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Dissanayake TDCK
Department of Chemistry,
Faculty of Applied Sciences,
University of Sri
Jayewardenepura, Nugegoda,
Sri Lanka

Abeysekera AM
Department of Chemistry,
Faculty of Applied Sciences,
University of Sri
Jayewardenepura, Nugegoda,
Sri Lanka

Salim N
Department of Botany, Faculty
of Applied Sciences, University
of Sri Jayewardenepura,
Nugegoda, Sri Lanka

Chandrika UG
Department of Biochemistry,
Faculty of Medicinal Sciences,
University of Sri
Jayewardenepura, Nugegoda,
Sri Lanka

Padumadasa C
Department of Chemistry,
Faculty of Applied Sciences,
University of Sri
Jayewardenepura, Nugegoda,
Sri Lanka

Correspondence
Dissanayake TDCK
Department of Chemistry,
Faculty of Applied Science,
University of Sri
Jayewardenepura, Nugegoda,
Sri Lanka

Evaluation of *Centella asiatica* morphotypes for high yields of asiaticoside

Dissanayake TDCK, Abeysekera AM, Salim N, Chandrika UG, Padumadasa C

Abstract

Centella asiatica (L) is a medicinal plant containing triterpenoid saponins including asiaticoside (ASTC) which currently incorporated on many cosmetic preparations. There are several morphotypes of the plant found in Sri Lanka. The ASTC content of five of the most common morphotypes were evaluated with the purpose of identifying a suitable morphotype for cultivation. A precise and accurate method based on HPLC analysis was developed. Of the five morphotypes studied, in field trials three were found to have significantly higher ASTC contents than the other two. Growing the same morphotypes in pots increased the ASTC content by nearly threefold.

Keywords: *Centella asiatica*, asiaticoside, HPLC, morphotypes, field trials

1. Introduction

C. asiatica (L.) is a small herbaceous perennial plant of the family Apiaceae native to India, China, Indonesia, Australia, the South Pacific, Madagascar, and Southern and middle Africa. The plant is used in traditional medicine for the treatment of skin diseases, and as a memory enhancing drug/ brain and nerve tonic [1-4]. These uses have been justified by modern pharmacological and clinical studies [1-4] and have led to the industrial use of the plant in herbal cosmetics and herbal dietary supplements.

Based on the numerous studies, the main biologically active compounds in the plant are believed to be the triterpenoids asiatic acid, madecassic acid, ASTC and madecassoside [1-4]. Of these ASTC has been shown high potential for development as a wound healing and skin toning agent [5-11].

Triterpenoid saponins of *C. asiatica* are used as biomarker compounds [12] and there are several literature reports on quantification of these compounds using modern techniques such as HPLC [13], HPTLC [14] and LC-MS [12].

Different morphotypes of *Centella asiatica* have been reported in Sri Lanka, which considerably differ morphologically and biochemically [15]. They show significant variation among them in mineral and carotenoid contents [16] and also differ in their susceptibility to the infestation by the root knot nematode, *Meloidogyne incognita* [15]. There are no reported data on triterpenoids components from different morphotypes of *C. asiatica* found in Sri Lanka.

In this study, a method was developed and validated to quantify ASTC in the leaves of *C. asiatica* and it was used to assess the ASTC content in five different morphotypes of *C. asiatica* growing in Sri Lanka with the aim of selecting a suitable morphotype for extended field trials.

2. Materials and methods

2.1 Instruments and accessories

HPLC was carried out on an Agilent 1260 infinity LC instrument fitted with a column-C18 Alltec ALLTIMA C18 5 MICRON (250 mm*4.5 mm) and a diode array detector.

2.2 Solvents and chemicals

Pure asiaticoside obtained from Link Natural Products pvt. Ltd, Sri Lanka was used as the standard ASTC.

Freshly distilled chloroform and methanol was used for defatting and extraction of ASTC. HPLC grade methanol and acetonitrile was used for chromatography. Water used for HPLC was freshly double distilled.

3. Plant materials

3.1 Plants for method development

A fresh sample of the commonly available morphotype from the local market was used to develop and validate a method to quantify ASTC in extracts of *C. asiatica* and to develop a method for the quantitative extraction of ASTC from *C. asiatica*.

3.2 Plants for ASTC analysis

Plants derived from five different morphotypes (G1, G2, G4, G5 and G6) from the collection of plants at the Department of Botany, University of Sri Jayewardenepura which had been previously identified by us (Wijekoon *et al.*, 2002) [15] were used.

3.2.1 Potted plants

C. asiatica plantlets at the growth stage of two leaves and a bud were selected from each morphotype (five plantlets per morphotype), and planted in pots of 10 cm × 10 cm (diameter × height), one plant per pot each containing 500 cm³ of potting medium. The potting medium contained a mixture of top soil: sand: compost: coir dust in the volume ratio of 2:3:1:1. The plants were grown under homogeneous conditions in a greenhouse at 31 ± 2 °C and harvested after 75 days growth.

