

Phytochemical Screening and In-Vitro Evaluation of Antioxidant and Anti- Inflammatory Potential of Hydroalcoholic and Ethyl Acetate Extracts of *Centella Asiatica*

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Abstract—*Centella asiatica* (L.) Urban (Apiaceous), commonly known as Got kola or Indian pennywort, is a traditional medicinal herb long used in Ayurvedic and folk medicine for wound healing, cognitive enhancement, and management of inflammatory skin conditions. The present study was undertaken to carry out phytochemical screening and to evaluate the in vitro antioxidant and anti-inflammatory potential of hydroalcoholic (HAE) and ethyl acetate (EAE) extracts of *C. asiatica* leaves. Shade -dried and powdered leaves were subjected to continuous hot Soxhlet extraction separately with ethyl acetate and a hydro alcoholic solvent system. Both extracts were screened for the presence of alkaloids, catechins, coumarins, phenols saponins, triterpenoids, glycosides (Salkowski test) and tannins (lead acetate test) using standard qualitative colour and precipitation reactions. Antioxidant activity was assessed by the nitric oxide (NO) radical scavenging assay, and anti-inflammatory potential was evaluated by the Griess- based NO scavenging assay, both over a concentration range of 20 - 80 µg /m L, with ascorbic acid as the reference standard. Phytochemical screening revealed the presence of alkaloids, coumarins, phenols, saponins, triterpenoids, glycosides and tannins in both extracts, whereas the catechin test was negative for both HAE and EAE. In the antioxidant assay, HAE exhibited the highest percentage inhibition (81.40% at 20 µg/mL), a value comparable to or exceeding that of the standard, while EAE showed a concentration-dependent rise in inhibition from 58.74% to 61.82%. In the anti-inflammatory assay, HAE (55.66-57.38%) performed comparably to ascorbic acid (51.82- 55.47%), whereas EAE displayed comparatively weaker activity that declined with increasing concentration (20.92% to 15.35%). These findings indicate that the hydroalcoholic extract of *C. asiatica* is a richer source of polar antioxidant and anti-

inflammatory phytoconstituents than the ethyl acetate extract and support its further exploration for nutraceutical and topical anti-inflammatory applications.

***Index Terms*—Centella asiatica; phytochemical screening; antioxidant activity; anti-inflammatory activity; nitric oxide scavenging assay; Soxhlet extraction.**

I. INTRODUCTION

Phytochemical screening and in vitro evaluation of antioxidants and anti-inflammatory potential of hydro alcohol and ethyl acetate extraction of *Centella asiatica* (L.) Urban is a medicinal herb belonging to the family Pinaceae and is widely recognized for its traditional and pharmacological importance. The plant is commonly known as Indian pennywort or Gotu kola and is used in several traditional systems of medicine, particularly for skin problems, wound healing and nervous-system-related applications. The literature describes *Centella asiatica* as a plant containing biologically active triterpenic acids, triterpenic saponins, flavonoids and other phenolic compounds. These constituents are associated with several biological activities, including antioxidant, anti-inflammatory, antimicrobial, wound-healing, neuro protective and skin-protective effects.^[1] The biological activity of a plant extract can be influenced by the extraction solvent because compounds with different chemical characteristics show different solubility and extraction behavior. Studies on *Centella asiatica* have demonstrated that solvent polarity can alter the amount and type of phytochemicals obtained and consequently influence antioxidant potential. This provides a scientific basis for comparing extracts prepared using different solvent systems. In the present project, ethyl acetate and hydroalcoholic extraction are considered for phytochemical screening and for evaluating the antioxidant and anti-inflammatory potential of *Centella asiatica*.^[2]

The proposed study is therefore focused on the systematic examination of *Centella asiatica*, beginning with its plant profile and therapeutic importance, followed by extraction, phytochemical screening and assessment of antioxidant and anti-inflammatory potential. The available literature supports the importance of solvent selection, phytochemical composition and biological activity in understanding the medicinal potential of this plant.



Fig no:1 centella asiatica

Plant Profile Of *Centella Asiatica*:

Centella asiatica (L.) Urban, with the synonym *Hydro cotyle asiatica* Linn., belongs to the family Apiaceae, formerly known as Umbelliferae. It is commonly called Indian pennywort or Gotu kola. The plant is also known by several vernacular names, including Vallarai in Tamil, Mandookaparni in Hindi and Thankuni in Bengali. It is a perennial, prostrate, stoloniferous and faintly aromatic creeping herb. The plant generally grows to about 15 cm in height and possesses slender stems that root at the nodes.^[3] The leaves arise from the nodes on long petioles and are generally orbicular to reni form in shape, with crenate margins. Flowers occur in fascicled umbels and may range from white to purple or pink.

The plant is commonly found in shady, marshy, damp and wet environments such as paddy fields and riverbanks. *Centella asiatica* is distributed widely in tropical and subtropical regions and is reported from India and other parts of South-East Asia as well as several other tropical regions.^[4] The plant has a long history of medicinal use. Traditional applications described in the reviewed literature include management of skin problems, wound healing, fever, jaundice, dysentery, diarrhea and certain neurological or mental-health-related conditions. Its continued investigation has been associated with the presence of biologically active secondary metabolites and its broad functional properties.^[5]

Chemical Constituents

Centella asiatica contains a range of secondary metabolites and other bioactive constituents. The most characteristic constituents are triterpenes and triterpenoid saponins. Important compounds include asiatic acid, madecassic acid, asiaticoside and madecassoside. Other reported triterpenoid constituents include brahmoside, brahmic acid, brahminoside, thankuniside, isothankuniside, centelloside, madasiatic acid, centic acid and related compounds. Asiatic acid, madecassic acid, and have been used as marker compounds in quality assessment.^[6]

The distribution and concentration of phytochemicals may vary with plant part, geographical origin and environmental conditions^[7]. Phytochemical screening is used to identify groups of secondary metabolites present in plant extracts and provides an initial indication of the chemical basis for biological activity. In *Centella asiatica*, the reviewed literature identifies Alkaloids, Flavonoids, Phenols, Tannins, Saponins, Terpenoids, Steroids, Glycosides^[8]

Therapeutic And Activities:

Centella asiatica has been reported to possess a wide range of pharmacological and functional activities^[9]. The reviewed literature describes antioxidant, antibacterial, antifungal, antiviral, antiprotozoal, antifilarial, antiulcer, antidiabetic, anti-inflammatory, cytotoxic and anti tumour, neuroprotective, cardioprotective, skin-protective, radio protective, immune modulatory memory- enhancing and wound-healing activities. These activities are associated with the plant's diverse phytochemical constituents and different extract preparations.^[10] Antibacterial activity has been reported for several *Centella asiatica* extracts against different Gram-positive and Gram-negative organisms, although the activity varies according to extraction solvent, organism and

experimental conditions. The reviewed evidence includes activity against organisms such as *Staphylococcus aureus*, *Bacillus* species and selected enteric pathogens. At the same time, some studies reported little or no activity against particular organisms, showing that antimicrobial activity is dependent on the extract and test system. Antifungal activity has also been reported. Ethanolic and petroleum-ether extracts have shown activity against selected fungal strains, while ethanolic fractions have demonstrated inhibitory effects on some yeasts, moulds and fungal spore germination. The variation between extracts again indicates that solvent selection can influence the recovery of active constituents. ^[11]

