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Preliminary phytochemical screening of bioactive compounds from leaves of *Centella asiatica* L. Urban

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Abstract
The present study described that the preliminary phytochemical analysis of leaf extracts of *Centella asiatica*. The organic solvents such as hexane, chloroform, ethyl acetate and ethanol were used for extracting bioactive compounds present in leaf. The phytochemical tests of the different extracts have revealed the presence of different quantities of alkaloids, glycosides, terpenoids, steroids, flavonoids, tannins, saponins and reducing sugars. Among all the extracts, ethanolic extract of leaf showed maximum yield of bioactive compounds than the other solvent extracts. Carbohydrates and falvonoides were present in all four extracts and hexane and chloroform extracts were yielded similar bioactive compounds Ethanol extracts showed the presence of all compounds. Proteins, antheroquinones and amino acids were absent in all the extracts. Thin layer chromatographic profiles yielded the different pattern of compounds and as well as different Rf values. The number of bands and Rf values of each extracts and in suitable solvent systems. Five different types of solvent systems were used to evaluate better elution of compounds. In specific compounds such as alkaloids detection from ethanol extract yielded three bands in solvent system of ethanol: NH_4OH (8.5:1.5), saponins yielded five bands in chloroform: glacial acetic acid: ethanol: water (6:2:1:1), terpenoids yielded eight bands in benzene: ethyl acetate (5:5) and flavonoids yielded ten bands in chloroform: ethanol (9:1).

Keywords: *Centella asiatica*, asiaticoside, madecassoside, thin layer chromatography

1. Introduction
Centella asiatica (L.) Urban is a small creeping perennial and profoundly branched prostrate tropical medicinal herb belongs to the family Apiaceae (Umbelliferare), it is native to Southeast Asian countries and commonly known as Asiatic pennywort, Indian pennywort, Indian water navelwort, is a tropical plant, which has been also cultivated successfully due to its medical importance. The plant has a long history of utilization in ayurvedic and Chinese traditional medicines since many centuries. It has been used widely in folk medicine for hundreds of years to treat a wide range of illness [1]. The leaves, which are edible, are in yellowish-green color, thin, alternate with long petioles, and quite characteristic reniform, orbicular, or oblong-elliptic shapes with seven veins [2]. The stem is slender, creeping stolon, green to reddish green in colour, connecting plants to each other. The rootstock consists of rhizomes growing vertically down. The plant has been used traditionally as brain tonic in ayurvedic medicine. This plant is used as brain tonic, and to treat chronic diseases and mental disorders. The plant contains several valuable bioactive compounds viz., centellasaponin, asiaticoside, madecassoside and sceffoleoside, pectin [3], castilliferol 1 and castillicetin 2 [4]. This plant is known as a source for various chemical constituents like carotenoids, vitamins B and C, bitter compound vellarin, fatty oils as glycerides of oleic, linolic, centoic, linolenic, palmitic and steric acids [5]. In classical Indian ayurvedic literauture it is considered to be one of 'Rasayana' (rejuvenator) drugs also widely used for its medicinal properties like sedative, analgesic, antidepressive, antimicrobial, antiviral and immunomodulatory. Previously triterpenoid acids, volatile and fatty oils, alkaloids, glycosides, flavonoids, and steroids have been isolated from the different parts of the plant [6].

Edible plants have traditionally occupied an important position in the socio-cultural, spiritual and health arena of rural and tribal lives of India. India has one of the oldest richest and most diverse cultural traditions associated with the use of traditional system of medicine. This plant possesses a wide range of pharmacological effects, being used for wound healing, mental disorders, antibacterial, antioxidant and anticancer purposes [7]. Leaf juice is rubbed on forehead to treat severe headache. To treat leprosy and other skin disorders it is used as ointment or dusting powder. Mixed with bath water, it is used in eczema [8]. The plant is highly effective in ulcer preventive, antidepressive sedative and ability to improve the venomous insufficiently. A bitter principle vellarine, pectic acid and resin present in the leaf and root; asiaticoside and oxyasiaticoside shown to be active in the treatment of leprosy and tuberculosis. The fatty oil isolated from the plant consists of glycerides of oleic, linolic, centoic, linolenic, lignoceric, palmitic, and steric acids; the leaves contain triterpene madasiatic acid as well as 3-glycosyl quercetin, 3-glycosyl kaempferol and 7-glycosyl kaempferol [9]. Asiaticoside is one of the prime triterpene saponin found in leaves in large amount is utilized commercially as a wound healing agent due to its potent anti-inflammatory effect and showed the potential use as anti-gastric ulcers drugs [10].

