a wide variety of ailments, both mental and physical. The plant is listed as a drug in the Indian Herbal Pharmacopoeia, the German Homoeopathic Pharmacopoeia (GHP), the European Pharmacopoeia, and the Pharmacopoeia of the People's Republic of China. Grown in swampy areas of India, Sri Lanka, Madagascar, Africa, Australia, and the tropics, it is used as a stimulant to treat and prevent mental breakdowns [1, 2]. In Ayurveda, *C. asiatica* is one of the spiritual herbs for improved meditation [3]. The leaves of the plant are widely used to treat leprosy, cancer, skin disorders, arthritis, varicose veins, ulcers, lupus, tuberculosis, and skin wounds [3–5].

Currently, four bioactive triterpenes have been used for the standardization of *C. asiatica*: a pair of saponins, asiatic acid (**1**) and madecassic acid (**2**), and a pair of trisaccharides, asiaticoside (**3**), and madecassoside (**4**) [6, 7].



1 R = H R' = OH
2 R = OH R' = OH
3 R = H R' = O-gluc-gluc-rha
4 R = OH R' = O-gluc-gluc-rha



5 R = OH R' = OH
6 R = OH R' = O-gluc-gluc-rha

While developing the HPLC separation, it became clear that two compounds were present in each of the purchased standard compounds of **2** and **4**, but the structures of the second compounds were not given. Structural confirmation of the two unknown compounds was done by ^{1}H- and ^{13}C NMR and MS analysis. The known compounds were terminolic acid (**5**) and asiaticoside-B (**6**); both having the oleanane triterpene skeleton [7, 8].

The literature has shown a variety of LC methods for the separation of the active secondary metabolites, **1**–**4**, but none have shown the separation of the oleanane triterpene isomers, **5** and **6**, from **2** and **4**, respectively [9–13]. Chromatograms respectively reported by Morganti et al. show peak splitting of **2** [12] and those reported by Gunther et al. show peak splitting of **4** [10], but neither achieved baseline separation. Morganti did indicate the peak splitting is from the isomers, but their research was only concerned about the quantity of the total triterpene concentration in an anti-cellulitis transdermal delivery system [12].

Baseline separation and identification of the isomers is needed for a more accurate standardization of *C. asiatica*. Although total triterpene content can be determineded by previous methods, it is important to determine the concentration of each individual component to yield a better understanding of each preparation's activity. Case in point, Gotu Kola is known for its wound healing properties, but asiaticoside (**3**), and not madecassoside (**4**), was found to be active *in vitro* and *in vivo* [5]. Thus, the total triterpene concentration does not represent an accurate description of the wound healing ability of the preparation if only one triterpene is active. Yet, the individual concentrations of the other five triterpenes are important as further biologi-

cal studies determine their role in the overall pharmacological activity of the preparations.
An improved, reproducible HPLC method is reported here in for the baseline separation of **1**–**6**. This method was then used to determine the quantity of the bioactive triterpenes in extracts and a number of dietary supplements on the market claiming to contain *C. asiatica* (Gotu Kola).

