

## Research Article



## Phytochemistry, *in vitro* Free Radical Scavenging, Chelating and Toxicity of *Centella asiatica* L. (Apiaceae) Ethanolic Leaf Extract

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### ABSTRACT

*Centella asiatica* L. (Apiaceae) has various pharmaceutical properties and has been widely used for treating various ailments in Ayurveda. The present study was carried out to identify the phytochemicals present in the ethanolic extract of *Centella asiatica* L. (Apiaceae) leaves using both qualitative and quantitative screening methods, GC-MS and LC-MS analysis. The ethanolic extract was also subjected to *in vitro* free radical scavenging and chelating activity. The phytochemical screening showed the presence of triterpenoids, saponins, glycosides, sterols and alkaloids. The major components identified by gas chromatography-mass spectrometry (GC-MS) analysis were Methyl pyromeconic acid (RT: 5.183), Methoxy vinyl phenol (RT: 8.617), 3',5'-Dimethoxyacetophenone (RT: 11.867), Beta-D-Ribofuranoside (RT: 10.675), Cyclohexanecarboxylic Acid (RT: 12.825), 5-methoxy-2,2,8,8-tetramethyl-acetate (RT: 25.733) and Nobiletin (RT: 27.275). The major components identified by liquid chromatography-mass spectrometry (LC-MS) analysis were Maltol, 3', 5'-Dimethoxyacetophenone, Papyriogenin A, Asiatic acid, Asiaticoside, Madecassoside and Madecassic acid. *Centella asiatica* ethanolic extract effectively scavenges hydroxyl (OH•), superoxide anion (O<sub>2</sub>•-), DPPH•, nitric oxide, ABTS•+ and chelates in a concentration-dependent manner (12.5, 25, 50, 100 and 200 µg/ml). The LC<sub>50</sub> values for *in vitro* toxicity and haemolytic activity was found to 69.17 ±3.2 and 476.19 ±5.9 µg/ml, respectively. The present study indicates the presence of strong antioxidant, free radical scavenging and chelating properties in the ethanolic extract of *Centella asiatica* L. (Apiaceae) leaves.

**Keywords:** Triterpenoids, Phytochemical, Toxicity, Haemolytic.

### INTRODUCTION

*Centella asiatica* L. (Apiaceae) is also commonly known as Asiatic pennywort, Indian pennywort, Mandukparni, Spadeleaf or Gutu kola. It belongs to the family Apiaceae, formerly known as Umbelliferae. It is a slender, prostrate, glabrous, perennial creeping herb rooting at the nodes, with simple petiolate, palmately lobed leaves.

It has been widely cultivated in Southeast Asia, India, China, Sri Lanka, Africa, etc., as vegetable or spice. *Centella asiatica* L. (Apiaceae) has various activities like memory enhancing, wound healing, anti-inflammatory, antioxidant, immun-stimulant, anti-anxiety (anti-hypertensive), anti-stress and anti-epilepsy.

The various health benefits of *Centella asiatica* L. (Apiaceae) has lead to the increased usage of this plant in food and beverages. It has been widely used for the treatment of skin diseases, rheumatism, inflammation, syphilis, mental illness, epilepsy, hysteria, dehydration, diarrhea, wounds and ulcers.<sup>1-5</sup>

The present study was conducted to identify the phytochemicals present in the ethanolic extract of *Centella asiatica* L. (Apiaceae) leaves using both qualitative and quantitative screening methods, GC-MS and LC-MS analysis. The ethanolic extract was also used to evaluate the *in vitro* toxicity, free radical scavenging and chelating activity.

### MATERIALS AND METHODS

#### Chemicals

Sodium carbonate, KMnO<sub>4</sub>, FeCl<sub>3</sub>, H<sub>2</sub>O<sub>2</sub> and 2, 6-dichlorophenolindophenol was purchased from E. Merck. BHT, trichloroacetic acid, potassium ferricyanide, 1, 1-diphenyl-2-picryl- hydrazyl (DPPH), and ascorbic acid were purchased from Sigma Chemical Co. Ltd, USA. All other chemicals and solvents used were of analytical grade.

