ABSTRACT

Objective: In this study isolation of asiatic acid was achieved by TLC and thus isolated asiatic acid was characterized by 1H NMR spectral analysis and LCMS. An isocratic RP-HPLC method was established for the assessment of asiatic acid from the methanol extract of Centella asiatica. The triterpenoid separations were achieved by RP-C18 column 250x4.6 mm (5µ Particle size) Shimadzu UFLC pump LC 20AD and mobile phase composed of 0.1% orthophosphoric acid: acetonitrile (50:50). The analysis of column effluents was achieved using a PDA detector (Photo Diode Array) at 210 nm, and the flow rate was recorded 1 ml/min.

Methods: Soxhlet extraction process followed by fractional extraction using different solvents was performed. Methanol extract was taken to isolate asiatic acid by TLC (RF 0.97) and base peak from mass spectra was found to be 489M. The analysis of column effluents was achieved using a PDA detector (Photo Diode Array) at 210 nm, and the flow rate was recorded 1 ml/min.

Results: Retention time of asiatic acid was found to be 9.6±0.22 min. This method obeyed linearity over the concentration range of 10-50 µg/ml and regression coefficient was obtained from mean assay values was found to be 1.02%. Since there were no marked changes in the performance characteristics of the method.

Conclusion: Isolation, characterization and identification of asiatic acid were achieved significantly which will be useful for the standardization of herbal formulation containing asiatic acid.

Keywords: Asiatic acid, RPHPLC, 1H NMR, LCMS, PDA

INTRODUCTION

Centella asiatica, generally known as Gotu kola is a small; herbaceous plant belongs to a family Apiaceae. It is a frost-tender perennial plant prevalent in tropical and sub-tropical countries. In India, it is well known as Brahmi, and since ancient times, it has been used as a memory enhancer [1]. Centella asiatica plants are the richest bio-resource of drugs. Separation of medicinally active portions of the plant was done by extraction method by using selective solvents [2]. The triterpenoid compounds are responsible for the pharmacological activity of Centella asiatica. The triterpenoid ingredients in Centella asiatica have been identified by thin layer chromatography using mass spectrometry and NMR to determine the compounds [3]. The triterpenes isolated from Centella asiatica are mainly pentacyclic triterpenic acids and that the asiatic acid, asiaticoside, madecassic acid, madecassoside, brahmoside, brahmic acid, brahminoside and other triterpenic glycosides of Centella asiatica are of the ursane-or oleanane-type. In triterpenoids, asiatic acid (AA), has gained substantial value due to its polypharmacological properties and therapeutic potential in various diseases. AA has been identified as the most active compound in the plant associated with the power of rejuvenating neuron [4]. It is used to control oxidative stress response that is associated with neurodegenerative changes in Alzheimer’s disease. Neuroprotective efficacy of AA was shown against glutamate-induced or beta-amyloid-induced neurotoxicity and focal cerebral ischemia. The useful application of AA was shown in wound healing, various skin lesions, treatment for inflammations, liver fibrosis, cerebral ischemia, dementia, hyperglycemia, metabolic syndrome, diarrhoea, asthma, and tuberculosis [5].

Chromatography is considered as one of the well-established analytical separation techniques. High-performance liquid chromatography has been the most comprehensive chromatographic technique initially employed for the analysis of phytoconstituents [6]. High-performance liquid chromatography/electrospray ionization tandem mass spectrometry (HPLC/ESI-MS/MS) techniques are adopted for quantifying triterpenoids from biological matrices [6]. Working of Reversed-phase high-performance liquid chromatography (RP-HPLC) includes the separation of molecules based on the hydrophobicity. The separation process involves the hydrophobic binding of the solute molecule from the mobile phase to the immobilized hydrophobic ligands connected to the stationary phase. The separations are prompt than the classical method and provide the high degree of sensitivity with enhanced accuracy [7].

The present study aimed at the development of suitable extraction, isolation and characterization procedure to obtain the purified compound and to establish suitable RP-HPLC method for the quantitation of asiatic acid from Centella asiatica.

MATERIALS AND METHODS

Materials

HPLC grade methanol, orthophosphoric acid, acetonitrile and water were procured from E. Merck Ltd., Mumbai, India. 10%v/v sulphuric acid was purchased from Sigma-Aldrich Chemise, USA. All the chemicals including solvents used were of analytical or HPLC grade.