2. Investigations, results and discussion

2.1. Standard separation

Originally, the standards of **1**, **2**, **3**, and **4** were purchased for this study, but while screening various stationary phases (Luna 5 µ RP18(2), Luna 5 µ C8(2), Aqua 5 µ C18 200 Å, Hypersil 5µ C18 BDS, Lichrosphere 5 RP18, Synergi MAX-RP 80 Å), the standards, **2** and **4**, showed peak splitting with Aqua 5µ C8(2). Further literature research reported the existence of structural isomers of **2** and **4**. Triterpenes **2** and **4** had an ursane skeleton while the isomer of each had an oleanane skeleton (**5** and **6**). With this knowledge, it was important to achieve baseline separation of **2** and **4** from its isomers for the most accurate qualitative and quantitative analysis of the bioactive compounds in *C. asiatica* extracts and commercial products.
Standards were isolated from *C. asiatica*. Purity was determined by the PDA data of all peaks of interest. Peak purity was greater than 95% for each standard. Isomer percentages in the madecassoside mixture (**4** and **6**) were 74% and 26% respectively, while in the madecassic acid mixture (**2** and **5**) they were 71% and 29% respectively. All standards and samples were injected in triplicate. The maximum standard deviation was 1.5% except for GK-10 and CA-3 (3.41% and 4.44% respectively).
The validity of the extraction procedure listed by Morganti et al. was confirmed [12]. A sample (GK-1) was spiked with a known amount of standard compounds. The recovery rates were consistent (between 99 and 100%).
Baseline separation was a challenge due to the variation in the structure of the standard compounds. The trisaccharides, **3** and **4**, have higher polarities than the aglycones, **1** and **2**. A solvent with high water content was needed in the beginning to ensure the sugars did not elute with the solvent peak. This was followed by a ramping up of the percentage of organic solvent to elute the acids in a reasonable amount of time.
Combinations of acetonitrile and water, both containing TFA, did not give baseline separation, only peak splitting of **2** and **4**. A high concentration of water (81%) was needed to elute the trisaccharides, then the acetonitrile concentration had to be ramped up to 45% in order to elute the aglycones in under 50 min. A linear gradient alone did not give baseline separation, manipulation of the gradient to a concave curve was needed. The initial concentrations are held steady and then at the end of the time allotted, the concentrations change rapidly to the end point. The modifier MTBE was then added to achieve final baseline separation. Baseline drift is sometimes observed due to the ramping of acetonitrile during the separation and the presence of MTBE.

2.2. Plant and product analysis

Standard compound composition of the three plant materials (CA-1, 2, and 3) varied. The highest concentration of **1**–**6** was present in CA-2, and the lowest in CA-3. Extracts from CA-1 and 2 contained higher concentrations of the glycosides (**3**, **4**, and **6**) over the aglycone (**1**, **2**, and **5**), while CA-3 contained higher concentrations of **1**, **2**, and **5** over **3**, **4**, and **6**. The concentrations of **1**, **2**, and **5** in CA-1 were at the limit of detection. Differences were also detected in the glycoside concentrations. In CA-2 and 3, the concentration of **5** was higher than that of **4**, while the concentration of **4** in CA-1 was greater than **5**.
Qualitative and quantitative composition of the commercial products varied greatly. Chromatograms shown in

