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Research Article

Comparative Analysis of Active Constituents in *Centella Asiatica* Varieties (Majjaposhak and Subhodak)

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Abstract

Centella asiatica is an important medicinal plant of subtropical to tropical region. It grows widely,in different habitats. A comparative quantitative analysis of chemical constituents in Majjaposhak and Shubodak varieties of *Centella asiatica* collected from CIMAP, Hyderabad was carried out by HPLC to evaluate the variability in the important constituents. Qualitative phytochemical analysis of the plant confirms the presence of various phytochemicals like alkaloids, flavonoids, tannins, terpenoids, saponin, steroids, carbohydrates and cardiac glycosides in its aqueous extracts. The two varieties of *C. asiatica* exhibited differences in terpenoid content especially of asiaticoside. The asiaticoside of interest was analysed and quantified by TLC and HPLC in crude ethanolic extracts. The chromatogram for the plant extracts of two varieties of *Centella asiatica (*Majjaposhak and subhodak*)* were developed using different solvent systems such as methanol: water (70:30) using isocratic program and Acetonitrile: water using Binary gradient program. Peak was not identified when methanol:water used as solvent system (isocratic elusion). Finally, the Acetonitrile: water system (gradient elusion) was used for the analysis of Asiaticoside A difference in bioactive triterpenoids was exhibited that was tissue specific and varied between the two varieties. Asiaticoside content was highest in leaves of Subhodak variety (1.42 ± 1.60% dw) than the leaves of Majjaposhak variety (0.78 ± 0.02 % dw). However, the difference was statistically significant for asiaticoside ($p < 0.05$). Comparative study of two varieties of *Centella asiatica* showed a correlation between genomic diversity and asiaticoside content. The leaves of Shubodak variety of *Centella asiatica* found to contain around two time's higher concentration of asiaticoside than those from Majjaposhak variety.

1. INTRODUCTION

Centella asiatica is used in different regions by diverse ancient cultures and tribal groups. In India, it is described under the name of Mandukaparni and used in Ayurveda medicine. It is also listed as one of the Traditional Chinese Medicine (TCM) in China (World Health Organization, 1989). It is used as a support for faster healing of small wounds[1-6]. The plant extract is incorporated into the Indian pharmacopoeia and recommended not only for wound healing but also for the treatment of skin diseases such as eczema, leprosy and psoriasis. In addition to the above, it is also used in the treatment of burns, itching and insect bites. In contrast with other medicinal plants, this plant has been subjected to quite extensive experimental and clinical investigations due to its ability to heal relieve and help in the recovery from various pain and sickness.[1] It is used to modulate oxidative stress response that has been implicated in the neurodegenerative changes that occur with Alzheimer's disease [2,7-11].

1.1 Chemical composition of *Centella asiatica*

Triterpene is a major and the most important component of *C. asiatica.* It is used as a as a marker constituent in quality control analysis. The triterpenes obtained from *Centella asiatica* are mainly pentacyclic triterpenic acids and their respective glycosides, belonging to ursane-or oleanane-type, including asiatic acid, asiaticoside, madecassic acid, madecassoside, brahmoside, brahmic acid, brahminoside, thankuniside, isothankuniside, centelloside, madasiatic acid, centic acid, cenellic acid, betulinic acid, indocentic acid etc[3,4, 12-18].

Centellosides such as asiaticoside, madecassoside, asiatic acid and madecassic acid are found to be active metabolites of Centella asiatica. They have many medicinal and therapeutic properties. They are used for diseases associated with the skin, nervous system and blood. Centellosides are ursane-type triterpene saponins resulting from the cyclisation of 2, 3-oxidosqualene by amyrin synthase.[5,6, 19-23]Among the three triterpenes, asiatic acid is the most important ingredient for biological activity. The active principle of Centella asiatica especially asiaticoside has been studied extensively for use as antidepressant.[7, 8, 24-30]

