Centellosides in *Centella Asiatica*: Bioconversion (Ratios), Elite Lines, Biomarkers

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Abstract

Centellosides *viz.*, asiatic acid (ASA), madecassic acid (MDA), asiaticoside (ASI), madecassoside (MAD), are the major bioactive molecules in *Centella asiatica* (CA). In this study MDA:ASA, MAD:ASI (C6-hydroxylation *versus* non-hydroxylation) and ASI:ASA, MAD:MDA (C28-glycoside *versus* aglycone) ratios in 50 CA accessions originally collected from wild locations in south India and grown under identical ecological conditions for six generations were determined using validated HPTLC-densitometry protocol(s). ASA, MDA, ASI and MAD ranged from 0.01-0.29% (average $0.06 \pm 0.08\%$, $n = 22$; 28 accessions recorded ASA content as zero), 0.02-0.72% ($0.12 \pm 0.13\%$), 0.04-2.41% ($0.44 \pm 0.52\%$) and 0.15%-5.27% ($1.59 \pm 1.26\%$, $n = 50$), respectively. Distinctly, C6-hydroxylated (MDA:ASA 4.00, MAD:ASI 3.61) and C28-glycosylated (ASI:ASA 7.33, MAD:MDA 13.25) centellosides dominated over the respective non-derivatized entities. Our results infer that both C6-hydroxylation by P450-dependent monooxygenases and C28-glycosylation by UDP-Glc glucosyltransferases (UGTs) are dominant biosynthetic steps in CA. This study also discovered six elite clones of CA from south India with their (ASI+MAD) contents above the industrial benchmark ($\geq 4\%$). Two elite clones with ASI contents $\geq 2\%$ were also identified. These elite lines can be multiplied and utilized for the industrial applications of CA. Besides, this study emphasizes the need for standardizing all four centellosides as biomarkers in CA raw drugs, pharmaceutical and cosmetic products.

Introduction

Centella asiatica (L.) Urban (CA, family Apiaceae) is a pharmaceutically important medicinal herb, known for its neuroprotective, memory enhancing, cardioprotective, antioxidant, anti-inflammatory, anticancer and wound healing activities^{1,2}. India and Madagascar are two major geographical sources of CA. So far, nearly 125 secondary metabolites were isolated from CA. The most prominent CA metabolites are the major centellosides (saponins: asiaticoside (ASI), madecassoside (MAD); sapogenins: asiatic acid (ASA), madecassic acid (MDA)) (**Scheme 1**). Centellosides are triterpene (6 isoprene units) saponins/sapogenins bearing an ursane or oleanane skeleton. The four major CA centellosides *viz.*, ASA, MDA, ASI, MAD are ursane-type triterpenes/glycosides. Of these MDA and MAD have hydroxyl (-OH) substitutions (hydroxylation) at C6 instead of the (-H) in ASA and ASI (**Scheme 1**). In both ASA and MDA, C28s are linked to -OH groups and the modifications in the saponins (ASI, MAD) are three identical sugar units (-Glu-Glu-Rha), and they are formed by glycosylation (at the C28s) of these aglycones³. The molecular formulae and mol. weights of CA aglycones and glycosides are ASA C₃₀H₄₈O₅, 488.70 Da, MDA C₃₀H₄₈O₆, 504.71 Da, ASI C₄₈H₇₈O₁₉, 959.12 Da and MAD C₄₈H₇₈O₂₀, 975.12 Da.

Studies on the biosynthesis of centellosides are gradually evolving³⁻¹². The triterpenoid saponin skeletons in plants, which include oleanane, ursane, lupane and dammarane types, are synthesized *via* the isoprenoid pathway through farnesyl diphosphate (FPP) followed by cyclization of 2,3-oxidosqualene by 2,3-oxidosqualene cyclase (OSC). In CA, the genes involved in the main pathway to triterpenoid formation include farnesyl diphosphate synthase (*CaFPS*), squalene synthase (*CaSQS*), oxidosqualene synthase (*CaOSQs*) and the putative β -amyrin synthase (*CabAs*), an oxidosqualene cyclase, subsequently

identified as a dammarenediol synthase (*CaDDs*)^{4,8,9}. Modifications to the triterpenoid backbone include oxidation, substitution or glycosylation by enzymes such as P450-dependent monooxygenases and UDP-Glc glucosyltransferases (UGTs). The aglycones (sapogenins) undergo oxidation at various positions in the C30 triterpenoid skeleton through P450 monooxygenase enzymes^{13,14}. CYP716A53v2 is reported to hydroxylate the C6 of the dammarane-type tetracyclic sapogenin, protopanaxadiol, in *P. ginseng* to form protopanaxatriol¹⁵. In CA, a recent study reported the conversion of oleanane and ursane pentacyclic triterpenoid skeletons to their 6 β -hydroxy derivatives by CYP716 enzymes^{13,16}. P450s and their functional roles in plants are least explored so far. UGTs are pivotal enzymes in the process of glycosylation in plants, contributing to the biosynthesis of medically important secondary metabolites. In saponin biosynthesis, UGT's catalyze the transfer of UDP linked sugar moieties to the triterpenoid skeleton^{4,9,17}. Glycosides, ASI and MAD, in CA are formed by glycosylation of ASA and MDA, respectively, catalyzed by UDP-glucosyltransferases (UGTs) which first link a Glu to the C28 carboxyl group followed by a second glycosylation and a consecutive rhamnosylation^{3,4,12}. Glycosylation and hydroxylation change the physicochemical properties and enhance the biological potentials of triterpenoids^{12,18,19}. In ASI and MAD, the triterpenoid structures (aglycone) are hydrophobic and are linked to hydrophilic sugar chains (glycone)^{12,18-20}. The surface-active properties of saponins (*sapo* (Latin) = soap; soap-like surfactants that form long-lasting bubbles on shaking an aqueous solution; glycosides: ASI, MAD) are distinguishing factors of these amphiphilic compounds from other glycosides. This is of considerable significance in drug design, and in CA, the glycosylated entities (ASI, MAD) are the prime target molecules in neuroprotection, memory enhancing, wound healing and skin protection.