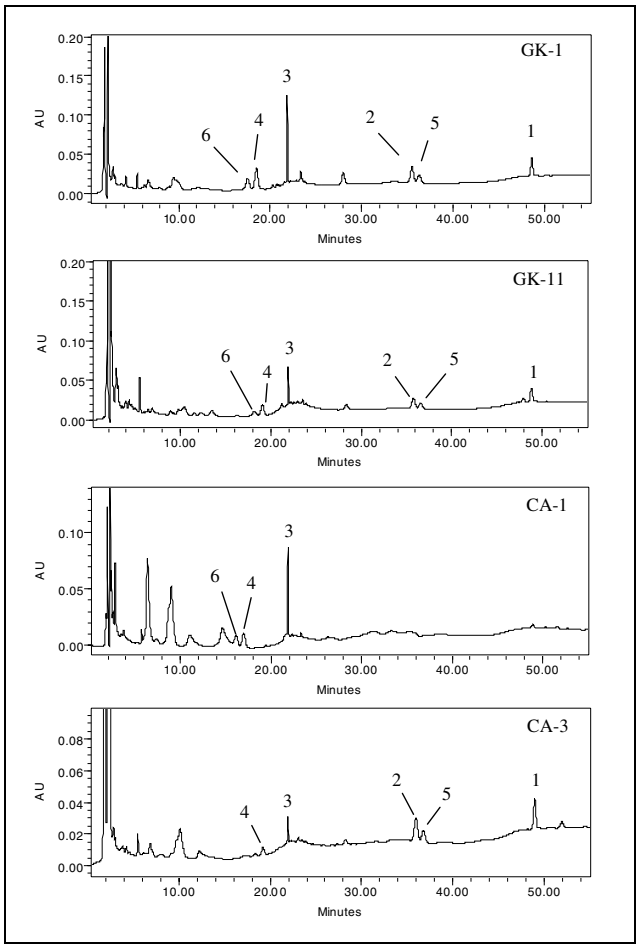


Fig. 1: Representative chromatograms of GK-1, GK-3, CA-1, and CA-3 at 206 nm

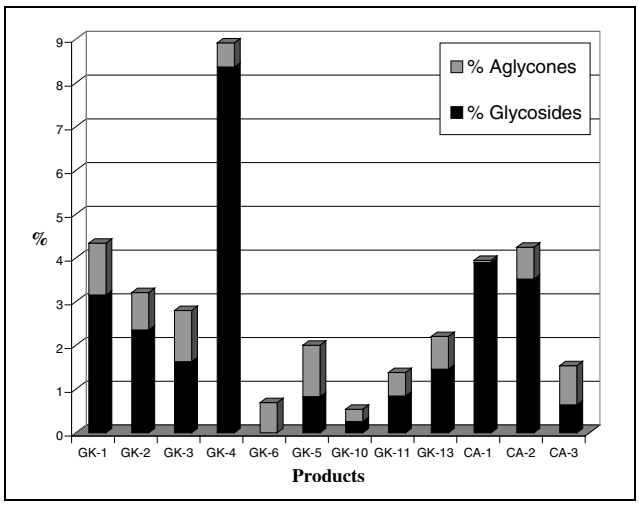


Fig. 2: Variations in the concentration of glycosides compared to the aglycones in the Gotu Kola samples tested

Table 1: Concentration (%) of triterpenes quantified in Gotu Kola and *C. asiatica* extracts

Product	1	2	3	4	5	6	Total
GK-1	0.24 (2.59)*	0.57 (2.83)	1.05 (2.66)	1.30 (4.02)	0.80 (1.56)	0.37 (3.72)	4.33
GK-2	0.23 (0.36)	0.37 (1.98)	0.90 (0.28)	0.84 (3.85)	0.61 (3.72)	0.25 (1.28)	3.20
GK-3	0.31 (0.52)	0.50 (1.55)	0.68 (1.42)	0.56 (0.84)	0.39 (1.28)	0.36 (2.48)	2.79
GK-4	0.21 (1.09)	0.17 (2.75)	3.84 (0.99)	1.82 (2.07)	2.70 (0.85)	0.17 (2.83)	8.91
GK-5	0.14 (0.65)	0.71 (0.37)	0.20 (3.25)	0.43 (2.18)	0.19 (3.99)	0.33 (2.14)	2.00
GK-6	ND	ND	0.12 (1.08)	0.42 (0.86)	ND	0.15 (2.74)	0.69
GK-7	ND	ND	ND	ND	ND	ND	–
GK-8	ND	ND	ND	ND	ND	ND	–
GK-9	ND	ND	ND	ND	ND	ND	–
GK-10	0.10 (3.20)	0.13 (4.34)	0.14 (3.56)	0.06 (3.89)	0.05 (3.08)	0.08 (3.50)	0.54
GK-11	0.14 (0.26)	0.25 (3.32)	0.31 (3.71)	0.32 (0.57)	0.20 (0.05)	0.14 (3.05)	1.38
GK-12	ND	ND	ND	ND	ND	ND	–
GK-13	0.17 (0.68)	0.34 (0.66)	0.51 (1.30)	0.56 (0.42)	0.38 (1.52)	0.23 (0.85)	2.21
CA-1	0.03 (0.93)	0.04 (2.00)	1.01 (1.10)	0.93 (1.84)	1.95 (1.01)	0.03 (1.91)	3.94
CA-2	0.15 (3.02)	0.37 (2.72)	0.76 (1.78)	1.49 (3.15)	1.26 (3.79)	0.21 (3.86)	4.24
CA-3	0.20 (3.64)	0.44 (2.58)	0.15 (3.50)	0.23 (3.62)	0.26 (1.51)	0.25 (2.56)	1.53

