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Qualitative and Quantitative analysis of Phytotochemicals in leaf extracts of *Centella asiatica* L.

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Abstract: *Centella asiatica is one of the chief medicinal herbs used for treating skin problems, wound, nervous disorders etc. and is found throughout tropical and sub tropical regions of India up to an altitude of 600m. Centella asiatica contains asiatic acid, asiaticoside and madecassoside as major phytochemical constituents that are responsible for pharmacological value apart from being rich in flavonoids and terpenoids.*
The present study was carried out on six solvent extracts of Centella asiatica to investigate the presence of medicinally important phytochemicals in their leaves. All the six extracts revealed the presence of various phytochemicals such as tannins, phlobatannins, saponins, terpinoids, diterpinoids, emodins, flavonoids, cardiac glycosides, anthraquinones, carotenoids, reducing sugars, alkaloids, anthocyanin, coumarins, steroids, phytosterols, phenol, fatty acids, proteins and amino acids. The leaves of Centella asiatica contained a significant amount of alkaloid, flavonoids, phenolic, saponins and tannin content. The amount of flavonoids was maximum (45.75mg/gm) followed by phenols (25.85mg/gm), alkaloids (17.75mg/gm), saponins (16.75mg/gm) and tannins (14.45mg/gm). The concentration of total alkaloids was maximum in distilled water extract (35.85mg/gm), followed by methanol extract (17.65mg/gm), ethanol extract (15.75mg/gm), petroleum ether extract (14.75mg/gm), acetone extract (12.45mg/gm) and benzene extract (11.75mg/gm). The concentration of total flavonoids was maximum in ethanol and methanol extracts (42.45mg/gm and 42.65mg/gm respectively), followed by distilled water extract (27.87mg/gm), benzene extract (13.35mg/gm), petroleum ether extract (14.65mg/gm) and acetone extract (13.55mg/gm). The amount of total phenol was maximum in ethanol and methanol extracts (15.35 and 15.45mg/gm respectively), followed by distilled water extract (12.33mg/gm), benzene and petroleum ether extracts (9.85 and 11.67mg/gm respectively) and acetone extracts (10.65mg/gm). Saponin concentration was maximum in ethanol (16.75mg/gm), benzene extract (15.25mg/gm) and distilled water extract (15.35mg/gm). Acetone and petroleum ether extracts contained relatively least amount of saponins (10.55 and 11.45mg/gm respectively). The total tannin concentration was maximum in ethanol extract (12.25mg/gm), followed by petroleum ether, acetone and benzene extracts (11.55, 10.35 and 10.35 mg/gm respectively. Methanol and distilled water extracts contained relatively low amount of total tannins, 9.25 mg/gm and 9.45 mg/gm respectively.
The data obtained in the present study is expected to serve as valuable tool for identification, authentication and detection of adulterants, standardization and quality control of the drugs. Hence it can be concluded that the results of the present study have given qualitative and quantitative information about the purity standards of the leaves of Centella asiatica.

Key words: *Centella asiatica, Phytochemicals, Acetone, Petroleum ether, Ethanol, Methanol, Benzene, Distilled water*

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I. Introduction

Centella asiatica L. (Syn Hydrocotyle asiatica Linn.) belonging to family Umbelliferae/Apiaceae of dicotyledonous angiosperm is a medicinal herb in India, China, Srilanka, Nepal and Madagascar. *Centella asiatica* is one of the chief herbs for treating skin problems, to heal wounds, for revitalizing the nerves and brain cells, and hence it is known as a "Brain food" in India. This herb is also known as Indian Pennywort, Gotu Kola, Asiatic pennywort, Spade leaf and Brahmi.

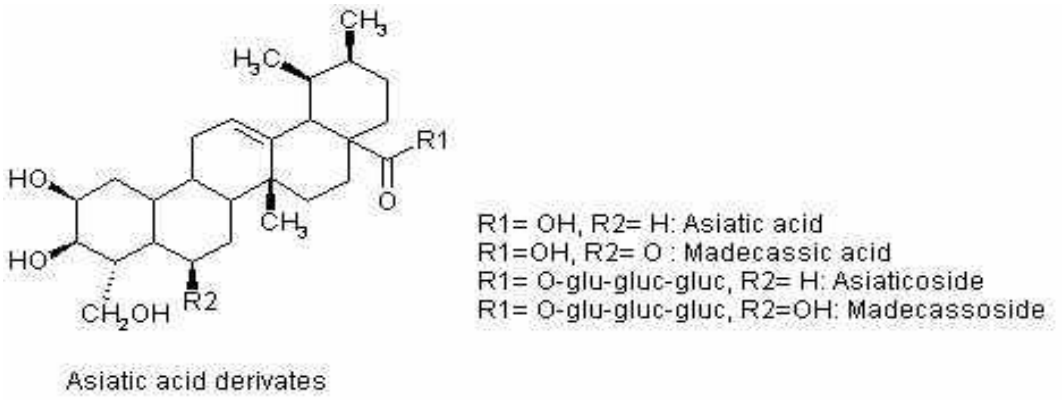
In Southeast Asia, it is traditionally used for the treatment of a wide variety of disorders such as skin diseases, rheumatism, inflammation, syphilis, mental illness, epilepsy, hysteria, dehydration, and diarrhea (Shanghai, 1977; Yu *et al*., 2006) [1, 2]. In Indian systems this plant is used as medicine for enhancing memory

and for the treatment of skin diseases and nervine disorders (Jamil *et al*., 2007) [3]. The plant medicinal properties have long been utilized by the people of Java and Indonesia. In China, it is indigenously called as Gotu kola, and it was one of the documented "miracle elixirs of life" (Diwan *et al*., 1991) [4]. Herbal medicines can be used as adaptogens, these plant derived drugs either reduce stress reactions in the alarm phase and provide a certain degree of safety against long-term stress (Wagner *et al*., 1994) [5]. *C. asiatica* is used to treat various ailments across India which includes body aches, headaches, insanity, asthma, leprosy, ulcers, eczemas, and wound healing (Mishra, 2003) [6]. *Centella asiatica* is an important medicinal herb used in the orient (Bown, 1995) [7], and is also popular in the West (Chevallier, 1996) [8]. It has been used as a medicine in the Ayurvedic tradition of India for thousands of years and listed in the historic '*Sushruta Samhita*', an ancient Indian medical text (Chopra *et al*., 1986; Diwan *et al*., 1991) [9, 4]. This medicinal plant has been listed as Threatened plant species by the International Union for Conservation of Nature and Natural Resources (IUCN) (Pandey *et al*., 1993) [10], and also as an endangered species (Singh, 1989; Sharma and Kumar, 1998) [11, 12].