Pentacyclic triterpenes (centellosides) are accumulated in CA in their glycoside (ASI, MAD) forms rather than as aglycones (ASA, MDA), and the glycoside to aglycone ratio influences the efficacy of CA extracts and its pharmaceutical and skin care products²¹⁻²³. Previous studies quantified the four centellosides (ASA, MDA, ASI, MAD) in CA by various analytical techniques^{20,21,24-26}. A few studies reported the quantification of only one or two (not all four) of these centellosides in CA (examples, Devkota et al., 2010²⁷; Thomas et al., 2010²⁸; Prasad et al., 2013²⁹). But, these quantification studies are on limited number of samples from various genetic/ecological origins. Genetic and environmental parameters significantly affect the production of secondary metabolites in plants, *viz.*, centellosides in CA^{20,26,28,30-32}. Therefore, the C6-hydroxylation (MDA, MAD) *versus* non-hydroxylation (ASA, ASI) and the resultant C28-glycoside (ASI, MAD) *versus* aglycone (ASA, MDA) ratios (**Scheme 1**) in CA are determined by the variations in the genes involved in their biosynthesis, and to a lesser extent by the ecological parameters^{4,5,20}. Otherwise, genetically these ratios depend on the presence and activity of enzymes involved in C6-hydroxylation and C28-glycosylation²⁰. In CA cell cultures, high production of centellosides is achieved by growth regulators and elicitors (examples, methyl jasmonate, salicylic acid), and they presumably modulate the expression of certain genes involved in their biosynthesis¹⁰. CA cell cultures are also capable of converting precursors like α -amyrin into centellosides with very high efficiency⁶. Plant cell cultures are able to carry out regio- and stereoselective hydroxylation, hydrogenation, glycosylation etc. of exogenous substrates, biotransforming them into other compounds with improved pharmacological

actions¹⁰. Therefore, the C6-hydroxylation *versus* non-hydroxylation and C28-glycoside *versus* aglycone ratios in CA cell cultures are influenced by the biotransformations induced by their growth conditions.

Here, we explore the ratios of the four major centellosides *viz.*, ASA, MDA, ASI, MAD, biosynthesized through C6-hydroxylation and/or C28-glycosylation (**Scheme 1**) in 50 CA accessions originally collected from wild locations in south India and grown under identical ecological conditions for six generations. The study is conceived to derive biosynthetic clues on the four centellosides by nullifying their ecological variations. We also address the significance of using the four centellosides as biomarkers in CA extracts and products. Moreover, these 50 CA accessions under study are scrutinized for elite lines based on industrial benchmarks of the contents of centellosides.

Materials And Methods

Chemicals and reagents

ASI (≥ 98.5 % HPLC), MAD (≥ 95 % HPLC) and ASA (≥ 98 % HPLC) were purchased from Sigma Aldrich (St. Louis, Missouri, US). MDA (≥ 96 %) was procured from Santa Cruz Biotechnology (Santa Cruz, Dallas, USA). Silica gel TLC plates (60 F₂₅₄, 20 x 10 cm, 0.2 mm thickness) were obtained from E. Merck, Germany. Solvents used for the HPTLC analysis were of analytical or HPLC grade.

Collection of plant materials, growing conditions

Fifty CA accessions were collected from its wild populations in various agro-climatic regions of Kerala, Tamil Nadu and Karnataka (**Table 1**), taxonomically authenticated by Dr. Mathew Dan, Senior Scientist of Jawaharlal Nehru Tropical Botanic Garden and Research Institute (JNTBGRI), Thiruvananthapuram and a voucher specimen (91008) is preserved at JNTBGRI Herbarium (TBGT) for future reference. These CA collections were made as part of one of the Programme Support projects hosted by JNTBGRI and funded by Department of Biotechnology (DBT), Government of India. All CA accessions were maintained under identical environmental conditions in the Field Gene Bank (FGB) of JNTBGRI for a minimum of three years. For the phytochemical analysis, aerial parts of six generation vegetatively propagated plants were collected in May 2019 and dried in an oven at 40°C and powdered.

Extraction of plant materials

CA plant powder (0.5 g each) was extracted in 50 ml methanol by refluxing for 90 min (16.5 ml x 30 min x 3), extracts were pooled together and concentrated to 5 ml using rotary vacuum evaporator (Buchi, Switzerland) at 40°C.

HPTLC-densitometry analysis, calibration of centellosides

Quantitative analyses of four major centellosides (ASI, MAD, ASA and MDA) were carried out by HPTLC-densitometry (CAMAG, Switzerland) made of an automatic Linomat V sample applicator, CAMAG twin trough plate development chamber, CAMAG TLC Scanner 3, Reprostar 3 and WinCATS software 4.03.

Stock solutions (1 mg/ml) of the standards, MAD, ASI, MDA and ASA, were prepared in methanol; ASI and MAD were applied in the range of 0.1-0.8 µg/band and 0.2-2.0 µg/band, respectively, and ASA and MDA were spotted in the range 0.05-1.5 µg/band and their calibration plots were generated by plotting against respective peak area(s). Amounts of ASI, MAD, ASA and MDA in CA extracts were determined by means of these calibration plots (**Table 2**).

Quantitative analysis of ASA and MDA

CA extracts (6 µl each) were applied onto silica gel TLC plates (60 F₂₅₄, 20 x 10 cm, 0.2 mm thickness) as 8 mm wide bands (7 tracks) with the automatic Camag Linomat V sample applicator, fitted with a Camag microsyringe under N₂ flow (application rate - 150 nl/s, space between two bands - 12.1 mm, slit dimension - 6.00 mm x 0.45 mm x mm, scanning speed - 20 mm/s). Plates were developed up to 80 mm in a CAMAG twin trough plate development chamber saturated with 20 ml of toluene:ethyl acetate:formic acid (4:5:1, v/v). Peaks of ASA and MDA were well resolved in this mobile phase. Plate(s) were derivatised by spraying with anisaldehyde-sulphuric acid reagent, heated at 110°C in a hot air oven for 5 min, scanned at 570 nm (tungsten lamp) using Camag TLC Scanner 3 and photo documented using Reprostar 3.

Quantitative analysis of ASI and MAD

CA extracts (2 µl each) were applied onto silica gel TLC plates as described in above section. Plates were developed up to 80 mm in a CAMAG twin trough plate development chamber previously saturated with 20 ml organic layer of butanol:ethyl acetate:water (4:1:5, v/v) for 30 min. The twin peaks of ASI and MAD in CA extracts were well resolved in this mobile phase. The plates were derivatised and photo documented as mentioned above.

Validation methods

HPTLC method was validated in terms of accuracy, precision, repeatability, reproducibility, linearity, limits of detection (LOD), limits of quantification (LOQ) and % recovery³³⁻³⁵. Calibration curves were generated by plotting amounts of analytes (standards: ASA, MDA, ASI, MAD) against peak response(s) (**Table 2**). Intra-day precision was performed by repeating the same assay four times on the same day (of each standard). Inter-day precision was performed by repeating the assay twice for consecutive five days. Recovery of ASI, MAD, MDA and ASA was carried out using standard addition method. Three different concentrations of ASA (0.1, 0.2, 0.3 µg), MDA (0.1, 0.2, 0.3 µg), ASI (0.25, 0.50, 0.75 µg) and MAD (0.25, 0.50, 0.75 µg) were added to pre-analyzed CA extracts and re-analyzed (**Table 3**). Instrumental precision was determined by applying a sample solution (CA extract, ASA and MDA 6 µl each, ASI and MAD 2 µl each,) on a TLC plate, developed as per the protocol mentioned earlier, track(s) were scanned repeatedly (ten times each) and % coefficient(s) of variations were determined (**Table 4**).