#### Plant Material collection and identification

*Centella asiatica* L. (Apiaceae) used in this study was collected freshly from outskirts of Chidambaram, Cuddalore District. The plant was identified at the herbarium of Department of Botany, Annamalai University. The leaves were washed under running tap water to remove dirt and other debris. It was then spread under a clean shade for drying. The dried leaves were milled to coarse power using a mechanical grinder and stored in an air-tight container.

#### Ethanolic extraction of plant material

Approximately 1 kg of powered *Centella asiatica* L. (Apiaceae) was used for ethanolic extraction using Soxhlet apparatus. The dark green extract obtained was subjected to ultracentrifugation followed by micro-filtration. The final clear dark extract was then concentrated in a rotary evaporator under reduced pressure. The final dried extract was lyophilized and was stored in a glass vials at -20°C for further use. This extract



was then subjected to preliminary qualitative and quantitative phytochemical analysis.

#### Percentage yield of plant extract

The percentage yield of the extract was determined gravimetrically using the dry weight of the crude extract obtained (X) and dry weight of plant powder used for the extraction (Y) by using the following formula:

$$\text{Percentage yield} = X/Y * 100$$

#### Qualitative and quantitative analysis

##### A. Qualitative screening

Phytochemical screening was carried out by using 1 gram of the dried ethanolic extract which was subjected to phytochemical test as described below (Harborne, 1973).<sup>6</sup>

##### Detection of alkaloids (Mayer's Test)

The extracts was dissolved in dilute Hydrochloric acid and filtered. The filtrate was treated with Mayer's reagent (potassium mercuric iodide). Formation of yellow coloured precipitate indicates the presence of alkaloids.

##### Detection of phenols (Ferric Chloride Test)

Extract was treated with 3-4 drops of 10% ferric chloride solution. Formation of green colour indicates the presence of phenols.

##### Detection of flavonoids (Alkaline Reagent Test)

Extracts were treated with few drops of sodium hydroxide solution. Formation of intense yellow colour, which becomes colourless on addition of dilute acid, indicates the presence of flavonoids.

##### Detection of quinones

The extract was treated with few drops of sulphuric acid. Formation of red colour indicates the presence of quinines.

##### Detection of tannins (Gelatin Test)

To the extract, 1% gelatin solution containing sodium chloride was added. Formation of white precipitate indicates the presence of tannins.

##### Detection of saponins (Foam Test)

0.5 gm of extract was shaken with 2 ml of water. If foam produced persists for ten minutes it indicates the presence of saponins.

##### Detection of terpenoids (Salkowski test)

The extract was added 2 ml of chloroform. Concentrated H<sub>2</sub>SO<sub>4</sub> (3 ml) was carefully added to form a layer. A reddish brown colouration of the interface indicates the presence of terpenoids.

##### B. Quantitative screening

##### Determination of Total Flavonoids (Aluminium chloride colorimetric assay method)

Total flavonoid contents were measured with the aluminum chloride colorimetric assay.<sup>8</sup> Aqueous and ethanolic extracts that has been adjusted to come under the linearity range i.e. (400µg/ml) and different dilution of standard solution of Quercetin (10-100µg/ml) were added to 10ml volumetric flask containing 4ml of water. To the above mixture, 0.3ml of 5% NaNO<sub>2</sub> was added. After 5 minutes, 0.3ml of 10% AlCl<sub>3</sub> was added. After 6 min, 2ml of 1 M NaOH was added and the total volume was made up to 10ml with distilled water. Then the solution was mixed well and the absorbance was measured against a freshly prepared reagent blank at 510 nm. Results are provided in (Table 2 and Figure 2). Total flavonoid content of the extracts was expressed as percentage of Quercetin equivalent per 100 g dry weight of sample.