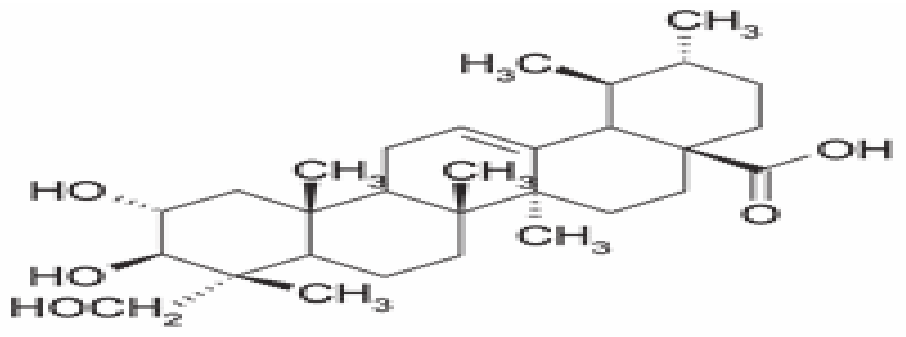
Botanical profile: *Centella asiatica* (L.) is a prostrate, faintly aromatic, stoloniferous, perennial, creeper herb, attaining a height up to 15cm. Stem is glabrous, striated, rooting at the nodes. This plant flourishes extensively in shady, marshy, damp and wet places such as paddy fields, river banks forming a dense green carpet and rather than clayey soil, the sandy loam (60% sand) is found to be the most fertile soil for its regeneration (Devkota Anjana and Jha, 2009) [13]. The leaves, 1-3 from each node of stems, long petioled, 2-6cm long and 1.5-5cm wide, orbicular-renniform, sheathing leaf base, crenate margins, glabrous on both sides. Flowers are in fascicled umbels, each umbel consisting of 3-4 white to purple or pink flowers, flowering occurs in the month of April-June. Fruits are borne throughout the growing season. It is about 6.5 cm long, oblong, globular in shape and has strongly thickened pericarp. Seeds have pendulous embryo which are laterally compressed.

Centella asiatica is found throughout tropical and sub tropical regions of India up to an altitude of 600m. The plant has been reported to occur also at high altitudes of 1550m in Sikkim and 1200m in Mount Abu (Rajasthan). The plant is indigenous to South-East Asia, India, Sri- Lanka, parts of China, the Western South Sea Islands, Madagascar, South Africa, South East USA, Mexico,Venezuela, Columbia and Eastern South America (Subban Ravi *et al*., 2008) [14].

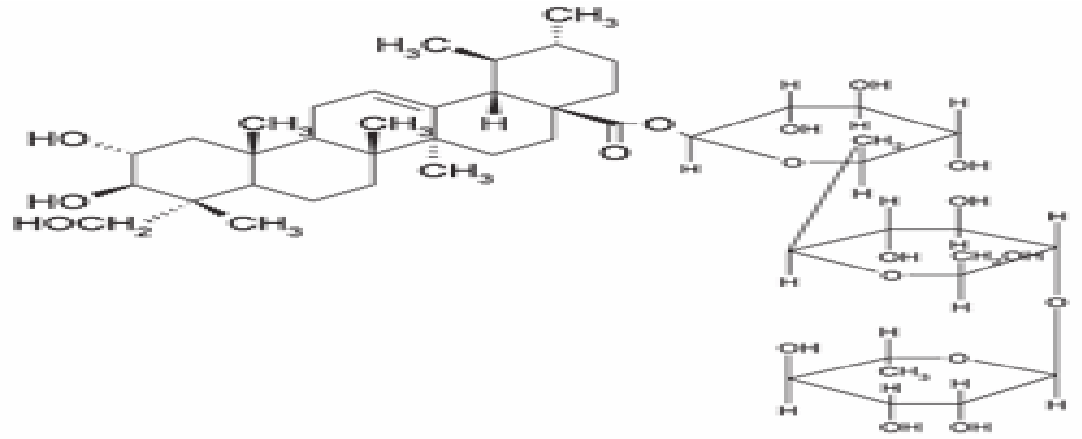
Phytochemicals of *Centella asiatica:* Centella asiatica contains asiatic acid, asiaticoside and madecassoside as major phytochemical constituents that areresponsible for pharmacological value apart from being rich in flavonoids and terpenoids (Roy *et al*., 2013) [15]. Centelloid was term given for different constituents of secondary metabolites produced by plant which mainly comprised of pentacyclic triterpenoid saponins (James and Dubery, 2009) [16]. Centellin, Asiatic and centellicin are also present in the aerial part of the plant (Siddiqui *et al*., 2007) [17]. From plant extract madecassoside, asiaticoside, madecassic acid and asiatic acid have been isolated in the significant amount (Inamdar et al., 1996) [18]. A quantitative estimation of triterpenoids shows highest asiaticoside content in leaf samples (Randriampionoma *et al*., 2007) [19]. New triterpene and a saponin, 2α,3β,23-trihydroxyurs-20-en-28-oic acid and 2α,3β,23- trihydroxyurs-20-en-28-oic acid O-α-l-rhamnopyranosy-(1→4)-O-β-d-glucopyranosyl(1→6)-O-β-d glucopyranosyl ester, have also been isolated from the aerial part of *C. asiatica* (Yu *et al*., 2007) [20]. Chemical structure of some major phytochemicals of *Centella asiatica* is illustrated below:



Asiatic acid derivates



Madecassic acid



Asiaticoside

Pharmacological importance: The whole plant is used for medicinal purposes (Singh and Singh, 2002) [21]. It is widely used as a blood purifier as well as for treating high blood pressure, for memory enhancement and promoting longevity. In Ayurveda, *Centella asiatica* is one of the main herbs for revitalizing the nerves and brain cells. Eastern healers relied on this plant to treat emotional disorders, such as depression, that were thought to be rooted in physical problems (PDR for herbal medicine, 1999; Hagemann et al., 1996) [22, 23]. In the Western medicine, during the middle of the twentieth century, *Centella asiatica* and its alcohol extracts were used in the treatment of leprosy (Baily, 1945) [24]. *Centella asiatica* shows a wide range of pharmacological activities viz.