Results

HPTLC-densitometry, validation. Fifty CA accessions were collected from the various agro-climatic regions of the south Indian states *viz.*, Kerala, Tamil Nadu and Karnataka, and maintained in a Field Gene Bank (FGB) for three years. These six generation vegetatively propagated CA specimens grown under identical environmental conditions ensured that the variations in their chemical profiles are genetically determined and are not influenced by environmental factors (**Table 1**). Mobile phases for the quantification of aglycones (ASA, MDA) and glycosides (ASI, MAD) were optimized as toluene:ethyl acetate:formic acid (4:5:1, v/v) and organic layer of butanol:ethyl acetate:water (4:1:5, v/v), respectively. R_f values of aglycones ASA and MDA were 0.45 ± 0.02 ($n = 22$) and 0.37 ± 0.02 ($n = 50$), respectively. Similarly, R_f values of ASI and MAD in CA extracts were 0.37 ± 0.02 ($n = 50$) and 0.47 ± 0.01 ($n = 50$), respectively. The typical HPTLC chromatograms of CA methanol extracts showed well resolved twin peaks of MDA and ASA (**Fig. 1A**) and MAD and ASI (**Fig. 1B**). The quantitative estimation data of the four centellosides are shown in **Table 1**. HPTLC-densitometry method was validated in terms of linearity, precision, accuracy, reproducibility, limits of detection (LOD), limits of quantification (LOQ) and % recovery. Reproducibility was assessed by the repeated application of standards, (ASI, MAD, ASA and MDA), and their R_f values were reproducible and same as the values observed in CA extracts. Linearity was analyzed using calibration curves of each analyte (ASA: 0.05 - 1.25 $\mu\text{g}/\text{band}$, MDA: 0.05 - 1.25 $\mu\text{g}/\text{band}$, ASI: 0.1 - 0.8 $\mu\text{g}/\text{band}$, MAD: 0.2 - 2.0 $\mu\text{g}/\text{band}$). **Table 2** represents the regression equations, correlation coefficients (R^2), limits of detection (LODs) and limits of quantification (LOQs) of standards. On repeated measurements 97.56-98.91% (ASA), 96.61-97.44% (MDA), 98.93-100% (ASI) and 98.69-100% (MAD) recoveries were observed for the standards (**Table 3**). Instrumental precision was determined by scanning the track(s) repeatedly (ten times each) and the % coefficient(s) of variation were acceptable (ASA 1.13, MDA 0.88, ASI 0.81, MAD 0.47) (**Table 4**).

Quantification of centellosides. Notably, of the 50 CA accessions, 28 recorded their ASA contents as zero or below the detectable level. Average ASA content in the rest of the 22 CA accessions was $0.06 \pm 0.08\%$ ($n = 22$), with the lowest and highest contents as 0.01% (Ca 13, Ca 15, Ca 22, Ca 33, Ca 41, Ca 44, Ca 92) and 0.29% (Ca 56), respectively (**Table 1**). The lowest MDA content was 0.02% (Ca 42, Ca 90) and highest 0.72% (Ca 56), and average $0.12 \pm 0.13\%$ ($n = 50$). MAD content ranged from 0.15% (Ca 124) to 5.27% (Ca 48), and average MAD content was $1.59 \pm 1.26\%$ ($n = 50$). Similarly, the lowest ASI content was displayed by Ca 124 (0.04%) and highest by Ca 47 (2.41%); and the average ASI content was $0.44 \pm 0.52\%$ ($n = 50$) (**Table 1**). The C6-hydroxylation:non-hydroxylation ratios in 50 CA accessions were MDA:ASA 0.12:0.03 (4.00, $n = 50$, including the 28 accessions with zero content) and MAD:ASI 1.59:0.44 (3.61, $n = 50$). Likewise, the C28-glycoside:aglycone ratios in these 50 CA accessions *viz.*, ASI:ASA and MAD:MDA were 0.44:0.06 (7.33) and 1.59:0.12 (13.25), respectively (**Scheme 1, Table 1**).

Biosynthetic clues. In CA, MDA and MAD are formed by hydroxylation at the C6 position (controlled by P450-dependent monooxygenases) of ASA and ASI, respectively. MAD also could be formed by the C6-hydroxylation and C28-glycosylation of ASA (**Scheme 1**). ASI and MAD in CA are formed by C28-glycosylation of ASA and MDA, respectively, (**Scheme 1**) catalyzed by UGTs which links two glucose units and one rhamnose unit to the C28 carboxyl group^{3,4,12}. In this study, we quantitatively estimated the four

major centelloses in 50 CA accessions, and all these accessions showed significant variability in their contents, and the average C28-glycoside:aglycone ratios ASI:ASA and MAD:MDA are 0.44:0.06 (7.33, n = 50) and 1.59:0.12 (13.25, n = 50), respectively (**Table 1**). The C6-hydroxylation:non-hydroxylation ratios in 50 CA accessions are MDA:ASA 0.12:0.03 (4.00, n = 50), MAD:ASI 1.59:0.44 (3.61, n = 50) (**Table 1**).

Elite lines. In this study, of 50 south Indian accessions, ASI, MAD and (ASA+ASI+MDA+MAD) contents ranged from 0.04-2.41%, 0.15-5.27% and 0.38-7.43%, respectively. Industries consider CA accessions (aerial parts) with biomarker (ASI+MAD) content $\geq 4.0\%$ as elite lines, and in this study, six accessions are satisfying this benchmark, viz., Ca 51 (7.23%), Ca 47 (7.02%), Ca 52 (6.83%), Ca 48 (5.98%), Ca 56 (5.64%) and Ca 55 (4.04%) (**Table 1**). Another industrial benchmark (CA aerial parts) is the biomarker (ASI) content $\geq 2.0\%$, and two accessions, viz., Ca 47 (2.41%) and Ca 51 (2.13%), out of these 50 accessions are satisfying this criterion. Ca 47 and Ca 51 are fulfilling both the industrial benchmarks for elite lines of CA (**Table 1**).