Being the chief bioactive substances in *C. asiatica*, triterpenoid derivatives play an important role in the aspect of medicinal application. Traditional uses of several triterpenoids have been scientifically validated and some of the active principles were reported. [9,10,31,32] Three major triterpenoid glycosides, i.e. asiaticoside, madecassoside and asiaticoside-B were isolated by thin layer chromatography method in *Centella asiatica* extracts.[11,33] Asiaticoside and madecassoside may be effective in treating arthritis. Asiatic acid induces apoptosis and cell cycle arrest in several types of cancer in rats.[12, 13,34-38] Other components isolated from *C. asiatica,* such as brahmoside and brahminoside, may be responsible for CNS and utero relaxant actions, but are yet to be confirmed by clinical studies[14,39-43]. Centelloside and its derivatives were found to be effective in the treatment of venous hypertension[15, 16,44] The present research work has been undertaken to isolate and identify Asiaticoside (Fig.1) from *Centella asiatica* plant extracts by High Performance Liquid Chromatography[17,45]. The study comprises the qualitative and quantitative analysis of the chemical components of *Centella asiatica* by qualitative tests followed by

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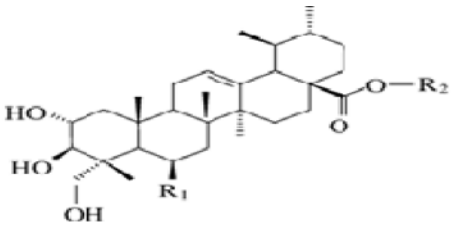
chemical analysis (especially Asiaticoside) by Thin Layer Chromatography (TLC) and High Performance Liquid Chromatography.



Asiatic acid $R_1 = H$ $R_2 = H$
Madecassic acid $R_1 = OH$ $R_2 = H$
Asiaticoside $R_1 = H$ $R_2 =$ Glu-Glu-Rha
Madecassoside $R_1 = OH$ $R_2 =$ Glu-Glu-Rha

Figure 1: Asiaticoside structure

2. MATERIALS AND METHODS

2.1 Materials required
Pre-coated silica gel (Aluminium sheet) as an adsorbent, Chloroform, Glacial acetic acid, Methanol, water, Acetonitrile and Sulphuric acid etc.

2.2 Chemicals and glassware
Chemicals required are Ethanol, methanol, acetone, acetonitrile, HPLC grade water, Liquid nitrogen, Chloroform, Standard asiaticoside etc.

2.3 Instruments
Freeze drier, Vortexes, HPLC equipment with RP C_{18} Column. In the present work, the modular LC (LC 20-A) from Shimadzu available in central facilities for research and development was used. The equipment consists of Dual plunger with modular flow line, Auto injectors (for injection of sample), RP C_{18} Column ovens (CTO 20A/20AC) and Photo Diode Array (PDA) detector.

2.4 Plant material
The two varieties of *Centella asiatica* (L.) Urban *(*syn.*:Hydrocotyle asiatica L*.) of the Apiaceae (Umbelliferae) family were collected from CIMAP (Hyderabad) and from the Forest Academy, Dhulapally, on the outskirts of Hyderabad and GHMC Herbal Garden, Hyderabad. and grown in the botanical and experimental garden, Department of Botany, Osmania University, Hyderabad. The two varieties have been referred to as Majjaposhak and Subhodak.

2.5 Processing of plant samples
The leaves of the *Centella asiatica* (L.) Urban plants were properly washed using tap water and then rinsed with distilled water. The rinsed leaves were dried in an oven at a temperature of 35-40°C for 3 days. The dried leaves of each plant were pulverized, using a sterile electric blender, to obtain a powered form. The powdered form of these plants was stored in airtight glass containers, protected from sunlight until required for analysis.

2.6 Preparation of aqueous extract of plant samples
The aqueous extract of each plant sample was prepared by soaking10 g of powdered samples in 200 ml of distilled water for 12 h. The extracts were then filtered using filter paper or Whatman filter paper.

