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Pharmacognostic and Phytochemical Investigation of Aerial Parts of *Centella asiatica* Linn

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Abstract

In Indo-Pak subcontinent, the traditional systems of medicine, both Ayurvedic and Unani are primarily based on the use of herbs and herb based preparation for therapy. Therefore, the importance of herbs identification process still remains the key factor in achieving the desired and successful therapeutic effect. To support the manufacturers and practitioners of both the systems, a huge quantity of herbs are still collected from wild source, as the herbal farming is not very much developed in this part of the world. During a survey program conducted in different areas of Pakistan, significant lacking and gaps were noted to be present in the identification & characterization of herbs which needs to be addressed and fulfilled as many species look alike apparently or physically but have different biological or pharmacological activity. Based on this objective and approach *Centella asiatica* was selected for pharmacognostic and preliminary phytochemical investigation to establish a better correlation and to provide useful methods in its identification as use of *Centella asiatica* is very common in Pakistan and other South Asian countries for CNS disorders therapy. Therefore, purpose of this study was to develop & report some and rapid identification method for *Centella asiatica*. The present study includes physical, physicochemical, preliminary phytochemical and fluorescence analysis. For the first time, in the present study NIR and FT-IR spectrum of *Centella asiatica* have been reported for identification. Finding of the present study are quite promising which can be helpful for the manufacturers and researchers in the identification and development of *Centella asiatica* based new drugs or formulations.

Keywords: *Centella asiatica*, fluorescence analysis, pharmacognostic, physicochemical, phytochemical, , NIR and FT-IR analysis.

Introduction

Centella asiatica Linn. belongs to family Apiaceae [1] and grows well in different parts of the world including Pakistan, Brunei, Burma, Cambodia, China, Columbia, East Timor, Eastern region of South America, India, Indonesia, Laos, Madagascar, Malaysia, Mexico, Philippines, Singapore, South Africa, Southeast of USA, Sri Lanka, Thailand, Venezuela, Vietnam and Western South Sea Islands [2,3]. The stems of *Centella asiatica* are cylindrical with internodes, leaves are kidney shaped (Reniform), glabrous arising from stem nodes,

flowers makes umbel and each umble bears 2 to 5 fruits. Fruits encircled with hard and thick pericarp [4]. Traditionally the plant is used for the treatment of asthma, bronchitis, cystitis, dehydration, epilepsy, headache, leprosy, and syphilis. It is also reported as carminative, diuretic, memory enhancer and soporific. Traditional Chinese Medicine and Ayurveda both used it for the treatment of anxiety and depression [5]. Conventionally whole plant is

suggested for animal therapy against colic pain, contagious abortion, foot and mouth disease, jaundice, and respiratory tract inflammation . The chemical literature search revealed the presence of amino acids [4], tannins [5], flavonoids, sterols, saponins, triterpenoids, volatile oil [6], alkaloids [7] and sugars [8]. Antibacterial, antidepressant, antiemetic, antigenotoxic, antineoplastic, antioxidant, antithrombotic, anxiolytic, gastroprotective, immunomodulatory, nerve-regenerative, radioprotective and wound healing are reported pharmacological activities [9].

The importance Pharmacognostic evaluation cannot be ignored as it provides and supports as well as authenticates the results of the biological or pharmacological studies. With these observations, when biological and pharmacological studies were performed on

Centella asiatica found in Pakistan it was also decided to perform the Pharmacognostic evaluation so that the results can be authenticated and used to formulate products. Based on this approach, the Pharmacognostic investigation and evaluation was taken into consideration which includes, some valuable physico-chemical test, microscopic examination, fluorescence analysis and qualitative tests. For optimum results, physicochemical test were performed on dried areal parts (untreated and grinded). Both organoleptic and physical parameters were taken into consideration and tests were performed to observe the color, odor and taste along with the characteristic features of stem, leaves, fruits and flowers, where as the chemical tests performed on treated material (grinded) includes solubility, saponins, chalk, fixed oil and NIR identification. The physico-chemical tests performed include, detection of foreign matters using dried aerial parts (untreated) and some other test such as loss on drying, total ash, water soluble ash, acid insoluble ash, residue on ignition, alcohol soluble extractive, water soluble extractive and crude fibers (grinded).