ANTICANCER ACTIVITY: Asiatic acid is the major phytochemical of Centella asiatica which shows anticancer activity, particularly against cell lines of human breast cancer (Wang *et al*., 2013; Pittella *et al*., 2009; Babykutty *et al*., 2009; Hussin *et al*., 2014; Wu *et al*., 2017; Park *et al*., 2005; Zhang *et al*., 2013; Kwon *et al*., 2014) [25-32].

ANTIBACTERIAL ACTIVITY: Methanol hot extract from *C. asiatica* leaves shows antibacterial activity against *Staphylococcus aureus* ATCC 25923, Bacillus subtilis, Escherichia coli, Bacillus cereus, Pseudomonas aeruginosa (Zaidan *et al*., 2005; Oyedeji *et al*., 2005; Pitinidhi Pat, 2015; Sekar *et al*., 2011) [33-36].

ANTIFUNGAL ACTIVITY: The petroleum ether, ethanol, chloroform, n-hexane, and aqueous extract of *C. asiatica* shows antifungal activity against *Aspergillus niger, Candida. Albicans and Aspergillus flavus* (Dash *et al*., 2011; Dhiman *et al*., 2016 ; Idris *et al*., 2017 ; Sultan *et al*., 2014) [37-40].

ANTI-INFLAMMATORY ACTIVITY: Plants with medicinal properties are rich in ceramide and different forms of terpenoids which show anti-inflammatory activity (Prakash, 2017) [41]. Pentacyclic triterpenoid and saponins are collectively known as centelloids that are responsible for therapeutic actions. The Centella asiatica extract showed moderate anti-inflammatory property on prostaglandin E2-induced inflammation in a dose-dependent manner (Somchit *et al.,* 2004) [42]. The aqueous and alcoholic extract of *C. asiatica* showed inhibition of edema (George *et al*., 2009) [43]. The asiatic acid reduced paw edema by regulation of catalase,

superoxide dismutase (SOD), and glutathione in the liver tissue (Huang *et al*., 2011) [44]. It was observed that the methanolic extract showed significant inhibition of oedema (Saha *et al*., 2013) [45].

NEUROPROTECTIVE ACTIVITY: Neuroprotection aspect of *C. asiatica* mainly involves enzyme inhibition, prevention of amyloid plaque formation in Alzheimer's disease, dopamine neurotoxicity in Parkinson's disease, and reducing oxidative stress (Orhan, 2012) [46]. Aqueous extract of *C. asiatica* was evaluated on the activity of subtypes of phospholipase A2 (PLA2) in primary cultures of rat cortical neurons, asiaticoside present in extract inhibited cPLA2 and sPLA2 activities (Defillipo *et al*., 2012) [47]. In male Sprague-Dawley rats, improved learning and memory were observed on acute administration of asiatic acid (Nasir *et al*., 2011) [48]. Neuroprotective potential of modern medicine constituents of the plant includes asiatic acid, madecassic acid, and brahmaside as well as flavonoids madecassoside and madesiatic acid (Thomas *et al*., 2015) [49]. *C. asiatica* was explored for neuroprotective effect on cell death and cognitive irregulation in aluminum-treated rat. Significant improvement in memory performance, oxidative defense was observed on chronic administration of CA (Prakash and Kumar, 2013) [50]. The plant is known to utilize neuroprotective effects by attenuating the changes in an animal model such as pathological neurobehavioral and neurochemical properties. Phosphoinositides-assisted cytodynamics and synaptic function show the neuroprotective effects of asiaticoside in the rat which includes mode of ROT-infused hemiparkinsonism (Gopi *et al*., 2017) [51].

ANTIOXIDANT PROPERTY: *C. asiatica* extract and powder was evaluated for reduction in oxidative stress in Sprague-Dawley rats. Results showed a decrease in the generation of ROS and oxidative stress in the rats. It was also noted that there was a significant decrease in SOD level (Hussin *et al*., 2007) [52]. Essential oil of *C. asiatica* extracted through steam distillation showed to be excellent antioxidant for food containing lipids. Its activity was quite comparable with the synthetic antioxidant butylhydroxyanisole (BHA) (Raza *et al*., 2009) [53]. Polyphenol, flavonoid, β-carotene, tannin, Vitamin C, and DPPH compounds are readily found in *C. asiatica* contributing to significantly higher antioxidant activity in the herb (Chandrika *et al*., 2015) [54]. Crude methanolic extract on continuous supplementation for 14 days resulted in increase in level of antioxidant enzymes and ascorbic acid level reduced in lymphoma-bearing mice (Jayashree *et al*., 2003) [55]. Extracts of *C. asiatica* in different solvents such as chloroform, hexane, acetone, ethyl acetate, methanol, and water were assessed for antioxidant potential. The DPPH and hydroxyl radical scavenging activity were tested for methanolic extract which showed the IC50 value of 0.07 mg/ml and 500 μg/ml, respectively (Anand *et al*., 2010) [56].

WOUND HEALING: The extract of Centella asiatica showed wound healing activity in a number of experimental animals (Shetty *et al*., 2006; Somboonwong *et al*., 2012; Yao *et al*., 2017) [57-59].

ANTIDEPRESSANT: Compared to diazepam *C. asiatica* possesses antianxiety effect but has no effect on behavioral despair. Total triterpenes and imipramine from *C. asiatica* were evaluated for antidepressant activity using forced swimming test, the result showed a reduction in stillness duration and regulated amino acid levels (Chen *et al*., 2003; Kalshetty *et al*., 2012; Ceremuga *et al*., 2015) [60-62].