2.7 Phyto chemical analysis
Chemical tests were conducted on the aqueous extract of each plant sample and also of the powdered form of the plant samples using standard methods.[18,19.] Test for tannins, phlobatannins, saponins, flavonoids, terpenoids and cardiac glycosides was performed. The plant extract was analysed for the estimation of several phytochemical constituents by quantitative analysis and for the estimation of asiaticoside by thin layer Chromatography (TLC) and High Performance Liquid Chromatography (HPLC).

2.8 Special method of extraction for TLC and HPLC analysis
The leaves of *Centella asiatica* were freeze-dried and 1 gram of powered tissue was dissolved in Ethanol (70ml): water (30ml) (7:3) and kept for 48 hours. The extract was filtered through Whatman No.1 filter paper and was transferred into separating funnel. To this extract, 20 ml petroleum ether was added, to separate the aqueous layer. The aqueous layer was collected, and acetone (20 ml) and 20 ml Di ethyl ether were added to it. After that, the aqueous layer was collected and vortexed for 3 minutes followed by centrifugation at 5800g for 1 minute to remove particulate matter. Supernatant was collected and filtered through 0.22µm membrane filter and then subjected to TLC, HPLC analysis.
The requirements of TLC includes adsorbent, mobile phase, TLC plates, developing tank and detecting agents.

a) TLC: TLC profile of leaf extract of two varieties of *Centella asiatica* was performed by using pre-coated silica gel (Aluminium sheet as an adsorbent). Chloroform: Glacial acetic acid: Methanol: water (60:32:12:8) was used as mobile phase. Standard is prepared by dissolving 10 mg of asiaticoside in 10 ml of methanol. 1 ml of *Centella asiatica* was dissolved in 10 ml methanol. 10 µl applied on TLC plate. Solvent front was run up to 9 cms. Anisaldehyde Sulphuric acid reagent was used for detection of spots. The RF Value of standard asiaticoside was 0.4(Green spot). The spots with same RF value were identified with leaf extract of two varieties of *Centella asiatica*.

b) HPLC: Standard asiaticoside is procured from Sigma™**.** Shimadzu's Prominence HPLC system was used for the analysis. The Column used in HPLC analysis was RP C18 RP-Reverse Phase and PDA (Photo Diode Array) as detector. The Mobile Phase used is Methanol: water (70:30), Acetonitrile: water (Gradient elution). The flow rate used was 1 ml/min., sample injection volume 20µl at a temperature of 25°C and the detector wavelength was 214nm.

c) Standard preparation
Weigh accurately 1 mg of asiaticoside in a 25 mL volumetric flask. Dissolve in 15 mL of Acetonitrile and make up to 25 mL with Acetonitrile.

d) Procedure
The mobile phase (mixture of solvents) was introduced into the column with the help of plunger reciprocating pump. The instrument consists of Dual plunger with tandem flow line (LC 20-A) to reduce pressure fluctuations. Temperature is maintained at 25°C with the help of column ovens. Initially standard asiaticoside is placed in auto injector (SIL 20-AC) and the chromatogram was run. Then the column was washed with HPLC grade water. Later the extract is auto injected. The components are separated in RP C_{18} Column and detected by PDA. Finally, the chromatograms were recorded.