The microscopic studies are under investigation and once authenticated by the herbarium authorities of the University of Karachi, will be reported accordingly.

Materials and Methods

Plant Material

The aerial part of *Centella asiatica* was collected from Tehsil Kahutta, District Rawalpindi, Pakistan. The sample specimen (G. H. No. 86232) was deposited in the University of Karachi Herbarium for future reference and study.

Preparation of Extract:

Test sample (4.0 g each) were transferred as coarsely powdered to 8 glass-stoppered conical flasks and 100 ml of hexane, ether, ethyl acetate, acetone, butanol, ethanol, methanol and water were added as single solvent in flasks and stoppers were inserted into the flasks. With the use of mechanical shaker, mixtures were continuously shaked during initial 8 hours and after that samples were allowed to stand for 18 hours. Samples were filtered with the help of vacuum pump assembly by taking precautions against losses. Preliminary phytochemical and fluorescence analysis of each extract was done separately.

Macroscopic examination

The macroscopic characters (such as color, odor, taste, shape and size) of fresh leaves, stem, flowers and fruit was studied as per protocols of W.H.O. 1998 [10].

Physicochemical analysis

Physicochemical tests were performed as per USP 34-NF 29 methods [11].

Qualitative Phytochemical screening

The qualitative phytochemical analysis of different solvent extracts of *Centella asiatica* was done using respective chemical tests [12-15].

Fluorescence studies

Determination of fluorescence characteristic of powdered aerial parts of *Centella asiatica* alone, and in combination with different reagents and in extract form with different solvents was determine under ordinary and UV light (254 and 366 nm) is based on protocols of Kokoski et al., 1958[16].

NIR and FT-IR Spectroscopy

NIR and FT-IR Spectrums of *Centella asiatica* obtained with the help of FOSS NIR (Rapid Content Analyzer) and Thermo Nicolet Avatar 370 DTGS Analyzer respectively.

Results and Discussion

Few pharmacognostic studies of *Centella asiatica* are already available but that are primarily based on microscopic, physic-chemical and preliminary phytochemical techniques. The identification methods with new analytical techniques like HPLC and GC are also available for *Centella asiatica* but they needed costly reference standards for the procedures [1]. Results of present studies have been interpreted as under.

Macroscopic examination

Results of the physical examination of C. asiatica (aerial parts) have been presented in Table-1. The physical description ascertains for dried C. asiatica includes color of leave as greenish gray, stem as yellowish-brown, flower as brownish yellow and fruit as brownish-gray. Leave, stem, and flower have slight aromatic odor except fruit that are odorless, however all aerial parts have slightly bitter taste. The size and shape description relating to stem, leave, fruit and flower are also recorded and reported in Table 1. C. asiatica stem as describe by USP 34-NF 29 [11] is cylindrical, yellowish to brown in color with long internodes and rooting at internodes. It further describes leave color as grayish green and fruit reported as brownish gray, orbicular cremocarp and flattened laterally with proportionate curved ridges. Earlier, Shakoor et al., 1994 [2] described color of powder drug as blackish green with slightly aromatic odor and bitter taste whereas WHO monographs[17] on selected medicinal plants depicted color of dried aerial parts as grayish green, odor as characteristic along with slightly bittersweet taste. Ling et al., 2009 [9] reported *Centella asiatica* as a creeping perennial herb with long cylindrical horizontal stolons, characterized by typical internodes. The fresh leave reported as green with fan shaped round-reniform with a crenate or dentate margin. Fresh flowers have been reported as umbles with white or slightly purple to pink petals with small oval

fruits. Results of the present study are in accordance with the previous results published by various investigators [2,9,11,17].