Methods

Collection and identification of plant materials

The plant specimen was collected early morning from fertile land in the month of June. The specimen was identified. Voucher specimen number of the whole plant 16PH005R and it was confirmed, authenticated by Dr. Krishna Kumar G, Professor, Department of...
saponins, quinone and phenolic compounds were assessed in various phytochemical analyses. The major secondary metabolites like Extracts were filtered, concentrated and subjected to various applied Botany, Mangalore University, Karnataka, India. The whole plant was used for the research activity.

Preparation of Centella asiatica extract

The extraction process was conducted by soxhlet extraction method with the repeated cycle by using fresh solvent. Fresh leaves of the Centella asiatica plants were sun-dried for two weeks and grinded to powder. 50 g of clean powder was subjected to extraction using methanol in a soxhlet apparatus. 44 cycles were run for the extraction at the temperature of 65°C [8].

Fractional extraction process

The dried powdered leaves extract was further fractionally extracted with five different solvents such as petroleum ether, chloroform, ethyl acetate, n-butanol and methanol subsequently throughout 24 h for 5 d. Extracts were filtered, concentrated and subjected to various pharmaceutical analyses. The major secondary metabolites like carbohydrate, tannins, steroids, terpenoids, alkaloids, flavonoids, saponins, quinone and phenolic compounds were assessed in various extractions was confirmed by standard protocols [8].

Isolation and purification of asiatic acid

Initially, preparative 0.2 mm silica gel coated TLC plates of 5×5 cm size (GF254, Merck, Germany) was procured. An aliquot of a standard solution of asiatic acid and a sample solution of Centella asiatica extract were spotted onto the silica gel plate and allowed to dry. The mobile phase which was used to develop TLC plate was ethyl acetate: methanol: water (8:1:1) in a saturated glass chamber. Later, the developed plate was dried and spots were visualized by spraying with a solution of 10%v/v sulphuric acid and dried under oven at 105°C. The RI values of isolated compounds and standard were calculated and compared [9]. For purification, the extract was applied manually on a TLC glass plate (20×20 cm; 1500 µm thickness) along with inorganic fluorescent indicator binder (Analtex, Sigma-Aldrich, Germany). The same mobile phase was taken which was used in the analytical TLC. Plate was kept for air drying for 30 to 40 min for the separation. Well-developed band was scratched and dissolved in HPLC grade methanol, centrifuged at 12000rpm for 15 min to remove silica. Supernatant was filtered from 0.22 µm filter and dried. All the samples were passed under nitrogen gas for 5 min and then dissolved in methanol for further characterization and quantitative HPLC analysis [9].

Characterization of isolated compound

Liquid chromatography-mass spectroscopy

Identification and integration of chromatographic peaks were achieved by using Thermo/Finnigan Surveyor system containing a degasser, binary pump, autosampler, and column heater. The column outlet was coupled to a Thermo (LOQ-Fleet) Ion Trap mass spectrometer equipped with an ESI ion source. By using Data Analysis software (Qual Browser; Thermo Electron, San Jose, CA) Data acquisition and mass spectrometric evaluation were carried out [10]. For the chromatographic separation, a phenomenex C8 column (250 × 4.6 mm) was used. The column was held for 1 min at 95% solvent A (0.1% orthophosphoric acid in water) and 5% solvent B (acetonitrile). Then followed by 11 min in step gradient from 5% B to 100% B, then 4 min with 100% B. Elution was achieved with a linear gradient from 100% B to 5% B for 2 min. The flow rate was kept 0.2 ml/min and 5 µl volume was injected. This condition was maintained throughout the MS experiment: for electrospray ionization with positive ion polarity the capillary voltage was set to 20 V, the capillary temperature to 300 °C, the nebulizer pressure to 40 psi, and the drying gas flow rate to 15 L/min. MS data were documented in the complete scan mode (m/z 50 to 1000). A quantitative analysis was performed by the monitoring of each protonated molecular ion in the positive ion mode [11].

\[ {^1}H \text{ NMR} \]

\[ {^1}H \text{ NMR} \] spectra of isolated asiatic acid were recorded on a NMR-400 MHz (with multi-nuclei analysis from 'H) and chemical shifts were recorded as δ values. Using TMS as an internal standard and deuterated methanol was taken as solvent [12].