In Asiatic countries, *C. asiatica* is used as an ingredient in traditional systems of medicine such as Ayurveda, Siddha and Unani. In accordance with its potential wound healing property, several report described the remarkable protective effect of the plant against several diseases of central nervous

system [11, 12]. It also involved in wide range of biological activities desired for human health such as anti-inflammatory [13], hepatoprotective [14], anticonvulsant [15], cardioprotective [16], cytotoxic and antitumor [17], antiviral [18] and antibacterial activities [19]. The antioxidant activity of the plant is comparable to that of commonly utilized plant and it has been reported that it possess very good potential to be explored as a source of natural antioxidants [20]. Biological effects of *C. asiatica* have been attributed to the existence of major triterpene derivatives including asiatic acid, madecassic acid, asiaticoside, madecassoside, and brahmic acid [21, 22]. The occurrence of several important flavonoid derivatives including quercetin, kaempferol and several important phenolic compounds has also been reported [23].

In addition to neuroprotective effect of *C. asiatica*, it has been reported to own a wide range of biological activities desired for human health such as anti-inflammatory, antipsoriatic [24], sedative [25], immunostimulant, cardioprotective [26], antidiabetic [27], cytotoxic and antitumor, antiviral [28], antibacterial, insecticidal and venous deficiency treatments [29]. Therefore, the present study has been carried out to evaluate the preliminary screening of bioactive compounds present in leaves of *C. asiatica*.

2. Materials and Methods

2.1 Plant Materials

The plant leaves of *Centella asatica* were collected during ethnobotanical survey studies in Vathal Hills in November 2016. Specimen was labeled, numbered, annotated with the date of collection, the locality and their medicinal uses. The voucher specimens were then identified, and deposited in the herbarium of PG and Research Department of Botany, Government Arts College, Dharmapuri, Tamil Nadu, India for the future reference. After authentification leaves and fruits were collected in bulk, washed, shade dried and extracted with different solvents such as hexane, chloroform and ethanol for 48 hrs in a Soxhlet assembly.

2.2 Preparation of plant extract

Fresh leaves were washed thoroughly under running tap water followed by sterile distilled water and dried under shade. They were ground into coarse powder by using mechanical pulveriser. The leaf powder, about 100 g were weighed and repeatedly extracted with hexane, chloroform, ethyl acetate and ethanol in a 500 mL round bottom flask containing 250 mL solvent individually. The reflux time for each solvent was varying with 25 to 40 cycles for complete extraction in soxhlet apparatus [30, 31]. The filtrate was collected and concentrated by using rotary evaporator under controlled condition of temperature and pressure. The extracts were concentrated to dryness to yield crude residue. These residues were stored at 4°C, used for preliminary phytochemical screening of secondary metabolites. The presence of different chemical constituents in crude drugs can be detected by subjecting them to successive extraction using solvents in the order of increasing polarity. In the present study were therefore, subjected to extraction followed by qualitative chemical tests in order to know the phyto profiles on a preliminary basis.

2.3 Phytochemical Screenings [32, 33]

Phytochemical screening were performed to assess the qualitative chemical composition of different samples of crude extracts using commonly employed precipitation and coloration reactions to identify the major secondary metabolites like alkaloids, glycosides, steroids, tannins and terpenoides. To identify the chemical constituents of sample extracts by standard producers have been followed. The crude extract was qualitatively tested for the presence of chemical constituents using the following reagents and chemicals. The extracts obtained in the successive extraction process were subjected to preliminary phytochemical screening for the identification of various phytoconstituents such as alkaloids, carbohydrates, steroids, cardiac glycosides, flavonoides, carbohydrates, amino acids, phenols, naphthoquinones and tannins according to standard methods.

2.4 Tests for Alkaloides

Dragendroff's Test: Little amount of the sample was treated with the Dragendroff's reagent; the appearance of reddish brown precipitate indicated the presence of alkaloids.

Mayer's Test: Sample (2-3 ml) was treated with few drops of Mayer's reagent. Appearance of white precipitate indicated the presence of alkaloids.

Wagner's Test: Sample (2-3 ml) was mixed with few drops of Wagner's reagent. Appearance of reddish brown precipitate indicated the presence of alkaloids.