Table 1: Macroscopic examination of dried aerial parts of *Centella asiatica*

Aerial parts (Dried)	Test	Observations
Leaf	Color	Grayish-green
	Odor	Slightly aromatic
	Taste	Slight bitter
	Shape and size	Fan-shaped with smooth surface, no hairs, variable in size (12 to 33 mm length & 20 to 41 mm width), mostly alternative but occasionally grouped together at nodes
Stem	Color	Yellowish-brown
	Odor	Slightly aromatic
	Taste	Slightly bitter
	Shape and size	Cylindrical with nodes, nodes have rooting, diameter is 0.5 to 2 mm and internodes length is 3.5 to 8.0 cm
Flower	Color	Brownish yellow
	Odor	Slightly aromatic
	Taste	Slightly bitter
	Shape and size	Single umble of 3 to 4 flowers , each flower is 1 to 2 mm in in length
Fruit	Color	Brownish-gray
	Odor	Odorless
	Taste	Slightly bitter
	Shape and size	Cremocarp orbicular, varied in size between 3 to 5 mm

Table 2: Physicochemical evaluation of dried aerial parts of *Centella asiatica*

Test	Standard limits (% w/w)	Average values ± SD
Foreign organic matter	NMT 7.0	4.1±0.15 % w/w
Underground organs	NMT 5.0	3.2±0.21 % w/w
Other foreign matter	NMT 2.0	0.9±0.43 % w/w
Loss on drying	NMT 12.0	6.47±0.14 % w/w
Total ash	NMT 12.0	6.87±0.11 % w/w
Acid-insoluble ash	NMT 3.5	1.45±0.08 % w/w
Water-soluble ash	NA	5.25±0.13 % w/w
Alcohol soluble extractive		
Hot extraction	NA	133.97±1.89 mg/g
Cold extraction	NA	154.43±2.56 mg /g
Water soluble extractive		
Hot extraction	NA	125.47±1.83 mg/g
Cold extraction	NA	147.61±1.58 mg/g
Crude fiber	NA	8.94±0.25 % w/w
NA = Limit not available in USP 34-NF 29 ; SD=Standard Deviation ; Number of observations= 5		

Table 3: Phytochemical screening of different extracts of *Centella asiatica*

Phytoconstituents	Test	Hexane Extract	Ether Extract	Ethyl acetate Extract	Acetone Extract	Butanol Extract	Ethanol Extract	Methanol Extract	Water Extract
Alkaloids	Mayer's test	-	-	-	-	+	-	-	-
	Dragendorff's test	-	-	-	-	+	-	-	-
Protein / Amino acid	Biuret test	-	-	+	+	-	+	+	-
	Xanthoprotein test	-	-	+	+		+	+	-
Reducing sugar	Fehling's test	-	-	-	+	+	+	+	+
	Benedict's test	-	-	-	-	+	+	+	+
Saponins	Foam test	-	-	-	-	+	+	+	+
Tannins	Lead acetate test	-	-	-	+	+	+	+	+
	Nitric acid test	-	-	-	+	-	+	+	-
Triterpenoids	Liebermann-Burchard test	+	+	+	+	+	+	+	-
Starch	Iodine test	-	-	-	-	-	-	-	-
Fixed Oil	Spot test	-	-	-	-	-	-	-	-
+ denotes presence and – denotes absence									

Table 4: Fluorescence characteristic of powdered aerial parts of *Centella asiatica*

Treatments	Under ordinary light	Under U.V. light	
		254 nm	366 nm
Powder as such	Greenish gray	No change	Green
Powder + Purified Water	Greenish gray	Black	Bluish black
Powder + 10% NaOH (aqueous)	Yellowish gray	Black	Bluish black
Powder +1N NaOH (methanol)	Greenish gray	No change	Brownish green
Powder + 0.1N Iodine solution	Yellowish green	Black	Black
Powder + NH_3	Greenish gray	Black	Green
Powder + 5% $FeC1_3$ solution	Yellowish green	Black	Black
Powder + Glacial acetic acid	Greenish gray	No change	Reddish brown
Powder + Oxalic acid	Greenish gray	Black	Brown
Powder + concentrated H_2SO_4	Blackish brown	Green	Green
Powder + concentrated HNO_3	Yellowish brown	Black	Black
Powder + concentrated HCl	Greenish gray	Black	Black
Powder + H_2SO_4 with water (1:1)	Blackish brown	Black	Black
Powder + HNO_3 with water (1:1)	Yellowish brown	Black	Black
Powder + HCl with water (1:1)	Greenish gray	Black	Bluish black