3. RESULTS AND DISCUSSION
Centella asiatica leaf extract was brownish green in coloration when treated with 0.1% FeCl3 that shows the presence of tannins. The frothing was converted into emulsion with 3 drops of olive oil, which indicates the presence of saponins. Plant extract has shown yellow coloration when treated with 1% NH3, which indicates the presence of flavonoid compounds. An interface with a reddish brown coloration is formed when plant extract was treated with concentrated H_2SO_4, which indicates the presence of tanins. Brown ring is appeared when plant extract was treated with FeCl3 and concentrated H_2SO_4, which indicates the presence of cardiac glycosides. The percentage of phytochemicals in Majjaposhak and Subhodak varieties of *Centella asiatica were* reported (Table 1and 2).
Standard asiaticoside and Leaf extract of Majjaposhak and Subhodak variety of *Centella asiatica* (C1, C2) were loaded on pre-coated silica gel. Solvent front was found to be 9 cms. The Rf value of standard asiaticoside was 0.4. The Rf values of C1 and C2 were identical to the Rf value of asiaticoside. All the spots were appeared in green colour. Hence, asiaticoside was confirmed in leaf extracts of *Centella asiatica.* Chloroform: Glacial acetic acid: Methanol: water (60:32:12:8) was used as mobile phase. In HPLC studies, initially the chromatogram was recorded for standard asiaticoside (procured from Sigma™) using Acetonitrile: water (70:30) solvent system. Standard Asiaticoside fraction was eluted in between 8-10

minutes and peak was identified (Figure 2). The chromatogram for the plant extracts of two varieties of *Centella asiatica (*Majjaposhak and subhodak*)* were developed using different solvent systems such as methanol: water (70:30) using isocratic program (polarity is constant) and Acetonitrile: water using Binary program (polarity increases gradually). Peak was not identified when methanol: water used as solvent system (isocratic elusion). Finally, the Acetonitrile: water system (gradient elusion) was used for the analysis of Asiaticoside (Table 3). The retention time of asiaticoside is around 8-10 minutes (Figure 3 and 4). The asiaticoside content present in two plant extracts were calculated from peak areas. The mean asiaticoside content in Majjaposhak variety was found to be (

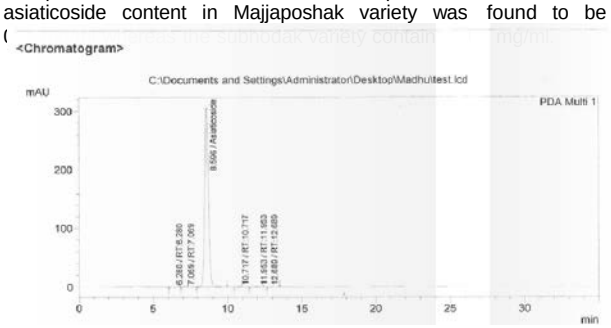


Figure 2: HPLC of standard Asiaticoside

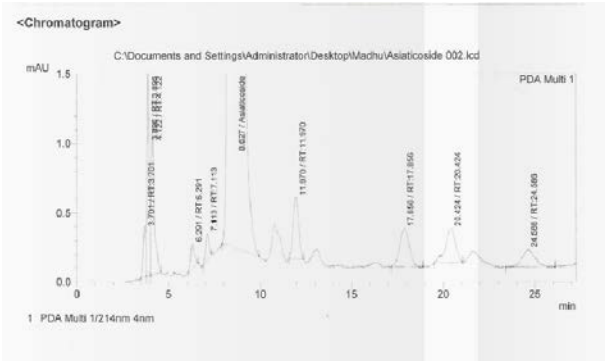


Figure 3: HPLC of *Centella asiatica* leaf extracts (Majjaposhak)

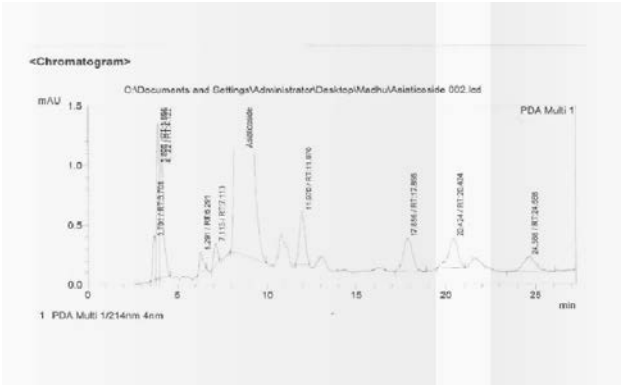


Figure 4: HPLC of *Centella asiatica* leaf extracts (Subhodak)