2.5 Tests for Carbohydrates

Molisch's Test: To a small amount of the extract few drops of Molish's reagent was added followed by the addition of concentrated H_2SO_4 along the sides of the test tube. The mixture was then allowed to stand for 2 min and then diluted with 5 ml of distilled water. Formation of red or dull violet colour at the inter phase of two layers indicates the presence of carbohydrates. First yellow then brick pink red precipitate was observed.

Fehling's Test: The extract was treated with 5 ml of Fehling's solution (A and B) and kept in boiling water bath for 5-10 min. The formation of yellow or red colour precipitate indicates the presence of reducing sugar.

Benedict's Test: Sample solution and equal volume of Benedict's reagent were mixed in the test tube. Heated in boiling water bath for 5 min solution appears green, yellow colour appeared based on the amount of reducing sugar present in test solution.

2.6 Test for Glycosides [34]

Free content of the sugar extract was determined. The sample was hydrolysed with mineral acid (dilute hydrochloric or dilute sulphuric acid). Again the total sugar content of the hydrolysed extract was determined. Increase in the sugar content indicated the presence of glycoside in the extract.

Glycoside -----H_2O----→ Aglycon (genin) + Glycon (sugar)

Baljet's Test: To 5 ml of the extract few drops of sodium picrate was added to observe yellow to orange colour.

Keller-killiani Test: To 5 ml of the extract few drops of ferric chloride solution was added and mixed, then sulphuric acid containing ferric chloride solution was added, it forms two layer showed reddish brown while upper layer turns bluish green indicates the presence of glycosides.

Legal's Test: Aqueous or alcoholic sample extract was mixed with 1 ml of pyridine sodium nitroprusside was added. Pink to red color appeared.

2.7 Test for steroids

Salkowski's Test: Sample (2 ml) was mixed with 2 ml of concentrated Sulphuric acid, it was well shaken then chloroform layer appeared red and acid layer shown greenish yellow fluorescene.

Liberman-Buchard reaction: Sample (2 ml) was mixed with chloroform. 1-2 ml of acetic anhydride was added and 2 drops concentration sulphuric acid was added from the sides of the tube. First red then blue and finally green colour appeared.

2.8 Test for Proteins [35]

Million's Test: Test sample (3ml) was mixed with 5ml of Million's reagent. White precipitate is formed. On warming precipitate turn's brick red or the precipitate dissolves giving red colored solution.

Biuret Test: Test sample (3 ml) was mixed with 4% NaOH and few drops of 1% $CuSO_4$ solution were added. Violet or pink color not appeared. To 3 ml of the extract few drops of 10% sodium chloride and 1% copper sulphate was added for the formation of violet or purple colour. On addition of alkali, it becomes dark violet.

2.9 Test for flavonoides

Alkaline Test: To 3 ml of the extract few magnesium ribbons are dipped and concentrated Hydrochloric acid was added over them and observed for the formation of magenta (brick red) colour indicating the presence of flavonoides.

Shinoda Test: Sample extract was treated with 5 ml of 95% ethanol; few drops of concentrated Hydrochloric acid and 0.5g of magnesium turnings were also added. Pink colour was observed. Addition of increasing amount of sodium hydroxide to the residue shown yellow coloration, which decolorizes after addition of acid.

2.10 Determination of Flavonoides as Quecetin Equivalent

Flavonoids contents in the extracts were determined by standard colorimetric method with minor modifications. To 1 ml leaf extract was added 0.3 ml 5% sodium nitrite; 4 ml distilled water and held for 5 minutes. To the mixture 0.3 ml of 10% aluminium chloride was and held for 6 minutes. Finally 2 ml of 1M sodium.

2.11 Test for Tannins

A fraction of the extract was dissolved in water and then it was subjected to water bath 37°C for 1 h and treated with ferric chloride solution and observed for the formation of dark green colour.

Lead acetate test: The sample was treated with 10% lead acetate solution; appearance of white precipitate indicated the presence of tannins. When the extract was treated with aqueous bromine solution, appearance of white precipitate indicated the presence of tannins.

Ferric chloride test: To 1 ml of extract, 2 ml of 5% ferric chloride was added. Formation of greenish black colour indicated the presence of tannins. A fraction of the extract was dissolved in water and then it was subjected to water bath 37°C for 1hr and treated with ferric chloride solution and observed and for the formation of dark green colour.

2.12 Test for Saponin

Foam Test: To 1 ml of the extracts 5 ml distilled water was added and shaken vigorously. Formation of foam indicated presence of saponins.

2.13 Test for anthraquinones: Weighted leaf powder, 0.5g, was boiled in 10% hydrochloride acid and filtered hot. To this, 2 ml chloroform and 10% ammonia solution each were added. Formation of pink color in the aqueous layer indicated presence of anthraquinones.