Table 5: Fluorescence characteristic of aerial parts extracts of *Centella asiatica*

Extracts	Consistency	Under ordinary light	Under U.V. light	
			254 nm	366 nm
Hexane	Non sticky	Yellowish green	Black	Red
Ether	Non sticky	Brownish green	Brown	Red
Ethyl acetate	Non sticky	Reddish green	Reddish brown	Red
Acetone	Non sticky	Grayish green	Black	Dark pink
Butanol	Non sticky	Blackish green	Bluish brown	Pink
Ethanol	Non sticky	Brownish green	Pinkish brown	Pink
Methanol	Non sticky	Reddish green	Black	Pink
Water	Non sticky	Turbid greenish yellow	Black	Blue

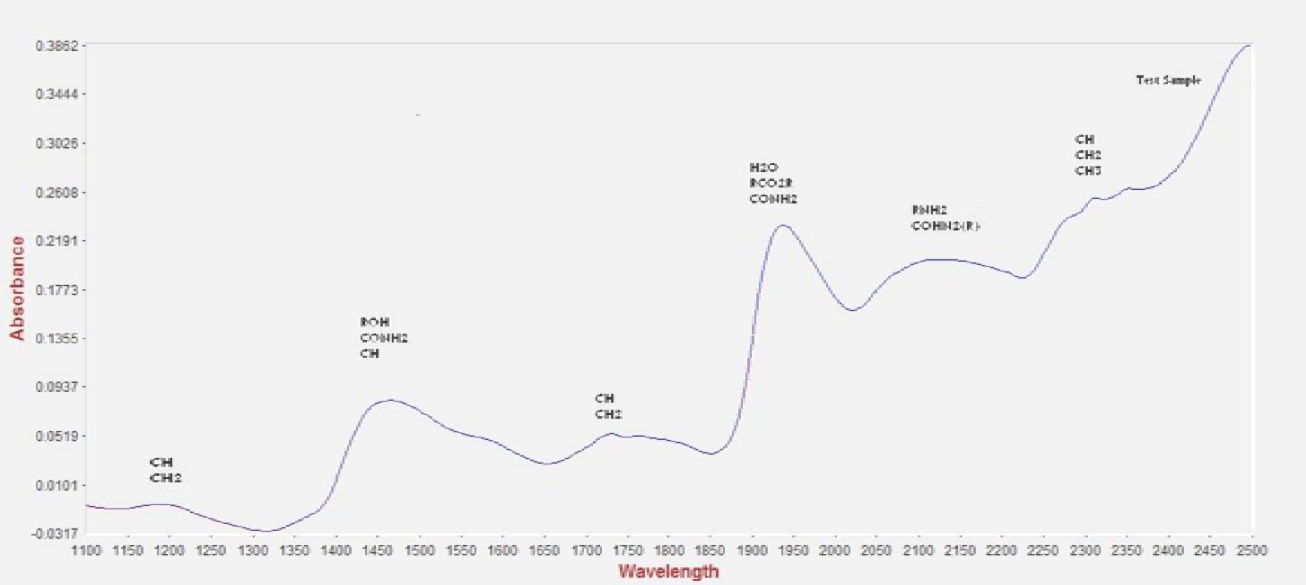


Figure 1: NIR spectrum of the powdered aerial parts of *Centella asiatica*

Physicochemical evaluation

Table 2, showing physicochemical analysis results, include various tests such as foreign organic matter (underground organs = 3.2% w/w and other foreign matter = 0.9% w/w), loss on drying = 6.47% w/w, total ash = 6.87% w/w, acid-insoluble ash = 1.45% w/w, water-soluble ash = 5.25% w/w, alcohol soluble extractive (hot extraction = 133.97 mg/g and cold extraction = 154.43 mg/g), water-soluble extractive (hot extraction 125.47 mg/g and cold extraction = 147.61 mg/g) and crude fiber = 8.94 % w/w. The results of the sample are found in accordance with the standard limits given in USP 34-NF 29 [11] that is quite promising because higher results of foreign organic matter in sample have the ability to reduce the therapeutic efficacy of the recommended dose and it may produce undesired side effect due to the presence of adulterated materials. The samples with less LOD limit mostly have good shelf life because high content of water have the ability to support microbial growth along chemical deterioration in sample materials. The total ash and acid insoluble ash results of our sample supports that sample is minimum polluted with inorganic compounds and earthy substance. Standard limit for water-soluble ash, alcohol soluble extractive, water soluble extractive and crude fiber are not available and due to this result are reported for information only.