* % Standard Deviation in Parentheses
ND = Not Detected

Fig. 1 are from two commercial products and two plant extracts (GK-1, GK-3, CA-1, and CA-3). All products, except GK-6 (liquid form), claimed to contain plant material (aerial), not an extract, of *C. asiatica*. Only one product guaranteed a minimum level of triterpene concentration (GK-4, 10%), but did not list which triterpenes were quantitated. The concentrations of **1**–**6** are shown in Table 1. GK-1 6 only contained one herbal, *C. asiatica*, while GK-7–9 were a combination of two herbals, Gingko and Gotu Kola, and GK-10 and 11 were a combination of Ginseng and Gotu Kola. GK-12 was a combination of various herbals and GK-13 was a combination of huperzine A and Gotu Kola. The triterpenes were not detected in any of the products containing Ginkgo (GK-7, 8, and 9) or GK-12. The Gotu Kola quantity in the capsule was low, and other extractants interfered in the chromatograms.

In conclusion, the qualitative and quantitative evaluation of Gotu Kola plant material and some Gotu Kola containing preparations are improved with this HPLC method. This method allows for the individual quantitation of **2** and **4** as well as **5** and **6**, which is not true in other methods. Accurate quantitation of active constituents in *C. asiatica* leads to a greater assurance of quality in the Gotu Kola preparations.

3. Experimental

3.1. Chemicals and reference compounds

HPLC grade acetonitrile, methyl tert-butyl ether (MTBE), and methanol were obtained from Fisher Scientific (Fair Lawn, NJ, USA). Trifluoroacetic acid (TFA) was purchased from Sigma (St. Louis, MO, USA). HPLC grade water was prepared by filtering nanopure water through a 45 μm membrane filter. Asiaticoside, asiatic acid, madecassoside, madecassic acid, terminolic acid and asiaticoside-B were isolated from *C. asiatica* (L.) Urban. in our laboratories (University, MS, USA). Identity and purity of the isolated compounds were confirmed by chromatographic (TLC, HPLC) and spectral (1D- and 2D-NMR, LC-MS) methods.

Table 2: Calibration data for compounds 1–6

Compd.	Regression equation	Correlation coefficient	Limit of detection (μg/ml)
1	$y = 2.03 \times 10^3 X$	0.998	0.01
2	$y = 2.12 \times 10^3 X$	0.998	0.01
3	$y = 2.03 \times 10^3 X$	0.999	0.01
4	$y = 2.93 \times 10^3 X$	0.995	0.01
5	$y = 2.48 \times 10^3 X$	0.999	0.01
6	$y = 5.31 \times 10^3 X$	0.999	0.01

3.2. Plant material and dietary preparations

Three plant samples were used in this study. The first (CA-1) was obtained from Botanical Liason (Boulder, CO, USA). *C. asiatica* (L.) Urban. (CA-2) was purchased from Frontier (Norway, IA, USA). The third (CA-3) was purchased at a local herbal store and was labelled *C. asiatica* Sri Lanka. Voucher specimens of the samples are deposited at the National Center for Natural Products Research, University, MS. Products were purchased from Nutri-Mart (Diamond Bar, CA, USA) and a local herbal store (Oxford, MS, USA).

3.3. Equipment

Sample analysis by HPLC was done on a Waters 600 E pump with WISP autoinjector and a Waters 996 PDA detector (Waters, Millford, MA, USA). The analysis software was Millennium[32] by Waters. The column was a Phenomenex Aqua 5μ C18 (200 Å) 150 × 4.60 mm and the guard column was a Security Guard C18 cartridge system (Phenomenex, Torrance, CA, USA). Extraction took place in a FS20H Ultrasonic Cleaner (VWR Scientific Products, West Chester, PA, USA).

3.4. Sample preparation

Twelve of the commercial products tested were encapsulated powder, while one product was in liquid form. Six of the products were *C. asiatica* and the other seven products were a combination of *C. asiatica* and other herbal products such as ginkgo and ginseng.

3.4.1. Encapsulated powder sample preparation

The powder (0.4–1.0 g) was dumped into a 15 ml screw capped polypropylene centrifuge tube (Falcon tubes from VWR Scientific Products) and extracted three times with 3.0 ml of methanol by sonication for 10 min. The emulsion was centrifuged (5.0 min at 3000 rpm) and the supernatants were combined to a 10 ml volumetric flask by pipette and diluted to the final volume with methanol and mixed thoroughly. All samples were filtered through a 0.45 μ PTFE syringe filter prior to injection.