2.14 Test for Amino Acids

Ninhydrin Test: Test sample (3 ml) and 3 drops of 5% ninhydrin solution were heated in boiling water for 10 min. Purple color appeared.

2.15 Test for phenols

Ferric chloride test: A fraction of the extract was treated with 5% ferric chloride solution and observed for the formation of deep blue or black colour.

To 1 ml of the extract, 2 ml of distilled water, 3 drops of 10% aqueous ferric chloride ($FeCl_3$) and 3 drops of potassium ferrocyanide were added. Formation of blue or green color showed the presence of polyphenols.

2.16 Test for total polyphenols: Phenolic compounds in the leaf extracts were estimated by a colorimetric assay, based on standard procedures with minor modifications. To 5 ml distilled water was added 0.5 ml Folin Ciocalteu's reagent. After 3 min, 1 ml 7.5% sodium carbonate solution, 1 ml extract were added to the mixture and made to 10 ml with distilled water. The mixture was kept in water bath maintained at 50°c for 16 minutes. UV Visible spectrophotometer (UV-Vis Shimadzu) was used to read the absorbance at 765 nm. Gallic acid was prepared in different concentrations and the absorbance equally read at 765 nm. The values obtained were used to generate the standard curve against which polyphenols in the leaves, stem bark, fruit pulp and seeds were calculated and expressed as Gallic acid equivalents (GAEs) per 100 g dwb.

2.17 Test for terpenoides

Chloroform Test: To 5 ml of the extract few drops of

chloroform and concentrated H_2SO_4 was added carefully along the sides of the test tube. Formation of brown color at interface was a positive indicator.

2.18 Test for reducing sugars: The residue was dissolved in water and kept in the water bath. Two ml of the solution in a test tube was added with 1 ml each of Fehling's reagent A and B. The mixture was shaken and heated in a water bath for 10 min. A brick red precipitate indicates a reducing sugar.

2.19 Identification of Compounds by Thin Layer Chromatography (TLC)

In 1958 Stahl [36] demonstrated application of TLC in analysis. It is at present an important analytical tool for qualitative analysis of a number of natural products. The plates were visualized for spot identification under iodine chamber and sprayed with spray reagent of the category given in table. The Rf value was calculated by using formula:

$$\text{Rf value} = \frac{\text{Distance travelled by solute from the base line}}{\text{Distance travelled by solvent from the base line}}$$

10 mg/ml of *Centella asiatica* in different extract was dissolved in ethanol solvent used for TLC examination. TLC plates were prepared by using Silica Gel-G as adsorbent. The different solvent systems of different polarities were prepared and TLC studies were carried out to select the solvent system capable of showing better resolution. The above prepared plant extracts were applied on pre-coated TLC plates by using capillary tubes and developed in a TLC chamber using suitable mobile phase. The developed TLC plates were air dried and observed under ultra violet light UV at both 254 nm and 366 nm. 100g Silica Gel-G was mixed with sufficient quantity of distilled water to makes Slurry. The slurry was immediately poured into a spreads and plates were prepared by spreading the slurry on glass plates of required size. Plates were allowed to air dry for one hour. They were later sprayed with different spraying reagents and some were placed in hot air oven for 1 min for the development of color in separated bands. The movement of the analyze was expressed by its retention factor (R*f*). Values were calculated for different sample. After drying the plates, they were exposed to Iodine vapours by placing in a chamber that was saturated with iodine vapours and also exposed to different spraying reagents. All plates were visualized directly after drying and with the help of UV at 254 nm and 366 nm in UV TLC viewer. The R*f* value of the different pots that were observed was calculated.

2.20 Statistical analysis

Phytochemical estimation and quantification were performed in five replicates under standard procedures to ensure consistency of all conclusions. Data of all experiments were statistically analysed and expressed as Mean ± Standard Deviation.

3. Results

The present study was carried out to analyze the presence of bioactive compounds in leaves of *Centella asiatica*. The presence of phytochemical compounds in the leaf extracts of *C. asiatica* was evaluated by hexane, chloroform, ethyl acetate and ethanol extracts. This investigation carried out through cold percolation as well as Soxhlet extraction methods have revealed the presence of bioactive compounds. The concentrated extracts of *C. asiatica* were carefully stored and analyzed. The colours of the extracts were pale green colour to yellow colour particularly chloroform extract was bright green in colour. The percentage yield of these extract were also measured, and it was the ethanolic extract 47.65% maximum yield in comparison with other solvent extracts. All the extracts were sticky semisolid in their consistency. The qualitative phytochemical screening of ethanol, ethyl acetate, chloroform, and hexane extracts of leaves of *Centella asiatica* L. and its secondary metabolites were shown in Table-1. The results showed the presence of phytochemical constituents, namely alkaloids, saponins, steroids, total phenols and tannins, triterpenoid, and absence of proteins and aminoacids, flavonoids, glycosides, anthoquinones, and steroids.