Antiulcer activity has been associated particularly with asiaticoside and *Centella* extracts in experimental models. The reviewed studies describe protection against experimentally induced gastric ulceration and effects associated with strengthening of gastric mucosal defence. Antidiabetic activity has also been reported for ethanolic and ethanolic extracts in experimental diabetic models, where reductions in elevated blood glucose and improved wound healing have been described. ^[12] Neuroprotective and memory-enhancing activities have been widely investigated. *Centella asiatica* has been described as capable of protecting against oxidative damage in neural tissue, supporting endogenous antioxidant defence and influencing learning and memory in experimental models. These effects are linked in part to its antioxidant constituents and triterpenoid compounds. ^[13]

Cardioprotective activity has been reported in experimental models of cardiac injury, including ischemia-reperfusion-related injury. Radio protective activity has also been described, with *Centella* extracts investigated for their ability to reduce radiation-associated tissue damage in experimental models. Immuno modulatory effects have been reported for triterpenoid saponins, pectin and extracts of the plant. ^[14] Skin-protective activity is particularly relevant to the present project. Skin ageing is associated with changes in collagen and extracellular-matrix structure, and asiaticoside has been reported to stimulate type I collagen synthesis in human dermal fibroblasts. More recent in vitro work also describes antioxidant, anti-inflammatory and collagen-modulating effects of standardized *Centella asiatica* extract in human dermal fibroblast models. Wound-healing activity is one of the characteristic therapeutic properties of *Centella asiatica*. Triterpenoid fractions and compounds such as Asiatic acid, madecassoside acid and asiaticoside have been associated with collagen synthesis, extracellular-matrix accumulation, cellular proliferation, wound contraction and epithelialization in experimental models. Asiaticoside has also been associated with increased antioxidant levels during the early stage of healing. ^[15]

Extraction of *Centella Asiatica*

Extraction is an important preliminary step because the solvent system determines which constituent is preferentially recovered from plant material. The reviewed literature states that organic solvents diffuse into plant material and solubilize compounds having compatible polarity. Therefore, the nature and polarity of the extraction solvent can influence both extraction yield and the biological activity of their suiting extract. ^[16]

Type of method:

- Ultrasonic Assisted Extraction (UAE)
- Microwave Assisted Extraction (MAE)
- Soxhlet Extraction
- Maceration extraction

Ultrasonic Assisted Extraction (UAE):

50 gm of *C. asiatica* soaked in 500 mL of water and introduced into Ultrasonic bath for 1hr at 45 °C at 40 KHz power and cooled for 1hr at room temperature. The above process was repeated for 5 times till a clear filtrate was obtained. Experiments were performed keeping the mass of material as 25, 50 and 75 g extracted for at different time periods 10, 15, 25 and 30 min. The yield of the extracts was monitored^[17]

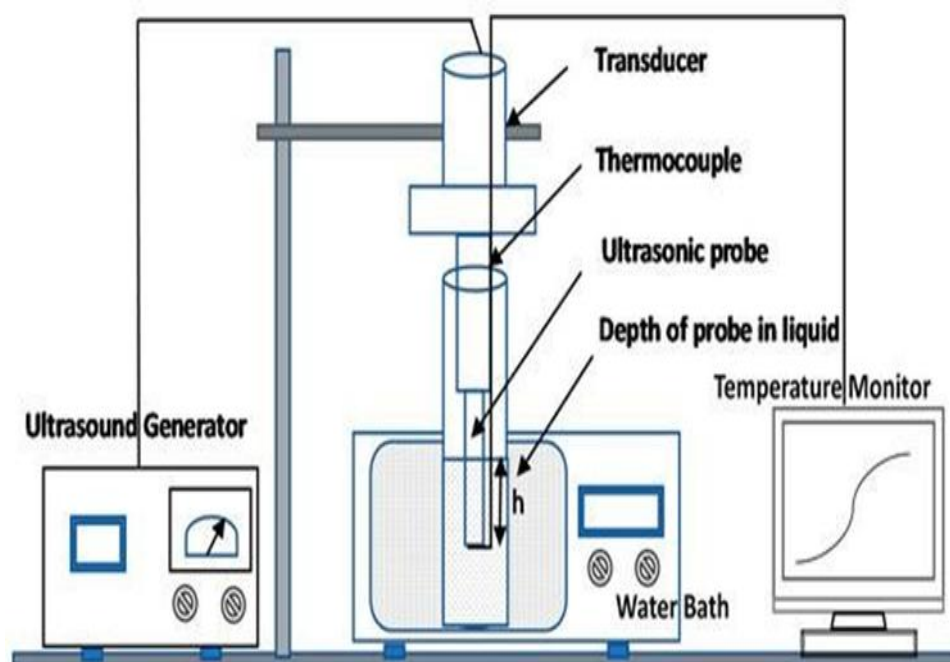


Fig no:2 Ultrasonic Assisted Extraction

Microwave Assisted Extraction (MAE):

50 gm of *C. asiatica* soaked in 500 mL of distilled water and introduced into Microwaves in an oven for 2 min and cooled for 15 min at room temperature. The above process was repeated for 5 times till a clear filtrate was obtained. The resulted extract was concentrated using rotary evaporator at 45 °C till dryness and the dried sample was dissolved using Methanol^[18]

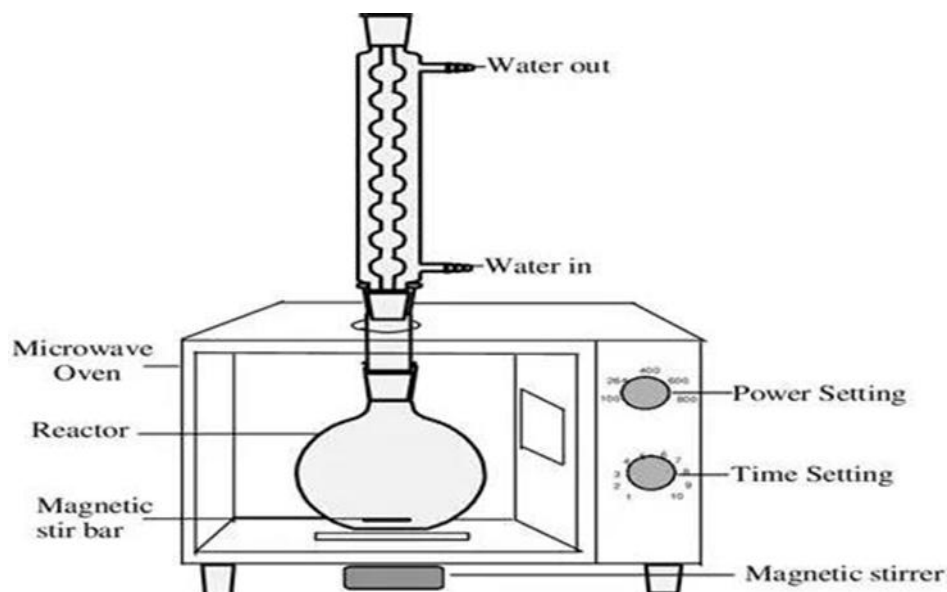


Fig no:3 microwave assisted extraction

Soxhlet Extraction



Fig no:4 soxhlet extraction

Soxhlet extraction was used for the preparation of methanol and ethanol extracts. 50g of *Centella asiatica* coarse plant powder was extracted with 1 liter of either methanol or ethanol for 24 hours and then filtered to collect the extracts. The extracts were then concentrated under vacuum and excess solvent were removed using rotary evaporator. For the preparation of aqueous extract, 50 g of coarse plant powder in 1 liter of distilled water was boiled for 1 hour and the extract filtered. Extract was then freeze-dried.^[19]