##### Determination of total phenolic content

The total phenolic content (TPC) assay was performed in accordance to Singleton *et al.*, 1999, with modifications.<sup>8</sup> An aliquot of 0.5 ml of each sample was mixed with 1 ml of Folin-Ciocalteu reagent (10% in distilled water) in a universal bottle covered with aluminum foil. After 3 min, 3 ml of 1% sodium bicarbonate was added to each sample bottle, the universal bottles were cap-screwed and vortex. The samples were then incubated for 2 hr at room temperature in darkness. The absorbance was measured at 760 nm spectrophotometrically (Genesys UV 20, US). A standard curve of gallic acid solutions (ranging from 0 µg ml<sup>-1</sup> to 250 µg ml<sup>-1</sup>) was used for calibration. The experiment was done in triplicate. Results were expressed as microgram of gallic acid equivalents (GAE) per milligram of extract (GAE; µg mg<sup>-1</sup> dry extract).

##### Quantitative Estimation of Saponins

Plant extract was dissolved in 80% methanol, 2ml of Vanillin in ethanol was added, mixed well and the 2ml of 72% sulphuric acid solution was added, mixed well and heated on a water bath at 60°C for 10min, absorbance was measured at 544nm against reagent blank.<sup>9</sup>

##### Quantitative estimation of Alkaloids

5 g of the sample was weighed into a 250 ml beaker and 200 ml of 10% acetic acid in ethanol was added and covered and allowed to stand for 4 h. This was filtered and the extract was concentrated on a water bath to one-quarter of the original volume. Concentrated ammonium hydroxide was added drop wise to the extract until the precipitation was complete. The whole solution was allowed to settle and the precipitated was collected and washed with dilute ammonium hydroxide and then filtered. The residue is the alkaloid, which was dried and weighed.<sup>10</sup>

### Gas chromatography-mass spectrometry fingerprinting of crude extract

GC-MS analysis was done at National Chemical Laboratory Pune, Maharashtra, India. GC-MS sample was prepared by dissolving about 1 mg of *Centella asiatica L. (Apiaceae)* extract in 5 mL of methanol. Active extract was dissolved in HPLC grade methanol and subjected to GC and MS JEOL GC mate equipped with secondary electron multiplier. JEOL GCMATE II GC-MS (Agilent Technologies 6890N Network GC system for gas chromatography). The column (HP5) was used with fused silica 50 m x 0.25 mm I.D. Analysis conditions were 20 minutes at 100°C, 3 minutes at 235°C for column temperature, 240°C for injector temperature, helium was the carrier gas and split ratio was 5:4. The sample (1µl) was evaporated in a split less injector at 300°C. Run time was 22 minutes. The components were identified by gas chromatography coupled with mass spectrometry. Interpretation of mass spectra of GC-MS was done using the database of National Institute Standard and Technology (NIST) library search which is having more than 62,000 drug formulation. The mass spectrum of the unknown component was compared with the spectrum of the known components stored in the NIST-08 and Wiley-08 libraries. The name, molecular weight and structure of the components of the test materials were validated.

### LC-MS metabolomic fingerprinting of crude extract

In the present work the RP-HPLC (Shimadzu model – LA 3000) with semi preparative C18 HPLC column of Phenomenex (250 x10mm, 4 µm particle size, and 90Å pore size) and analytical column Zorbax C18 (4.6 x250mm, 5 µm particle size, and 80Å pore size) were used for the purification. Solvent system used here is Acetonitrile and Water containing (0.1%) TFA. A three step gradient elution was performed using of 0.1% TFA/water and 0.1% (v/v) TFA in 50% acetonitrile: 0–100% (60 min) held at 100% for 5 min and brought back to 0% (100–0%). Injection volume was 0.5 microlitres. Fractions were collected automatically using the fraction collector (FRC-10-A, Shimadzu model). The absorbance of the fractions was monitored at 280 nm and the peaks were compared with the spectrum of known components in NIST-08, Wiley-08, NAPRALET and CHEMSPIDER databases. The compound names, molecular weight and the structure of the compound identified in the crude extract were validated.

### In vitro free radical scavenging activity

Hydroxyl radical scavenging activity was measured by studying the competition between deoxyribose and the ethanolic leaf extract of *Centella asiatica L. (Apiaceae)* by  $\text{Fe}_3^+$ -Ascorbate-EDTA- $\text{H}_2\text{O}_2$  system (Fenton reaction) according to the method of Elizabeth and Rao<sup>11</sup>. The generation of  $\text{OH}^\bullet$  is detected by its ability to degrade deoxyribose to form products, which on heating with TBA forms a pink colored chromogen. The absorbance of the supernatant was read in a spectrophotometer at 535 nm.

