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Phytochemical Screening of Micropropagated *Centella asiatica* L.

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Abstract

The objective of the present study was to find out the presence of phytochemicals in the ethanol, methanol, chloroform extracts of different parts [leaf, stem, root] of micropropagated *Centella asiatica* L. In vitro propagation of *C. asiatica* using nodal explant. Nodal explants inoculated on MS medium supplemented with various concentrations of BA 1.8, 2.0 mg/L, with combination of 1 mg/L each IBA and IAA gives maximum growth. Micropropagated *Centella asiatica L.* was analyzed by qualitative screening methods. In qualitative analysis, the phytochemical compounds such as steroids, reducing sugars, triterpenoids, sugars, alkaloids, phenolic compounds, flavonoids, saponins, tannins, alkaloids, amino acids, etc. were screened by using standard methods. The methanol extract of the each part showed positive results for most phytochemical tests while chloroform extracts of different parts showed less phytochemical. The generated data from the different extracts of *Centella asiatica* L., provided the basis for its wide uses in the traditional & folk medicines.

Keywords: *Centella asiatica* L., Phytochemicals, Qualitative, In Vitro Propagation.

Introduction

Centella asiatica L. is a small herbaceous plant belonging to Apiaceae (Umbelliferae) family. Centella comprises some 50 species[1], inhabiting tropical and sub-tropical regions. This perennial creeper flourishes abundantly in moist areas and is in the subfamily Mackinlaya[2], previously included in hydrocotyle occurring in swampy areas of India, Sri Lanka, Madagascar, Africa, and Australia[3]. *Centella asiatica* L. known as Brahmi, Indian Pennywort and Mandookaparni is a small herbaceous annual plant of the family Apiaceae. *Centella asiatica* L. L. has been used as a medicinal herb for thousands of years in India, China, Sri Lanka, Nepal and Madagascar. *Centella asiatica* L. is one of the chief herbs for treating skin problems, healing wounds, revitalizing nerves and brain cells, hence it is primarily known as a "Brain food" in India[4]. *Centella asiatica* L. has been used for wound healing, better blood circulation, memory enhancement, anti-carcinogenic, Apoptosis Induction of *Centella asiatica* L. on Human Breast Cancer Cells[5] and also has been used for respiratory ailments, detoxifying the body, treatment of skin disorders (such as psoriasis and eczema), revitalizing connective tissue, burn and scar treatment, clearing up skin infections, slimming and edema, arthritis, rheumatism, treatment of liver and kidneys, periodontal disease, strengthening of veins (varicose veins), blood purifier, high blood pressure, sedative, anti-stress, anti-anxiety, an aphrodisiac, immune booster, anabolic etc.[6] Phytochemicals are the natural bioactive compounds found in plants. These phytochemicals work with nutrients and fibers to form an integrated part of defense system against various diseases and stress conditions[7]. The most important of these bioactive constituents of plants are alkaloids, tannins, flavonoids, steroid, terpenoids, carbohydrate and phenolic compounds[8]. The medicinal value of these plants lies in some chemical substances that have definite physiological processes in the human body. The most important of these bioactive constituents of plants are alkaloids, terpenoids, carbohydrates and proteins compounds.[9].The primary constituents of *C. asiatica* is the triterpenic fractions which showed wide range of defensive and therapeutic effects, most prominently influencing of collagen production and deposition in wound healing. Titrated Extract of *Centella asiatica* L. (TECA) is used to treat several microcirculatory problems.[10].

Efforts to produce large quantities of active secondary compounds by plant tissue culture techniques have been developed for the rapid, large scale production of cells and their secondary compounds[11]. Therefore, the present study has been carried out to evaluate the preliminary phytochemical screening of bioactive compounds and its quantification of in vitro developed *C. asiatica.*

Material and Methods

Collection of Plant Material

The fresh parts of *Centella asiatica* (L.) Urb. were collected in flowering period from Amrutkund Tq. Basavkalyan, Dist. Bidar near Maharashtra-Karnataka border. The plant material were properly washed with tap water and then rinsed with distilled water. The nodal segment of *Centella asiatica* (L.) was used as explant for in vitro propagation.

Surface Sterilization

The explants were washed under running tap water for 15 min to remove the surface contaminants and soil particles and immersed in detergent (laboline) for 5 min and rinsed with distilled water for four times. The explants were deeped in 70% ethanol for 1 min. Then the explants were soaked in 0.1% (w/v) mercuric chloride solution for 1-2 min and thoroughly rinsed with sterile distilled water for four times. The explants were cultured on Murashige and Skoog basal medium supplemented with different concentrations of plant growth regulators.