3.4.2. Liquid sample preparation

Ten drops of the liquid product was weighed in a 10 ml volumetric flask and then diluted to the mark with methanol. Once the sample was mixed thoroughly, filtration was done prior to injection as in the above sample preparation. Plant sample preparation.

3.4.3. Plant sample preparation

Approximately 500 mg of the ground plant material (leaves, stems, and roots) was placed in a 15 ml screw capped polypropylene centrifuge tube. The extraction procedure was the same as shown in the encapsulated powder sample preparation above.

3.5. Calibration

Approximately 5.0 mg of each standard compound was placed in a 10 ml volumetric flask and diluted in methanol (stock solution). Further calibration levels were prepared by diluting the stock solution with methanol. Within the range of concentrations injected (500.0–5.0 μg/ml) the detector response was linear. The combined calibration data (regression equation, correlation coefficient, limit of detection) is listed in Table 2. The recovery rates were as follows: **1**, 99.84%; **2**, 100.02%; **3**, 98.39%; **4**, 100.01%; **5**, 99.51%; **6**, 99.68%.

3.6. Analytical conditions

The following conditions were optimal: solvent A was water (0.01% TFA), solvent B was acetonitrile (6.0% MTBE and 0.01% TFA), solvent C was acetonitrile (12.0% MTBE and 0.01% TFA), and solvent D was acetonitrile. The HPLC mobile phase composition is shown in Table 3. Total run time was 70 min. The flow rate was held at 1.0 ml/min and analysis carried out on a 10 μl injection at ambient temperature and a detector wavelength of 206 nm.

Table 3: The HPLC mobile phase composition

Time (min)	% A	% B	% C	% D	Curve
0–15	8167	1933	–	–	10
15–20	6767	330	033	–	6
20–40	6759	–	3341	–	10
40–50	5955	–	4145	–	6
50–60 wash	–	–	–	100	–
60–70 equilibrate	81	19	–	–	–

A: water (0.01% TFA), B: acetonitrile (6.0% MTBE and 0.01% TFA), C: acetonitrile (12.0% MTBE and 0.01% TFA), and D: acetonitrile

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References

1 Bonati, A.: Medicinal plants of Madagascar and Senegal: A comment. J. Ethnopharmacol. **2**, 91, (1980)
2 Kartnig, T.: Clinical Applications of *Centella asiatica* (L.) Urb. in Herbs, Spices and Medicinal Plants, Vol. 3 Oxyx Press, Phoenix, AZ 1988
3 Castiglioni, A.: A History of Medicine, 2nd ed. Alfred A Knopf, New York 1958
4 Arpaia, M. R.; Ferrone, R.; Amitrano, M.; Nappo, C.; Leonardo, G.; del Guercio, R.: Int. J. Clin. Pharmacol. Res. **10**, 229 (1990)
5 Shukla, A.; Rasik, A. M.; Jain, G. K.; Shankar, R.; Kulshrestha, D. K.; Dhawan, B. N.: J. Ethnopharmacol. **2**, 1 (1999)
6 Castellani, C.; Marai, A.; Vacchi, P.: Boll. Chim. Farm. **120**, 570 (1981.)
7 Sahu, N. P.; Roy, S. K.; Mahato, S. B.: Phytochemistry **28**, 2852 (1989)
8 King, F. E.; King, T. J.: J. Chem. Soc. 4469 (1956)
9 Inamdar, P. K.; Yeole, R. D.; Ghogare, A. B.; de Souza, N. J.: J. Chrom. A **742**, 127 (1996)
10 Gunther, B.; Wagner, H.: Phytomedicine **3**, 59 (1996)
11 Laugel, C.; Baillet, A.; Ferrier, D.: J. Liq. Chrom. Rel. Rechnol. **21**, 1333 (1998).
12 Morganti, P.; Fionda, A.; Elia, U.; Tiberi, L.: J. Chrom. Sci. **37**, 51 (1999)
13 Verma, R. K.; Bhartariya, K. G.; Gupta, M. M.; Kumar, S.: Phytochem. Anal. **10**, 191 (1999)