Among the four solvents used, ethanol extracts were yielded maximum bioactive compounds followed by ethyl acetate extract, chloroform extract and minimum amount of compounds present in hexane extract. The result of preliminary phytochemical analysis of *C. asiatica* leaf extracts were revealed the presence bioactive components namely alkaloids, amino acids, cardiac glycosides, phytosterols, triterpenoids, reducing sugars, steroids, saponins, flavonoids, phenols, tannins, anthraquinones in different concentrations except carbohydrates. *Centella asiatica* is considered as an enriched source of different active compounds presented in Table 1. The main active compounds are pentacyclic triterpenes (Asiatic acid, madecassic acid, asiaticoside and madecassoside).

The main active principle compounds are triterpenoids, glycosides such as asiatic acids. Carbohydrates and falvonoides were present in all four extracts in varying concentrations. Hexane and chloroform extracts were yielded similar bioactive compounds such as alkaloids, carbohydrates, steroids and flavonoids. Ethyl acetate extract yield the compounds are carbohydrates, flavonoids, tannins, saponins, phenols, terpenoids and reducing sugars. Ethanol extracts showed the presence of all compounds of ethyl acetate extract and additionally glycosides. Proteins, antheroquinones and amino acids were absent in all the extracts. Further, the ethyl acetate and ethanol extracts were showed the absence of anthraquinones.

For quantification of phytochemicals and its Rf values were calculated by thin layer chromatographic analysis. Further individual bands and its colour and Rf value based on corresponding authentic samples of each bioactive compounds have been identified. Thin layer chromatographic profiles yielded the different pattern of compounds and as well as different Rf values. The extracted bioactive compounds were tested followed by calculate their Rf value by analyzing thin layer chromatographic techniques with five different solvent systems. The number of bands and Rf values of each extracts and in suitable solvent systems were presented in Table 2. Five different types of solvent systems were used to evaluate better elution of compounds. The ethanol extracts of leaves have yielded four bands were yielded in solvent system 1 [Benzene : Ethanol : Water (7:2:1)] (Rf = 0.21, 0.48, 0.56,

0.78), similarly solvent system 3 [Benzene : Chloroform : Ethanol (6:2:2)] (Rf = 0.20, 0.43, 0.58, 0.77), solvent system 4 [Chloroform : Ethyl acetate : Ethanol : Water (5:2:2:1)] (Rf = 0.30, 0.52, 0.61, 0.78), solvent system 5 [Chloroform : Ethyl acetate : Ethanol (6:3:1)] (Rf = 0.38, 0.62, 0.74, 0.86), respectively. Five bands were yielded in solvent system 2 [Benzene: Chloroform: Acetone: Ethanol (6:2:1:1)] (Rf = 0.24, 0.48, 0.53, 0.68, 0.92). In ethyl acetate extract of leaves were yielded four bands in solvent system 2 (Rf = 0.31, 0.54, 0.66, 0.85) in other solvent systems yield only three bands in solvent system 1 (Rf = 0.32, 0.54, 0.72), solvent system 3 (Rf = 0.25, 0.58, 0.75), solvent system 4 (Rf = 0.33, 0.58, 0.75) and solvent system 5 (Rf = 0.33, 0.60, 0.71). In chloroform extract of leaves were yielded three bands in solvent system 2 (Rf = 0.52, 0.66, 0.83) in other solvent systems yield only two bands in solvent system 1 (Rf = 0.44, 0.57), solvent system 3 (Rf = 0.48, 0.60), solvent system 4 (Rf = 0.39, 0.62) and solvent system 5 (Rf = 0.40, 0.58). In hexane extract of leaves was yielded only one band in all the solvent systems in different Rf values (Rf = 0.86, 0.91, 0.88, 0.78 and 0.75). In specific compounds such as alkaloids detection from ethanol extract yielded three bands in [ethanol : NH_4OH (8.5:1.5)] solvent system (Rf = 0.68, 0.72, 0.85), saponins yielded five bands in [chloroform : Glacial Acetic acid : Ethanol : Water (6:2:1:1)] solvent system (Rf = 0.38, 0.46, 0.55, 0.64, 0.90), Terpenoids yielded eight bands in [Benzene : Ethyl acetate (5:5)] (Rf = 0.35, 0.39, 0.48, 0.53, 0.57, 0.75, 0.87, 0.92) and Flavonoids yielded ten bands in [chloroform : Ethanol (9:1)] (Rf = 0.18, 0.19, 0.23, 0.32, 0.42, 0.48, 0.65, 0.74, 0.79, 0.85) (Table 3).