3.2.2 Field plants

A plot of evenly shaded, flat grass land was cleared and dug to depth of 15 cm. fifty isolated raise beds of 45 cm × 45 cm × 15 cm (length × width × height) were created and each was fertilized with 250 cm³ of compost.

Plantlets at the growth stage of 2 leaf 1 bud were planted (one plant per one plot, ten plants of each morphotype). The plants were then raised under homogeneous conditions and harvested after 75 days growth.

3.3 Method development for quantification of ASTC in the methanol extract of leaves of *C. asiatica*

3.3.1 HPLC

Air dried and ground leaves without petioles (0.10 g) was defatted with chloroform (2.5 ml × 30 min × 3) and extracted with methanol (2.5 ml × 30 min × 3). The extracts were pooled and the solvent was evaporated, and the residue was dissolved in 1.00 ml of HPLC grade methanol and filtered and 5 µl of the filtrate was chromatographed on HPLC in triplicate at 26 °C using an isocratic solvent system, acetonitrile: water (70:30) at a flow rate of 1 ml/min. the chromatogram was monitored at 210 nm wave length. Asiaticoside eluted at 5.7 minutes and the peak identity was confirmed by comparing with HPLC chromatographs of standard ASTC and plant extracts spiked with standard ASTC.

3.4 Optimum conditions for quantitative extraction of ASTC

3.4.1 Ratio of plant material: solvent and number of sequential extractions

Twelve Samples of 0.10 g of dried, ground *C. asiatica* leaves without petioles were defatted and divided into four groups A, B, C, and D containing 3 samples each. The defatted samples in each group A, B, C and D were individually extracted with 1 ml, 2.5 ml, 5 ml and 10 ml of methanol respectively by periodic manual swirling for 30 minutes. Each sample was extracted 5 times with the appropriate volume of methanol. Each extract was individually evaporated and the residue was dissolved in 1.00 ml of methanol and subjected to HPLC in

triplicate as given above, and the area under the peak of ASTC was measured.

For all samples, first extracts (extracts of the first extraction) showed the presence of ASTC and the area under the peak was almost similar. Second extracts (extracts of the second extraction) showed a small peak around the base line no ASTC was detected after the 2nd extract.

The volume of 5 ml of methanol to extract 0.10 g of ground *C. asiatica* leaves was chosen for further experiments considering the convenience of handling.

3.5 Method of extraction

A further twelve samples of 0.10 g of dried leaf powder of *C. asiatica* was defatted and divided into four groups E,F,G and H. The samples in each group were individually extracted with methanol (5 ml × 30 minutes × 3) using magnetic stirrer assisted extraction, sonicator assisted extraction, mechanical shaker assisted extraction and by periodic manual swirling respectively. Each extraction was carried out in 10 ml stoppered conical flasks. The size of the stirrer magnet was 15 mm (length). The three extracts of each sample were pooled and evaporated. The residue was suspended in 1.00 ml of methanol and subjected to HPLC in triplicate as given above, and the area under the peak of ASTC was measured at 210 nm.

The peak area obtained for method of periodic manual swirling was about one third lower than the other methods which were approximately equal. The method of magnetic stirrer assisted extraction was chosen for further experiments considering the convenience of handling.

3.6 Calibration curve

A standard stock solution of ASTC containing 0.0500 g of standard ASTC in 10.00 ml was prepared. Asiaticoside standard solutions of concentrations (mg/ml) of 0.01, 0.05, 0.10, 0.20, 0.50, 1.00, 1.60, 2.00, 3.00, 4.00 and 5.00 were prepared by diluting the appropriate volume of the stock solution. An aliquot of 5 µl was subjected to HPLC in triplicate as given above. Average instrument signal (peak area) at 210 nm wave length was plotted against asiaticoside concentration.

3.7 Validation of the extraction and quantification method

3.7.1 Precision

Six samples of 0.10 g of dried *C. asiatica* leaves were defatted with chloroform and extracted with methanol (5 ml × 30 min × 3). The extracts were individually evaporated. The residues were individually dissolved in 1.00 ml of HPLC grade methanol and subjected to HPLC in triplicate. The area under the peak of ASTC of each sample was measured and relative standard deviation was calculated.

3.7.2 Accuracy

An aliquot of 0.10 g (S) of a *C. asiatica* leaf sample was extracted and quantified the asiaticoside content as described above.

Another two aliquots of 2.0 g from the same *C. asiatica* leaf sample were weighed and 20% of the estimated content of asiaticoside was added to one aliquot (S1) as a solution in 2 ml of methanol. To the other aliquot (S2) 2 ml of methanol was added.

Both S1 and S2 were dried and 3 samples of 0.10 g from each sample (S1 and S2) were extracted and 5 µl aliquots were subjected to HPLC in triplicate. And percentage recovery of added ASTC (R) was calculated as follows.

$$100 \times \frac{100}{20} \times \frac{(C1 - C2)}{C2} = R$$

R= percentage recovery

C1 = average asiaticoside peak area of samples of S1

C2 = average asiaticoside peak area of samples of S2

3.8 Investigation of ASTC content of different morphotypes of *C. asiatica*

Leaves of the five different morphotypes grown in pots under greenhouse conditions were washed, dried, and ground separately using a mortar and pestle.