ANTIDIABETIC ACTIVITY: Antidiabetic properties of leaf extract of *C. asiatica* was evaluated in alloxan-induced rat model and showed reduction in blood glucose level (Rahman *et al*., 2012) [63]. Effect of ethanol extract was tested in streptozotocin (50 mg/kg)-induced Wistar rats. Studying the serum glucose, urea cholesterol, lipid, liver glycogen level, and body weight, the antidiabetic activity of extract at concentration of 200 mg/kg was noticed (Gayathri *et al*., 2011; Supkamonseni *et al*., 2014; Haque *et al*., 2013; Kabir *et al*., 2014; Maulidiani *et al*., 2016) [64-68]. Asiatic acid was found to reduce blood glucose level in Goto-Kakizaki (GK) rat by enhancing fibrosis of islets in diabetes which plays a vital role in the prevention of islets dysfunction (Wang *et al*., 2015) [69]. In diabetic Wistar rat model, asiatic acid showed to preserve and restore beta cell mass (Liu *et al*., 2010) [70].

COGNITIVE FUNCTION: Asiatic acid was found to prevent spatial working memory and reduction of neurogenesis defects in the hippocampal region caused by 5-FU chemotherapy (Chaisawang *et al*., 2017) [71]. Water extract of *C. asiatica* was observed to enhance synaptic differentiation and dendritic arborization with reference to Aβ which causes cognitive improvement (Gray *et al*., 2017) [72]. In a study, gotu-kola extract was supplemented for weeks in defined concentration results showed to be effective in the treatment of cognitive function impairment after stroke (Farhana *et al*., 2016) [73]. Asiatic acid has potential to restore the impairment of cell proliferation, spatial working memory caused by treatment with valproic acid (Umka *et al*., 2016) [74]. Water extract helped to improve cognitive function by activation of antioxidant response gene and mitochondrial biogenesis (Gray *et al*., 2016) [75], normalized calcium homeostasis (Gray *et al*., 2015) [76].

HEPATOPROTECTIVE: Effect of methanolic extract of *Centella asiatica* was evaluated in Type 2 diabetes mellitus, and showed reduction in hepatic concentrations of interleukin-1β, MCP-1, and tumor necrosis factor alpha in diabetic control rats (Oyenihi *et al*., 2017) [77]. In dimethylnitrosamine-induced liver injury *C. asiatica* noticeably enhanced fibrosis of liver tissues by mass periportal±bridging necrosis, intralobular degeneration, and focal necrosis (Choi *et al*., 2016; Ghosh *et al*., 2017; Duggina *et al*., 2015) [78-80]. Asiatic acid protects liver injury by onset of Smad7-dependent inhibition of TGF-beta/Smad-assisted fibrogenesis (Tang *et al*., 2012) [81]. Conventionally, used plants to get rid of liver dysfunction might, therefore, could be potential source for new hepatoprotective compounds for development as pharmaceutical entities (Rajalingam *et al*., 2016) [82].

The pharmacological activities of *Centella asiatica* can be summarized by a Diagram:

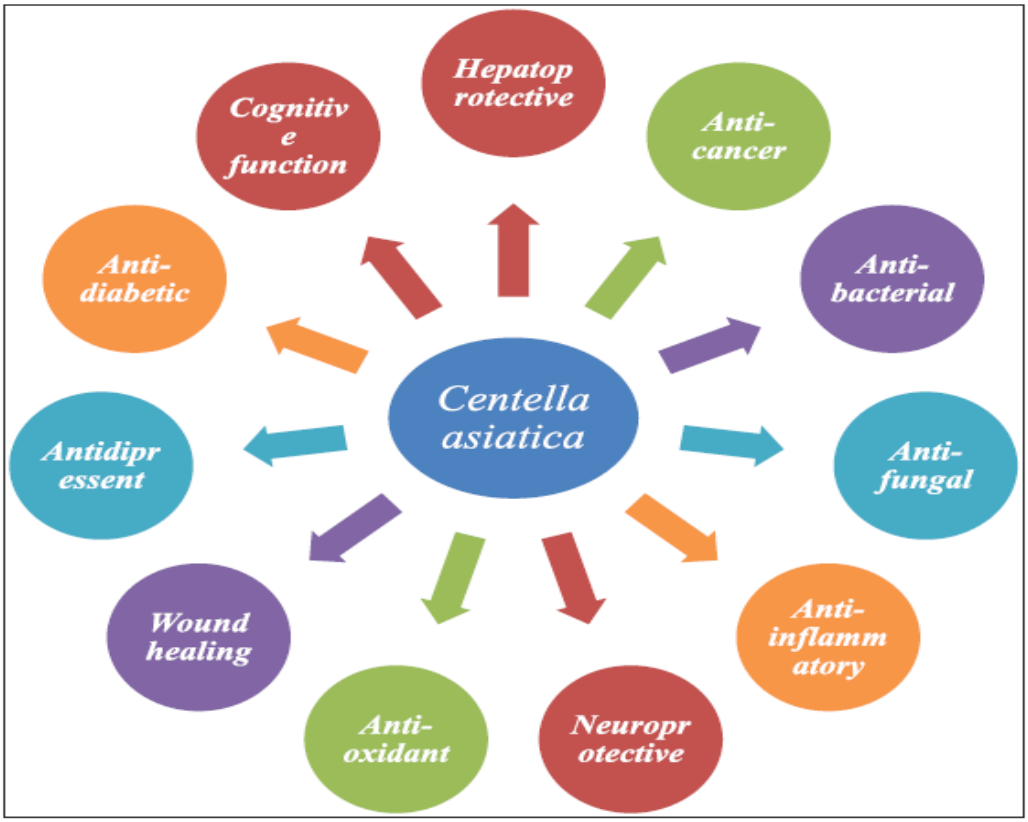


Diagram: Pharmacological activities of *Centella asiatica*

In the present investigation phytochemicals of leaves of *Centella asiatica* were analyzed qualitatively and quantitatively in six solvent extracts viz. Acetone, Petroleum ether, Ethanol, Benzene and Methanol and the Distilled water.