Discussion

The four major centelloses in CA viz., 2 aglycones (ASA, MDA) and 2 glycosides (ASI, MAD), were quantified by various authors using HPLC, HPTLC, LC-MS and other techniques. In an early study, the contents of the 4 major centelloses were quantified by HPLC in five CA accessions grown at different locations in Germany (4) and India (1) (all 5 accessions were originally from India)³⁶. Glycosides, ASI and MAD contents ranged from (0.18-0.52%) and (0.74-4.02%) and aglycones, ASA and MDA varied from (0.14-0.49%) and (0.53-0.80%), respectively. Average ASI, MAD, ASA and MDA contents were 0.37%, 1.92%, 0.24%, 0.67%. Overall, C6-hydroxylation:non-hydroxylation (MDA:ASA and MAD:ASI) ratios were 0.67:0.24 (2.79) and 1.92:0.37 (5.19) and C28-glycoside:aglycone ratios ASI:ASA and MAD:MDA were 1.54 and 2.87. The total centelloside contents ranged from 1.98-5.26%³⁶. Again, Schaneberg and co-workers (2003) analyzed ASI, MAD, ASA and MDA in extracts of three CA accessions obtained from Boulder (USA), Norway, (USA) and Sri Lanka by HPLC. The average contents of ASI, MAD, ASA and MDA were 0.64%, 0.88%, 0.13% and 0.28%, respectively (MDA:ASA 2.15; MAD:ASI 1.38 & ASI:ASA 4.92; MAD:MDA 3.14)²⁵. Randriamampionona and co-workers (2007) quantified the four centelloses in seven CA leaf samples collected from various habitats in Madagascar using HPLC, and the average ASI, MAD, ASA and MDA contents were 3.85%, 3.63%, 0.30% and 0.28% (dry wt.) (MDA:ASA 0.93; MAD:ASI 0.94 & ASI:ASA 12.83; MAD:MDA 12.96)²⁶. Rafamantanana et al., 2009 analyzed three CA samples collected from the East and High Plateau regions of Madagascar using HPLC. Average ASI, MAD, ASA and MDA contents were 1.79%, 1.54%, 0.85% (n = 2) and 0.84% (n = 2) (dry wt.) (MDA:ASA 0.99; MAD:ASI 0.86 & ASI:ASA 2.11; MAD:MDA 1.83). One CA sample showed below detectable levels of ASA and MDA²¹. Again, ASI (0.21%), MAD (0.18%), ASA (0.46%) and MDA (0.31%) (dry wt.) contents in CA leaves obtained from Bad Grund, Germany were quantified by Raj and Kielisz (2019) by HPTLC-densitometry (MDA:ASA 0.67; MAD:ASI 0.86 & ASI:ASA 0.46; MAD:MDA 0.58)³⁷.

Randriamampionona and co-workers (2007) also reported the average ASI, MAD, ASA and MDA contents in *in vitro* propagated CA leaves as 1.06%, 0.84%, 0.14% and 0.14% (n = 5) (dry wt.) (MDA:ASA 1.00; MAD:ASI 0.79 & ASI:ASA 7.57; MAD:MDA 6.00)²⁶. James and co-workers determined the average ASI, MAD, ASA and MDA contents in two *in vitro* propagated CA leaf samples by HPLC as 4.88%, 4.52%, 1.84%, 1.93% (dry wt.) (MDA:ASA 1.05; MAD:ASI 0.93 & ASI:ASA 2.65; MAD:MDA 2.34)²⁰. Prasad and co-workers (2012) quantified the average ASI, MAD, ASA and MDA contents in hydroponically grown (42nd day of growth) CA whole plants by HPLC as 0.17%, 1.10%, 0.63%, 3.66% (dry wt.) (MDA:ASA 5.81; MAD:ASI 6.47 & ASI:ASA 0.27; MAD:MDA 0.30). In these hydroponically grown plants the glycoside contents are low compared to the aglycones³⁸. Fourteen CA accessions grown under identical glass house conditions minimizing the effect of environmental parameters were analyzed for their centellosides by HPLC and found average ASI, MAD, ASA and MDA contents as 0.17% (n = 11), 0.10% (n = 11), 0.05% (n = 13), 0.14% (n = 14) (fresh wt.) (MDA:ASA 2.80; MAD:ASI 0.59 & ASI:ASA 3.4; MAD:MDA 0.71)³¹. In another study, Prasad and co-workers (2016) estimated the average ASI, MAD, ASA and MDA contents in three sets of *in vitro* cultures developed on the basis of an artificial neural network-based model by HPLC as 0.12%, 0.05%, 0.86%, 2.03% (dry wt.) (MDA:ASA 2.36; MAD:ASI 0.42 & ASI:ASA 0.14; MAD:MDA 0.02)³².

In these quantification studies, the number of CA samples is limited, and they were originally from markedly varying environmental (wild locations)^{21,25,26,36,37} and growth (*in vitro*)^{26,31,32,38} conditions. Along with the genetic origins, the variation in collection (ecological)/growth conditions influence the C6-hydroxylation (MDA:ASA; MAD:ASI) and C28-glycosylation (ASI:ASA; MAD:MDA) reactions^{4,39}. Moreover, the extraction and analytical parameters vary between these studies.

In previous studies, the C6-hydroxylation:non-hydroxylation ratios in wild CA accessions were (MDA:ASA 2.79; MAD:ASI 5.19)³⁶, (MDA:ASA 2.15; MAD:ASI 1.38)²⁵, (MDA:ASA 0.93; MAD:ASI 0.94)²⁶, (MDA:ASA 0.99; MAD:ASI 0.86)²⁰, (MDA:ASA 0.67; MAD:ASI 0.86)³⁷. Similarly, C6-hydroxylation:non-hydroxylation ratios in *in vitro* or controlled conditions were (MDA:ASA 1.00; MAD:ASI 0.79)²⁶, (MDA:ASA 1.05; MAD:ASI 0.93)²⁰, (MDA:ASA 5.81; MAD:ASI 6.47)³⁸, (MDA:ASA 2.80; MAD:ASI 0.59)³¹, (MDA:ASA 2.36; MAD:ASI 0.42)³².

In most of the previous studies, the glycosides are dominating CA tissues compared to the aglycone contents. Glycoside:aglycone ratios (wild accessions) were (ASI:ASA 1.54, MAD:MDA 2.87)³⁶, (ASI:ASA 4.92, MAD:MDA 3.14)²⁵, (ASI:ASA 12.83; MAD:MDA 12.96)²⁶, (ASI:ASA 2.11; MAD:MDA 1.83)²¹. Glycoside:aglycone ratios in *in vitro* or controlled conditions were (ASI:ASA 7.57; MAD:MDA 6.00)²⁶, (ASI:ASA 2.65; MAD:MDA 2.34)²⁰. In a few cases, aglycone contents were relatively high. Glycoside:aglycone ratios (wild accessions): (ASI:ASA 0.46; MAD:MDA 0.58)³⁷. Glycoside:aglycone ratios (*in vitro* or controlled conditions): (ASI:ASA 0.27; MAD:MDA 0.30)³⁸, (ASI:ASA 3.4; MAD:MDA 0.71)³¹, (ASI:ASA 0.14; MAD:MDA 0.02)³².

In the present study, 50 CA accessions collected from various agroclimatic conditions were grown under identical ecological conditions and their C6-hydroxylation:non-hydroxylation (MDA:ASA; MAD:ASI) and C28-glycoside:aglycone (ASI:ASA; MAD:MDA) ratios were analyzed under standardized (identical) parameters. Therefore, these ratios *viz.*, MDA:ASA (4.00, n = 50, including the 28 accessions with zero content), ASI:ASA (7.33, n = 50), MAD:MDA (13.25, n = 50), MAD:ASI (3.61, n = 50), are genetically determined (controlled), and reliable pharmaceutical and biosynthetic clues can be derived from these data. Thereby, our study indicates that the C6-hydroxylation and C28-glycosylation driven by P450-dependent monooxygenases and UDP-Glc glucosyltransferases (UGTs), respectively, are dominant biosynthetic steps in CA.