The efficiency of *Centella asiatica L. (Apiaceae)* ethanolic leaf extract was compared with dimethyl sulphoxide (DMSO) as standard. Superoxide anion ( $\text{O}_2^{\bullet-}$ ) scavenging activity of *Centella asiatica L. (Apiaceae)* ethanolic leaf extract was determined by the method of Liu *et al.*, 1997.<sup>12</sup> Superoxide anion that is derived from dissolved oxygen through the PMS/NADH coupling reaction reduces NBT and absorbance was read in spectrophotometer at 560 nm. The efficiency of *Centella asiatica L. (Apiaceae)* ethanolic leaf extract was compared with ascorbic acid as standard. The effect of *Centella asiatica L. (Apiaceae)* ethanolic leaf extract on DPPH<sup>•</sup> was assayed using the method of Brand-Williams *et al.*, 1995.<sup>13</sup> DPPH<sup>•</sup> is a stable free radical and accepts an electron, or hydrogen radical to become a stable diamagnetic molecule. DPPH<sup>•</sup> reacts with an antioxidant compound that can donate hydrogen and gets reduced. The change in color (from deep violet to light yellow) was measured at 517 nm. The efficiency of *Centella asiatica L. (Apiaceae)* ethanolic leaf extract was compared with BHT as standard. The nitric oxide radical scavenging capacity of the *Centella asiatica L. (Apiaceae)* ethanolic extract was measured by Griess reaction.<sup>14</sup>

Various concentrations of *Centella asiatica L. (Apiaceae)* ethanolic leaf extract (12.5, 25, 50, 100 and 200 µg/ml in 95% ethanol) were prepared. Sodium nitroprusside (1.5 mL, 10 mM) in phosphate buffer was added to 0.5 mL different concentrations of the extract. The reaction mixture was incubated at 25°C for 150 min. After incubation, 0.5 mL aliquot was removed and 0.5 mL of Griess reagent (1% (w/v) sulfanilamide, 2% (v/v)  $\text{H}_3\text{PO}_4$  and 0.1% (w/v) naphthylethylene diamine hydrochloride) was added. The absorbance was measured at 546 nm. Ascorbic acid was used as reference standard and was treated the same way as that of the extract. Sodium nitroprusside in PBS (2 mL) was used as control.

The improved technique for the generation of  $\text{ABTS}^{\bullet+}$  involves the direct production of the blue/green  $\text{ABTS}^{\bullet+}$  chromophore through the reaction between  $\text{ABTS}^{\bullet+}$  and potassium persulphate.<sup>15</sup> The reaction mixture consisted of 0.5 mL of 15 µM  $\text{H}_2\text{O}_2$ , 0.5 mL of 7 mM ABTS and 50 mM sodium phosphate buffer, pH 7.5 and varying concentrations of *Centella asiatica L. (Apiaceae)* ethanolic leaf extract (12.5, 25, 50, 100 and 200 µg/ml). The blank contained water in place of *Centella asiatica L. (Apiaceae)* ethanolic leaf extract. The absorbance was read in spectrophotometer at 734 nm and compared with standard ascorbic acid. Decreased absorbance of the reaction mixture in all the assays indicated increased radical scavenging activity. The % of scavenging or inhibition was calculated according to the following formula:

$$\% \text{ of scavenging or inhibition} = [(A_0 - A_1)/A_0] \times 100$$

Where  $A_0$  was the absorbance of the control and  $A_1$  was the absorbance in the presence of *Centella asiatica L. (Apiaceae)* ethanolic leaf extract or ascorbic acid/DMSO.



### Iron chelating activity

The method of Benzie and strain (1996) was adopted for the assay.<sup>16</sup> The principle is based on the formation of O-Phenanthroline-Fe<sub>2</sub><sup>+</sup> complex and its disruption in the presence of chelating agents. The reaction mixture containing 1 ml of 0.05% O-Phenanthroline in methanol, 2 ml ferric chloride (200µM) and 2 ml of various concentrations ranging from 10 to 500µg was incubated at room temperature for 10 min and the absorbance of the same was measured at 510 nm. EDTA was used as a classical metal chelator. The experiment was performed in triplicates.