HPLC method development

Preparation of standard stock solution

Accurately weighed 10 mg of standard asiatic acid was transferred to a 10 ml volumetric flask and dissolved in methanol. Volume was made with methanol to obtain standard stock solution of concentration 1000 µg/ml. This stock solution was further diluted for the studies [12].

Calibration of asiatic acid

A stock solution consisting of asiatic acid (1000µg/ml) was prepared. From this 10, 20, 30, 40, 50µg/ml aliquots were made by adjusting with methanol in 10 ml volumetric flasks and from each 10 µl of the solution was injected into the RP-HPLC system. Linearity was established by triplicate injections of solutions containing standard asiatic acid. The developed RP-HPLC method was validated by determination of selectivity, linearity, limit of quantitation and detection, precision, accuracy, recovery, robustness and stability [13].

System suitability studies

System suitability was achieved by injecting six replicate injections of standard solution of asiatic acid and followed by determination of % relative standard deviation (% RSD) of peak areas, resolution factor, tailing factor and theoretical plates [13].

LoD and LoQ

As per ICH of technical requirements for the registration of pharmaceuticals for human use recommendations, Limit of Detection (LoD), and Limit of Quantitation (LoQ) are terms used to illustrate the minute concentration of an analyte that can be consistently measured by an analytical procedure. From the calibration curves LoD and LoQ values were calculated by using k*SD/b where, k=3 for LoD and 10 for LoQ, SD stands for standard deviation of the response of the minimum detectable drug concentration and b represents the slope of calibration [13].

Accuracy (Recovery)

Accuracy of the process was determined by recovery experiments by considering standard addition method at three different levels (80, 100 and 120%). Known volumes of standard solutions containing asiatic acid (8, 10, and 12 µg) were added to pre-quantified sample solutions (isolated asiatic acid) to reach the 80, 100 and 120% levels. These samples were analysed in triplicate and recovery was calculated. Then the difference between the spiked and un-spiked sample was determined for different recovery [13].

Precision (Repeatability)

Method Precision (intraday study) of the assay method was demonstrated by analysing six different sample solutions containing isolated asiatic acid equivalent to 10µg/ml and from the area attained, concentration was determined and the results were exhibited as % RSD (Relative Standard Deviation) [13].

Intermediate precision (Ruggedness)

Intermediate precision (intraday study) of the method was validated by performing an experiment on different days by different analysts and on different C18 columns [13].

Robustness

Robustness of the method was determined by intentionally varying the chromatographic conditions. The flow rate of the mobile phase was changed from 1.0 to 0.9 ml/min and 1.1 ml/min. The composition of mobile phase was changed from 50:50 (0.1 % orthophosphoric acid:acetonitrile) to 47.5:52.5 and 52.5:47.5 (0.1 % orthophosphoric acid:acetonitrile) (i.e. 5% change). Three times standard solution was injected into the column to study for the robustness and the responses were noted [13].

RESULTS AND DISCUSSION

After identification of the plant, leaves were collected, dried and subjected to soxhlet extraction process. The dark green liquid extract gets concentrated, and methanol was evaporated resulted in
dried powder which was then preserved in a dessicator [14]. One part of extraction is further subjected to fractional extraction process by using petroleum ether, chloroform, ethyl acetate, n-butanol and methanol to assess phyto constituents. The extracts of Centella asiatica showed different phyto profiles with reference to the solvents. Out of the five solvents (petroleum ether, chloroform, ethyl acetate, n-butanol, methanol) used methanol extract demonstrated the maximum occurrence of phytoconstituents. It is because of amphilic nature methanol dissolves a larger portion of polar compounds and a certain group of non-polar compounds. Phytoconstituents' screening was shown in table 1. Hence methanol extract was selected for further analytical study [14].