Maceration extraction:

The powdered material from each accession (100 g) was subjected to maceration using three solvents of increasing polarity: n-hexane (non-polar), ethyl acetate (semi polar), and ethanol (polar) at a 1:10 (w/v) plant-to-solvent ratio for 72 h at room temperature with continuous agitation (150 rpm). After maceration, mixtures were filtered through Whatman No. 1 filter paper, and filtrates were concentrated using a rotary evaporator at 40°C under reduced pressure. Crude extracts were further dried in a vacuum desiccator to constant weight, and percentage yield was calculated as $(\text{dry extract mass}/\text{initial dry plant mass}) \times 100$. Bulk extracts for each accession solvent combination were stored in amber vials at 4°C until analysis; downstream assays were performed with technical replicates as described in the statistical analyses section



Fig no: 5 maceration extraction

Antioxidant Activity:

The production of reactive oxygen species and free radicals exceeds the antioxidant defense capacity of a biological system. Reactive species can damage lipids, proteins and nucleic acids and are associated with several pathological processes. Antioxidants help protect biological systems by scavenging free radicals and limiting oxidative damage.^[21]

Centella asiatica has demonstrated substantial antioxidant potential in different experimental systems. The reviewed evidence indicates that leaves can possess high antioxidant activity and that phenolic compounds may make an important contribution.^[22]

Ferric Reducing Antioxidant Power (FRAP) Assay:

Working FRAP reagent was prepared by mixing 25 ml of acetate buffer pH3.6, 25 ml of TPTZ (2,4,6-tripyridyl-striazine) solution, 2.5 ml of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ solution and 3 ml of distilled water

(Benzene & Strain 1996). The reaction of mixture of aliq out of CA extracts and bioactive compounds (25µl) with working FRAP reagent (175µl) was monitored for up to 4 minutes and the optical density changes were read at 593 nm. FRAP values were obtained by comparing the absorbance change at 593 nm in test reaction mixtures with those containing ferrous ion in known concentration using FeSO₄.7H₂O solution [23].

DPPH radical scavenging assay:

The slightly modified method of Brand-Williams et al. (1995) was used for the DPPH radical scavenging assay. Approximately 1 mL of the extract was added with 4 mL DPPH solution in a tube. The tubes were vortexed well and placed in a standing position for 30 mins in the dark environment. Hereafter, the absorbance reading of the mixtures was estimated at 517 nm by Jen way 6405 Spectrophotometer (Germany). A DPPH solution without adding the aliquot was considered as a control. The DPPH radical scavenging effect was estimated using the following calculation, where the results were expressed as a percentage [24]

DPPH Radical Scavenging Effect (%) = $(A_0 - A_s) \times 10$ Anti Inflammatory Activity:

Inflammation is a biological response associated with tissue injury, infection and other harmful stimuli. Excessive or persistent inflammatory signaling can contribute to tissue damage. The reviewed literature identifies *Centella asiatica* as possessing anti-inflammatory potential and reports activity for both crude extracts and individual constituents [25].

Centella asiatica water extract and asiaticoside have been reported to exert anti-inflammatory effects through inhibition of nitric oxide synthesis in experimental ulcer-healing models. Crude *Centella* extract has also shown anti-inflammatory activity in experimental paw-edema models. Bioactive terpene acids such as asiatic acid and madecassoside acid have been suggested as contributors to the observed activity.

The literature also contains a specific study on phytochemical screening together with in vitro anti-inflammatory activity of an ethanolic *Centella asiatica* extract. In addition, a more recent in vitro study using human dermal fibroblasts reported that a standardized *Centella* extract reduced inflammatory signaling, including IL-6, while also influencing collagen-related parameters. These Findings connect phytochemical composition, anti-inflammatory activity and skin-protective potential.

The anti-inflammatory potential of *Centella asiatica* is therefore relevant to the present investigation because antioxidant and anti-inflammatory mechanisms are biologically interconnected. Oxidative stress can contribute to inflammatory responses, while antioxidant constituents may help reduce oxidative stimuli associated with inflammation. Evaluation of both activities can provide a broader understanding of the biological potential of different *Centella* extracts.

COX Enzymes Assay:

Anti-inflammatory activity of CA extracts and bioactive compounds was evaluated by its

ability²⁰³ to inhibit the inflammatory pathway enzyme, cyclo-oxygenase (COX) which catalyses the production of PGE₂. This was determined using COX Inhibitor Screening Assay Kit (Cayman Chemical ACE, USA). 20 µl of extract solutions (30 µg/ml) or bioactive compounds (5 µg/ml) were added to the COX-1 and COX-2 tubes respectively. The mixtures were incubated for 10 minutes at 37°C. The reaction was initiated by the addition of 10 µl of arachidonic acid to all the test tubes. The tubes were shaken vigorously and allowed to stand for another 2 minutes at 37°C. After that, 50 µl of 1 M HCl was added to each test tube to stop the reaction. Test tubes were removed from the water bath and 100 µl of the saturated stannous chloride solution was added to each test tube. The tubes were then shaken vigorously before leaving them to stand for 5 minutes at room temperature. The prostaglandin E₂ (PGE₂) produced were then quantified using a commercial Prostaglandin E₂ Monoclonal-EIA Kit (Cayman Chemical ACE, USA)

Nitric Oxide Assays:

To assess their potential anti-inflammatory effects, the ability of the ethanolic extracts to decrease the production of nitric oxide (NO) induced by lipopolysaccharide (LPS) in RAW

264.7 cells was examined. The cell suspension was seeded in a 24-well microplate and treated with 72.28 µg/L of the extract with or without 1 µg/mL of LPS obtained from Sigma-Aldrich (St. Louis, USA). Cells treated with 1 µg/mL of LPS alone were used as a positive control. Aspirin was prepared in DMSO before being added to the well as a negative control. The final concentration of DMSO in the medium was 1/1000 (v/v). After a 24-hour incubation at 37°C in 5% CO₂, the nitric oxide production was quantified by treating the supernatant with an equal volume of Griess reagent (Sigma-Aldrich, St. Louis, USA). The OD was measured at 570 nm using a microplate reader. Each test was performed in triplicate. The

concentration of nitric oxide production was calculated using the following equation: OD of test / OD of standard concentration of standard.^[26]

II. METHODOLOGY

Extraction:

Collection:

- Selection of plant: Fully matured, healthy leaves were selected Damaged and infected leaves were discarded.
- Separation of leaves: Leaves were manually separated from after drying processing Care was taken to avoid contamination with residues. Washing:

removed impurities.

Collected leaves were washed thoroughly with distilled water This

- Shade Drying:

Washed leaves separate spread on clean trays Dried under shade at room temperature to prevent degradation of heat sensitive phenolic compounds.



Fig no:9 Shade of drying

- **Oven Drying:**

Further dried in a hot air oven at 60°C for 30 minutes Ensured complete removal of moisture.

- **Storage:**

Completely dried leaves were stored in airtight, light-resistant containers Kept in a cool and dry place until extraction.

Ethyl acetate Soxhlet Extraction of centella asiatica

- Place powdered Centella asiatica in a Soxhlet apparatus.
- Use Ethyl acetate as the extracting solvent.
- Heat gently so the solvent repeatedly passes through the plant material.
- Continue until adequate extraction is achieved.
- Filter and evaporate the solvent.
- Gives relatively exhaustive extraction.
- Useful for continuous extraction.