II. Materials and Methods

In the present investigation the dried leaves of *Centella asiatica* were powdered using a mixture grinder and stored in air-tight container for future use. Six different solvents (five non polar viz. Acetone, Petroleum ether, Ethanol, Benzene and Methanol and one polar solvent, the Distilled water) were used for preparation of solvent extracts. The dried plant sample was soaked separately with acetone, petroleum ether, ethanol, benzene, methanol and distilled water under reflux condition for the solvent extract preparation. About 1 gm of the dried sample of leaves was added respectively into the test tubes containing 5 ml of solvents, and was extracted at room temperature. In the present investigation the important phytochemicals of leaves of *Centella asiatica* have been qualitatively and quantitatively analyzed for alkaloids, flavonoids, tannins, saponins and total phenols.

Phytochemical Analysis: Phytochemicals in leaves of *Centella asiatica* were analyzed qualitatively and quantitatively in all the six solvent extracts

Qualitative Phytochemical Analysis

The extracts in all the six solvents of leaves of *Centella asiatica* were tested for the presence of biological compounds by using following standard methods.

Test for Carbohydrates

Fehling's test: Equal volume of Fehling A and Fehling B reagents were mixed together and 2ml of it was added to crude extract and gently boiled. A brick red precipitate appeared at the bottom of the test tube indicated the presence of reducing sugars.

Benedict's test: Crude extract when mixed with 2ml of Benedict's reagent and boiled, a reddish brown precipitate formed which indicated the presence of the carbohydrates.

Iodine test: Crude extract was mixed with 2ml of iodine solution. A dark blue or purple coloration indicated the presence of the carbohydrate.

Test for Phenols and Tannins

Crude extracts were mixed with 2ml of 2% solution of $FeCl_3$. A blue–green or black coloration indicated the presence of phenols and tannins.

Test for Flavonoid

Alkaline reagent test: Crude extracts were mixed with 2ml of 2% solution of NaOH. An intense yellow color was formed which turned colorless on addition of few drops of diluted acid which indicated the presence of flavonoids.

Test for Saponins (Frothing test): Crude extracts were mixed with 5ml of distilled water in a test tube and it was shaken vigorously. The formation of stable foam was taken as an indication for the presence of saponin.

Test for Glycosides

Liebermann's test: Crude extracts were mixed with each of 2ml of chloroform and 2ml of acetic acid. The mixture was cooled in ice. Carefully concentrated H_2SO_4 was added. A colour change from violet to blue to green indicated the presence of steroidal nucleus, i.e., glycone portion of glycoside.

Salkowski's test: Crude extracts were mixed with 2ml of chloroform. Then 2ml of concentrated H_2SO_4 was added carefully and shaken gently. A reddish brown color indicated the presence of steroidal ring, i.e., glycone portion of the glycoside.

Keller-kilani test (Cardiac Glycosides): Crude extracts were mixed with 2ml of glacial acetic acid containing 1-2 drops of 2% solution of $FeCl_3$. The mixture was then poured into another test tube containing 2ml of concentrated H_2SO_4. A brown ring at the inter phase indicated the presence of cardiac glycoside.

Test for Alkaloids: The crude extract of all the six solvents was boiled in 10 ml methanol and filtered separately. 1% HCl was added followed by 6 drops of Dragendroff reagent, and the brownish-red precipitate was taken as evidence for the presence of alkaloids.

Phlobatannins: The deposition of a red precipitate denoted the presence of phlobatannins when crude extract of all the six solvent of plant material was dissolved in 10 ml of aqueous extract and few drops of 1% HCl were added in the boiling tube.

Anthraquinones: All the six solvent extracts of leaves were boiled in 10% HCl for 5 mins separately and the filtrate was allowed to cool. An equal volume of $CHCl_3$ with few drops of 10% NH_3 was added to the 2ml filtrate. The formation of rose-pink colour implies the presence of anthraquinones.

Quantitative estimation of phytochemicals

Determination of Alkaloids: Alkaloids content was measured by method suggested by Harborne (Harborne, 1973) [83]. A suspension was prepared by dispersing 5 gm of the dried leaves in 10% acetic acid solution in ethanol and kept at 28^0C for 4hrs which was further filtered through Whatman No. 42. Thereafter alkaloid was precipitated by concentrating the filtrate to one-quarter of its original volume and drops of conc. aqueous NH_4OH were added. Finally, the precipitate was washed with 1% ammonia solution and dried at 80^0C in the oven. The content of alkaloid was calculated and expressed as mg/g of sample.

Determination of Flavonoids: The flavonoids content was also determined by Harborne (Harborne, 1973) [83] method. 5 gm of leaves were boiled in 2M HCl for 30 min under reflux condition and filtered after cooling. An equal volume of ethyl acetate was then added drop wise to the filtrate. The weight of precipitated flavonoid was determined and recorded as mg/g.

Determination of Tannins: The finely powdered leaves of *Centella asiatica* were kept in a beaker containing 20 ml of 50% methanol covered with parafilm and then heated at 80oC in a water bath for 1 hr with continuous stirring. The extract was quantitatively filtered using a double layered Whatman No.1 filter paper and rinsed with 50% methanol. 1 ml of sample extract was treated with 20 ml distilled water, 2.5 ml Folin-Denis reagent and 10 ml of 17% Na_2CO_3 for the development of a bluish-green colour and was allowed to stand for 20 mins. The absorbance was measured at 760 nm and the amount of tannin was calculated by comparing it with a standard curve prepared in the range of 0-10 ppm.

Determination of Saponins: 100 ml Isobutyl alcohol was added to 1 gm of the finely powdered sample and stirred for 5 hrs. 20 ml of 40% saturated solution of Magnesium carbonate was added to the mixture and filtered. 2 ml of 5% FeCl3 solution and 50ml volume of distilled water was added to 1ml of colourless solution and kept for 30 mins for colour (blood red) development The absorbance of the samples as along with the standard were read at 380 nm and calculated in mg/g. Standard saponin solution was prepared in the reference range of 0-10 ppm.