Qualitative Phytochemical screening

The qualitative chemical examination results have been presented in Table 3. This includes Mayer's test (showing white creamy precipitate for the presence of alkaloid), Dragendorff's test (showing orange brown precipitate for the presence of alkaloid), Biuret test (showing violet or pink color for the presence of Protein / Free amino acid), Xanthoprotein test (showing deep orange color for the presence of free amino acid/protein), Fehling's test (showing first yellow followed by brick red precipitate for the presence of reducing sugar), Benedict's test (showing yellowish precipitate for the presence of reducing sugar), foam test (showing persistent foam for the presence of saponins), lead acetate test (showing yellowish white precipitate for the presence of tannin), Nitric acid test (showing reddish to yellow color for the presence of tannin), Liebermann-Burchard test (showing reddish brown color for the presence of triterpenoids), iodine test (showing blue color due to the presence of starch) and spot test (translucent/waxy spot for the presence of fixed oil). The qualitative analysis result supports the previous reported constituents of C. asiatica while majority of tests were found positive in methanolic and ethanolic extracts.

Fluorescence studies

The fluorescence analysis of the powdered aerial part of *Centella asiatica* indicated no difference between the test and reference sample, when observed under visible and UV light at 254 and 366 nm (Table 4). Under visible light the powdered drug was noted as greenish gray and no difference in color was noted when purified water or 1N sodium hydroxide in methanol or ammonia or glacial acetic acid or oxalic acid or hydrochloric acid (concentrated and diluted) were added to powder drug. Similarly when powdered drug was treated with 0.1N iodine solution or 5% $FeCl_3$ solution a yellowish green color was observed in both test and reference samples.

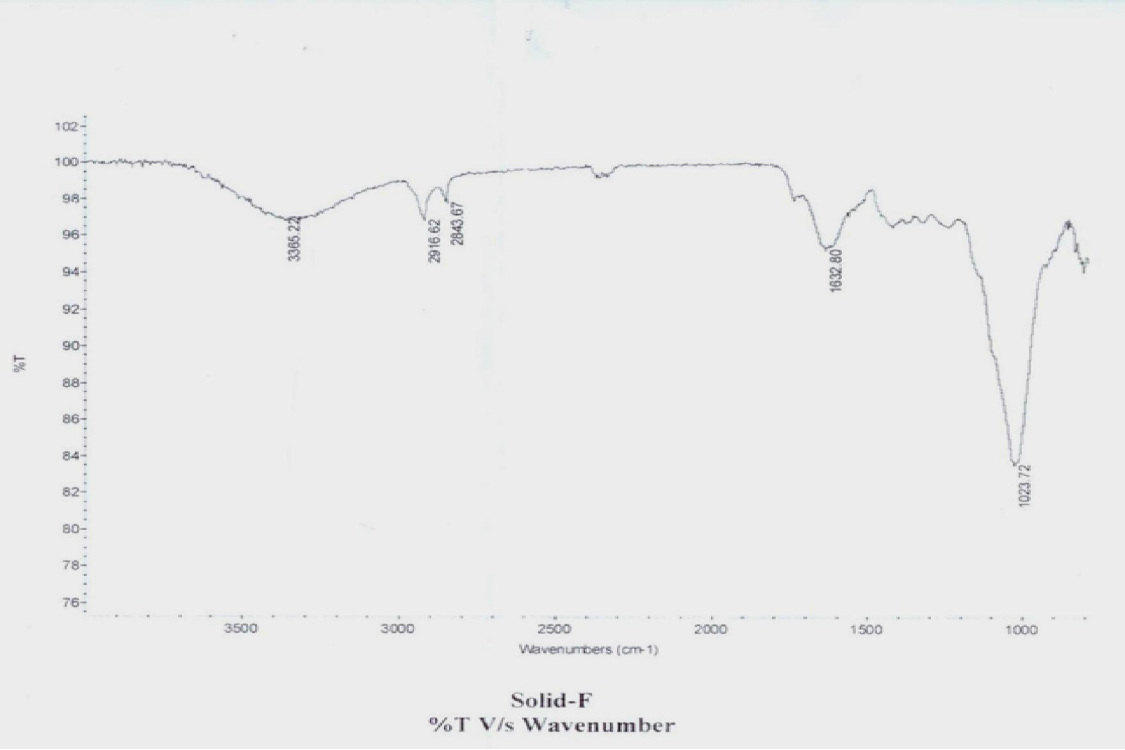
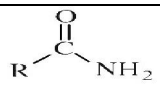