Culture Media and Culture Conditions

The surface sterilized explants were inoculated on Murashige and Skoog[12] basal medium containing 30gm/L sucrose, and 1.5gm/L clerigel. The pH of all media was adjusted to 5.8 prior to autoclaving at 121^0C for 20 min. MS media (Murashige and Skoog, 1962) supplemented with different concentration of phyto-hormones like, BA (Benzyl Adenine), IBA (Indol -3-butyric acid) and IAA (indol -3-acetic acid). Nodal explants inoculated on MS medium supplemented with various concentrations of BA 1.8, 2.0 mg/L, with combination of 1 mg/L each IBA and IAA gives maximum growth.

Extraction

Preparation of Ethanolic or Methanolic or Chloroform Extracts

For preliminary phytochemical analysis, extract was prepared by weighing the dried and powdered material of leaf, stem & root of micropropagated plant. For preparation of ethanolic or methanolic or chloroform extracts, a modified method of **Abdulrahman et.al (2004)**[13] was used. 5 gram of each leaf, stem and root powder were then macerated in 50 ml of absolute ethanol or methanol or chloroform for 72 hrs. & properly covered with aluminium foil & labeled. After 72 hrs of extraction, each extract was filtered through Whatman's filter paper no.1. The filtrate was evaporated to dryness at room temperature & store at 5^0C in refrigerator.

Qualitative Analysis

Extracts were tested for the presence of active principles. Following standard procedures were used. [14, 15]

Test for Alkaloids

Ethanolic extract was warmed with 2% H_2SO_4 for two minutes. It is filtered and few drops of reagents were added and indicated the presence of alkaloids.

1. **Mayer's Reagent**- A creamy- white colored precipitation positive.
2. **Wagner's Reagent**-A reddish-brown precipitation positive.
3. **Picric Acid (1%)**- A yellow precipitation positive.

Test for Steroids Terpenoid and Triterpenoids

a) Liebermann Burchard Test

Crude extract was mixed with few drops of acetic anhydride, boiled and cooled. Concentrated sulphuric acid was then added from the sides of the test tube and observed for the formation of a brown ring at the junction of two layers. Green coloration of the upper layer and the formation of deep red color in the lower layer would indicate a positive test for steroids terpenoid and triterpenoids respectively.

b) Salkowski Test

The extract was mixed with 2ml of chloroform and concentrate H_2SO_4 (3ml) is carefully added to form a layer. A reddish brown coloration of the interface is formed to show positive result of the presence of steroids terpenoid and triterpenoids respectively.

Test for Saponins

Crude extract was mixed with 5ml of distilled water in a test tube and it was shaken vigorously. The formation of stable foam was taken as an indication for the presence of saponins.

Test for Phenols and Tannins

Crude extract was mixed with 2ml of 2% solution of $FeCl_3$. A blue-green or black coloration indicated the presence of phenols and tannins.

Test for Flavonoids

A small quantity of the extracts is heated with 10 ml of ethyl acetate in boiling water for 3 minutes. The mixture is filtered differently and the filtrates are used for the following test.

a) Ammonium Test

The filtrate was shaken with 1 ml of dilute ammonia solution (1%). The layers were allowed to separate. A yellow coloration was observed at ammonia layer. This indicates the presence of the flavonoid.

b) Aluminum Chloride Test

The filtrates were shaken with 1 ml of 1% aluminum chloride solution and observed for light yellow color. It indicated the presence of flavonoids and diluted NaOH and HCl was added. A yellow solution that turns colorless indicated positive.

Test for Carbohydrate

Benedict's Test

Test solution was mixed with few drops of Benedict's reagent (alkaline solution containing cupric citrate complex) and boiled in water bath, observed for the formation of reddish brown precipitate to show a positive result for the presence of carbohydrate.

Test for Glycosides

Fehling's Test

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

Test for Proteins

Millon's Test

Crude extract when mixed with 2ml of Millon's reagent, white precipitate appeared which turned red upon gentle heating that confirmed the presence of protein.

Test for Free Amino Acids

Ninhydrin Test

Test solution when boiled with 0.2% solution of Ninhydrin, would result in the formation of purple color suggesting the presence of free amino acids.

Test for Vitamin C

DNPH Test

Test solution was treated with Dinitrophenylhydrazine dissolved in concentrated sulphuric acid. The formation of yellow precipitate would suggest the presence of vitamin C.

For Carboxylic acid, test for NH_2, Nitrogen, Sulphur, Halogen, Amides, test for Unsaturation, test for Aromaticity.

Result and Discussion

Nodal explants inoculated on MS medium supplemented with various concentrations of BA 1.8, 2.0 mg/L, with combination of 1 mg/L each IBA and IAA gives average percentage of shoot multiplication. Highest shoot multiplication is observed 2.0 mg/L BA in combination of 1 mg/L IBA & IAA. However, root initiation was achieved from the bases of excised shoots in the presence of IAA or NAA. The 1.5 mg/L IAA produced maximum number of roots after 2-3 weeks incubation with basal callusing.