Determination of total phenols: Five gms of the powdered leaves were boiled with 50 ml of ether for 15 mins and distributed in the ratio 1:2 (extract: distilled water). 2ml of ammonium hydroxide followed with 5ml of pentanol was added to it and incubated at the room temperature for 30mins. The absorbance was read at 505 nm wavelength.

For measuring alkaloids a suspension was prepared by dispersing 5 gm of the dried leaves in 10% acetic acid solution in ethanol and kept at 28^0C for 4hrs which was further filtered through Whatman No. 42. Thereafter alkaloid was precipitated by concentrating the filtrate to one-quarter of its original volume and drops of conc. aqueous NH_4OH were added. Finally, the precipitate was washed with 1% ammonia solution and dried at 80^0C in the oven. The content of alkaloid was calculated and expressed as mg/g of sample.

For determining flavonoids 5 gm of leaves were boiled in 2M HCl for 30 min under reflux and filtered after cooling. An equal volume of ethyl acetate was then added drop wise to the filtrate. The weight of precipitated flavonoid was determined and reported as mg/g.

For measuring tannin the finely powdered leaves of *Centella asiatica* were kept in a beaker containing 20 ml of 50% methanol covered with parafilm and then heated at 80^0C in a water bath for 1 hr with continuous stirring. The extract was quantitatively filtered using a double layered Whatman No.1 filter paper and rinsed with 50% methanol. 1 ml of sample extract was treated with 20 ml distilled water, 2.5 ml Folin-Denis reagent and 10 ml of 17% Na_2CO_3 for the development of a bluish-green colour and was allowed to stand for 20 mins. The absorbance was measured at 760 nm and the amount of tannin was calculated by comparing it with a standard curve prepared in the range of 0-10 ppm.

For determining saponin content 100 ml Isobutyl alcohol was added to 1 gm of the finely powdered sample and stirred for 5 hrs. 20 ml of 40% saturated solution of Magnesium carbonate was added to the mixture and filtered. 2 ml of 5% $FeCl_3$ solution and 50ml volume of distilled water was added to 1ml of colourless solution and kept for 30 mins for colour (blood red) development The absorbance of the samples as along with the standard were read at 380 nm and calculated in mg/g. Standard saponin solution was prepared in the reference range of 0-10 ppm.

For determining total phenolic content five gms of the powdered leaves were boiled with 50 ml of ether for 15 mins and distributed in the ratio 1:2 (extract: distilled water). 2ml of ammonium hydroxide followed with 5ml of pentanol was added to it and incubated at the room temperature for 30mins. The absorbance was read at 505 nm wavelength.

Quantitative analysis of phytochemical constituents in six different solvent extracts

Six solvent extract of leaves of *Centella asiatica* viz. acetone, petroleum ether, ethanol, methanol, benzene and distilled water were prepared by soaking 10gm of the powdered sample in 200 ml of each of the solvent separately for 12 hrs. The extracts were then filtered using filter paper. The extracts were then concentrated to ¼ of the original extracts i.e. 50 ml.

The amount of total phenolics in extracts was determined by the Folin–Ciocalteu method. Gallic acid was used as a standard by using different concentrations of (20-200µg) from which the total phenol content in the extract was expressed in terms of gallic acid equivalent (mg GAE /gm) extract. Different aliquots of 0.1 to 1.0 ml of plant extract were also prepared in methanol and 0.5 ml of each sample were introduced into test tubes and mixed with 2.5 ml of a 10-fold dilute Folin- Ciocalteu reagent and 2 ml of 7.5% sodium carbonate. The mixture was allowed to stand for 30 mins at room temperature. Phenols react with the phosphomolybdic acid in Folin- Ciocalteau reagent in alkaline medium and produce blue coloured complex (Molybdenum blue). The absorbance of the resulting solutions was measured at 760 nm against reagent blank. A standard calibration

curve was prepared by plotting absorbance against concentration and it was found to be linear over this concentration range. The concentration of total phenol in the test sample was determined from the calibration graph. The assay was carried out in triplicate and the mean values with ± SD are presented.

The aluminium chloride colorimetric method was used for flavonoids determination. Each solvent extract (0.5 ml of 1:10 gm ml^{-1}) was separately mixed with 1.5 ml of methanol, 0.1 ml of 10% aluminium chloride, 0.1 ml of 1 M potassium acetate and 2.8 ml of distilled water. It was kept at room temperature for 30 min; the absorbance of the reaction mixture was measured at 418 nm. The percentage of total flavonoids were calculated from the calibration curve of Quercetin (200-1000µg) plotted by using the same procedure and total flavonoids was expressed as Quircetin equivalents (QE) in mg per gm sample.

The results obtained have been presented in Table-1, 2 and 3; Figure-1 and 2.

Table- 1: Phytochemicals of *Centella asiatica* analysed qualitatively in leaves in six different solvent extracts

Solvent extracts of leaves	Ta	Phl	Sap	Terp	Dter	Emd	Fla	Car	Anth	Crt	Res	Alk	Anc	Cou	Str	Pst	Phe	Fa	Prt	Aa
Acetone	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Petroleum ether	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Ethanol	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+
Methanol	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+
Benzene	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Distilled water	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+

Ta= Tannin; Phl= Phlobatannin; Sap= Saponin; Ter= Terpinoid; Dtr = Diterpinoid ; Emd= Emodin ; Fla= Flavonoid; Car= Cardiac glycoside; Anth= Anthraquinones; Crt= Carotenoids; Res= Reducing sugar; Alk= Alkaloid; Anc= Anthocyanin; Cou= Coumarin; Str= Steroids; Pstr= Phytosterol; Phe= Phenol; FA= Fatty acids; Prt= Protein; Aa= Aminoacids

Table- 2: Quantitative estimation of Phytochemicals in leaves of *Centella asiatica*

Phytochemicals	Amount in mg/gm
Alkaloids	17.75±0.25
Flavonoids	45.75±0.45
Phenols	25.85±0.65
Saponins	16.75±0.25
Tannins	14.45±0.25