Three aliquots of 0.10 g of the leaf samples of each morphotype were defatted with chloroform and extracted with methanol (2.5 ml × 30 min × 3). The solvent from each extract and the residues were each dissolved in 1.00 ml of HPLC grade methanol and subjected to HPLC analysis in triplicate as given above.

The leaves of each plant of the five different morphotypes grown in open field condition (ten plants per morphotype) were washed, dried, and ground separately using mortar and pestle.

Three aliquots of 0.10 g of each leaf sample were defatted with chloroform and extracted with methanol (2.5 ml × 30 min × 3). The solvent from each extract and the residues were each dissolved in 1.00 ml of HPLC grade methanol and subjected to HPLC analysis in triplicate as given above.

(MINITAB 14)

4. Results and discussion

4.1 Method development

For all four experimental groups with different ratios of plant material: solvent, the first extractions (extracts of the first extraction) showed a clear peak for asiaticoside whereas the second extractions (extracts of the second extraction) showed only a very small peak about the baseline. 3rd, 4th and 5th extractions of all ratios did not show the presence of asiaticoside. The areas of the peak for asiaticoside for the four ratios, 1: 10, 1: 25, 1: 50 and 1: 100 were 80, 80, 81 and 80.15 mAU respectively. The similarity of the values indicates that the solvent was not saturated even at the lowest ratio of plant material: solvent. Considering the convenience of handling the ratio of 1:50(0.10 g of plant material with 5 ml of methanol) was selected.

The peak areas for asiaticoside obtained for the different methods of extraction, magnetic stirrer assisted extraction, Sonicator assisted extraction, mechanical shaker assisted extraction and manual extraction by frequent swirling using a plant material: solvent ratio 1: 50 were 120, 118, 117 and 80 mAU respectively. The results show clearly that manual extraction by frequent swirling is not an efficient method of extraction of ASTC and that the other three methods gave very similar yields. Considering the convenience of handling, magnetic stirrer assisted extraction method was chosen for the extraction of ASTC of test samples.

The selected extraction conditions produced a relative standard deviation of 0.38% (n=6) and 99.8% recovery of added ASTC.

Thus the method of defatting 0.10 g of dried leaf powder of *C. asiatica* with chloroform, extracting the defatted sample with methanol (5.00 ml × 30 min × 3), pooling the three extracts and evaporating, dissolving the residue in 1.00 ml of methanol, filtering, subjecting to HPLC by injecting 0.25 µl samples, eluting with solvent system acetonitrile: water 30: 70 at 26 °C and measuring area under the ASTC peak is a

convenient, precise and accurate method of Quantitative extraction and quantification of ASTC in *C. asiatica* leaves.

The calibration curve was linear ($r^2 = 0.99$) over the range of 0.01 to 5 mg/ml and fitted the equation

$y = 670.01x + 20.82$; where x = concentration of ASTC/ (mg/ml); y = area under the peak of ASTC at 210 nm/ mAu*s.

4.2 Investigation of ASTC content of different morphotypes of *C. asiatica*

The asiaticoside content as a mass percentage of dry leaf of pot grown plants for the morphotypes G1, G2, G4, G5 and G6 were 1.46, 2.70, 2.75, 2.98 and 3.53 respectively.

The asiaticoside content of field grown plants are as shown in table 01

Table 1: Asiaticoside content of different morphotypes of *C. asiatica* grown under field conditions

Morphotype	Content of asiaticoside as a mass percentage of dry leaf
G1	0.46±0.07(n=30) ^c
G2	1.19±0.14(n=27) ^a
G4	0.71±0.13(n=24) ^b
G5	1.13±0.12(n=30) ^a
G6	0.96±0.20(n=30) ^a

Asiaticoside content is given as mean percentage ± SD. a, b, c: Means having same letter (s) are not significantly different by Tukey's pair wise comparison test ($P \leq 0.05$).

For all morphotypes, field grown plants gave lower yields of ASTC than for potted plants grown under greenhouse conditions. In both field grown and potted plants, the poorest source of ASTC among the morphotypes tested was G1, of which, the field grown plants of G1 showed the lowest content. There was no significant difference in ASTC content among the morphotypes G2, G5 and G6 in field grown plants. Many abiotic factors such as temperature, UV stress, nutrient stress and space restriction can affect on the content of secondary metabolites in plants [17-20]. Some of these factors may have contributed to the difference observed between the ASTC content of pot and field grown plants.

It is reported that the content of ASTC in the leaves of *C. asiatica* is 0.4% to 1.4% (dry weight) in chemotypes from South Africa [21]. ASTC contents of field grown plants in the current study lies around this range. However Jacinda *et al* (2008) [13] reports a higher ASTC content for two distinct south African phenotypes as 5.23% and 4.52%. In contrast it is reported ASTC contents ranging from 0.006% to 0.114% for 10 genotypes in India [22].

5. Conclusion

Of the five morphotypes studied, G2, G5 and G6 are the suitable candidates for an extended field trial to evaluate the agronomic factors influencing ASTC content.

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