Principle:

A Soxhlet extractor works on the principle of continuous hot solvent extraction. The solvent is heated to boiling, and its vapour condenses into the extraction chamber containing the powdered drug. When the solvent level reaches the siphon tube, it automatically flows back into the flask carrying the extracted constituents. This cycle repeats continuously.

Procedure:

1. Dry the Centella asiatica leaves and grind them into a coarse powder.
2. Weigh about 25–50 g of powdered drug.
3. Place the powder inside the Soxhlet thimble.
4. Add Ethyl acetate to the round-bottom flask.
5. Assemble the Soxhlet apparatus with the condenser.

6. Heat gently so that methanol evaporates, condenses, and repeatedly passes through the plant material.
7. Continue extraction for approximately 6–8 hours or until the siphon solvent becomes nearly colourless.
8. Concentrate the extract using a rotary evaporator or gentle evaporation.
9. Store the concentrated extract in an airtight container at 2–8°C.



Fig no: 10 Soxhlet extraction of Ethyl acetate

Assembly:

- Connect the round bottom flask, Soxhlet extractor, and Condenser properly
- Ensure continuous water flow through the condenser.

Heating :

- Heat the system using a heating mantle.
- Maintain temperature at 60–65°C

Extraction Cycle:

- Solvent evaporates → condenses → fills extraction.
- When the chamber fills to siphon level, it automatically
- empties back into the flask.
- This cycle repeats continuously.
- Duration on Continue extraction for 6–8 hours.
- Approximately 10–15 siphon cycles are complete

Filtration:

- Pour the extracted solvent containing dissolved phytoconstituents into the funnel.

- Apply vacuum to facilitate rapid filtration.
- Ensure complete separation of liquid extract from insoluble residues



Fig no:11 filtration

Collection of Filtrate:

- Collect the clear filtrate in the receiving flask.
- If required, repeat filtration to ensure clarity

Hydroalcoholic Soxhlet Extraction of centella asiatica:

- Place powdered Centella asiatica in a Soxhlet apparatus.
- Use Hydroalcoholic as the extracting solvent.
- Heat gently so the solvent repeatedly passes through the plant material.
- Continue until adequate extraction is achieved.
- Filter and evaporate the solvent.
- Gives relatively exhaustive extraction.
- Useful for continuous extraction.

Principle:

A Soxhlet extractor works on the principle of continuous hot solvent extraction.

The solvent is heated to boiling, and its vapour condenses into the extraction chamber containing the powdered drug. When the solvent level reaches the siphon tube, it automatically flows back into the flask carrying the extracted constituents. This cycle repeats continuously.

Procedure:

1. Weigh about 25–50 g of powdered drug.
2. Place the powder inside the Soxhlet thimble.
3. Add Hydroalcoholic to the round-bottom flask.
4. Assemble the Soxhlet apparatus with the condenser.

5. Continue extraction for approximately 6–8 hours or
6. until the siphon solvent becomes nearly colorless.
7. Concentrate the extract using a rotary evaporator or gentle evaporation.
8. Store the concentrated extract in an airtight container at 2–8°C.



Fig no:12 Soxhlet extraction of Hydroalcoholic

Assembly:

- Connect the round bottom flask, Soxhlet extractor, and Condenser properly
- Ensure continuous water flow through the condenser.

Heating :

- Heat the system using a heating mantle.
- Maintain temperature at 60–65°C

Extraction Cycle:

- Solvent evaporates → condenses → fills extraction.
- When the chamber fills to siphon level, it automatically
- empties back into the flask.
- This cycle repeats continuously.
- Duration on Continue extraction for 6–8 hours
- Approximately 10-15 siphon cycles are complete

Filtration:

- Pour the extracted solvent containing dissolved phytoconstituents into the funnel.
- Apply vacuum to facilitate rapid filtration.
- Ensure complete separation of liquid extract from insoluble residues



Fig no:13 filtration

Collection of Filtrate:

- Collect the clear filtrate in the receiving flask.
- If required, repeat filtration to ensure clarity

Phytochemical screening of Ethyl acetate:

1. Test for alkaloids:

The test solution was mixed with little amount of dil. HCl and Mayer's reagent formulation of a white P.P.T indicates the presence of alkaloids.^[39]



Fig no:14 Test for alkaloids

2. Test for Catechin:

Take 2ml of test solution, a few drops of Ehrlich reagent and concentrate HCL were added appearance of pink color indicates the presence of catechin.



Fig no:15 Test for catechin

3. Test for coumarin:

Take 2ml of test solution, a few drop of alcoholic sodium hydroxide were added appearance of yellow color indicates the presence of coumarin.^[40]



Fig no:16 Test for coumarin

4. Test for phenol:

Take 2ml of the test solution a few drops of (ferric chloride) solution were added bluish green or

red color indicator the presences of phenol^[41]



Fig no:17 Test for phenol

5. Test for saponin:

The test solution was shaken with water; copious lather formation indicates the presence of saponin.



Fig no:18 Test for saponin

6. Test for triterpenoid:

Add 1ml of extract, 2ml of chloroform, and addition of 2ml acetic anhydride with 2 drops of conc. H₂SO₄ from the side of the test tube; then appearance of a brown ring at the junction and presence of blue-green color^[42]



Fig no:19 Test for triterpenoid

7. Test for Salkowski test:

Add 1ml extract and 2ml of chloroform shake and add 2ml H₂SO₄ carefully to form a layer appearance of Reddish-brown colour at interface.^[43]



Fig no:20 Test for Salkowski test

8. Test for Lead acetate:

Add 1ml of extract and 3-4 drops of 10% lead acetate appearance of form of yellow colour.



Fig no:21 Test of lead acetate

Phytochemical screening of Hydroalcoholic:

1. Test for alkaloids:

The test solution was mixed with little amount of dil. HCl and Mayer's reagent formulation of a white P.P.T indicates the presence of alkaloids.



Fig no:22 Test for alkaloids

2. Test for Catechin:

Take 2ml of test solution, a few drops of Ehrlich reagent and concentrate HCL were added appearance of pink color indicates the presence of catechin.^[44]



Fig no:23 Test of catechin

3. Test for coumarin:

Taking 2ml of test solution, a few drops of alcoholic sodium hydroxide were added appearance of yellow color indicates the presence of coumarin.



Fig no:24 Test of coumarin

4. Test for saponin:

The test solution was shaken with water copious Lather formation indicates the presence of saponin^[45]



Fig no:25 Test of saponin

5. Test for phenol:

Taking 2ml of the test solution a few drops of (ferric chloride) solution were added bluish green or red color indicator the presences of phenol.



Fig no:26 Test of phenol

6. Test for triterpenoid:

Add 1ml of extract 2ml of chloroform and addition of 2ml acetic anhydride with 2 drops of conc.H₂SO₄ from side of test tube then appearance of brown ring at junction and presence of blue-green color.^[46]



Fig no:27 Test of Triterpenoid

7. Test for Salkowski test:

Add 1ml extract and 2ml of chloroform shake and add 2ml H₂SO₄ carefully to form a layer appearance of Reddish brown colour at interface.^[47]



Fig no:28 Test of Salkowski

8. Test for Lead acetate:

Add 1ml of extract and 3-4 drops of 10% lead acetate appearance of form of yellow colour.^[48]



Fig no:29 Test of lead acetate

Antioxidant activity:

D.P.P.H Method: Principle:

The nitric oxide radical scavenging assay is based on the generation of nitric oxide (NO) from sodium nitroprusside in an aqueous solution. Nitric oxide reacts with oxygen to form nitrite ions, which react with Griess reagent to produce a pink-colored azo compound. Antioxidant compounds present in the test sample scavenge nitric oxide radicals and decrease the formation of the colored product. The decrease in absorbance is measured spectrophotometrically at 546 nm and is proportional to the nitric oxide scavenging activity of the sample.

