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Research Paper

Synthesis of silver nanoparticles from *Centella asiatica*(L.) plant and *in vitro* derived callus culture: Assessment of antibacterial activity

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ABSTRACT

Callus raised from the leaf explants of *Centella asiatica*(L) was evaluated in the present study. The plant is pharmacologically very important and its consumption as underutilized green leafy vegetable affluent in micronutrients is communally conscientious for its threatened status. Therefore, there is an importunate need to preserve its germplasm so that pharmacologically active constituent can be made available all over the year without causing loss of species. Maximum callusing was observed in MS + benzylaminopurine (BAP, 0.5 mg/l) + α-napthalene acetic acid (NAA, 0.3 mg/l) in leaf explants with callus induction frequency of 75%. The callus and leaf parts of *in vivo* plant were used for qualitative primary metabolites in its methanolic extracts. This study evaluates the significance of antibacterial activity of silver nanoparticles synthesized from plants extracts and *in vitro* derived callus extracts of *C. asiatica*(L.) and characterized by UV spectroscopy, FTIR analysis. Antibacterial activity was evaluated using the well diffusion assay, and zone of inhibition of synthesized silver nanoparticles which were tested against the gram-positive bacteria and the gram-negative bacteria.

Key words: *Centella asiatica*(L), callus culture, primary metabolites, $AgNO_3$, antibacterial activity, AgNPs (silver nanoparticles).

INTRODUCTION

Plants have been used as treatments for thousands of years all over the world based on folk remedies and continue to draw wide attention for their role in the treatment of diseases. At present, interest on plant research has increased and evidence has been accumulated to highlight the immense potential of medicinal plants used in various traditional systems of medicine (Gohil et al., 2010). India is one of the mega diversity countries of the world with a rich diversity of biotic resources and has two major hotspots, namely the Eastern Himalayas and the Western Ghats. India harbours about 47, 000 species of plants, 17,000 of which are angiosperms (Kapai et al., 2010). The discovery of medicinal plants has usually depended on the experience of the users based on long and dangerous self-experimentation(Chhetri et al., 2008). Biosynthesis of nanoparticles have received considerable attention due to the growing need to develop cost effective, clean, easily affordable, nontoxic chemicals, environmental friendly biodegradable solvents and renewable materials (Harris and Bali, 2008) (Gericke and Pinches, 2006). As a result, bio-nano researchers in the field of nanoparticle biosynthesis have turned towards biological system, such as plant extracts, bacteria, yeast and fungi for the synthesis of biocompatible nanoparticles without need of toxic chemicals(Kasthuri et al., 2009; Lee and Park, 2011; Shankar et al., 2003a). Biosynthesis of silver nanoparticles using a plant extracts is a useful technology in research with many advantages over traditional methods of nanoparticles synthesis and also has many practical applications in the modern medicine(Malabadi et al., 2012a; Xia et al., 2010). Silver nanoparticles synthesized using plant extracts are highly toxic to microorganisms, showing good antimicrobial activity and exhibits important role in drug delivery (Chopade et al., 2012; Malabadi et al., 2012b;

Rao and Savithramma, 2013; Shankar et al., 2003b; Shekhawat et al., 2012; Song and Kim, 2009). Synthesis of silver nanoparticles has been reported using several plant species including tissue culture-derived callus and leaf of the *Sesuvium portulacastrum* (L.) (Ankamwar et al., 2005; Bar et al., 2009; MubarakAli et al., 2011; Nabikhan et al., 2010; Song and Kim, 2009).