In all 50 CA accessions, MAD contents are higher than ASI (including the ASI-rich accessions: Ca 47, Ca 51, Ca 52, Ca 55, Ca 56), except in one (Ca 92: ASI 0.55%, MAD 0.36%). Similarly, in our previous study a decade ago²⁸, on 60 CA accessions originally collected from a wide range of locations in south India (as in the present study) and grown under identical ecological conditions for 3 generations, ASI and MAD contents were quantified using similar HPTLC-densitometry protocol and average MAD:ASI ratio was $1.86:0.37 = 3.96$, n = 60 (which is close to our current ratio, 3.61). Only one of these accessions showed higher ASI content (0.80%) compared to MAD (0.29%). Of these 60 CA accessions, one showed absence of both the glycosides (ASI, MAD) and two other accessions showed the absence of ASI²⁸. In another recent study, we screened 106 CA accessions collected from various wild locations in south India (i.e., directly from different ecological conditions) using similar protocol, and the observed MAD:ASI average ratio was $1.22:0.55 = 2.71$ (unpublished data). This ratio (2.71) is considerably different from the two screening studies *viz.*, current study 3.61, Thomas and co-workers (2010) 3.96^{28} , under identical ecological conditions. In these 106 CA accessions, 6 showed higher ASI contents compared to MAD levels (unpublished data). These extensive data (under identical and varying ecological conditions) demonstrate MAD as the prominent constituent of the four centellosides in CA. This is clearly supporting the bioconversion possibilities portrayed in **Scheme 1**.

On further evaluation of the data, among the four major centellosides in CA, ASA has three biosynthetic conversion probabilities, *viz.*, ASA to MDA (C6-hydroxylation), ASA to ASI (C28-glycosylation) and ASA to MAD (C6-hydroxylation + C28-glycosylation) (**Scheme 1**), whereas MDA and ASI have only one (each) possibility of getting converted to MAD by C28-glycosylation and C6-hydroxylation, respectively. In 50 CA accessions under identical environmental conditions, the average contents of ASA, MDA, ASI and MAD are 0.03, 0.12, 0.44 and 1.59%, respectively. As anticipated, ASA (aglycone) which has highest probability of enzymatic conversion(s), showed the lowest content in CA. MDA (aglycone) % content is 4 times that of ASA, and the highest content was displayed by MAD (1.59%) which has no further conversion prospects (**Scheme 1**). The average content of MAD (1.59%, n = 50) is 2.69 times the total average contents of the other three centellosides (ASA+MDA+ASI = 0.59%, n = 50). These data are inferring high rates (probabilities) of one or more transformations of the three centellosides as depicted in **Scheme 1**. The enzymes involved, sequence of these hydroxylation/glycosylation reactions, reaction rates etc. are to be further established by biosynthetic studies.

ASI and (ASI+MAD) have been widely assigned as the major biomarkers for quality evaluation of CA raw drugs, pharmaceutical and cosmetic products^{23,28}. But the four centellosides, ASA, MDA ASI and MAD, exert varying effects in the biological (neuroprotection, wound healing, skin protection) activities of CA, and thereby display disproportionate influences (roles) in its industrial applications. In our study, CA accessions with their ecological variations nullified (and only the genetic traits governing centelloside biosynthesis), showed considerable fluctuations (even absence) in their ASA, ASI, MDA and MAD contents. Therefore, efficient quality control practices of CA raw drugs warrant the quantification of a set of major triterpenoids (four centellosides) as biomarkers²³.

This study also discovered six elite clones of CA from south India with their (ASI+MAD) contents above the industrial benchmark ($\geq 4\%$). Two elite clones with ASI contents $\geq 2\%$ were also identified. The agricultural practices of these CA elite lines can be standardized and utilized for the industrial purposes.

Conclusion

In summary, the high demand of CA is leading to its overexploitation at an uncontrolled rate and destruction of its wild genotypes. Our study discovered six elite lines of CA from south India, and these high yielding genotypes can be multiplied and utilized for its pharmaceutical and cosmetic purposes. The four major centellosides *viz.*, ASA, ASI, MDA and MAD, have uneven effects on the bioactivities of CA based extracts/drugs. In this study, ASA was below detectable levels in 28 of the 50 screened CA accessions. These facts emphasize the need for quantifying the contents of all the four centellosides (as biomarkers) in CA extracts, pharmaceutical and cosmetic products. In an ecologically uniform environment, 50 CA accessions showed C28-glycosylation (ASI:ASA and MAD:MDA) ratios as 7.33 and 13.25, respectively, displaying relatively high contents of glycosides (MAD, ASI) in them. Similarly, the C6 hydroxylation (MDA:ASA and MAD:ASI) ratios in 50 CA accessions were 4.00 (n = 50), 3.61 (n = 50). Consequently, our data on 50 CA accessions grown under identical ecological conditions infer that both C6-hydroxylation driven by P450-dependent monooxygenases and C28-glycosylation by UDP-Glc glucosyltransferases (UGTs) are dominant biosynthetic steps in CA. Further studies are required to unravel the specific enzymes and enzymatic processes involved in these biosynthetic steps in CA.

Abbreviations

ASA	Asiatic acid
ASI	Asiaticoside
CA	<i>Centella asiatica</i>
CYP	Cytochrome P450
MDA	Madecassic acid

MAD	Madecassoside
Glu	Glucose
HPLC	High performance liquid chromatography
HPTLC	High performance thin layer chromatography
Rha	Rhamnose
UGT	UDP-Glc glucosyltransferase
LOD	Limit of detection
LOQ	Limits of quantification

Declarations

Data availability

All data generated or analyzed during this study are included in this article.

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Author contributions

R.K.¹ and S.B. developed the concept; R.K.¹, R.K.² and A.J.J. carried out the chemical and data analyses; S.B. interpreted the data and wrote the manuscript with inputs from R.K.¹, R.K.² and A.J.J.

Competing interests

The authors declare no competing interests.

References

1. Kunjumon, R. *et al.* Madecassoside encapsulated in alginate chitosan nanoparticles exerts anti-excitotoxicity effects in pilocarpine-induced seizure. *Phytomedicine Plus*. **1**, 100004 (2021).
2. Sun, B. *et al.* Therapeutic potential of *Centella asiatica* and its triterpenes: a review. *Front. Pharmacol.* **11**, 568032 (2020).

