Quantitative Estimation of Asiatic acid, Asiaticoside & Madecassoside in two accessions of *Centella asiatica* (L) Urban for Morpho-chemotypic variation

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ABSTRACT

Aim and Scope: Two morphologically distinct accessions of Centella asiatica (SL and LL) from Indo-Gangetic plains of India were compared in relation to the levels of triterpenoid saponins. The plant was evaluated through its morphology, quantitative microscopy and physico-chemical tests. **Materials and Methods:** The metabolites madecassoside, asiaticoside and its sapogenin asiatic acid were analyzed and quantified by HPTLC. A comparison and evaluation of different parameters together with triterpenoid content in these morphotypes are reported. **Results:** Quantitative microscopical studies doesn't show any significant difference except in terms of stomatal number, which was found to be higher in LL. Total sugar, starch, tannins and phenols doesn't showed any significant variation in both accessions. Concentration of asiatic acid, asiaticoside and made cassoside found in SL accession were 0.04%, 0.34% and 0.38% respectively, while in LL it was 0.05%, 0.31% and 0.31% respectively. Thus, showing closely similar quantity of metabolites in both the morphotypes. **Conclusion:** It can be concluded that the leaf size is not a deciding factor for the concentration of secondary metabolites present in a plant. Reported data will contribute to the establishment of knowledge about the triterpenoidal saponin composition of different morphotypes of C. asiatica found in Indo-Gangetic plains of India and lays a foundation for future studies on Chemotypic variations.

Keywords: Asiatic acid, asiaticoside, madecassoside, C. asiatica, chemotype, morphotype.

INTRODUCTION

Centella asiatica (L) Urban (Apiaceae) is claimed to possess various healing effects and antioxidant properties. It has been reported to be used in the treatment of asthma, ulcers, lepsory, vein diseases,[1] memory improvement,[2] antidepressant,[3] antibacterial, antifungal,[4] psoriasis.[5] The medicinal values of this plant are mainly attributed to the presence of triterpenes like asiatic acid, madecassic acid, asiaticoside and madecassoside.[6] Triterpenes being the major components of *C. asiatica*, they have been regarded as its biomarker components.[7] Quantification of triterpenes of *C. asiatica* has been successfully established by several researchers using HPLC-UV[8–10] and HPTLC[11] however, the triterpene components in *C. asiatica* are known to vary depending on its growth, location and the diverse environmental conditions.[12] Analytical studies have shown that *C. asiatica* contains triterpenoids, essential oils and amino acids. The plant contains asiaticoside, centelloside, madecassoside, brahmoside, brahminoside, thankuniside, centellose and terminolic, asiatic, brahmic, centic, centoic, centellic and madecassic acids.[13] A study on *C. asiatica* found that out of two accessions that were evaluated, the accession with larger leaf contained higher amount of triterpenoids.[14] This study was done to check the chemotypic variations of active biomarkers in correlation with morphotypic changes in *C. asiatica* collected from Indo-Gangetic plains of India.

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MATERIALS AND METHODS

Chemicals

Standard asiatic acid, asiaticoside and madecassoside was procured from Sigma-Aldrich (Steinheim). All other chemicals used were from Merck (Germany).

Plant Materials

Whole plants of *C. asiatica* were collected from two locations of Varanasi, India in 2011 and authenticated by Dr. AKS Rawat, NBRI, Lucknow. Voucher specimen (262541; 262542) has been submitted in institute's repository.

Quantitative Microscopical Studies

The leaf microscopic characters like stomatal number, stomatal index, vein islet number and vein termination number were determined. The quantitative determination of some pharmacognostic parameters is useful for setting standards for crude drugs. The vein termination, vein islet, stomatal number and stomatal index parameters determined in the quantitative microscopy, are relatively constant for plants and can be used to differentiate closely related species.[15]

Physicochemical and Phytochemical Studies

Physicochemical and phytochemical studies viz. extractive values, total ash, acid insoluble ash, total sugar, starch, tannin and phenols were estimated from shade-dried and powdered plant material.[16-18]

Preparation of Plant Extracts

Fresh plant material was thoroughly washed with water to remove all debris and then shade dried; the dried material was then powdered using electric grinder at 100 mesh size. Extraction was performed by soxhlation process in two steps. Firstly the powdered material was defatted under soxhlet assembly using 250mL of 98% ether for 6 hours. This is followed by 9 hours soxhlation of defatted powder by using 250mL of methanol as solvent. The final extracts were passed through Whatman No. 1 filter paper. The filtrates obtained were concentrated under vacuum in a rotary evaporator at 40 °C and stored at 4 °C for further use.