Table 1: Qualitative analysis of leaf extract

S. No	Test	Inference
1.	Test for tannins	Positive
2.	Test for phlobatannins	Positive
3.	Test for saponins	Positive
4.	Test for flavonoids	Positive
5.	Test for terpenoids	Positive
6.	Test for cardiac glycosides	Positive
7.	Test for alkaloids	Positive

Table 2: Quantitative analysis of leaf extract

S. No.	Test	Majjaposhak	Subhodak
1.	Test for tannins	10.1±0.14	0.9±0.13
2.	Test for phlobatannins	0.9±2.03	0.7±2
3.	Test for saponins	2.3±0.12	2.6±0.11
4.	Test for flavonoids	0.53±0.21	0.50±0.22
5.	Test for terpenoids	0.7±1.21	0.6±1.01
6.	Test for glycoside	O.5±O.7	0.6±0.9
7.	Test for Phenols	0.71±0.20	0.720±0.19

Table 3: Gradient conditions used in HPLC analysis of *C. asiatica* leaf extract

Time (minutes)	Water (%)	Acetonitrile (%)
0-10	20	80
10-15	30	70
15-20	40	60
20-25	60	40
25-35	70	30

3.1 Data analysis

The results were analyzed to know the difference in measured attributes among the two varieties by one way analysis of variance (ANOVA) and the Duncan's homogeneity test using Statistical Package for Social Science, version 11.5 (SPSS 2002). Content of asiaticoside varied greatly in different varieties. However, the difference was statistically significant for asiaticoside ($p< 0.05$) (Table 4). Samples collected from Majjaposhak variety showed the highest asiaticoside. Asiaticoside was the most dominant constituent (mean 1.42% dw); its value ranged from 1.42% in Majjaposhak to 0.78% in Shubodak variety (Table 4).

Table 4: Asiaticoside content of *Centella asiatica* varieties

Centella asiatica variety	Asiaticoside content (dw).%	Mean (dw).%	F value	P value
CA-M1	1.42 ± 1.10	1.42 ± 1.60	10.334	0.030
CA-M2	1.31 ± 1.03			
CA-M3	1.51 ± 1.03			
CA-S1	0.83 ± 0.01	0.78 ± 0.02	0.604	0.012
CA-S2	0.78 ± 0.02			
CA-S3	0.73 ± 0.11			

For each parameter, significant difference between mean among the sites are indicated by different letters (Duncan homogeneity test, á = 0.05). F and P values were obtained by one way analysis of variance (ANOVA).

Therefore, the present study revealed that the asiaticoside content in leaves of the two ecotypes varied. Asiaticoside was not detected in undifferentiated cells including cultured cell suspensions and calluses. The distribution of asiaticoside throughout the plant was organ specific; with leaves of both varieties, containing the higher content of this compounds.The distribution of asiaticoside is more in leaves of *Centella asiatica*. The phytochemical screening on qualitative and quantitative analysis shows that the leaves of the *C.asiatica* are rich in alkaloids, tannins, saponins, terpenoids and flavonoids, which are popular phytochemical constituents. Significant difference in contents of asiat coside has been observed in samples of *C. asiatica* originating from different varieties, such as Shubodak and Majjaposhak. Comparative study of two varieties of Centella asiatica from different regions of India showed a correlation between genomic diversity and asiaticoside content. In present study, mean asiaticoside content in samples of Majjaposhak variety *Centella asiatica* was 1.42% (dw). The leaves of Majjaposhak variety of *C.asiatica* found to contain around two time's higher concentration of asiaticoside than those from Shubodak variety.

4. CONCLUSION

Although the present study provides a lead, many more studies are needed to confirm the efficacy of the use of *Centella asiatica* as a source of phytomedicine. There is a long way to go before its establishment as a phytomedicine since studies on animals and human clinical research must be completed successfully.

5. ACKNOWLEDGEMENTS

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