Centella, belonging to the Apiaceae family, is a diverse genus encompassing approximately 50 species including the medicinally eminent plant *Centella asiatica*(L.), which is the only asiatica species that are put into commercial use today. The areal parts of the plant are used for medicinal purposes (James and Dubery, 2009; Zainol et al., 2008). *Centella* grows in tropical swampy areas along ditches and in low, wet areas. In India, elevation reaches 600 to 1800 m above the sea level (Patra et al., 1998). The stems are creeping, green to reddish-green in color, connecting plants to each other having long-stalked, green leaves with rounded apices which have smooth texture. The roots grow vertically down and they are creamish in color and covered with root hairs (Das, 2011). They are commonly known as Asmandukparni or Asiatic pennywort or Indian pennywort or Jalbrahmiin Europe, Gotukola in America and Tung Chain in China, and are used as a medicine in the Ayurvedic tradition of India for thousands of years (Schaneberg et al., 2003). Phytochemicals are primary and secondary metabolites found in plants and work with nutrients and fibers to form an integrated part of defense system against various diseases and stress conditions. The most important of these bioactive constituents of plants are alkaloids, tannins, flavonoids, steroid, terpenoid, carbohydrate and phenolic compounds (James and Dubery, 2009; Pascaline et al., 2011; Singh et al., 2016).The aqueous extract of Asiatic pennywort exhibited the strongest antimicrobial activity(Rattanachaikunsopon and Phumkhachorn, 2010). *C. asiatica* are used in medicine due to their wide spectrum of pharmacological activities associated with the secondary metabolites, such as anticellulite agent (Sondari et al., 2011). A novel triterpene, 2α,3β,20,23-tetrahydroxyurs-28-oic acid (1), was isolated from the aerial part of *C. asiatica* (Yu et al., 2006). *C. asiatica* methanolic extract has the ability to induce apoptosis in different cancer cell lines. MCF-7 cells emerged as the most sensitive cell line for *in vitro* growth inhibitory activity (Babykutty et al., 2009). A triterpenoid, asiatic acid isolated from *C. asiatica*, has shown biological effects, such as antioxidant and anti-inflammatory (Krishnamurthy et al., 2009). As compared with conventional mass, multiplication method tissue culture tool is considered expensive when it comes to commercial production needs. In present study, callus was raised from leaf explants of *C. asiatica* and primary metabolites of callus. Also, plant parts were compared and the antibacterial activity of silver nanoparticles synthesized from plants extracts and *in vitro* derived callus extracts of *C. asiatica*(L.) was evaluated. This evaluation was done using the well diffusion assay and zone of inhibition of synthesized silver nanoparticles.

The extracts were tested against the gram-positive bacteria *Bacillus subtilis* and *Staphylococcus aureus*, and the gram-negative bacteria *Escherichia coli* and *Klebsiella pneumoniae.*

MATERIALS AND METHODS

Plant material and chemicals

Healthy plants of *C. asiatica*(L.) were collected from herbal garden of Shoolini University, Bhajol, Solan, Himachal Pradesh (India). All the chemicals and growth regulators used were of analytical grade and purchased from HiMedia Pvt. Ltd. Mumbai, India.

Callus induction

The leaves were washed with Tween-20 for 30 min and then surface sterilized by 0.1% Bavistin for 2 h, followed by 0.1% $HgCl_2$ for 10 min and then rinsed thoroughly with distilled water. Thereafter, explants were inoculated in the MS medium (Murashige and Skoog, 1962) supplemented with different concentrations of NAA and BAP. The pH of the media was adjusted to 5.8 using 0.1 N HCL or 0.1 N NaOH solutions before autoclaving at 121ºC for 15 min. The cultures were incubated in growth room at temperature of 25±2°C and 60±10% relative humidity for 16-h photoperiod. All experiment was repeated twice and performed with 20 replicate cultures and the cultures were observed at regular intervals of period.

Extraction

Healthy leaves of *C. asiatica* were properly washed with tap water and then rinsed with distilled water to remove dust. The rinsed leaves were shed dried and crushed to obtain powder. A quantity of 50 g of the dried powder of plant leaves and callus were separately extracted with 95% methanol (Merck) using maceration process and kept for one week to obtain extract. Then, the extract was kept in water bath at 50°C to obtain crude extracts for phytochemical analysis.

Test for qualitative phytochemical analysis

Test for alkaloids

Extract was warmed with H_2SO_4 for two minutes and filtered in the filtrate. Few drops of Dragendroff's reagents were added and red precipitation indicated the presence of alkaloids.

Test for flavonoids

Some amount of the extracts was heated with 10 ml of ethyl acetate in boiling water for 2-3 min. The mixture was filtered, and the filtrate was shaken with 1 ml of dilute ammonia solution and yellow coloration was observed at ammonia layer which indicates the presence of the flavonoid.

Test for terpenoids

The extract was mixed with 2 ml of chloroform, and 3 ml of concentrated H_2SO_4 was carefully added to form a layer. At the interface, reddish brown coloration was formed to show positive result of the presence of terpenoids.

Test for steroids

Extract was warmed with 2 ml of H_2SO_4 for two minutes and 2 ml of acetic anhydride was added to the extract. Color change from violet to blue or green indicated the presence of steroids.

Test for saponins

Small quantity of extract was diluted with 5 ml of distilled water. The mixture was shaken vigorously and then observed on standing for stable forth for at least 10 min. The disappearance of color indicated the presence of saponins.

Test for tannins

Small quantity of the extract was boiled with 5 ml of 50% ethanol for 5 min. Each of the mixture was cooled and filtered. In 1 ml of the filtrate, three drops of lead sub acetate solution were added. A cream gelatinous precipitation indicates presence of Tannins.

Test for glycosides

Extract was warmed with 5 ml of diluted H_2SO_4 for fifteen minutes in a water bath and then cooled and neutralized with potassium hydroxides solution. 10 ml mixture of Fehling's solution A and B were added and boiled for five minutes. Dense red precipitate indicates the presence of glycosides.