% of chelating activity = sample OD/Control OD x 100.

### In vitro toxicity tests

#### Drug preparation

The *Centella asiatica* L. (*Apiaceae*) extract was dissolved in 0.05% (v/v) of Dimethyl Sulfoxide (DMSO) and it did not affect cell survival.

#### PBMC proliferation test

Blood samples from healthy volunteers were collected by venepuncture and transferred into 15 ml heparin coated test tubes. It was diluted at 1:1 ratio with PBS, layered onto Ficoll-Histopaque 1077 at a volume ratio of 3:1 and centrifuged at 1,000 x g for 30 min. During the centrifugation the PBMCs moved from the plasma and were suspended in the density gradient, isolating them from erythrocytes and granulocytes. The PBMCs layer was removed and then washed twice with PBS. The supernatant was then removed and the cells were resuspended in complete CDMEM medium supplemented with 1 mM L-glutamine, 100 Units/ml penicillin and 0.1 mg/ml streptomycin, 10% inactivated FCS, and adjusted

to pH 7.2 by the addition of 15 mM HEPES. Cell viability was determined by the trypan-blue dye exclusion method. (Results not included in paper)

#### In vitro Cell viability test

The viability of cells was assessed by MTT assay using primary lymphocyte cells.<sup>17</sup> The PBMC cell density used in the cell viability study was 1 x 10<sup>5</sup> cells/ well of the 96-well tissue culture plate. Dose-response 1- 1000 µg/ml between percentage of cell viability and concentrations of the extracts were constructed.

#### In vitro hemolytic assay

In vitro haemolytic activity assay was performed according to the method described by Bulmus *et al.*, 2003.<sup>18</sup> Briefly freshly collected human red blood cells were taken and washed three times with 150 mM NaCl by centrifugation method at 2500 rpm for 10 minutes. The serum was removed and the cells were suspended in 100 mM sodium phosphate buffer. Nine different concentrations (0,5,10,30,50,100,200,300, 400µg/ml) of extracts were mixed with 200 µL of RBC solutions and the final reaction mixture volume was made up to 1 ml by adding sodium phosphate buffer. The reaction mixture was then placed in water bath for 1 hour at 37°C. After the incubation time the reaction was collected and the optical density was measured at 541 nm.

#### Statistical analysis

The data were expressed as mean ± SD (n = 3). Statistical analysis of the data was carried out by one-way analysis of variance (Anova) followed by Duncan's Multiple Range Test (DMRT) using a statistical package program (SPSS v11.5 for Windows) *p* < 0.05 were considered as statistically significant.

**Table 1:** Percentage yield of plant extract

Plant	Solvent	Method	Weight of crude extract (g)	% yield
<i>Centella asiatica</i>	Ethanol	Soxhlet extraction	10.89	1.089

**Table 2:** Qualitative analysis of *Centella asiatica* ethanolic extract

Secondary metabolites	Test	<i>Centella asiatica</i>
Alkaloids	Mayer's Test	+
Phenols	Ferric Chloride Test	+
Flavonoids	Alkaline Reagent Test	+
Quinones	Sulphuric acid test	+
Tannins	Gelatin Test	-
Saponins	Foam Test	+
Terpenoids	Salkowski test	+

+ Presence; - Absence

**Table 3:** Quantitative analysis of Secondary Metabolites in *Centella asiatica* ethanolic extract.

Plant	Phenols <sup>#</sup>	Flavonoids <sup>*</sup>	Saponins <sup>*</sup>	Alkaloids <sup>*</sup>
<i>Centella asiatica</i> ethanolic extract	12.40	2.70	11.00	3.20

<sup>#</sup>GAE µg mg<sup>-1</sup> dry extract; <sup>\*</sup> mg/g of crude extract