3. Kim, O. T., Jin, M. L., Lee, D. Y. & Jetter, R. Characterization of the asiatic acid glucosyltransferase, UGT73AH1, involved in asiaticoside biosynthesis in *Centella asiatica* (L.) Urban. *Int. J. Mol. Sci.* **18**, 2630 (2017).
4. Haralampidis, K., Trojanowska, M. & Osbourn, A. E. Biosynthesis of triterpenoid saponins in plants. *Adv. Biochem. Eng. Biotechnol.* **75**, 31–49 (2002).
5. Kim, O. T. *et al.* Enhanced production of asiaticoside from hairy root cultures of *Centella asiatica* (L.) Urban elicited by methyl jasmonate. *Plant Cell Rep.* **26**, 1941–1949 (2007).
6. Hernandez-Vazquez, L. *et al.* Conversion of alpha-amyrin into centellosides by plant cell cultures of *Centella asiatica*. *Biotechnol. Lett.* **32**, 315–319 (2010).
7. Bonfill, M., Mangas, S., Moyano, E., Cusido, R. M. & Palazón, J. Production of centellosides and phytosterols in cell suspension cultures of *Centella asiatica*. *Plant Cell Tiss. Organ Cult.* **104**, 61–67 (2011).
8. Gallego, A. *et al.* Biotechnological production of centellosides in cell cultures of *Centella asiatica* (L.) Urban. *Eng. Life Sci.* **14**, 633–642 (2014).
9. de Costa, F. *et al.* Molecular cloning of an ester-forming triterpenoid: UDP-glucose 28-O-glucosyltransferase involved in saponin biosynthesis from the medicinal plant *Centella asiatica*. *Plant Sci.* **262**, 9–17 (2017).
10. Hidalgo, D. *et al.* An optimized biotechnological system for the production of centellosides based on elicitation and bioconversion of *Centella asiatica* cell cultures. *Eng. Life Sci.* **17**, 417–419 (2017).
11. Prasad, A., Mathur, A. K. & Mathur, A. Advances and emerging research trends for modulation of centellosides biosynthesis in *Centella asiatica* (L.) Urban - A review. *Ind. Crops Prod.* **141**, 111768 (2019).
12. Rahimi, S. *et al.* Triterpenoid-biosynthetic UDP-glycosyltransferases from plants. *Biotechnol. Adv.* **37**, 107394 (2019).
13. Miettinen, K. *et al.* The ancient CYP716 family is a major contributor to the diversification of eudicot triterpenoid biosynthesis. *Nature Commun.* **8**, 14153 (2017).
14. Seki, H., Tamura, K. & Muranaka, T. P450s and UGTs: Key players in the structural diversity of triterpenoid saponins. *Plant Cell Physiol.* **56**, 1463–1471 (2015).
15. Han, J. Y., Hwang, H. S., Choi, S. W., Kim, H. J. & Choi, Y. E. Cytochrome P450 CYP716A53v2 catalyzes the formation of protopanaxatriol from protopanaxadiol during ginsenoside biosynthesis in *Panax ginseng*. *Plant Cell Physiol.* **53**, 1535–1545 (2012).
16. Shang, Y. & Huang, S. Multi-omics data-driven investigations of metabolic diversity of plant triterpenoids. *Plant J.* **97**, 101–111 (2019).
17. Augustin, J. M., Kuzina, V., Andersen, S. B. & Bak, S. Molecular activities, biosynthesis and evolution of triterpenoid saponins. *Phytochemistry*. **72**, 435–457 (2011).
18. Kren, V. & Martinkova, L. Glycosides in medicine: “The role of glycosidic residue in biological activity”. *Curr. Med. Chem.* **8**, 1303–1328 (2001).

19. Křen, V. Glycoside vs. Aglycon: The Role of Glycosidic Residue in Biological Activity; in: Glycoscience. (eds. Fraser-Reid B.O., Tatsuta K. & Thiem J.) 2590–2644(Springer, Berlin, 2008).
20. James, J. T., Meyer, R. & Dubery, I. A. Characterisation of two phenotypes of *Centella asiatica* in Southern Africa through the composition of four triterpenoids in callus, cell suspensions and leaves. *Plant Cell Tiss. Organ Cult.* **94**, 91–99 (2008).
21. Rafamantanana, M. H. *et al.* An improved HPLC-UV method for the simultaneous quantification of triterpenic glycosides and aglycones in leaves of *Centella asiatica* (L.) Urb (APIACEAE). *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* **877**, 2396–2402 (2009).
22. Puttarak, P. & Panichayupakaranant, P. Factors affecting the content of pentacyclic triterpenes in *Centella asiatica* raw materials. *Pharm. Biol.* **50**, 1508–1512 (2012).
23. Gajbhiye, N. A., Makasana, J., Saha, A., Patel, I. & Jat, R. S. LC-ESI-MS/MS method for simultaneous determination of triterpenoid glycosides and aglycones in *Centella asiatica* L. *Chromatographia.* **79**, 727–739 (2016).
24. Inamdar, E. K., Yeole, R. D. & Ghogare, A. B. & de Souza. N. J. Determination of biologically active constituents in *Centella asiatica*. *J. Chromatogr. A.* **742**, 127–130 (1996).
25. Schaneberg, B. T., Mikell, J. R., Bedir, E. & Khan, I. A. An improved HPLC method for quantitative determination of six triterpenes in *Centella asiatica* extracts and commercial products. *Pharmazie.* **58**, 381–384 (2003).
26. Randriamampionona, D. *et al.* Comparative analysis of active constituents in *Centella asiatica* samples from Madagascar: Application for ex situ conservation and clonal propagation. *Fitoterapia.* **78**, 482–489 (2007).
27. Devkota, A., Dall'Acqua, S., Comai, S., Innocenti, G. & Jha, P. K. *Centella asiatica* (L.) urban from Nepal: Quali-quantitative analysis of samples from several sites, and selection of high terpene containing populations for cultivation. *Biochem. Syst. Ecol.* **38**, 12–22 (2010).
28. Thomas, M. T. *et al.* Elite genotypes/chemotypes, with high contents of madecassoside and asiaticoside, from sixty accessions of *Centella asiatica* of south India and the Andaman Islands: For cultivation and utility in cosmetic and herbal drug applications. *Ind. Crops Prod.* **32**, 545–550 (2010).
29. Prasad, A. *et al.* Fungal elicitor-mediated enhancement in growth and asiaticoside content of *Centella asiatica* L. shoot cultures. *Plant Growth Regul.* **69**, 265–273 (2013).
30. Hashim, P. *et al.* Triterpene composition and bioactivities of *Centella asiatica*. *Molecules.* **16**, 1310–1322 (2011).
31. Singh, J. *et al.* Enhanced secondary metabolite production and pathway gene expression by leaf explants-induced direct root morphotypes are regulated by combination of growth regulators and culture conditions in *Centella asiatica* (L.) urban. *Plant Growth Regul.* **75**, 55–66 (2015).
32. Prasad, A. *et al.* Biomass and centellosides production in two elite *Centella asiatica* germplasms from India in response to seasonal variation. *Ind. Crops Prod.* **94**, 711–720 (2016).
33. Reich, E. & Schibli, A. *High-Performance Thin-Layer Chromatography for the Analysis of Medicinal Plants* (Thieme Medical Publishers Inc., New York, 2007).

34. Pulikkalpura, H., Kurup, R., Mathew, P. J. & Sabulal, B. Levodopa in *Mucuna pruriens* and its degradation. *Sci. Rep.* **5**, 11078 (2015).
35. Renjith, R. *et al.* HPTLC-based quantification of camptothecin in *Ophiorrhiza* species of the southern Western Ghats in India. *Cogent Chem.* **2**, 1275408 (2016).
36. Günther, B. & Wagner, H. Quantitative determination of triterpenes in extracts and phytopreparations of *Centella asiatica* (L.) Urban. *Phytomedicine.* **3**, 59–65 (1996).
37. Raj, D. & Kielisz, P. High-performance thin-layer chromatographic-densitometric determination of the major triterpene saponins and their aglycones from *Centella asiatica*. *JPC-J. Planar Chromat.* **32**, 25–30 (2019).
38. Prasad, A. *et al.* Growth and centelloside production in hydroponically established medicinal plant-*Centella asiatica* (L.). *Ind. Crops Prod.* **35**, 309–312 (2012).
39. Nguyen, K. V., Pongkitwitoon, B., Pathomwichaiwat, T., Viboonjun, U. & Prathanturarug, S. Effects of methyl jasmonate on the growth and triterpenoid production of diploid and tetraploid *Centella asiatica* (L.) Urb. hairy root cultures. *Sci. Rep.* **9**, 18665 (2019).