Reagents:

The major reagents required are sodium nitroprusside (SNP), phosphate-buffered saline (PBS, pH 7.4), sulphadiazine, phosphoric acid, N-(1-naphthyl) ethylenediamine dihydrochloride (NED), methanol/distilled water or suitable solvent, and ascorbic acid as the standard antioxidant.

Procedure:

Different concentrations of the test extract are prepared, such as 20, 40, 60, 80 $\mu\text{g/ml}$. The test sample is mixed with sodium nitroprusside solution and incubated at room temperature in the dark for about 2.5 hours. After incubation, an aliquot of the reaction mixture is mixed with freshly prepared Griess reagent and allowed to stand for about 30 minutes at room temperature. The development of pink colour is measured at 546 nm using a UV-visible spectrophotometer. A control without the test extract and ascorbic acid as the standard are used for comparison. The percentage inhibition of nitric oxide is calculated to determine the antioxidant activity of the test sample.^[49]

D.P.P.H method of test ethyl acetate:

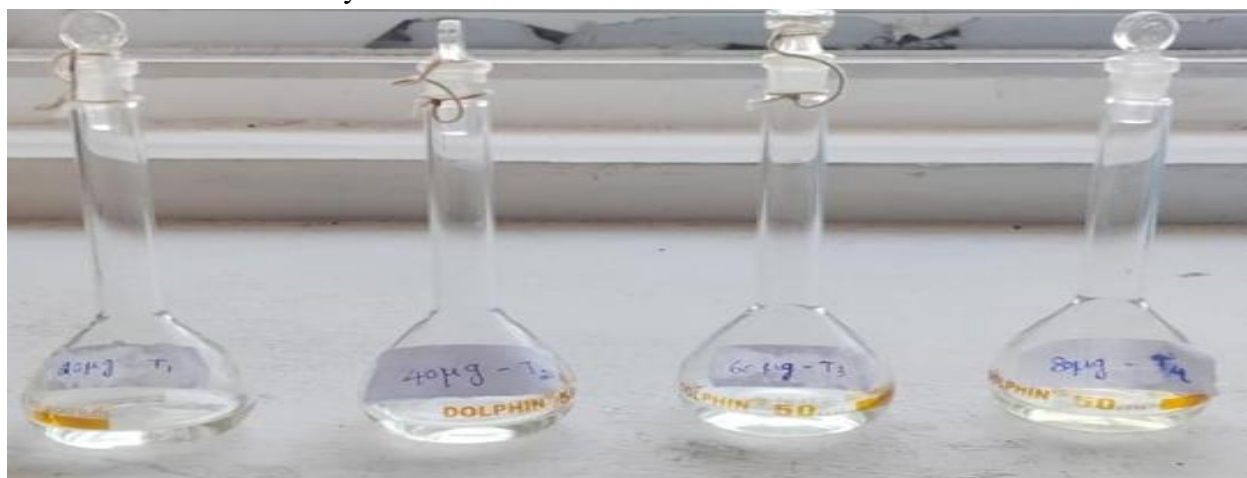


Fig no:30 D.P.P.H of test ethyl acetate

D.P.P.H method of test hydroalcoholic:

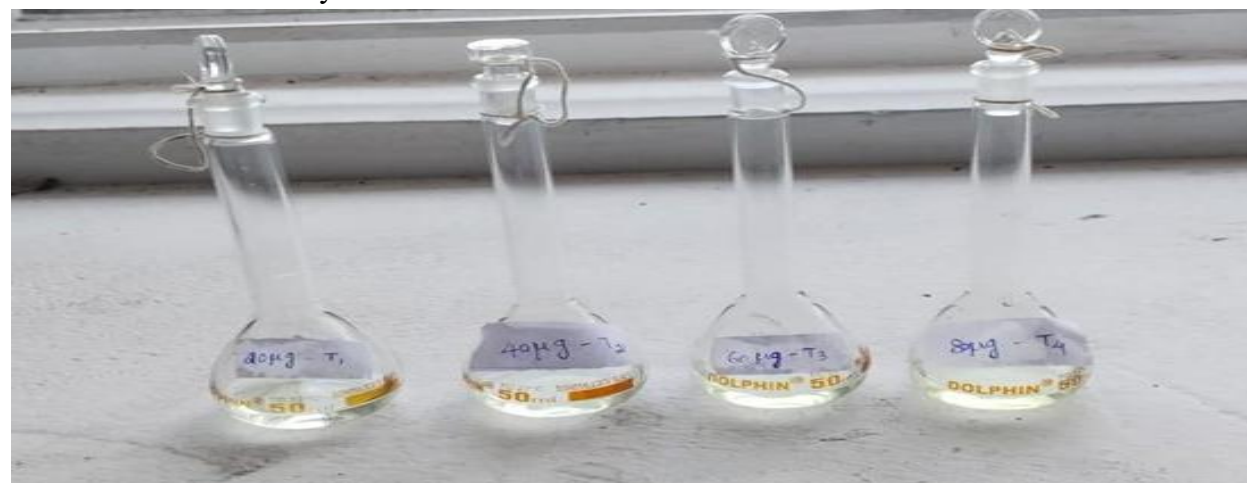


Fig no:31 D.P.P.H method of test hydroalcoholic

D.P.P.H method of standard ascorbic acid:



Fig no:32 D.P.P.H method of standard ascorbic acid

Anti inflammation activity:

Protocol for Free Radical Scavenging Assay (Nitric Oxide NO Assay):

Principle:

Nitric oxide (NO) is generated from sodium nitroprusside in an aqueous solution at physiological pH. It reacts with oxygen to produce nitrite ions (NO_2^-) which

Can be quantified using the Griess reagent. In the presence of antioxidants, nitric oxide is scavenged, leading to a decrease in nitrite formation. The reduction in absorbance at 546-550 nm reflects the NO scavenging ability of the compound.

Reagents:

Sodium nitroprusside (5 mM in PBS, pH 7.4)

Griess reagent (prepared fresh):

○ 1% sulphanilamide in 5% phosphoric acid

○ 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride (NED) Phosphate buffer (0.1 M, pH 7.4)

Ascorbic acid (standard, 1 mg/ml)

Test sample (dissolved in DMSO/methanol/water)

Procedure:

1. Add 1 ml of sample or standard at different concentrations (e.g., 20-100 $\mu\text{g/ml}$).
2. Incubate the mixture at 25° C for 2.5 hours under light (to generate NO).
3. After incubation, take 1 ml of the reaction mixture and add 1 ml of Griess reagent.
4. Incubate for 30 minutes in the dark at room temperature.
5. Measure the absorbance at 546 nm using a UV-Vis Prepare 1 ml of 5 mM sodium nitroprusside in phosphate buffer.
6. spectrophotometer.^[50]

D.P.P.H method of test ethyl acetate



Fig no:33 D.P.P.H of test ethyl acetate

D.P.P.H method of test hydroalcoholic:



Fig no:34 D.P.P.H method of test hydroalcoholic

D.P.P.H method of standard ascorbic acid:



Fig no:35 D.P.P.H method of standard ascorbic acid

Result and conclusion:

Phytochemical Analysis:

The qualitative phytochemical screening results of the ethyl acetate extract (EAE) and hydroalcoholic extract (HAE) obtained from *Centella asiatica* leaves are presented.