HPTLC Studies

Extract as prepared above were redissolved in methanol, filtered and finally made up to100 ml with methanol prior to HPTLC analysis.

Chromatographic Conditions

Chromatography was performed on Merck HPTLC precoated silica gel 60GF_{254} (10X10 cm) plates. Methanolic solution of samples and standard compounds asiatic acid, asiaticoside and madecassoside of known concentrations were applied to the layers as 6 mm-wide bands positioned 10 mm from the bottom and 15 mm from side of the plate, using Camag Linomat V automated TLC applicator with nitrogen flow providing a delivery speed of 150nl/s from application syringe. These conditions were kept constant throughout the analysis of samples. Following sample application, layers were developed in a Camag twin trough glass chamber which was pre-saturated with mobile phase of toluene: ethyl acetate: formic acid (5:5:1) for asiatic acid and n-butanol: ethyl acetate: water (4:1:5) for asiaticoside and madecassoside till proper separation of bands up to 8 cm height. After development, layers were dried with an air dryer. Asiatic acid, asiaticoside and madecassoside were simultaneously quantified using Camag TLC scanner model 3 equipped with Camag Wincats IV software. Following scan conditions were applied: slit width, 6 mm x 0.45 mm; wavelength 600 nm; and absorption-reflection mode. In order to prepare calibration curves, stock solution of asiatic acid, asiaticoside and madecassoside (0.1 mg/ml) was prepared and various volumes of the solution were analyzed through HPTLC, calibration curves of peak area vs. concentration were also prepared.

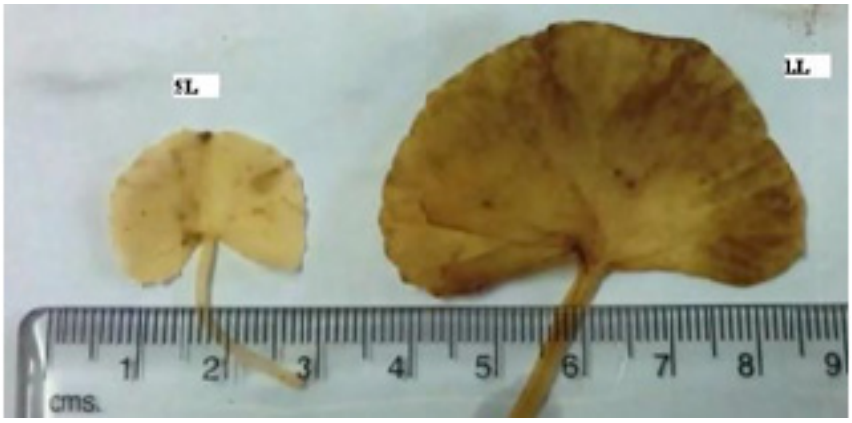

Figure 1: Accessions of *C. asiatica* Leaf (SL & LL)

RESULTS AND DISCUSSION

Quantitative Microscopical Studies

Stomatal number, stomatal index, vein-islet and vein termination number was determined and results are shown in Table 1.

Physicochemical Studies

Parameters such as extractive values (Water and alcohol soluble), total ash, acid insoluble ash, total sugar, starch, tannins and phenolics were determined and results are shown in Figure 2.

Table 1: Quantitative microscopical parameters of *C. asiatica* accessions

Accession	V.T	V.I	S.I (lower)	S.I (upper)	S.N (lower)	S.N (upper)
SL	3.0-7.0	2.0-5.0	18.81-20.16	8.0-11.0	100-175	50-75
LL	4.0-8.0	2.0-6.0	19.96-22.45	9.0-11.0	125-225	75-100