Table 1: Quantitative phytochemical analysis of leaf extracts of *Centella asiatica*

Phytochemicals	Biochemical tests	*Centella asiatica* Leaf			
		Hexane extract	Chloroform extract	Ethyl Acetate extract	Ethanol extract
Alkaloids	Mayer's Test	+	+	+	+
	Wagner's Test	+	+	+	+
	Dragendroff's Test	+	+	+	+
Carbohydrates	Molisch's Test	+	+	+	+
	Fehling's test	+	+	+	+
	Benedict's Test	+	+	+	+
Glycosides	Baljet's Test	-	-	-	+
	Keller-Killiani test	-	-	-	+
	Legal's test	-	-	-	+
Steroids	Liebermann-Buchard Test	+	+	-	-
	Salkowskis Test	+	+	-	-
Proteins	Biruet test	-	-	-	-
	Millions test	-	-	-	-
Flavonoides	Alkaline Test	+	+	+	+
	Shinoda Test	+	+	+	+
Tannins	Lead acetate Test	-	-	+	+
	Ferric chloride Test	-	-	+	+
Saponins	Foam Test	-	-	+	+
Anthraquinones	NH_4OH Test	-	-	-	-
Amino acid	Ninhydrin test	-	-	-	-
Phenols	Ferric chloride	-	+	+	+
Terpenoids	Chloroform Test	-	-	+	+
Reducing sugars	Fehling's Test	-	-	+	+

(Note: H=Hexane; C=Chloroform; EA=Ethyl Acetate; ET=Ethanol; (+) = Present; (-) = Absent)

Table 2: Thin Layer Chromatographic analysis of *Centella asiatica* leaves extracts with their Rf values in different solvent systems

S. No	Solvent systems	Leaf extracts	No. of bands	Rf values
1.	Benzene : Ethanol : Water (7:2:1)	Hexane	1	0.86
		Chloroform	2	0.44, 0.57
		Ethyl acetate	3	0.32, 0.54, 0.72
		Ethanol	4	0.21, 0.48, 0.56, 0.78
2.	Benzene : Chloroform : Acetone : Ethanol (6:2:1:1)	Hexane	1	0.91
		Chloroform	3	0.52, 0.66, 0.83
		Ethyl acetate	4	0.31, 0.54, 0.66, 0.85
		Ethanol	5	0.24, 0.48, 0.53, 0.68, 0.92
3.	Benzene : Chloroform : Ethanol (6:2:2)	Hexane	1	0.88
		Chloroform	2	0.48, 0.60
		Ethyl acetate	3	0.25, 0.58, 0.75
		Ethanol	4	0.20, 0.43, 0.58, 0.77
4.	Chloroform : Ethyl acetate : Ethanol : Water (5:2:2:1)	Hexane	1	0.78
		Chloroform	2	0.39, 0.62
		Ethyl acetate	3	0.33, 0.58, 0.75

		Ethanol	4	0.30, 0.52, 0.61, 0.78
5.	Chloroform : Ethyl acetate : Ethanol (6:3:1)	Hexane	1	0.75
		Chloroform	2	0.40, 0.58
		Ethyl acetate	3	0.33, 0.60, 0.71
		Ethanol	4	0.38, 0.62, 0.74, 0.86

Table 3: Thin Layer Chromatographic analysis of specific bioactive compounds in leaf extracts of *Centella asiatica* leaves with their Rf values in different specific systems

S. No.	Phytochemicals	Solvent systems	No. of bands	Rf values
1.	Alkaloids	Ethanol : NH_4OH (8.5:1.5)	3	0.68, 0.72, 0.85
2.	Saponins	Chloroform : Glacial Acetic acid : Ethanol : Water (6:2:1:1)	5	0.38, 0.46, 0.55, 0.64, 0.90
3.	Terpinoids	Benzene : Ethyl acetate (5:5)	8	0.35, 0.39, 0.48, 0.53, 0.57, 0.75, 0.87, 0.92
4.	Flavonoids	Chloroform : Ethanol (9:1)	10	0.18, 0.19, 0.23, 0.32, 0.42, 0.48, 0.65, 0.74, 0.79, 0.85