**Table 1: Phytochemical screening of Centella asiatica**

| Test             | Solvents       |
|------------------|----------------|
|                  | Petroleum ether| Chloroform | Ethyl acetate | n-butanol | Methanol |
| Alkaloids        | -              | +          | -             | +         | +        |
| Carbohydrate     | -              | -          | +             | +         | +        |
| Flavonoids       | -              | +          | -             | +         | +        |
| Glicosides       | -              | +          | +             | +         | +        |
| Phenolic compounds| -             | -          | +             | -         | +        |
| Protein          | -              | +          | -             | -         | -        |
| Saponins         | -              | -          | +             | +         | +        |
| Steroids         | -              | +          | +             | +         | +        |
| Tannins          | -              | -          | -             | -         | +        |
| Terpenoids       | -              | -          | -             | -         | +        |

+ indicates presence, - indicates absence

**Fig. 1: Preparative TLC run with standard asiatic acid and Centella asiatica extract**

**TLC extraction of asiatic acid**

The Rf value of both standard and sample were found to be similar (0.97) which confirmed that the isolated compound is structurally related to the reference standard of asiatic acid [15]. The TLC profile of the compound is represented in fig. 1

**Characterization of isolated compound**

Characterization of isolated compound was done by LCMS and NMR spectra analysis. Mass spectral data showed the molecular ion peak and base peak at m/z 489 M+ which confirmed successful isolation of asiatic acid from Centella asiatica. (fig. 2) [15]. The 1H NMR spectra of isolated component was recorded at 400 MHz frequency on δ-scale and 1H shifts are reported in parts per million (ppm). It showed the chemical shift values as follows: 1.257-1.198(2H, t, J=10.8Hz, J=12.8Hz, H-1), 3.525-3.475(2H, H-2,3, br, J=9.6Hz, J=5.6Hz), 4.221-4.211 (1H, d, J=4Hz, H-4), 4.168-4.159 (1H, d, J=3.6Hz, H-3), 1.579-1.469(H, m, H-5,6,7,15,16), 1.429 (1H, s, H-9), 1.392(H, s, H-18), 1.782-1.772(1H, d, J=4Hz, H-11a), 1.813-1.804(1H, d, J=3.6Hz, H-11b), 4.421-4.396 (1H, br t, J = 4 Hz, H-12); 2.139-2.111(1H, d, J = 11.2 Hz, H-18); 3.073 and 3.036 (1H each, d each, J = 10.4 Hz, H-23a and H-23b); 1.307(3H, s, CH3-24); 1.952-1.948(3H, d, CH3-25); 1.981(H, s, CH3-26); 1.922(3H, s, CH3-27); 1.894-1.876(3H, d, J=7.2Hz, CH3-29); 1.839(H, s, CH3-30). The obtained δ values matches with the described values of asiatic acid [15]. From this result it was further confirmed that the isolated component is asiatic acid represented in fig. 3.

**Fig. 2: LC MS spectra of isolated asiatic acid**
RP-HPLC Method development

Analysis was done by HPLC method using the conditions given in table 2. Several trials are conducted to develop a suitable method and to select a suitable mobile phase. It is because of the complexity of the chemical composition of the herbals and the affinity of the components towards various solvents [16].

### Table 2: Optimized chromatographic conditions

| Parameter             | Description                                                                 |
|-----------------------|-----------------------------------------------------------------------------|
| Instrument            | Shimadzu UFLC pump LC20AD                                                   |
| Autosampler           | S/L 20AC HT                                                                |
| Software used         | lab solutions 5.81                                                         |
| Column                | Phenomexuna C18 250×4.6 mm (5µ Particle size)                               |
| Flow rate             | 1 ml/min                                                                    |
| Injection volume      | 10 µl                                                                       |
| Column oven temperature | 30°C                                                                        |
| Detector              | PDA detector (SPD-M20A)                                                     |
| Wavelength            | 210 nm                                                                      |
| Mobile Phase          | 0.1% Orthophosphoric acid: Acetonitrile (50:50%)                             |

*PDA = Photo Diode Array

The proportions of the organic and aqueous phases were adjusted to obtain a rapid and simple assay method with the reasonable runtime, suitable retention time and sharp peak. Well-developed peak symmetry were obtained by using the mobile phase composition 0.1% orthophosphoric acid: acetonitrile (50:50 v/v).

Calibration of asiatic acid

HPLC profile for both standard and isolated asiatic acid indicated a single peak at retention time of 9.5 min (fig. 4) (fig. 5). Linearity regression data, summarized in table 3. This showed a good linear relationship between concentration and peak areas over a concentration range of 10-50 µg for asiatic acid (fig. 6) [16]. The correlation coefficient \( r^2 \) was found to be 0.9987.

### System suitability study

System suitability tests were carried out on freshly prepared standard solutions \( n = 6 \) asiatic acid. System suitability parameters obtained with 10 µl injection volumes are summarized in table 4.