Test for carbohydrates

Water was added to the extract and shaken vigorously and then filtered. Thereafter, few drops of Molisch's reagents were added to the aqueous filtrate, followed by vigorous shaking again. 1 ml concentrated H_2SO_4 was carefully added to form a layer at the bottom. A brown ring formed at the interface indicates the presence of carbohydrates.

Test for proteins

To the extract, 5 ml water was added and left to stand for three hours and then filtered. To 2 ml portion of the filtrate, 0.1 ml Millon's reagent was added and shaken vigorously and kept for observation. A yellow precipitation indicates the presence of proteins.

Synthesis of silver nanoparticles

The procedure for the preparation of silver nanoparticles has been adopted with slight modifications (Gardea-Torresdey et al., 2003; Rao and Savithramma, 2012). The callus and leaf material of *C. asiatica* were oven dried at 50°C and grind to make a fine powder. Further, 20 g of powdered callus and leaf were taken separately in 250 ml beaker and 100 ml of sterile distilled water was added and then boiled for 10 min at 100°C. The extracts were filtered using whatman filter paper and collected separately in two beakers.10 ml of callus and leaf extracts were taken in beaker separately and 90 ml of 1 mM $AgNO_3$ solution was added to the beakers drop wise with constant stirring at 60°C. Color change was observed indicating the formation of nanoparticles. The silver ions reduced to silver nanoparticles (AgNPs) within few minutes by metabolites present in the leaf, and callus extracts were regularly monitored through UV-vis spectrophotometer.

Characterization of phytosynthesized silver nanoparticles

UV-vis spectra of synthesized silver nanoparticles were recorded as a function of time at 5 min, 15 min, 30 min, 1 h and 24 h at different wavelength (350- 700 nm) operated at a resolution of 1 nm [Model-2202 SYSTRONICS]. The functional groups in the synthesized silver nanoparticles were analyzed by FTIR using spectral range of 600-4000 cm^{-1}(Pike Technologies, Gladi ATR for FTIR with diamond crystal).

Antibacterial screening

The antibacterial activities of silver nanoparticles were studied by agar well diffusion method against pathogenic gram-positive bacteria, such as *B. subtilis, S. aureus* and gram-negative bacteria *Pseudomonas aeruginosa, E. coli*. Bacterial strains grown on nutrient agar at 37°C for 18 h were suspended in a saline solution (0.85% NaCl) and

Table 1: Effect of different concentrations of growth regulators for callus induction from leaf explants.

Growth regulators concentration	Callus induction days	Callus induction factors%	Fresh weight of callus	Color of callus	Growth of callus
MS control	-	-	-	-	**
BAP (0.3) + NAA (0.5)	16	60.33 ± 0.36	0.843	Green	++
BAP (0.5) + NAA (0.3)	14	72 ± 0.22	1.152	Green	+++
BAP (0.5) + NAA (1.0)	25	64.66 ± 0.31	0.634	Green	++
BAP (1.0) + NAA (0.5)	18	60 ± 0.00	0.668	Green	++
BAP (1.0) + NAA (1.0)	26	70 ± 0.42	0.849	Green	+++
BAP (1.5) + NAA (2.0)	19	30.33 ± 0.12	0.406	Green	++
BAP (2.0) + NAA (1.5)	22	45.26 ± 0.34	0.443	Green	++

** no response; + fair; ++ average; +++ good.

adjusted to MacFarland standard (10^8 CFU/ml). Mueller–Hinton agar media was used to perform sensitivity assay (Wagner, 1996) and diameter of zone of inhibition was produced by the extract and compared with those produced by the commercial control antibiotics Streptomycin (25 µg/ml).

RESULTS AND DISCUSSION

Callus induction

Callus was observed in MS medium supplemented with different concentrations of BAP and NAA growth regulators except in the media free of growth regulators. The induced callus was compact and greenish yellow in color. Leaf showed maximum callus formation on MS medium with benzylaminopurine (BAP, 0.5 mg/l) + α-napthalene acetic acid (NAA, 0.3 mg/l) in leaf explants with callus induction frequency of 72%, and biomass 1.152 g was further evaluated for the presence of metabolites in callus and leaf of plant (Table 1).

Qualitative phytochemical analysis

The methanolic extracts of leaves and callus of *C. asiatica* were subjected to qualitative phytochemical analysis for the detection of phytoconstituents, such as alkaloids, flavonoids, terpenoids, steroids, saponins, tannin, glycosides, carbohydrates and proteins. The medicinal value of the plant lies on chemical substances that function as primary and secondary metabolites, which have a definite physiological action on the human body. The result revealed the presence of alkaloids, terpenoids, steroids, saponins, glycosides, carbohydrates and proteins compounds in the methanolic extracts of leaves, as well as in the callus of the *C. asiatica* (Table 2).