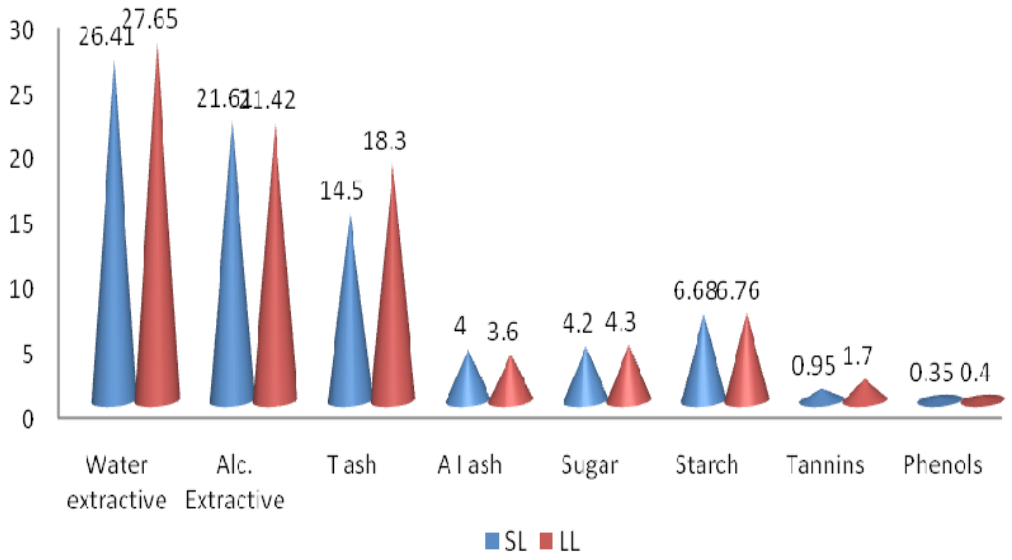

Figure 2: Physicochemical parameters of *C. asiatica*

HPTLC Studies

The calibration plots were obtained after densitometric analysis of the three authentic standards i.e., asiatic acid, asiaticoside and madecassoside. Chromatographic details are shown in Table 2. Quantification of asiatic acid, asiaticoside and madecassoside in the samples of *C. asiatica* has been performed and shown in Table 3. HPTLC banding pattern and densitograms obtained from extracts are shown in Figure 3.

Table 2: Chromatographic details

Parameters	Asiatic acid	Asiaticocide	Medecassoside
Rf	0.51±0.005	0.44±0.01	0.33±0.01
Linearity range	100-1000 ng	100-1000 ng	100-1000 ng
Regression via area	y=436.218+6.020*x	y=638.777+6.375*x	y=164.551+3.504*x
r	0.999	0.992	0.998
Slope	6.020	6.375	3.504
Intercept	436.218	638.777	164.551
LOD	40 ng	40 ng	40 ng
LOQ	100 ng	100 ng	100 ng

Table 3: Concentration of different markers in *C. asiatica* samples

Markers	R^2	R_f	Amount Quantified (%) in Crude Drug	
			SL	LL
Aisatic acid	0.98	0.51±0.004	0.04±0.00	0.05±0.01
Asiaticoside	0.99	0.44±0.005	0.34±0.03	0.31±0.04
Madecassoside	0.99	0.33±0.004	0.38±0.02	0.31±0.05