Tables

Table 1

Quantitative analyses of ASA, MDA, ASI and MAD in fifty accessions of CA, originally collected from various agro-climatic regions of south India and vegetatively propagated and maintained in a Field Gene Bank under identical environmental conditions for six generations.

Accession no.	Collection location	State	Altitude	ASA (%)	MDA (%)	ASI (%)	MAD (%)
1/Ca 07	Perumathura, Thiruvananthapuram	Kerala	150	ND	0.37 ± 0.02	0.11 ± 0.01	0.40 ± 0.01
2/Ca 08	Madanvila, Thiruvananthapuram	Kerala	240	ND	0.30 ± 0.03	0.36 ± 0.01	1.02 ± 0.01
3/Ca 09	Anakunnam, Pallikal, Thiruvananthapuram	Kerala	120	ND	0.34 ± 0.03	0.25 ± 0.01	0.83 ± 0.01
4/Ca 10	Pallikal, Thiruvananthapuram	Kerala	120	ND	0.05 ± 0.01	0.40 ± 0.01	2.32 ± 0.03
5/Ca 11	Parippally, Kollam	Kerala	90	0.03 ± 0.01	0.41 ± 0.02	0.49 ± 0.01	1.63 ± 0.02
6/Ca 12	Kazhuthurutty, Aryankavu, Kollam	Kerala	200	0.05 ± 0.00	0.14 ± 0.01	0.65 ± 0.02	0.84 ± 0.04
7/Ca 13	Kaithakkadu, Kulathupuzha, Kollam	Kerala	170	0.01 ± 0.00	0.03 ± 0.00	0.13 ± 0.01	0.79 ± 0.01
8/Ca 15	Kulathupuzha, Kollam	Kerala	170	0.01 ± 0.02	0.39 ± 0.00	0.25 ± 0.01	1.03 ± 0.02
9/Ca 17	Aryankavu, Kollam	Kerala	284	0.08 ± 0.00	0.05 ± 0.00	0.58 ± 0.03	1.57 ± 0.05
10/Ca 18	Arippa, Kollam	Kerala	197	ND	0.06 ± 0.01	0.14 ± 0.01	1.17 ± 0.04
11/Ca 19	Nedungolam, Kollam	Kerala	16	0.18 ± 0.01	0.05 ± 0.00	0.29 ± 0.03	2.02 ± 0.05
12/Ca 21	Pandalam, Pathanamthitta	Kerala	100	0.02 ± 0.01	0.05 ± 0.00	0.67 ± 0.03	1.57 ± 0.05

Accession no.	Collection location	State	Altitude	ASA (%)	MDA (%)	ASI (%)	MAD (%)
13/Ca 22	Ranni, Pathanamthitta	Kerala	160	0.01 ± 0.00	0.04 ± 0.01	0.55 ± 0.02	2.97 ± 0.05
14/Ca 23	Koodal, Pathanamthitta	Kerala	140	ND	0.05 ± 0.00	0.11 ± 0.01	0.48 ± 0.02
15/Ca 25	KGMOA House, Pathanamthitta	Kerala	60	ND	0.03 ± 0.00	0.82 ± 0.07	1.61 ± 0.16
16/Ca 26	Tholuzham, Thattayil, Adoor, Pathanamthitta	Kerala	15	0.05 ± 0.03	0.04 ± 0.01	0.35 ± 0.02	1.09 ± 0.01
17/Ca 27	Edathitta, Adoor, Pathanamthitta	Kerala	34	ND	0.12 ± 0.03	0.15 ± 0.02	1.04 ± 0.02
18/Ca 29	Kudassanadu, Alappuzha	Kerala	95	ND	0.09 ± 0.01	0.23 ± 0.01	1.48 ± 0.01
19/Ca 30	Kunnamkari, Alappuzha	Kerala	2	ND	0.12 ± 0.01	0.11 ± 0.01	0.78 ± 0.05
20/Ca 31	Muttar, Kaithathod, Alappuzha	Kerala	14	ND	0.16 ± 0.02	0.06 ± 0.00	0.56 ± 0.01
21/Ca 32	Kainery, Alappuzha	Kerala	8	ND	0.10 ± 0.01	0.32 ± 0.02	1.60 ± 0.11
22/Ca 33	Mannanchery, Alappuzha	Kerala	10	0.01 ± 0.00	0.09 ± 0.00	0.25 ± 0.02	1.50 ± 0.03
23/Ca 34	Thalavadi, Alappuzha	Kerala	22	0.02 ± 0.00	0.07 ± 0.01	0.15 ± 0.00	0.92 ± 0.01
24/Ca 35	Poovam, Changanassery, Kottayam	Kerala	20	ND	0.08 ± 0.01	0.22 ± 0.01	1.14 ± 0.01
25/Ca 38	Vaningery, Ancharakotty, Changanassery, Kottayam	Kerala	19	ND	0.07 ± 0.00	0.19 ± 0.01	1.42 ± 0.02

Accession no.	Collection location	State	Altitude	ASA (%)	MDA (%)	ASI (%)	MAD (%)
26/Ca 39	Aluva, Ernakulam	Kerala	58	ND	0.05 ± 0.01	0.12 ± 0.04	0.91 ± 0.02
27/Ca 40	Thattakkad, Ernakulam	Kerala	39	ND	0.08 ± 0.01	0.18 ± 0.01	1.10 ± 0.04
28/Ca 41	Bhoothathankett, Ernakulam	Kerala	77	0.01 ± 0.01	0.04 ± 0.00	0.67 ± 0.02	1.30 ± 0.01
29/Ca 42	Wagamon, Idukki	Kerala	1100	ND	0.02 ± 0.01	0.20 ± 0.02	1.18 ± 0.03
30/Ca 43	Nallathanni, Idukki	Kerala	860	0.03 ± 0.01	0.07 ± 0.01	0.11 ± 0.02	0.81 ± 0.03
31/Ca 44	Murinjapuzha, Idukki	Kerala	800	0.01 ± 0.02	0.05 ± 0.02	0.28 ± 0.01	1.75 ± 0.05
32/Ca 45	Pattumala, Peerumade, Idukki	Kerala	1100	ND	0.09 ± 0.03	0.22 ± 0.01	1.54 ± 0.08
33/Ca 47	KFIDC Garden, Elappara, Idukki	Kerala	1180	0.02 ± 0.00	0.13 ± 0.00	2.41 ± 0.06	4.61 ± 0.12
34/Ca 48	Kattappana, Idukki	Kerala	900	0.02 ± 0.00	0.06 ± 0.00	0.71 ± 0.02	5.27 ± 0.13
35/Ca 51	Kumaly, Idukki	Kerala	800	0.06 ± 0.00	0.14 ± 0.02	2.13 ± 0.09	5.10 ± 0.26
36/Ca 52	Kuttikanam, Idukki	Kerala	1064	0.04 ± 0.01	0.12 ± 0.00	1.71 ± 0.09	5.12 ± 0.22
37/Ca 55	Ramakkalmedu, Idukki	Kerala	911	0.27 ± 0.01	0.35 ± 0.00	1.48 ± 0.03	2.56 ± 0.03
38/Ca 56	Periyakanal, Sooryanelli, Idukki	Kerala	1332	0.29 ± 0.01	0.72 ± 0.02	1.23 ± 0.02	4.41 ± 0.06