### Table 3: Regression analysis of the calibration curves for asiatic acid

| Parameter             | Values                      |
|-----------------------|-----------------------------|
| Linearity range of Asiatic acid | 10-50µg/ml                  |
| Regression equation    | \( y = 3790.1 \times x - 3001.9 \) |
| Correlation coefficient \( r^2 \) | 0.9987                       |
| Slope                 | 3790.1                      |

*Acceptance criteria \( r^2 > 0.9 \)
Fig. 4: Chromatogram, UV spectrum and peak purity of asiatic acid

Fig. 5: Chromatogram of isolated asiatic acid
Fig. 6: Calibration curve of asiatic acid

Table 4: System suitability test parameters for asiatic acid

| Parameter                      | Values          |
|--------------------------------|-----------------|
| % RSD                          | 0.529%          |
| Standard deviation             | 979.90          |
| Retention time                 | 9.6±0.22        |
| Tailing Factor                 | 0.907±0.353     |
| Theoretical Plates             | 11225.5±0.38    |

*%RSD= Relative standard deviation. n=6 samples

LoD and LoQ
LoD and LoQ were obtained to be 0.784507 µg/ml and 2.615 µg/ml respectively. These values represent that the method is sensitive [17].

Accuracy (Recovery)
Accuracy studies indicated that the mean recovery of the added standard drug was 99.80% for isolated asiatic acid.

Precision (Repeatability)
In the precision studies, RSD of mean assay values was found to be 1.02%. The obtained value is satisfactory repeatability of the method [18].

Intermediate precision (Ruggedness)
The intermediate precision study indicates that the method is rugged with RSD values of 0.35% and 0.48% on different days and by different analysts respectively. By specificity study it was interpreted that there was no interference from impurities or degradation of products. It specifies that the peak response was due to asiatic acid only.

Robustness
Robustness studies showed that the results of the method remained uninfluenced by minute, intentional changes in the flow rate and column temperature [19]. The RSD of mean assay values was found to be 0.20% with a flow rate of 0.9 ml/min, and 0.20% with a flow rate of 1.1 ml/min. Also, RSD of mean assay values was found to be 0.10% and 0.12% for the mobile phase compositions 47.5:52.5 v/v and 52.5:47.5 v/v respectively. Since there were no marked changes in the performance characteristics of the method. It was considered being the well applicable method for validation and quantification of asiatic acid [20]. Summary of the study given was below table 5

Table 5: Summary of validation parameters for the proposed HPLC method for isolated asiatic acid

| Parameter                        | Value                 |
|----------------------------------|-----------------------|
| LoD µg/ml                        | 0.784507              |
| LoQ µg/ml                        | 2.615                 |
| Accuracy (% recovery)            | 99.80%                |
| Precision (%RSD)                 | 1.02                  |
| Intermediate precision           |                       |
| 1. different day                  | 99.83±0.35%           |
| 2. different analyst              | 98.62±0.48%           |
| Robustness (47.5:52.5 v/v)       | 99.03±0.106           |
| Robustness (52.5:47.5 v/v)       | 100.79±0.26           |
| Robustness (0.9 ml/min flow rate) | 111.28±0.23           |
| Robustness (1.1 ml/min flow rate) | 91.00±0.181           |

*Formula for LoD and LoQ=k*SD/b where, k=3 for LoD and 10 for LoQ, SD= Standard deviation, b= slope. Number of injections n=6, n=3 (Recovery studies)
CONCLUSION
Isolation and characterization of asiatic acid were accomplished which will be helpful for standardization of herbal formulations containing this active phytoconstituent. 'H NMR and LCMS spectra confirmed the successful isolation of asiatic acid. The proposed isolation method was found to be effective as the practical yield was satisfactory and the isolated compound was found to be relatively pure. The proposed RP-HPLC method was found to be simple, rapid, specific, accurate and precise for the determination of asiatic acid. The proposed method was found to be practical yield was satisfactory and the isolated compound was found to be relatively pure. The developed method can be adopted for the routine quantification and quality control of asiatic acid in various samples from formulations. This method was found to be reliable, robust, and better than the reported HPLC method for asiatic acid.

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AUTHORS CONTRIBUTIONS
All the authors have contributed equally

CONFLICT OF INTERESTS
Declared none

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