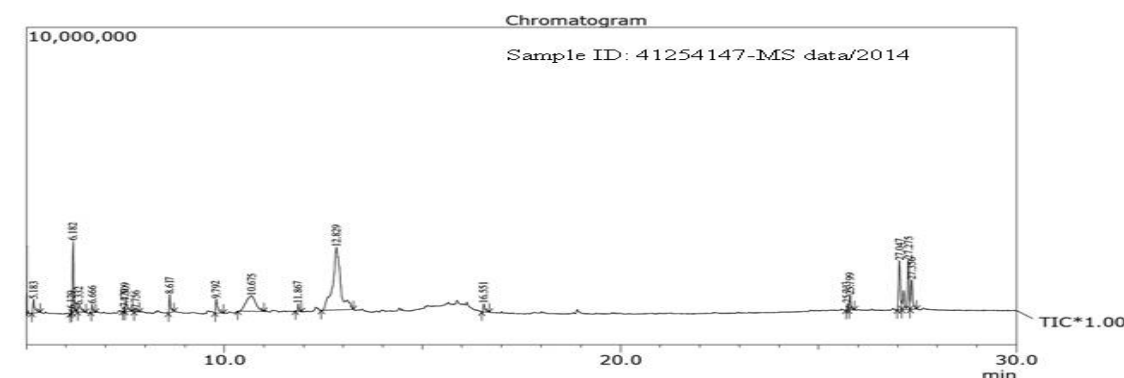
**Table 4:** Phytochemicals identified in the ethanolic extract of *Centella asiatica* by GC-MS.

R.T	Compound name	Molecular weight	Peak area %	Molecular formula	Pharmacology
5.183	Methyl pyromeconic acid (maltol)	126	2.24	C <sub>6</sub> H <sub>6</sub> O <sub>3</sub>	Catalyst, anti-proliferative, antioxidant, flavor additive
8.617	Methoxy vinyl phenol	150.17	3.06	C <sub>9</sub> H <sub>10</sub> O <sub>2</sub>	Anti gastritis and blood purifier
11.867	3',5'-Dimethoxyacetophenone	180	1.12	C <sub>10</sub> H <sub>12</sub> O <sub>3</sub>	-
10.675	Beta-D-Ribofuranoside	283.24	12.59	C <sub>10</sub> H <sub>13</sub> N <sub>5</sub> O <sub>5</sub>	-
12.825	Cyclohexanecarboxylic Acid	192	28.02	C <sub>7</sub> H <sub>12</sub> O <sub>6</sub>	Anti-inflammatory
25.733	5-methoxy-2,2,8,8-tetramethyl-acetate	372	1.01	C <sub>22</sub> H <sub>28</sub> O <sub>5</sub>	Antioxidant
27.275	Nobiletin	402.39	0.87	C <sub>21</sub> H <sub>22</sub> O <sub>8</sub>	Antioxidant and anti-inflammatory