Figure 2: FT-IR spectrum of the powdered aerial parts of *Centella asiatica*

Table 6: NIR spectrum of the powdered aerial parts of *Centella asiatica*

Peak's wavelength (nm)	Possible functional group	Compound that may be responsible for peak
1200	CH and CH_2	Aspartic acid, arginine, histidine, glutamic acid, sitosterol, stigmasterol, tyrosine and others
1460	CH, $CONH_2$ and ROH	Aspartic acid, arginine, histidine, glutamic acid, Sitosterol, stigmasterol, astragalin, tyrosine, ascorbic acid and others
1725	CH and CH_2	Aspartic acid, arginine, histidine, glutamic acid, sitosterol, stigmasterol, tyrosine and others
1940	$CONH_2$, RCO_2R and H_2O	Asiaticoside, madecassosid, asiaticin, centellicin, water and others
2125	$CONH_2(R)$ and RNH_2	Tyrosine, aspartic acid, arginine, histidine, glutamic acid and others
2300	CH , CH_2 and CH_3	Aspartic acid, arginine, histidine, glutamic acid, sitosterol, stigmasterol, tyrosine and others
2350	CH , CH_2 and CH_3	Aspartic acid, arginine, histidine, glutamic acid , Sitosterol, stigmasterol, tyrosine, centellicin and others

Table 7: FT-IR spectrum of the powdered aerial parts of *Centella asiatica*.

Peak's wavelength (cm^{-1})	Possible Functional group	Compound that may be responsible for peak
3365.2	OH and COOH	Tyrosine, astragalin, kaempferol, sitosterol, ascorbic acid, stigmasterol, arginine, glutamic acid, aspartic acid, water and others
2916.6	CH	Glutamic acid, arginine, histidine, sitosterol, stigmasterol, tyrosine and others
2843.6	CH	Glutamic acid, arginine, histidine, sitosterol, stigmasterol, tyrosine and others
1632.80	C=C, 	Centellicin, stigmasterol and others
1023.7	CO	Centellicin, asiaticin, tyrosine, arginine, glutamic acid, aspartic acid and other

Powder drug treated with sulfuric acid (concentrated and diluted) and nitric acid (concentrated and diluted) was developed blackish brown and yellowish brown color respectively in both test and reference samples, while 10% NaOH solution with powder drug gives a yellowish gray color in both test and reference samples. While under UV light (254 and 366 nm) the powder drug shows no change and green color; powder drug with purified water; powder drug in diluted HCl with water (1:1) indicated black and bluish black; powder drug with 10% sodium hydroxide solution indicated black and bluish black; powder drug with 1N sodium hydroxide in methanol showed no change and brownish green; powder drug with 0.1N iodine solution, powder drug with 5% $FeCl_3$ solution, powder drug with concentrated HNO_3, powder drug with concentrated HCl, powder drug with diluted H_2SO_4 with water (1:1), powder drug with diluted HNO_3 with water (1:1) shows black color at both wavelengths; powder drug with NH_3 indicated black and green; powder drug with glacial acetic acid showed no change and reddish brown; powder drug with oxalic acid shows black and brown; powder drug with concentrated H_2SO_4 shows green color on both wavelength.