Mean ± SD of five measurements

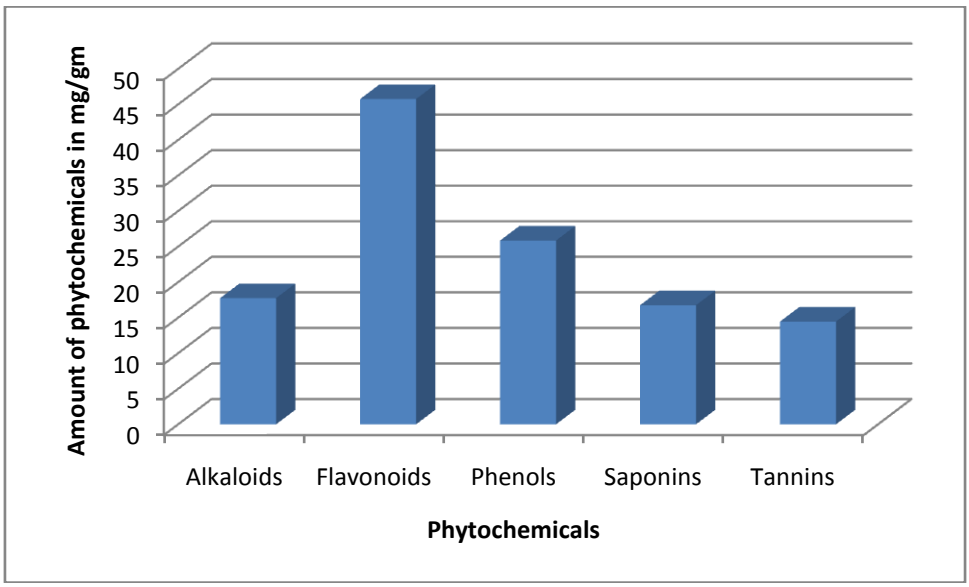


Figure-1: Phytochemicals observed in leaves of *Centella asiatica*

Table- 3: Comparative analysis of total Alkaloids, Flavonoids, Phenol, Saponins and Tannins in six different solvent extracts of leaves of *Centella asiatica* (amount in mg/gm)

Solvent Extracts	Total Alkaloids	Total Flavonoids	Total Phenol	Total Saponins	Total Tannin
Acetone	12.45±0.35	13.55±0.43	10.65±0.65	10.55±0.34	10.35±0.17
Petroleum ether	14.75±0.25	14.65±0.17	11.67±0.61	11.45±0.23	11.55±0.15
Ethanol	15.75±0.35	42.45±0.15	15.35±0.24	16.75±0.21	12.25±0.21
Methanol	17.65±0.17	42.65±0.16	15.45±0.21	13.38±0.31	9.25±0.12
Benzene	11.75±0.16	13.35±0.41	9.85±0.19	15.25±0.41	10.35±0.16
Distilled water	35.85±0.17	27.87±0.25	12.35±0.42	15.35±0.40	9.45±0.18

Tatal phenol in mg/gm is measured as Gallic Acid Equivalent (GAE/g extract); Total flavonoids in mg/gm is measured as Quercetin Equivalent (QE)/g extract.
Mean ± SD of five measurements

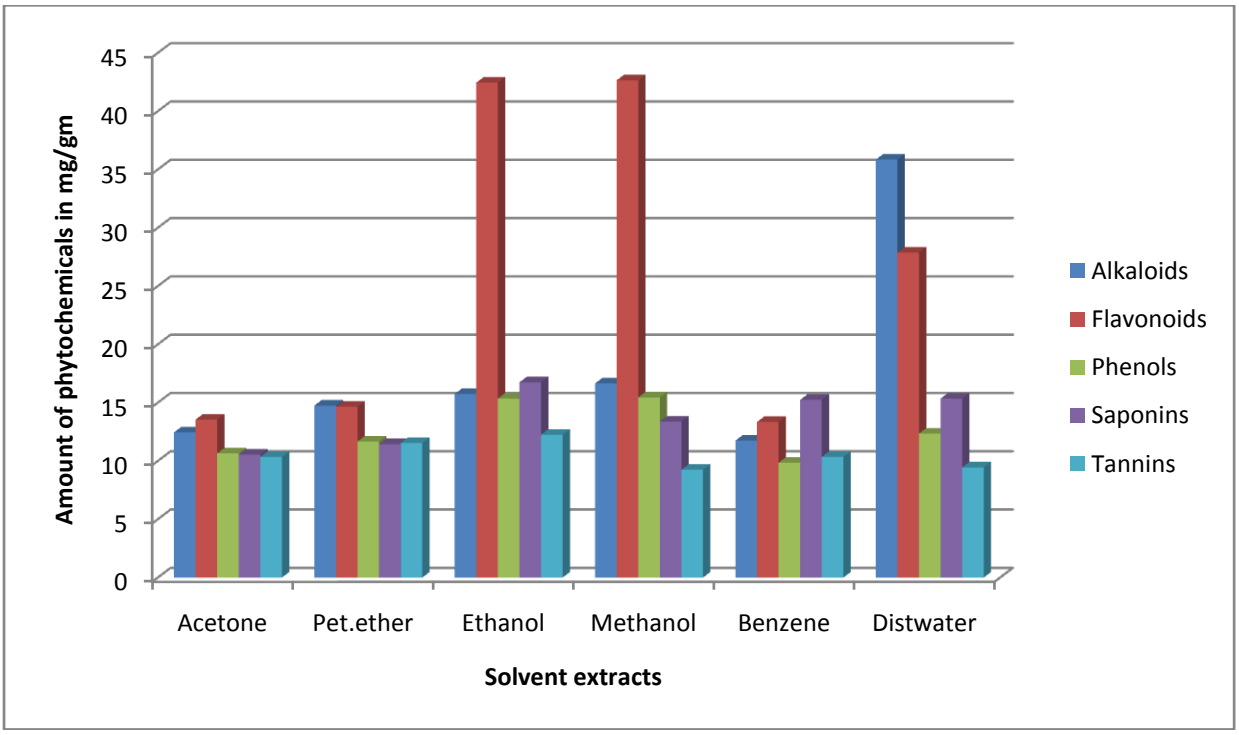


Figure-2: Comparative analysis of phytochemicals in six different solvent extracts of leaves of *Centella asiatica*