4. Discussion

Phytochemical analysis is of pivotal importance in identifying new and potent sources of therapeutically and industrially valuable phytocompounds in plants. It is an important tools in the determination of the bioactive components of medicinal plants, hence the fundamental scientific basis of their impact on health management. Phytochemical screening of the leaf and callus extracts of *C. asiatica* have revealed the presence of alkaloids, glycosides, terpenoids, steroids, flavonoids, tannins and reducing sugars. These compounds have significant application against human pathogens, including those that cause enteric infections and are reported to have curative properties against several pathogens and therefore could suggest their use in the treatment of various diseases [37]. Diterpenes, triterpenes as madecassic acid, or brahmic acid were proved as highly potential against various diseases. Alkaloids, flavonoids detected in the extracts are compounds that have been documented to possess medicinal properties and health promoting effects. Phenolics are the largest group of phytochemicals and have been said to account for most of the antioxidant activity of plant extract. In general, the total phenolic compounds found in the leaf, root and petiole are the major contributions to the antioxidant activities of the plant [38]. Saponin is not detected in *C. asiatica* in the present study, whereas alkaloids are present in all the tested extracts. Asiatic acid and asiaticoside found in *C. asiatica* showed great promise in prevention and treatment of cancer either as a plant alone or in combination with other forms of chemotherapy such as vincristine from *Catheranthus roseus* [39]. As phytochemicals often play an important role in plant defense against prey, microorganism, stress as well as interspecies protections, these plant components have been used as drugs for millennia and hence, screening of phytochemicals serves as the initial step in predicting the types of potential active compounds from plants [40].

There seem to be variation in the qualitative detectability of phytochemicals which may due to the processing procedure used on the extracts which may have resulted in the absence, increased or reduced presence of some phytochemicals. The notable differences between this study and previous works could be as a result of differences in reagents used and methods employed in the analysis, the time of the year when the plant materials were collected and geographical location as well as time lag between collection of plant materials and time of extraction. It is usually advisable to collect plant materials and extract almost immediately to forestall the physiological changes that may occur over time but this is usually not practiced especially as most researchers take to drying their plants before extraction. In the present study, most of the biologically active compounds such as flavonoids, alkaloids, glycosides, steroids, phenols, saponins, terpenoids, cardiac glycosides and tannins were found to be present in the ethanolic extracts of *Centella asiatica* leaves. The medicinal properties of *C. asiatica* leaf extracts may be due to the presence of above mentioned phytochemicals. Studies on the efficiency of medicinal plants with respect to the control of infectious diseases are more essential to know their therapeutic value and hence in pharmaceutical arenas.

The various phytochemical compounds detected are known to have beneficial importance in medicinal sciences. For instance, flavonoids have been referred to as nature's biological response modifiers, because of their inherent ability to modify the body's reaction to allergies and virus and they showed their anti-allergic, anti-inflammatory, anti-microbial and anti-cancer activities [41, 42, 43]. Plant steroids are known to be important for their cardiotonic activities and also possess insecticidal and antimicrobial properties. They are also used in nutrition, herbal medicine and cosmetics. Tannins were reported to exhibit antiviral, antibacterial and anti-tumour activities. It was also reported that certain tannins were able to inhibit HIV replication selectively and was also used as diuretic [44]. Saponin is used as mild detergents and in intracellular histochemistal staining. It is also used to allow antibody access in intracellular proteins. In medicine, it is used in hypercholestrolaemia, hyperglycaemia, antioxidant, anticancer, antiinflammatory, weight loss, etc. It is also known to have antifungal properties [45].

Moreover, acting by several different mechanisms, particular flavonoid can extract significant anticancer activity, amongst other modes of action. Certain flavonoids possess potent inhibitory activity against a wide array of enzymes. Evidence suggests that only activated cells are responding to a stimulus. So the presence of this type of phytochemical compounds in the screened medicinal plants has a wide range of applications and could be certainly used for a variety of applications [46]. Several workers have reported the analgesic [47] antispasmodic and antibacterial properties of alkaloids [48]. Glycosides are known to lower the blood pressure according to many reports [49]. The terpinoids have been shown to decrease blood sugar level in animal studies [50]. Plant saponins help humans to fight

fungal infections, combat microbes and viruses, boost the effectiveness of certain vaccines and knock out some kinds of tumor cells, particularly lung and blood cancers. These compounds served as natural antibiotics, which help the body to fight infections and microbial invasion [51]. Triterpenoids are reported to have useful for antibacterial activity and can be applied against various bacterial pathogens like *S. aureus, Shigella flexneri, Pasteurella multocida, E. coli*, *Salmonella* and etc [52].