Each percentage value is an average of six values±SD

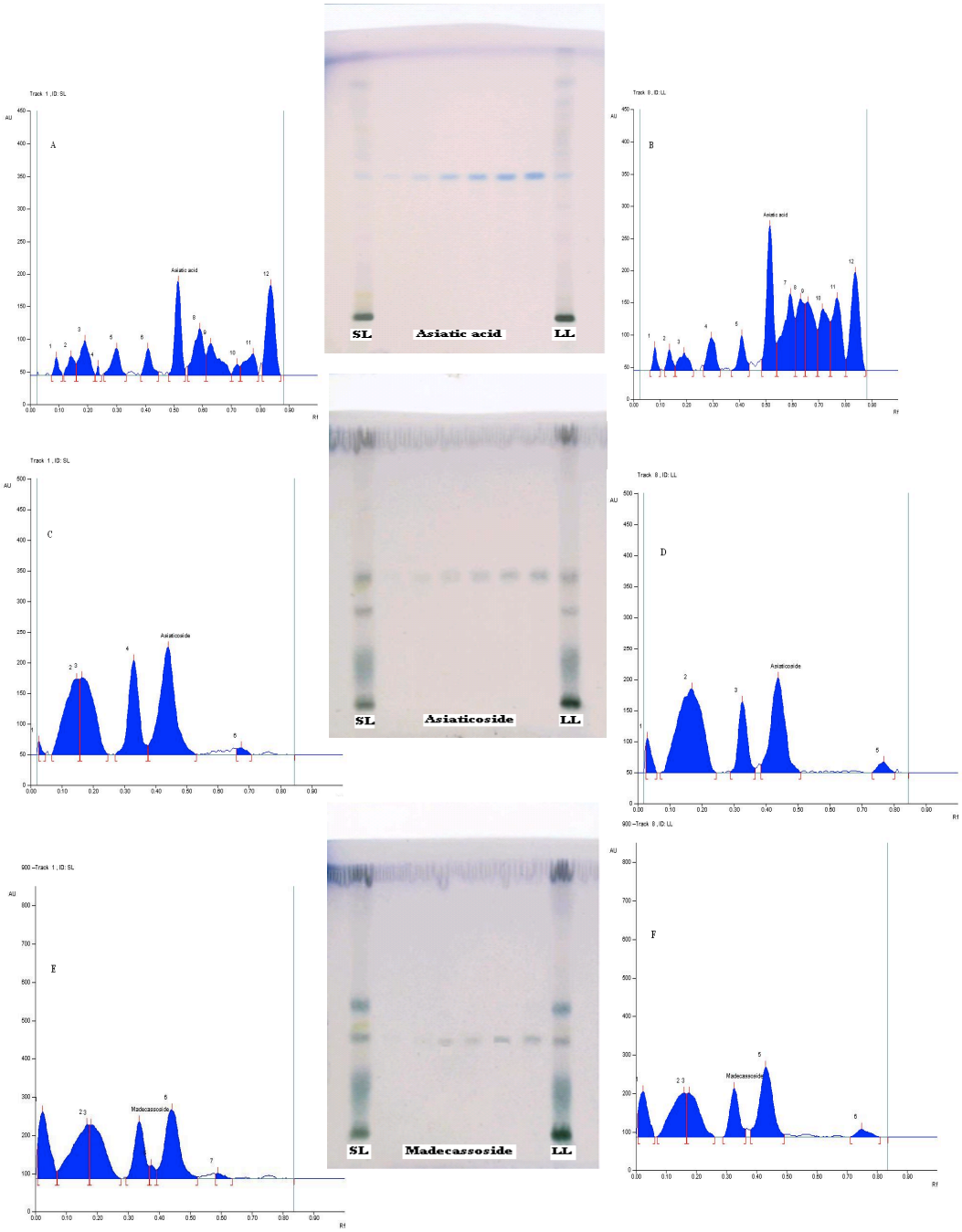

Figure 3: HPTLC Profile and Densitogram showing C. asiatica extracts-SL & LL with Asiatic acid (A & B), Asiaticoside (C & D) and Madecassoside (E & F)

CONCLUSION

There was no marked variation found in botanical descriptors, physicochemical parameters as well as in bioactive metabolites asiatic acid, asiaticoside and madecassoside quantified through HPTLC, this leads to a conclusion that morphotypic variations does not always lead to chemotypic variations in *C. asiatica*. The presence of asiatic acid, asiaticoside and madecassoside quantified in these accessions may be utilized for the proper identification of elite chemotypes of the drug. The concentrations of asiatic acid, asiaticoside and madecassoside showed no significant variation in both accessions. It can be concluded that the leaf size is not a deciding factor for the concentration of secondary metabolites present in a plant. This finding is however different from a similar work published earlier.[14] This may be due to the different determination methods and parameters used by those workers. Reported data will contribute to the establishment of knowledge about the triterpenoid saponin composition of different morpho-types of *C. asiatica* found in Indo-Gangetic plains of India and lays a foundation for future studies on chemo-typic variations of *C. asiatica*.

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CONFLICT OF INTEREST

The authors declare no conflict of interest

ABBREVIATIONS

SL: Small Leaf; LL: Large Leaf; S.N: Stomatal Number; S.I: Stomatal Index; V.T: Vein Termination Number; V.I: Vein Islet Number; HPTLC: High Performance Thin Layer Chromatography

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