The fluorescence analysis was also performed on various extract such as hexane, ether, ethyl acetate, acetone, butanol, ethanol, methanol and aqueous (Table 5). Turbid greenish yellow color of aqueous extract was observed under visible light and black & blue color under UV light at 254 and 366 nm respectively. Similarly with methanol and ethyl acetate extracts, reddish green color was noted under visible light while black and pink under UV light at 254 and 366 nm for methanol extract and reddish brown and red under UV light at 254 and 366 nm for ethyl acetate extract. A brownish green color was noted for ethanol and ether extracts under visible light while pinkish brown and pink under UV light at 254 and 366 nm for ethanol and brown and red under UV light at 254 and 366 nm for ether extract. The hexane, acetone and butanol extracts shown quite distinct color development under visible light that is yellowish green, grayish green and blackish green respectively. While under UV light at 254 and 366 nm indicated black and red, black and dark pink, Bluish brown and pink respectively for hexane, acetone and butanol extracts. The fluorescence study help us for identification of herbal drugs when they become powdered mostly due to the

over stacking in less storage area or due to improper handling of dried herbs.

NIR and FT-IR Spectroscopy

Two new identification methods (NIR and FT-IR) were used first time during this study for *Centella asiatica*. NIR and FT-IR techniques may be regarded as highly reliable and rapid techniques that will help herbal user for proper identification of material and will reduce chances of adulteration as well.

Figure1 shows the NIR spectrum of *Centella asiatica*, while Table-6 shows the brief explanation of the spectrum. In spectrum peaks appeared at 1200 nm, 1725 nm, 2300 nm and 2350 nm indicated the presence of CH group, where as the peak at 1460 nm shows the possible presence of CH, $CONH_2$ and ROH groups. The peak of 1940 nm shows the possibility of $CONH_2$, RCO_2R and H_2O presence; while peak of 2125 nm may be due to the presence of $CONH_2(R)$ or RNH_2 groups in sample molecular structure. Figure-2 shows FT-IR spectrum along with brief explanation in Table-7. Peak appeared at 3365.2 cm^{-1} shows the presence of OH and COOH functional groups. Two peaks at 2916.6 cm^{-1} and 2843.6 cm^{-1} shows the presence of CH stretching. These peaks also show the presence of a number of hydrocarbons. The peak at 1632.80 cm^{-1} shows the presence of alkene and amide functional groups. These is a characteristic peaks at 1023.7 cm^{-1} due to C-O stretching. In conclusion, the presence of various compound / constituents that may be responsible for various peaks of NIR and FT-IR have been depicted in Table – 6 and 7. The minima and maxima in the NIR and FT-IR spectrums will remain constant for *Centella asiatica* and on the basis of this we will get an easy and rapid identification of the sample.

Conclusions

Present study revealed quite promising and interesting results and finding relating to NIR and FT-IR along with the other physico-chemical parameters used to investigate and identify *Centella asiatica* for manufacturers and researchers, who further interested to study *Centella asiatica* as a drug for different activity. The present pharmacognostic and preliminary phytochemical investigation was under taken with a view to lay down standards which could be helpful for standardization and sample identification of *Centella asiatica* Linn., aerial parts.

List of Abbreviations

%: Percent
CH: Hydrocarcarbon
CNS: Central Nervous System
CO: Carbonyl group
COOH: Carboxylic
DTGS: Deuterated triglycine sulfate
$FeCl_3$: Ferric Chloride
FT-IR: Fourier transform infrared spectroscopy
g: Gram
H_2SO_4: Sulphuric acid
HCl: Hydrochloric acid
HNO_3: Nitric acid
LOD: Loss on Drying
ml: Milli litre
N: Normal
NaOH: Sodium hydroxide
NF: National Formulary
NH_3: Ammonia
NIR: Near Infra red
OH: Hydroxyl
USP: United States Pharmacopoeia
UV: Ultra violet
W.H.O: World Health Organization

Author's contributions:

Rafi Akhtar Sultan is the Ph.D research fellow responsible for experimental work and writing manuscript, Iqbal Azhar is the research supervisor and assisted experimental work & studies, Zafar Alam Mahmood is the co-supervisor and research coordinator of the project. Advised and edited manuscript. Muhammad Mohtasheem ul Hasan coordinated with R. Sultan in NIR and FT-IR Spectroscopy. Salman Ahmed contributed by providing assistance in various experimental work to R. Sultan.

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Conflict of interest:

No

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