Qualitative phytochemical analysis of EAE and HAE from *Centella asiatica* leaves

s.no	Phytoconstituent	Test performed	Ethyl acetate extract	Hydroalcoholic extract
1.	Alkaloids	Mayer's test	+	+
2.	Catechin	Ehrlich's test	—	—

3.	Coumarins	Alcoholic NaOH test	+	+
4.	Phenols	Ferric chloride test	+	+
5.	Saponins	Froth test	+	+
6.	Triterpenoids	Acetic anhydrous/H ₂ SO ₄	+	+
7.	Glycosides	Salkowski test	+	+
8.	Tannins	Lead acetate test	+	+

+: Present (positive); - : absent (negative)

Table no: 6 Qualitative phytochemical analysis of EAE and HAE from *Centella asiatica* leaves

Both the ethyl acetate and hydroalcoholic extracts of *Centella asiatica* showed positive results for alkaloids, coumarins, phenols, saponins, triterpenoids, glycosides (Salkowski-positive), and tannins. However, the catechin test was negative in both extracts. The similar phytochemical profiles observed in the two extracts suggest that most of the major secondary-metabolite groups reported in this species (1,2,5) can be extracted over a relatively broad polarity range, extending from ethyl acetate to hydroalcoholic solvents. This finding agrees with earlier phytochemical investigations of *C. asiatica*, which have also described a diverse range of constituents (1,2,13). The lack of a positive catechin reaction in either extract is consistent with studies indicating that flavan-3-ol compounds, particularly catechin-type constituents, occur at relatively low or variable concentrations in *C. asiatica* compared with its major triterpenoid saponins and other phenolic compounds (1,2). Furthermore, the results demonstrate that a positive ferric chloride test for general “phenols” does not necessarily confirm the presence of every specific phenolic subclass.

Antioxidant activity – Nitric Oxide radical Scavenging Assay

The percentage inhibition of nitric oxide radical by the standard (ascorbic acid), the hydroalcoholic extract (HAE) and the ethyl acetate (EAE), at concentrations of 20,40,60 and 80ug/ml is presented in Table 4 .

Table 4: Percentage inhibition (NO radical scavenging assay – antioxidant evaluation)

Concentration (ug/ml)	Standard (Ascorbic Acid) %	HAF%	EAE%
20	72.41	81.40	58.74
40	73.52	78.81	60.96
60	73.27	68.22	61.33
80	73.02	68.84	61.82

Table no: 7 Antioxidant activity – Nitric Oxide radical Scavenging Assay

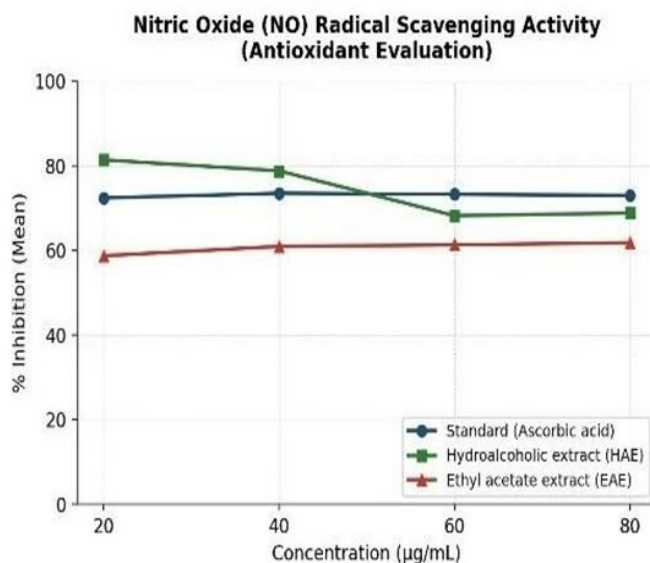


Fig. 6: Dose-response curve for nitric oxide radical scavenging (antioxidant) activity

Fig no: 36 Dose-response curve for nitric oxide radical scavenging (antioxidant) activity

The ascorbic acid standard product a relatively flat, near – maxim al inhibition profile across the tested range (72.42 -73.52%), consistent with reference antioxidant approaching saturation of the available nitric oxide radical even at the lowest concentration tested 981.40%at 20µg/ml),exceeding the standard, with a gradual decline to 68.22-68.84%at the two higher concentrations; this inverse concentration-activity pattern at the upper end of the tested range may reflect a degree of self-association or reduced solubility of the more polar hydroalcoholic constituents at higher loading, a phenomenon occasionally reported for polyphenol- rich plant extract in radical-scavenging assays (19,42) the ethyl acetate extract, in contract, displayed a conventional concentration-dependent increase in activity, rising steadily from 58.74% at 20ug/ml to 61.82% at 80ug/ml, though its overall inhibition remained lower than that of both the standard and the hydroalcoholic extract across the tested range. Because percentage inhibition for all three test articles exceeded 50% already at the lowest concentration examined (20ug/ml), the concentration range used in this study did not bracket the turn IC50 of the standard or of either extract, and a reliable interpolated IC50 of standard of either extract and a reliable interpolated IC50 value could not be calculated from the present data; wider ,lower concentration dilution series (for example,1-20ug/ml) would be required in follow-up work to determine IC50 value with confidence. Taken together, these results indicate that the hydroalcoholic extract of asiatic contains a higher concentration of nitric-oxide-scavenging and by extension anti-oxidant phytoconstituents- most plausibly phenolics and flavonoid glycosides that partition preferentially into the more polar hydroalcoholic solvent system- than the ethyl acetate extract, a pattern that parallels previous reports of solvent-polarity-dependent antioxidant activity in this species(42) and in other medicinal plants(48,50).

Anti-inflammatory activity -Griess-based nitric oxide scavenging assay:

The percentage inhibition of nitric oxide, used as a surrogate measure of Anti-inflammatory potential, for the standard, HAE and EAE at concentration of 20,40,60 and 80 ug/ml is presented in table 5 and fig.

Table 5: percentage inhibition (Griess-based NO scavenging assay-inflammatory evaluation)

Concentration(ug/ml)	Standard (Ascorbic acid)%	HAE%	EAE%
20	55.47	57.38	20.92
40	55.27	55.66	18.80
60	53.55	57.00	17.08
80	51.82	56.62	15.35

Table no:8percentage inhibition (Griess-based NO scavenging assay- inflammatory evaluation)

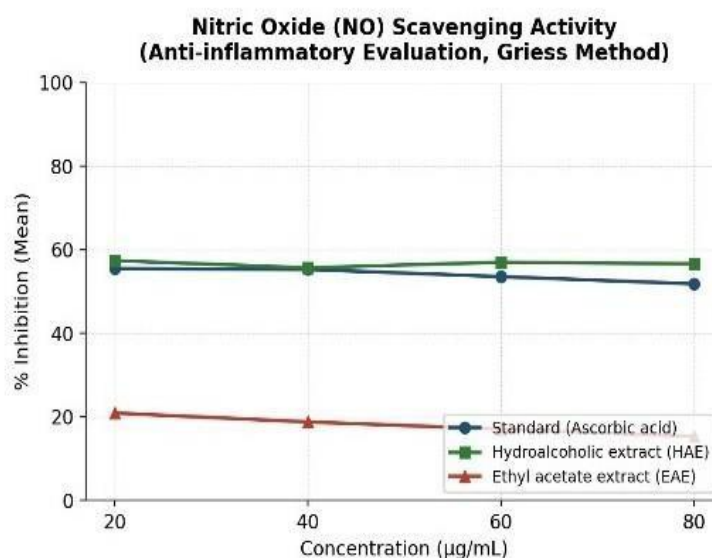


Fig. 7: Dose-response curve for Griess-based nitric oxide scavenging (anti-inflammatory) activity

