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Determination of biologically active constituents in *Centella asiatica*[1]

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Abstract

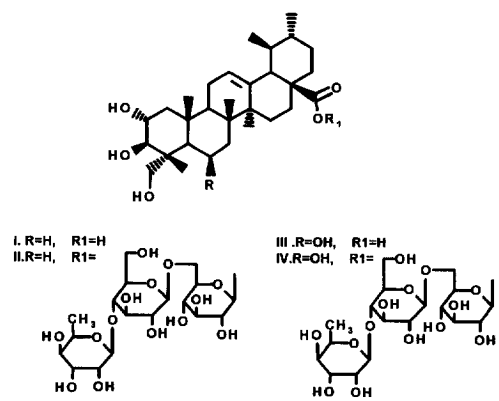
A high-performance liquid chromatographic method is described, using an octadecylsilylated silica column for simultaneous quantitative determination of the bioactive terpene acids: asiatic acid, madecassic acid and their respective glycosides, asiaticoside and madecassoside, in the plant *Centella asiatica*. The method is suitable for estimating these compounds in plant material. The method is also suitable for the routine assay of pharmaceutical preparations containing a *C. asiatica* extract.

Keywords: *Centella asiatica*; Madecassoside; Asiaticoside; Madecassic acid; Asiatic acid; Terpenes

1. Introduction

Centella asiatica (Linn) is an ethnomedical plant used in different continents by diverse ancient cultures and tribal groups. In India, it is usually described under the name of Mandukaparni in the Ayurvedic system of medicine [1]. Different uses are claimed for the plant, the more common being its use as a wound healing agent [2], constituent of brain tonics for the mentally retarded [3,4]. These properties have been ascribed to the active principles: asiatic acid (I), asiaticoside (II), madecassic acid (III) and madecassoside (IV). These are pentacyclic triterpenes, found to display chronic venous insufficiency [5], varicose vein and wound healing properties. They belong to the β-amyrin ursolic acid group, as shown in Scheme 1.

Madecassol, a formulation based on *C. asiatica* plant extract, when applied locally on wounds in rats prompted the proliferation of granulation and increased tensile strength [6]. It decreased the wound area of the skin necrosis induced by burn [7,8].



Scheme 1. Structures of asiatic acid (I), asiaticoside (II), madecassic acid (III) and madecassoside (IV).

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[1]This paper is dedicated to Dr. E. Baltin, Managing Director, Hoechst India Ltd., on the occasion of his 60th birthday.

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For the estimation of the terpene acids (I, III) and the glycosides (II, IV) of *C. asiatica* the titrimetric method of analysis is reported [9]. The method determines only total terpene acids content. For the glycosides determination, the extract must first be hydrolysed to convert them to acids and then the total terpene acids are chemically estimated. Hence this method is non-selective, non-specific and lacks precision and accuracy.

A reversed-phase gradient HPLC method which is superior to the reported analytical method is developed to assay the four (I–IV) constituents in the crude plant extracts. The method is further used for galenical preparations and the monitoring of the isolation process of these products from the plants obtained from various locations in India. The new method of analysis is precise, accurate and rapid.

2. Experimental

2.1. Reagents

Acetonitrile and methanol (HPLC grade) were from E. Merck (India), asiatic acid (I) and asiaticoside (II) were obtained from Carl Roth (Karlsruhe, Germany). Spectral data for madecassic acid (III) and madecassoside (IV), isolated by Kilo-scale laboratory, Hoechst (Bombay, India), were in agreement with reported values. A stock solution of all the compounds I–IV was prepared (10 mg/ml of each) in methanol–water (90:10). The stock solution was diluted to obtain solutions 0.5–7.5 mg/ml for determination of the linearity range. Double distilled water was filtered through a 0.45-μm membrane filter. The plant material was collected from the factory garden and identified as *C. asiatica* (HOE 924) by Dr. V. Shah of Hoechst India. A voucher specimen is preserved at the Herbarium of the Research Centre, Hoechst India. Centellase tablets were from Scharper (Italy) and madecassol ointment was from Laboratories Syntex (France).