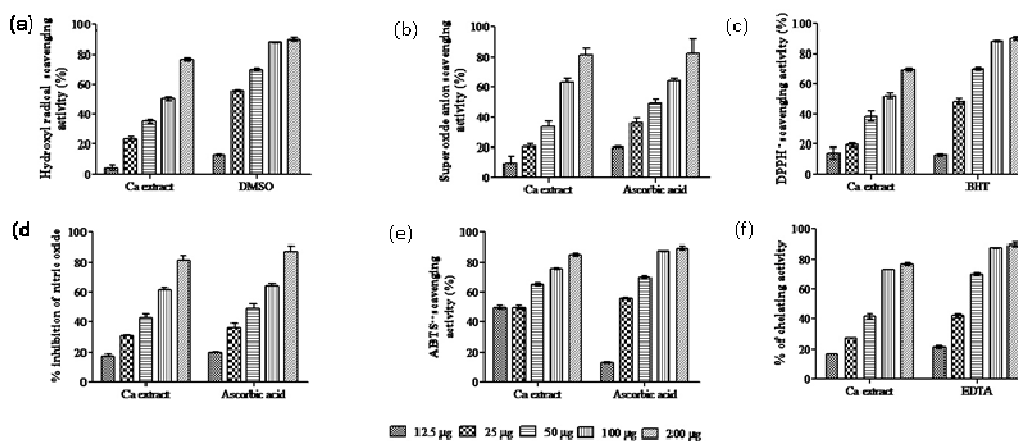
RT: Retention Time

**Table 5:** LC-MS mass library results for the ethanolic leaf extract of *Centella asiatica*.

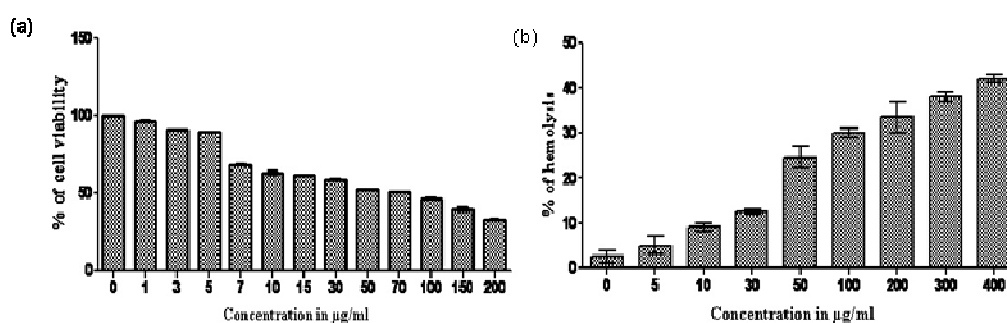
S. No.	Compound name	Molecular weight	Pharmacology
1	Maltol	150.17	Anti gastritis and blood purifier
2	3',5'-Dimethoxyacetophenone	180	Antioxidant
3	Papyriogenin A	466.308	Hepatoprotective
4	Asiatic acid	488.70	Neuro protective, anti-bacterial, anti-fungal, antioxidant, wound healing
5	Asiaticoside	959.12	wound healing
6	Madecassoside	975.1	wound healing
7	Madecassic acid	504.17	Anti-inflammatory



**Figure 1:** GC-MS spectrum of *Centella asiatica* ethanolic leaf extract.



**Figure 2:** Effect of *Centella asiatica* (Ca) extract on (a): hydroxyl (OH•); (b): superoxide anion (O<sub>2</sub>•-); (c): DPPH•; (d): Nitric oxide and; e: ABTS•+ radical scavenging ability. (f): Chelating activity of *Centella asiatica* extract. The values are given as mean ± SD of three experiments in each group.



**Figure 3:** (a) Percentage cell viability of *Centella asiatica* extract in PBMC. Lethal concentration 50 ( $LC_{50}$ ) value for *Centella asiatica* extract was found to be  $69.17 \pm 3.2$  µg/ml. (b) Percentage of hemolysis induced by *Centella asiatica* extract at concentrations ranging from 5-400 µg/ml. Lethal concentration 50 ( $LC_{50}$ ) value for *Centella asiatica* extract was found to be  $476.19 \pm 5.9$  µg/ml. The values are given as mean  $\pm$  SD of three experiments in each group.

## RESULTS AND DISCUSSION

This study was designed to determine the phytochemicals, *in vitro* toxicity, free radical scavenging and chelating activity in the ethanol leaf extract of *Centella asiatica* L. (*Apiaceae*). Table 1 shows the percentage yield of *Centella asiatica* (CA) ethanolic leaf extract and was found to be 1.089. The preliminary phytochemical screening of the ethanolic leaf extract of CA showed the presence of triterpenoids, saponins, glycosides, sterols and alkaloids (Table 2). The quantitative analysis of the extract (Table 3) showed the presence of phenols at a concentration of 12.4 GAE µg mg<sup>-1</sup> dry extract. The concentrations of flavanoids, saponins and alkaloids were found to be 2.70, 11.00 and 3.20 mg/g of crude extract respectively. In GC-MS analysis 7 phytochemical compounds were identified in the ethanolic leaf extract of *Centella asiatica* (CA). The identification of the phytochemical compounds is done by comparing the mass spectrum of the unknown component with the spectrum of the known components stored in the NIST-08 and Wiley-08 libraries. Activities of components identified in ethanolic extract were found from Dr. Duke's phytochemical and ethnobotanical databases [Online database]. Figure 1 shows GC-MS Spectrum of ethanolic leaf extract of *Centella asiatica*. The major components which were found in the leaves of the CA leaf ethanolic extract are (Table 4): Methyl pyromeconic acid (maltol) (RT: 5.183), Methoxy vinyl phenol (RT: 8.617), 3',5'-Dimethoxyacetophenone (RT: 11.867), Beta-D-Ribofuranoside (RT: 10.675), Cyclohexanecarboxylic Acid (RT: 12.825), 5-methoxy-2,2,8,8-tetramethyl-acetate (RT: 25.733) and Nobiletin (RT: 27.275). LC method was used for determining the non-volatile phytochemical contents constituents of *C. asiatica* ethanolic leaf extract. In LC-MS analysis 7 phytochemical compounds were identified in the ethanolic leaf extract of *C. asiatica* (CA). Activities of components identified in ethanolic leaf extract were found from Dr. Duke's phytochemical and ethnobotanical databases [Online database]. The major components which were found in the leaves of the CA leaf ethanolic extract are (Table 5): Maltol, 3', 5'-