Accession no.	Collection location	State	Altitude	ASA (%)	MDA (%)	ASI (%)	MAD (%)
39/Ca 58	Munnar, Idukki	Kerala	1521	ND	0.07 ± 0.02	0.22 ± 0.05	0.79 ± 0.03
40/Ca 59	Korandikadu, Munnar, Idukki	Kerala	1645	ND	0.20 ± 0.01	0.68 ± 0.02	1.54 ± 0.05
41/Ca 83	Vellanikkara, Thrissur, Kerala	Kerala	30	ND	0.07 ± 0.01	0.15 ± 0.01	0.95 ± 0.01
42/Ca 89	Thenjippalam, Malappuram	Kerala	160	ND	0.04 ± 0.00	0.16 ± 0.02	0.88 ± 0.03
43/Ca 90	Calicut University Campus, Malappuram	Kerala	200	0.02 ± 0.00	0.02 ± 0.00	0.07 ± 0.00	1.65 ± 0.02
44/Ca 92	Kizhakkumpadam, Mukkam, Kozhikode	Kerala	12	0.01 ± 0.00	0.04 ± 0.00	0.55 ± 0.00	0.36 ± 0.01
45/Ca 93	Payyannur, Kannur	Kerala	130	ND	0.03 ± 0.02	0.15 ± 0.00	2.58 ± 0.02
46/Ca 94	Kalpatta, Wayanad	Kerala	760	ND	0.05 ± 0.02	0.15 ± 0.03	1.24 ± 0.18
47/Ca 98	Thirunandikkara, Kanyakumari	Tamil Nadu	110	ND	0.07 ± 0.07	0.11 ± 0.01	0.56 ± 0.01
48/Ca 100	Chittar Dam, Kanyakumari	Tamil Nadu	140	ND	0.08 ± 0.00	0.18 ± 0.01	1.07 ± 0.03
49/Ca 121	Indian Institute of Horticulture Research, Bangalore	Karnataka	883	ND	0.07 ± 0.01	0.07 ± 0.06	0.53 ± 0.01
50/Ca 124	Pilikula, Nisargadhama, Mangalore, Dakshina Kannada	Karnataka	108	ND	0.19 ± 0.06	0.04 ± 0.01	0.15 ± 0.00

ND - not detected; each percentage is an average of six values ± SD.

Table 2

Validation parameters of the four centellosides ASA, MDA, ASI and MAD.

Compounds	Regression equation	Correlation coefficient (R^2)	Linearity range ($\mu\text{g}/\text{band}$)	LOD ($\mu\text{g}/\text{band}$)	LOQ ($\mu\text{g}/\text{band}$)
ASA	$4137.1 x + 247.46$	0.995	0.05 to 1.25	0.040	0.122
MDA	$3662.4 x + 241.83$	0.999	0.05 to 1.25	0.022	0.069
ASI	$6724.1 x + 465.11$	0.998	0.1 to 0.8	0.076	0.230
MAD	$2404.8 x + 473.76$	0.997	0.2 to 2.0	0.057	0.174

Table 3
Recovery data of ASA, MDA, ASI and MAD.

Accession no.	ASA + Extract (spiked) (µg)	ASA (µg)	ASA in extract (µg)	% Recovery
ASA				
CA 56	0.72	0.0	0.72	100
CA 56 + 0.1	0.80	0.1	0.82	97.56
CA 56 + 0.2	0.91	0.2	0.92	98.91
CA 56 + 0.3	1.00	0.3	1.02	98.03
Accession no.	MDA + Extract (spiked) (µg)	MDA (µg)	MDA in extract (µg)	% Recovery
MDA				
CA 56	0.29	0.0	0.29	100
CA 56 + 0.1	0.38	0.1	0.39	97.44
CA 56 + 0.2	0.48	0.2	0.49	97.96
CA 56 + 0.3	0.57	0.3	0.59	96.61
Accession no.	ASI + Extract (spiked) (µg)	ASI (µg)	ASI in extract (µg)	% Recovery
ASI				
CA 55	0.74	0.0	0.74	100
CA 55 + 0.25	0.84	0.10	0.84	100
CA 55 + 0.50	0.93	0.20	0.94	98.93
CA 55 + 0.75	1.03	0.30	1.04	99.03
Accession no.	MAD + Extract (spiked) (µg)	MAD (µg)	MAD in extract (µg)	% Recovery
MAD				
CA 55	1.28	0.0	1.28	100
CA 55 + 0.25	1.51	0.25	1.53	98.69
CA 55 + 0.50	1.76	0.50	1.78	98.88
CA 55 + 0.75	2.03	0.75	2.03	100

Table 4
Intra-day and inter-day precision of ASA, MDA, ASI and MAD.

Compounds	Concentration (µg/band)	Intra-day precision ^a		Inter-day precision ^b	
		SD in area	% CV	SD in area	% CV
ASA					
	0.2	31.16	1.99	40.02	2.63
	0.4	40.49	1.79	58.88	2.58
	0.6	20.87	0.70	70.80	2.35
MDA					
	0.2	15.91	1.62	26.77	2.85
	0.4	32.45	1.86	42.09	2.40
	0.6	48.67	1.81	69.25	2.59
ASI					
	0.4	40.61	1.35	50.30	1.55
	0.6	70.94	1.74	79.65	1.89
	0.8	76.11	1.49	96.85	1.82
MAD					
	0.4	34.34	1.83	29.31	1.99
	0.6	37.19	1.42	40.62	1.87
	0.8	39.92	1.24	54.61	1.97

^aMean SD of four trials on same day; ^b Mean SD of two trials for consecutive five days; % CV: Coefficient of variation (SD*100/mean).

Figures

Figure 1

Representative HPTLC chromatograms of methanol extracts CA *C. asiatica* accessions, (A) Ca-56 (ASA, MDA) and (B) Ca-52 (ASI, MAD).

Figure 2

Four major centellosides in *C. asiatica*, asiatic acid (ASA), a madecassic acid (MDA), asiaticoside (ASI) and madecassoside (MAD), and their probable C6-hydroxylation and C28-glycosylation reactions.