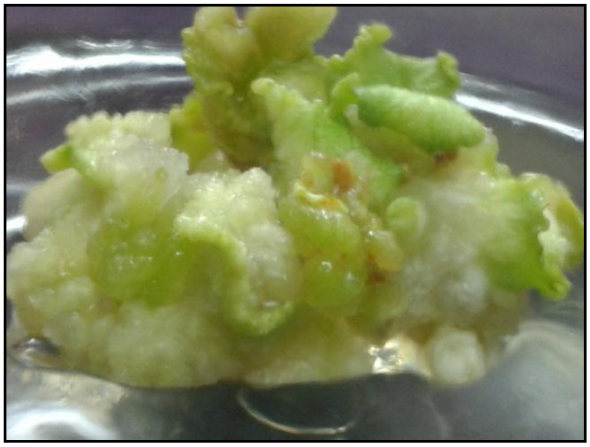

Fig: A

Fig: B

Fig:C

Fig: D

Fig:E

Fig: F

Fig:G

Figs A-G A.Callus formation B. initiation of shoot from the explant. B. multiplication of shoots. C&D. multiplication of shoot and basal callus formation. And intiation of roots E. initiation of roots from regenerated shoots. F. induction of rooting and basal callus formation G. multiplication of roots.

Phytochemical screening is of paramount importance in identifying new source of therapeutically and industrially valuable compound having medicinal significance, to make the best and judicious use of available natural wealth.

The phytochemical analysis of in vitro developed *Centella asiatica L.* tested is summarized in Table.1.Which revealed that presence of medicinally active compound in plant Leaf, stem and roots. For extraction of phytochemicals, ethanolic, methanolic and chloroform extracts were used.

Phytochemical compounds such as steroids, reducing sugars, triterpenoids, sugars, alkaloids, phenolic compounds, flavonoids, saponins, tannins, amino acids, etc were screened in different extracts of *Centella asiatica L.* Among these compounds alkaloids, phenolic compounds, flavonoids and tannins are important secondary metabolites and are responsible principles for medicinal values of the respective plant.

Table 1

Preliminary (Qualitative) Phytochemical Analysis of Different Extracts of *Centella asiatica*

Sr. No	Compound	Test	Plant part								
			Leaf extract			Stem extract			Root extract		
			E	M	C	E	M	C	E	M	C
1.	Alkaloid	Mayer's reagent	++	+	+	+	++	+	+	−	+
		Wagner's Reagent	++	++	+	++	+	−	+	+	−
		Picric acid	++	+	−	++	+	−	+	+	−
2.	Amides	Hydrolysis with alkali	+	+	+	+	+	+	+	+	+
3.	Amines	Amines test	+	+	+	+	+	+	+	+	+
4.	Ascorbic acid	DNPH test	++	++	++	+	+++	+	+	+++	+
5.	Carbohydrates	Benedict test	++	+	+	+	++	+	+	−	−
6.	Carboxylic acid	Sodium bicarbonate test	+	+	+	+	+	+	+	+	+
7.	Flavonoids	Ammonium Test	++	+++	+	+ +	+++	++	++	++	+
		Aluminum chloride test	++	++	++	+	++	+	++	+	+
8.	Glycosides	Fehling solution	++	+++	++	++	+++	++	+	+++	++
9.	Phenol	Ferric chloride test	++	+++	++	++	+	++	++	+	+
10.	Proteins	Millons Reagent test	+	++	+	++	+	+	+	++	−
11.	Reducing Sugar	Fehling solution test	+	++	+	++	+	++	++	+	+
12.	Saponin	Frothing test	+	+	−	+	+	+	+	−	−
13.	Starch	Starch test	+	+	+	+	+	+	+	+	+
14.	Steroids	Liebermann - Burchard's test	++	+++	++	+++	++	+	++	++	+
		Salkowski's Test:	+	+++	++	++	++	++	++	++	+
15.	Tannin	Ferric chloride test	+++	++	+	+ +	++	+	++	++	−
16.	Terpenoides and triterpenoids	Liebermann - Burchard's test	++	++	++	+	+++	++	+	++	++
		Salkowski's Test:	+	+++	++	+	+++	++	+	++	++
17.	Amino acid	Ninhydrin Reagent test	+	+	+	+	+	+	++	+	−

Note- − =absent, + =Presence, ++ = Moderate, +++ = Maximum

Conclusion

In the present study leaf, stem and root showed the presence of bioactive compound such as alkaloids, flavonoids, terpenoids, saponins, etc. This study also leads to the further research in the way of isolation and identification of the active compound from the leaf, stem and root of *Centella asiatica L. L.* using chromatographic and spectroscopic techniques.

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