2.2. Sample preparation

For extraction, 1 g dried, finely powdered material was stirred with methanol–water (9:1) (2×10 ml) for 5 h at ambient temperature and filtered. The filtrate was evaporated to dryness to obtain a dark brown extract (0.15 g). The dried crude extract was accurately weighed (≈100 mg), dissolved in methanol–water (90:10) and made up to 10 ml. The solution was filtered through a 0.45-μm filter (Waters Millipore) and the clear filtrate was used for HPLC analysis.

Table 1
Gradient conditions for HPLC

Time (min)	Flow-rate (ml/min)	Pump A water(%)	Pump B acetonitrile(%)	Curve
00	1.4	80	20	
30	1.4	45	55	6
35	1.4	45	55	
45	1.4	80	20	6

Five tablets of centellase, each weighing approx. 70 mg, were powdered. A quantity equivalent to one tablet was accurately weighed and was transferred to a 25-ml centrifuge tube. A 10-ml volume of methanol–water (90:10) was added and vortexed for 5 min. The suspension was centrifuged for 10 min. The procedure was repeated three times. The supernatant was evaporated under vacuum. The residue obtained was dissolved in 10 ml methanol–water (90:10) and used for the HPLC assay. A 500-mg sample of ointment was vortexed with 10 ml methanol–water (90:10) for 20 min, centrifuged and filtered through a 0.45-μm filter (Waters Millipore) and the clear solution was used for the HPLC assay.

2.3. Chromatography

The Waters HPLC system consisted of a high-pressure constant flow pump (Model 510), auto injector (WISP 712), a UV spectrophotometric detector (490 multiwavelength detector) and a data station (840 with Digital 350 computer). Chromatographic separation was performed with a μBondapak, C_{18} 10 μm, 30×0.39 cm, S.S. column (Waters) with a water–acetonitrile mobile phase, with UV detection at 220 nm and attenuation of 0.1 AUFS. Gradient conditions are described in Table 1. A 20-μl volume of sample was injected onto the column.

3. Results and discussion

To accomplish optimal separation of the *C. asiatica* extract, different experimental variables like

Table 2
Features of the developed HPLC method

Compound	LOD (μg)	L (mg/ml)	t_R (min)
Madecassoside	3	0.5–3.5	10.60
Asiaticosside	3	0.5–3.5	12.70
Madecassic acid	2.5	0.5–2.5	22.23
Asiatic acid	2.5	0.5–4.0	26.87

eluent strength, composition and flow-rate were attempted. Isocratic HPLC was first used to achieve a separation of the four compounds by using different variants. However, due to the large difference in polarity of the triterpene acids and their glycosides, either the two acids (I and III) (with high concentration of acetonitrile or methanol), or the two glycosides (II and IV) (with low concentration of acetonitrile or methanol) were separated. Hence, it was decided to use a linear gradient, so that all the four compounds (I–IV) could be separated in a single run. The optimum separation was obtained by using a linear gradient of acetonitrile in water from 20 to 55% in 30 min. The retention times (t_R) of the compounds I–IV are presented in Table 2 along with the limits of detection (LOD) and linearity range (L). The LOD was calculated as the ratio of peak height to three-times the baseline noise. The linearity range was determined by injecting the solutions of the concentrations ranging from 0.5 to 7.5 mg/ml each. The standard curves exhibited excellent linearity for the, admittedly rather short, concentration range given in Table 2 and regression analysis showed correlation coefficient greater than 0.99 with intercept values that did not deviate significantly from origin for all the four constituents.

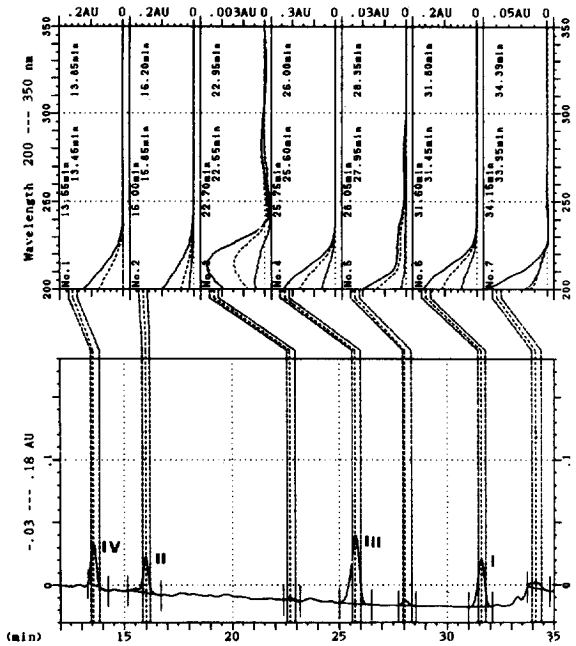

Fig. 1. Spectrum index plot of madecassoside (IV), asiaticaside (II), madecassic acid (III) and asiatic acid (I).