Fig no:37 Dose-response curve for Griess-based nitric oxide scavenging (anti-inflammatory) activity

The standard ascorbic acid showed a modest, gradual decline in NO inhibition with increasing concentration (55.47% to 51.82%) remaining above 50% throughout the tested range. The hydroalcoholic extract maintained a consistently high and comparatively stable level of NO scavenging across all four concentrations marginally exceeding the standard. This is consistent with the anti-inflammatory activity reported for polar centella asiatica extract and fractions in macrophage-based NO/iNOS suppression assays system (24.31.32), and support the interpretations that the phenolic and saponin-rich hydroalcoholic fraction retains meaningful anti-inflammatory potential in this assays system. The ethyl acetate extract by contract, showed distinctly weaker activity that declined progressively with increased concentration, from 20.92% at 20ug/ml

to 15.38% at 80ug/ml because EAE remained below 50% inhibition across the entire tested range. Its NO scavenging (anti-inflammatory) IC₅₀ could not be determined from the present data and would require testing at concentration beyond 80ug/ml.

The markedly weaker and inversely concentration-related anti-inflammatory activity of the ethyl acetate extract, relative to its comparatively stronger and concentration-dependent performance in the antioxidant assay (section 7.2). suggests that the phytoconstituent responsible for direct free-radical scavenging in EAE are not equally effective at intercepting inflammatory nitric oxide generation.

III. STANDARD ASCORBIC ACID

S.NO	CONTROL	20ug	40ug	60ug	80ug
1	0.811	0.224	0.215	0.216	0.221
2	0.813	0.223	0.215	0.217	0.219
3	0.812	0.224	0.216	0.217	0.218
AVG	0.812	0.224	0.215	0.217	0.219
%	-	72.41	73.52	73.27	73.02

Table no:9 standard ascorbic acid

IV. TEST SAMPLE (HYDROALCOHOL)

S.NO	CONTROL	20ug	40ug	60ug	80ug
1	0.811	0.151	0.173	0.253	0.252
2	0.813	0.153	0.171	0.258	0.256
3	0.812	0.151	0.172	0.258	0.252
AVG	0.812	0.515	0.172	0.258	0.253
%		81.40	78.81	68.22	68.84

Table no:10 test sample hydroalcoholic

V. TEST SAMPLE (ETHYL ACETATE)

S.NO	CONTROL	20ug	40ug	60ug	80ug
1	0.811	0.337	0.317	0.317	0.310
2	0.813	0.335	0.311	0.314	0.310
3	0.812	0.334	0.223	0.312	0.311
AVG	0.812	0.335	0.317	0.312	0.310
%		58.74	60.96	61.33	61.82

Table no:11 test sample ethyl acetate

Standard Ascorbic Acid:

CONCENTRATION	OD1	OD2	OD3	MeanOD	%
CONTROL	0.521	0.521	0.523	0.521	
20ug	0.231	0.232	0.234	0.232	55.47
40ug	0.232	0.233	0.234	0.233	55.27
60ug	0.241	0.242	0.243	0.242	53.55
80ug	0.251	0.253	0.251	0.251	51.82

Table no:12 standard ascorbic acid

VI. TEST SAMPLE (HYDROALCOHOL)

CONCENTRATION	OD1	OD2	OD3	MEAN OD	%
CONTROL	0.521	0.521	0.523	0.521	
20ug	0.222	0.221	0.223	0.222	57.38
40ug	0.231	0.231	0.231	0.231	55.66
60ug	0.224	0.225	0.224	0.224	57.00
80ug	0.226	0.226	0.227	0.226	56.62

Table no:13 test sample hydroalcoholic

VII. TEST SAMPLE (ETHYL ACETATE)

CONCENTRATION	OD1	OD2	OD3	MEANOD	%
CONTROL	0.521	0.521	0.523	0.521	
20ug	0.412	0.413	0.412	0.412	20.92
40ug	0.423	0.423	0.424	0.423	18.80
60ug	0.431	0.432	0.433	0.432	17.08
80ug	0.441	0.442	0.441	0.441	15.35

Table no:14 test sample ethyl acetate

REFERENCES

- [1] Biswas D, Mandal S, Chatterjee Saha S, Tudu CK, Nandy S, Batha GE, et al. Ethnobotany, phytochemistry, pharmacology, and toxicity of *Centella asiatica* (L.) Urban: a comprehensive review. *Phytotherapy Res.* 2021;35(12):6624-6648. doi:10.1002/ptr.7248.
- [2] Mohapatra P, Ray A, Jena S, Nayak S, Mohanty S. Influence of extraction methods and solvent system on the chemical composition and antioxidant activity of *Centella asiatica* L. leaves. *Bicoastal Agric Biotechnology.* 2021; 33:101971. doi: 10.1016/j.bcab.2021.101971.
- [3] Jamil SS, Nizami Q, Salam M. *Centella asiatica* (Linn.) Urban: A review. *Nat Prod Radiance.* 2007;6(2):158-170.
- [4] Botanical Survey of India. *Flora of Agra District.* Kolkata: Botanical Survey of India. The flora

description records *C. asiatica* as a creeping herb with long-petiolate, orbicular-reniform, crenate leaves and flowers in umbels.