Dimethoxyacetophenone, Papyriogenin A, Asiatic acid, Asiaticoside, Madecassoside and Madecassic acid. Medicinal plants are rich source of secondary metabolites. The triterpene form the major constituent of *C. asiatica*. Compounds like Asiatic acid, Asiaticoside, Madecassoside and Madecassic acid are already reported to be present in *C. asiatica*.<sup>19</sup> Papyriogenin A, a triterpenoid, was first time identified in *C. asiatica* extract. It is a potent hepato-protective compound.<sup>20</sup>

Figure 2 (a-e) shows the hydroxyl radical, superoxide anion, DPPH•, nitric oxide and ABTS•<sup>+</sup> scavenging ability of CA ethanolic leaf extract. CA ethanolic leaf extract inhibits radical formation and the percentage of inhibition was observed in a concentration-dependent manner. The effective concentration 50 ( $EC_{50}$ ) values of CA ethanolic leaf extract in hydroxyl radical, superoxide anion, DPPH•, nitric oxide and ABTS<sup>++</sup> were  $99.29 \pm 4.2$ ,  $78.98 \pm 5.4$ ,  $95.88 \pm 8.3$ ,  $80.90 \pm 3.5$  and  $38.26 \pm 7.8$  µg/ml, respectively and the free radical scavenging property was comparable to standard DMSO (Dimethyl sulfoxide), BHT (Butylated hydroxytoluene) and ascorbic acid, with  $EC_{50}$  values  $22.34 \pm 2.9$ ,  $35.67 \pm 8.3$ ,  $77.72 \pm 3.4$  for SOD and  $77.72 \pm 6.9$  µg/ml for nitric oxide radical scavenging assays, respectively. Iron binding capacity of CA ethanolic extract was carried out at various concentrations (12.5, 25, 50, 100 and 200 µg/ml). The metal chelator EDTA was used as a standard. Figure 2 (f) shows the percentage of iron chelating activity of CA ethanolic leaf extract compared to standard EDTA. The  $EC_{50}$  values of CA extract and EDTA was found to be  $68.13 \pm 7.9$  and  $35.67 \pm 6.8$  µg/ml, respectively. Figure 3(a) shows the percentage cell viability of *Centella asiatica* extract in PBMC. Lethal concentration 50 ( $LC_{50}$ ) value for *Centella asiatica* extract was found to be  $69.17 \pm 3.2$  µg/ml. Figure 3(b) shows the percentage of hemolysis induced by *Centella asiatica* extract at concentrations ranging from 5-400 µg/ml. Lethal concentration 50 ( $LC_{50}$ ) value for *Centella asiatica* extract was found to be  $476.19 \pm 5.9$  µg/ml. The presence of various antioxidant and anti-inflammatory compounds may be the reason for the presence of strong antioxidant, free radical scavenging and chelating properties of CA ethanolic leaf extract.

**CONCLUSION**

The present study revealed the presence of secondary metabolites of various therapeutical active compounds in the *Centella asiatica* ethanolic leaf extract. The presence of various bioactive compounds supports the use of the whole plant for various ailments in Ayurveda. This study further provides the scope of isolating and understanding the characteristics of each compound, alone or in combination, for its pharmacological properties.

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