The peak purity of the individual compounds I–IV was examined between 200 and 350 nm using a photodiode array detector (Waters 991 detector). Fig. 1 represents the spectrum index plot. None of the peaks showed absorbance beyond 230 nm.

Fig. 2 represents a chromatogram of the plant extract which demonstrates the optimum separation and ideal quantitative evaluation of individual constituents in the plant extract. In some of the plant extracts, an unidentified peak eluting just before asiatic acid is observed, but it does not interfere with the analysis of asiatic acid.

Extraction efficiency was studied by adding known amounts of pure compounds to the previously

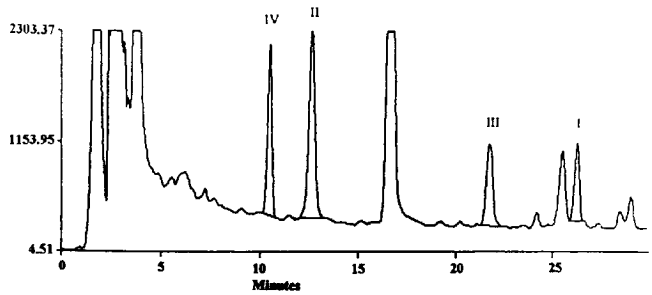

Fig. 2. HPLC analysis of the crude extract of *C. asiatica*.

Table 3
Results of centellase tablet assay

Tablet $n=(4\times3)$	Asiaticoside	Madecassic acid	Asiatic acid
Mean amount (mg)	13.2	10.1	4.03
S.D.	0.1	0.1	0.04
% Amount found	44.1	33.8	13.5
% Amount labelled	40.0	60.00 (madecassic acid+asiatic acid)	

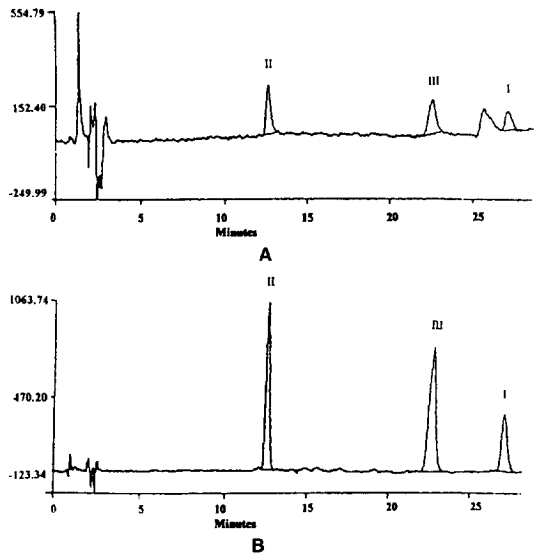

Fig. 3. HPLC analysis of ointment (A) and tablet (B) formulations containing *C. asiatica* extract.

analysed plant extract. The extraction efficiency ($n=4$) for madecassoside, assiaticoside, madecassic acid and asiatic acid was found to be 98.1% (R.S.D. 0.4%), 97.3% (R.S.D. 0.5%), 95.7% (R.S.D. 0.8%) and 96.4% (R.S.D. 0.6%) respectively.

The method was further employed to assess the four active products in the bulk isolation and purification process of active constituents from plant material.

The method was also used to assay the active components I–IV in the commercial formulations which are available in the European market. Fig. 3 shows the typical analyses of Madecassol, an ointment, and Centellase, a tablet formulation containing *C. asiatica* extract. None of the formulations showed the presence of madecassoside (IV). The results of the Centellase tablet assay are given in Table 3. The results are based on external standard quantitation method.

The developed method is also used for the estimation of the four active constituents in pharmaceutical preparations containing *C. asiatica* plant extracts, viz. tablets and ointments (products under development) for quality control and their stability studies [10].

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