III. Results

The present study was carried out on six solvent extracts of *Centella asiatica* to investigate the presence of medicinally important phytochemicals in their leaves. All the six extracts revealed the presence of various phytochemicals such as tannins, phlobatannins, saponins, terpinoids, diterpinoids, emodins, flavonoids, cardiac glycosides, anthraquinones, carotenoids, reducing sugars, alkaloids, anthocyanin, coumarins, steroids, phytosterols, phenol, fatty acids, proteins and amino acids. Of these 20 phytochemicals tannin, saponin, terpenoid, diterpinoid, emodin, flavonoid, cardiac glycoside, anthraquinone, carotenoid, reducing sugar, alkaloids, anthocyanin, coumarin, phenol, fatty acid, protein and amino acids were detected in all the six solvent extracts. Phlobatannin, steroid and phytosterol were not detected in ethanol, methanol and distilled water extracts. Emodin was detected in all extracts except petroleum ether and benzene (Table-1).

From the results (Table- 2; Fig- 1) it is evident that the leaves of *Centella asiatica* contained a significant amount of alkaloid, flavonoids, phenolic, saponins and tannin content. The amount of flavonoids was maximum (45.75mg/gm) followed by phenols (25.85mg/gm), alkaloids (17.75mg/gm), saponins (16.75mg/gm) and tannins (14.45mg/gm) (Table- 2; Fig- 1).

The comparative analysis of phytochemicals viz. total alkaloids, flavonoids, phenols, saponins and tannins in six different solvent extracts from leaves of *Centella asiatica* has been presented in Table- 3 and Fig- 2. From the results it is evident that the concentration of total alkaloids was maximum in distilled water extract (35.85mg/gm), followed by methanol extract (17.65mg/gm), ethanol extract (15.75mg/gm), petroleum ether extract (14.75mg/gm), acetone extract (12.45mg/gm) and benzene extract (11.75mg/gm). The concentration of total flavonoids was maximum in ethanol and methanol extracts (42.45mg/gm and 42.65mg/gm respectively),

followed by distilled water extract (27.87mg/gm), benzene extract (13.35mg/gm), petroleum ether extract (14.65mg/gm) and acetone extract (13.55mg/gm). The amount of total phenol was maximum in ethanol and methanol extracts (15.35 and 15.45mg/gm respectively), followed by distilled water extract (12.33mg/gm), benzene and petroleum ether extracts (9.85 and 11.67mg/gm respectively) and acetone extract (10.65mg/gm). Saponin concentration was maximum in ethanol (16.75mg/gm), benzene extract (15.25mg/gm) and distilled water extract (15.35mg/gm). Acetone and petroleum ether extracts contained relatively least amount of saponins (10.55 and 11.45mg/gm respectively). The total tannin concentration was maximum in ethanol extract (12.25mg/gm), followed by petroleum ether, acetone and benzene extracts (11.55, 10.35 and 10.35 mg/gm respectively. Methanol and distilled water extracts contained relatively low amount of total tannins, 9.25 mg/gm and 9.45 mg/gm respectively (Table- 3; Fig- 2).

IV. Discussion

The leaf extracts of *centella asiatica* showed the presence of terpenoids, steroids and phytosterols, tannins, alkaloids, glycosides, saponins, reducing sugars, phenols and flavonoids. The extraction of various phytochemicals was seen to be more effectively done in polar solvents (ethanol, methanol and distilled water) than the non polar (Acetone, petroleum ether, benzene) solvents. Especially, ethanolic, methanolic and distilled water leaf extracts showed presence of most of the tested phytochemicals. Hence, it can be reported that alcoholic extract was the best one for extracting the active principle than others. Flavonoids are water-soluble polyphenolic compounds which are extremely common and widespread in the plant kingdom as their glycosides. The flavonoids are known to act through scavenging or chelating process. The present findings gain support from the work of Arpita Roy *et al*., (2018) [84] who have found a more or less similar phytochemicals qualitatively and quantitatively in *Centella asiatica*. They observed similar phytochemicals in extracts of whole plant, shoot culture, callus culture and suspension culture. A more or less similar results was also observed by Rupa *et al*., (2017) [85]. According to reports, *Centella asiatica* extracts by ultrasonic assisted extraction showed Total Phenolic Content of 1350 mg GAE/100 g Dry Weight and Total Flavonoid Content, 599 mg QE/100 g Dry weight (Nithyanadam *et al*., (2014) [86]. The polyphenols in 100% ethanol extract was 21.1 ± 0.1 Pyrogallol Equivalent and flavanoid is 9.3 ± 0.3 Quercetin Equivalent (Rahman *et al*., (2013) [87]. The polyphenols in *Centella asiatica* was found to be 150 mg tannic acid/100 g for *C. asiatica* (Gupta *et al*., (2013) [88].

V. Conclusions

The results obtained in the present investigation are encouraging and can be used as reference data for the standardization of leaves of *Centella asiatica* and the formulations containing these plant leaves as a main ingredient. The evaluation of the various proximate parameters for the leaves of *Centella asiatica* has given a clear idea about the specific characteristics of these crude drugs under examination, in their powder form. The preliminary screening tests may be useful in the detection of the bioactive principles and subsequently may lead to the drug discovery and development. Such screening experiments form a primary platform for further phytochemicals and pharmacological studies that may open the possibility of finding new clinically effective compounds. Thus, the present study has authenticated the usefulness of the *Centella asiatica* plants for medicinal purposes. This species could also be seen as potential sources of useful drugs due to their rich contents of phytochemicals. The data obtained in the present study is expected to serve as valuable tool for identification, authentication and detection of adulterants, standardization and quality control of the drugs. Hence it can be concluded that the results of the present study have given qualitative and quantitative information about the purity standards of the leaves of *Centella asiatica.*

Conflict of interest: Authors declare no conflict of interest directly or indirectly.

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