The initial phytochemical screening tests may be helpful in the screening of the bioactive compounds and eventually may help to detection and development of new drugs. Further, these tests make easy their qualitative separation and quantitative estimation of pharmacologically active chemical compounds [53]. The phytochemical screening in the present study has publicized the presence of alkaloids, saponins, total phenols and tannins, and triterpenoids in the leaves. Further the presence of different phytochemicals in ethanol and chloroform solvent extracts may be responsible for the therapeutic properties of *C. asiatica*.

Tannins is a phenolic compounds that are acting as principal antioxidants or free radical scavengers. Since these phenolic compounds were originated to be present in the extracts, it might be accountable for the potent antioxidant capacity of *C. asiatica*. These phytochemicals of medicinal plants have primarily reported for their medicinal value, which can be valuable for therapeutic index. For instance, saponins proved as hypotensive and cardiodepressant properties [54], which are helpful for the treatment of congestive heart failure and cardiac myopathy [55]. The occurrence of saponins in ethanol extracts of leaves of *C. asiatica* might play a role in the cardioprotective potential. Alkaloids and tannins have the potential of hypoglycemic and anti-inflammatory activities [56]. Moreover, the terpenoids have also been revealed to decrease blood sugar level in animal studies. In addition, the steroids and triterpenoids demonstrated the analgesic properties and central nervous system activities [57, 58]. Hence the preliminary phytochemical investigation are actually obliging in finding chemical ingredients in the plant that may help to their quantitative evaluation and also in locating the source of pharmacologically active principle.

The reducing power of ethanolic extract was also significantly higher in CA-4 compared to other samples. It has been reported that there is a positive correlation between the total phenolic content and antioxidant activity [59, 60]. In India, the Ayurvedic system has described a large number of medicines based on plantys or plant products. Alkaloids rank among the most proficient and pharmaco-therspeutically significant plant substances and the largest single class of secondary plant metabolites. Saponins are function as potent antimicrobial agents. Tannins are complex phenolic polymers, which will bind to the proteins and carbohydrate molecules resulting in reduction and inhibition of microbial growth [61]. Our reports were accordance with previous investigations of same plant species *Centella asiatica* [62]. Triterpenoids of *Centella* was reported to exhibit antitumor activity. This plant was proved to be efficient in the treatment of rheumatic disorders. Asiaticoside is a major triterpene and posses anti-inflammatory and gastric ulcer drugs [63]. In the present study our results showed that the leaf extracts possess bioactive molecules with maximum antibacterial activity against all tested strains. Owing to the high therapeutic potential, it is of high stipulate in pharmaceutical industries which escorts to its overexploitation resulting in the relapsing of the population of *C. asiatica* to a precarious level that a ban on its collection from their natural habitat has been recommended [64]. It is suggested that the ethanol extract of leaf revealed a significant scope to develop a novel broad spectrum of important bioactive formulation and can be used to carry out further pharmacological evaluation to be used as antibacterial drugs.

5. Conclusion

Since the study was conducted in a controlled manner, the phytochemical results can be used for the standardization of the above mentioned drugs. A preliminary screening and more research has to be undertaken to explore the wonderful therapeutic properties of these medicines. The results of the present study revealed the presence of several phytochemical compounds like saponins, tannins and steroids. In the ethanolic extract shows the presence of maximum number of bioactive compounds yield such as alkaloids, carbohydrates, proteins, amino acids, Phenolic compounds and tannins. Similary alkaloids, carbohydrates, proteins, amino acids, Phenols were present in all four extracts. To conclude the presence study, we have found that most of the biologically active phytochemicals were present in ethanol and chloroform extracts of leaves of *C. asiatica*. In conclusion the ethanolic extracts of *Centella asiatica* showed varying inhibitory activities against all tested organisms. The results were encouraging enough to pursue fractionation of this extracts and to find out the functional properties of phytochemical compounds in view to discover useful potential chemotherapeutic agents. These phytochemical compounds may contribute as useful source of herbal and ayurvedic pathway for effective treatment of various diseases considering its tremendous potential pharmacological activites.

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7. References

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