- [5] Gohil KJ, Patel JA, Gajjar AK. Pharmacological review on *Centella asiatica*: a potential herbal cure-all. *Indian J Pharm Sci.* 2010;72(5):546-556
- [6] [6]. James JT, Dubery IA. Pentacyclic triterpenoids from the medicinal herb, *Centella asiatica* (L.) Urban. *Molecules.* 2009;14(10):3922–3941.
- [7] Orhan IE. *Centella asiatica* (L.) Urban: From traditional medicine to modern medicine with neuroprotective potential. *Evid Based Complement Alternat Med.* 2012; 2012:946259. doi:10.1155/2012/946259.
- [8] Gray NE, Alcazar Magana A, Lak P, Wright KM, Quinn J, Stevens JF, et al. *Centella asiatica* – phytochemistry and mechanisms of neuroprotection and cognitive enhancement. *Phytochemical Rev.* 2018;17(1):161-194. doi:10.1007/s11101-017-9528-y
- [9] Orhan IE. *Centella asiatica* (L.) Urban: From traditional medicine to modern medicine with neuroprotective potential. *Evid Based Complement Alternat Med.* 2012; 2012:946259. doi:10.1155/2012/946259.
- [10] Biswas D, Mandal S, Chatterjee Saha S, Tudu CK, Nandy S, Batha GE, et al. Ethnobotany, phytochemistry, pharmacology, and toxicity of *Centella asiatica* (L.) Urban: a comprehensive review. *Phytotherapy Res.* 2021;35(12):6624-6654. doi:10.1002/ptr.7248.
- [11] Jagtap NS, Khanabad SS, Ghorpade DS, Banaras NB, Nahide SS. Antimicrobial and antifungal activity of *Centella asiatica* (L.) Urban, Umbellifer. *Res J Pharm Technol.* 2009;2(2):328-330
- [12] Orhan IE. *Centella asiatica* (L.) Urban: From traditional medicine to modern medicine with neuroprotective potential. *Evid Based Complement Alternat Med.* 2012; 2012:946259. doi:10.1155/2012/946259.
- [13] Sun B, Wu L, Wu Y, Zhang C, Qin L, Hayashi M, et al. Therapeutic potential of *Centella asiatica* and its triterpenes: A review. *Front Pharmacol.* 2020; 11:568032. doi:10.3389/fphar.2020.00568.
- [14] Selvaratnam V, Banumathi P, Premalatha MR, Sundaram SP, Arumugam T. Functional properties of *Centella asiatica* (L.): a review. *Int J Pharm Pharm Sci.* 2012;4(Suppl 5):37-40.
- [15] Nagore Meeran MF, Goyal SN, Suchil K, Sharma C, Patil CR, Ojha SK. Therapeutic properties and pharmacological activities of Asiatic side and madecassoside: a review. *J Cell Mol Med.* 2023;27(5):593-608. doi:10.1111/jcmm.17635.
- [16] Lefebvre T, Destandau E, Lesellier E. Selective extraction of bioactive compounds from plants using recent extraction techniques: a review. *J Chromatogr A.* 2021; 1635:461770. doi: 10.1016/j.chroma.2020.461770.
- [17] Beatrice A, et al. Antioxidant and acetylcholine esterase inhibition activity of the extract from *Centella asiatica* obtained by ultrasound pre-treatment followed by microwave-assisted extraction method. *J Phytol.* 2023; 15:116-120.
- [18] Beatrice A, Sivaneshwaran MR, et al. Antioxidant and acetylcholine esterase inhibition activity of the extract from *Centella asiatica* obtained by ultrasound pre-treatment followed by

- microwave-assisted extraction method. *J Phytol.* 2023; 15:116-120.
- [19] Ullah R, et al. *Centella asiatica* (L.) Urb.: From traditional medicine to modern medicine—A review. [Use as a supporting review for extraction and phytochemical preparation.]
- [20] Pistanty MA, Astirin OP, Marliyana SD, Solichatun S. Geographical variation in *Centella asiatica* (L.) Urban: morphological adaptations associated with distinct phytochemical signals and bioactivity of extracts. *ScientificWorldJournal.* 2026; 2026:3298125. doi:10.1155/tswj/3298125.
- [21] Sies H, Berndt C, Jones DP. Oxidative stress. *Annu Rev Biochem.* 2017; 86:715-748. doi:10.1146/annurev-biochem-061516-045037
- [22] Ariffin F, Chew SH, Bhupinder K, Othman A, Karim AA. Antioxidant capacity and phenolic composition of *Centella asiatica* (L.) Urban. *Food Chem.* 2011;127(2):445-450.
- [23] Benzie IFF, Strain JJ. The ferric reducing ability of plasma (FRAP) as a measure of “antioxidant power”: the FRAP assay. *Anal Biochem.* 1996;239(1):70-76. doi:10.1006/abio.1996.0292.
- [24] Brand-Williams W, Cuvelier ME, Berset C. Use of a free radical method to evaluate antioxidant activity. *LWT-Food Sci Technol.* 1995;28(1):25-30. doi:10.1016/S0023-6438(95)80008-5.
- [25] Sun B, Wu L, Wu Y, Zhang C, Qin L, Hayashi M, Kudo M, Gao M, Liu T. Therapeutic potential of *Centella asiatica* and its triterpenes: a review. *Front Pharmacol.* 2020; 11:568032. doi:10.3389/fphar.2020.568032
- [26] Chanut P, Ramasamy C, Kanaye A, Niyomtham N, Goodla L, Mordmuang A, et al. Potential activities of *Centella asiatica* leaf extract against pathogenic bacteria-associated biofilms and its anti-inflammatory effects. *Adv Pharmacol Pharm Sci.* 2024; 2024:5959077. doi:10.1155/2024/5959077.
- [27] Government of India, Ministry of AYUSH. The Ayurvedic Pharmacopoeia of India. Part I, Vol IV. New Delhi: Department of AYUSH, Ministry of Health and Family Welfare; 2004. p. 69.
- [28] Prakash V, Jaiswal N, Srivastava M. A review on medicinal properties of *Centella asiatica*. *Asian journal Pharmaceutical Clinical Research.* 2017;10(10):69-74. doi:10.22159/ajpcr.2017.v10i10.20760.
- [29] Witkowska K, Paczkowska-Walendowska M, Garbiec E, Cielecka-Piontek J. Topical application of *Centella asiatica* in wound healing: recent insights into mechanisms and clinical efficacy. *Pharmaceutics.* 2024;16(10):1252. doi:10.3390/pharmaceutics16101252.
- [30] Aszrin FN, Adam SH, Abdul Mutalib M, Tang HC, Tang SGH. An overview of *Centella asiatica* and its therapeutic applications. *Life Science Biomedical science .* 2024;8(1). doi:10.28916/lmb.8.1.2024.146.3 ntella asiatica.
- [31] *asiatica*. *Indian Journal Pharmacology.* 2004;36(6):377-80.
- [32] Nagoor Meeran MF, Javed H, Al Tae H, Azimullah S, Ojha SK. Pharmacological properties of *Centella asiatica*: A review. *Phytomedicine.* 2017; 24:1-10
- [33] Sultana N, Saify ZS. Naturally occurring and synthetic agents as potential anti-

- inflammatory and immunomodulants. *Antiinflamm Antiallergy Agents Med Chem.* 2012;11(1):3-19. doi:10.2174/187152312803476264
- [34] Orhan IE. *Centella asiatica* (L.) Urban: from traditional medicine to modern medicine with neuroprotective potential. *Evid Based Complement Alternat Med.* 2012; 2012:946259. doi:10.1155/2012/946259.
- [35] Gray NE, Sampath H, Zweig JA, Quinn JF, Soumyanath A. *Centella asiatica* attenuates β -amyloid-induced oxidative stress and mitochondrial dysfunction. *J Alzheimers Dis.* 2015;45(3):933-946. doi:10.3233/JAD-142217
- [36] Gohil KJ, Patel JA, Gajjar AK. Pharmacological review on *Centella asiatica*: a potential herbal cure-all. *Indian J Pharm Sci.* 2010;72(5):546-556. doi:10.4103/0250-474X.78519.
- [37] Jagadeesan S, Chioma SM, Mohd Miklas MA, Baharuldin MTH, Mat Taib CN, Amom Z, et al. *Centella asiatica* L. Urban protects against morphological aberrations induced by chronic unpredictable mild stress in rat's hippocampus via attenuation of oxidative stress. *Egypt J Basic Appl Sci.* 2022;9(1):324-339. doi:10.1080/2314808X.2022.2091265.
- [38] Balamurugan V, et al. Phytochemical profile and pharmacological studies of plant extracts.
- [39] Prabakaran K, Rajalakshmi S. Phytochemical screening and larvicidal activity of plant extracts. *Int J Zolo Appl Biosci.* 2018;3(2):168-173.
- Shiva MP, et al. Phytochemical screening and evaluation of medicinal plant extracts. *World J Pharm Res.* 2016;5(8):1128-1138.
- Mandal S, et al. Analysis of phytoc