Synthesized silver nanoparticles

The leaf and callus extracts of *C. asiatica* were taken in conical flask, respectively and 1 m$MAgNO_3$ solution was added. The conical flasks were incubated at room temperature and color change from light yellow to light brown was checked periodically in case of leaf extracts and light yellow to brown in case in the case of callus extracts of the *C. asiatica*. The change in color visually indicated the formation of AgNPs which was used for further characterization (Figures 1 and 2).

UV-visible spectroscopy

Samples of the reaction mixture were diluted with double distilled water and then subjected to the spectral measurement. The color changes can be ascribed to the formation of silver nanoparticles due to the excitation of surface plasmon vibrations, which indicates the formation of silver nanoparticles directly. Figure 3 (a and b) shows the absorption spectra of reaction mixtures of *C. asiatica* leaf extract and callus extract containing silver nanoparticle solution after 5 min, 30 min, 1 h and 16 h, respectively. After the reduction, absorption maxima was observed at 420 nm range wavelength in both *C. asiatica* leaf extract and callus extract silver nanoparticles solution.

FTIR analysis

FTIR analysis was carried out to identify the possible bio molecules responsible for the reduction of Ag+ ions and capping of the bio-reduced AgNPs. Figure 4 represents the FTIR spectrum of AgNPs synthesized using *C. asiatica* leaf and callus extracts. The absorbance bands of FTIR spectrum of the *C. asiatica* aqueous leaf extract were observed at 3340, 2225, 1686 and 1078 cm^{-1}. The intense band absorbance at 3340 cm^{-1} is the characteristic of the OH functional group in alcohols and at 1686 cm^{-1} assigned to the amide I band of aromatic rings. Thus some weak bands were centered at 1078 and 2225 cm^{-1} characteristic to the amide III and amide I band. The FTIR spectrum obtained from callus extract displays several peaks and multiple

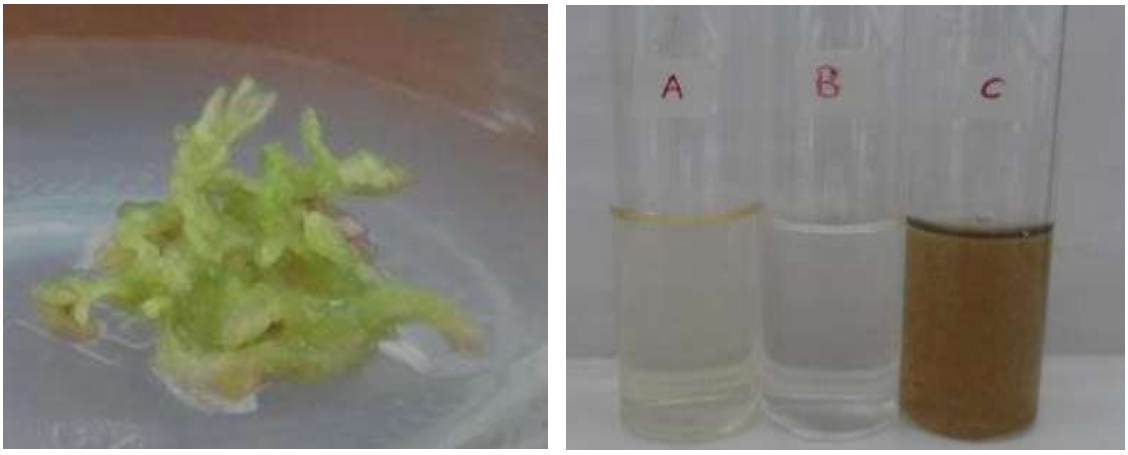
Table 2: Qualitative Phytochemical analysis of leaf and callus extracts of *Centella asiatica*.

Phytoconstituents	Leaf extract	Callus extract
Alkaloids	+	+
Flavonoids	-	-
Terpenoids	+	+
Steroids	+	+
Saponins	+	+
Tannins	-	-
Glycosides	+	+
Carbohydrate	+	+
Proteins	+	+

+ Presence of chemical constituents; - absence of chemical constituents.

(a) **(b)**

Figure 1(a): *Centella asiatica* plant **(b)** Tube A- contain *Centella asiatica* leaf extract, Tube B- contain $AgNO_3$ solution, Tube C- contain light brown colored silver nanoparticles solution.



(a) **(b)**

Figure 2(a): Callus of *Centella asiatica* **(b)** Tube A- contain *Centella asiatica* callus extract, Tube B- contain $AgNO_3$ solution, Tube C- contain brown colored silver nanoparticles solution.

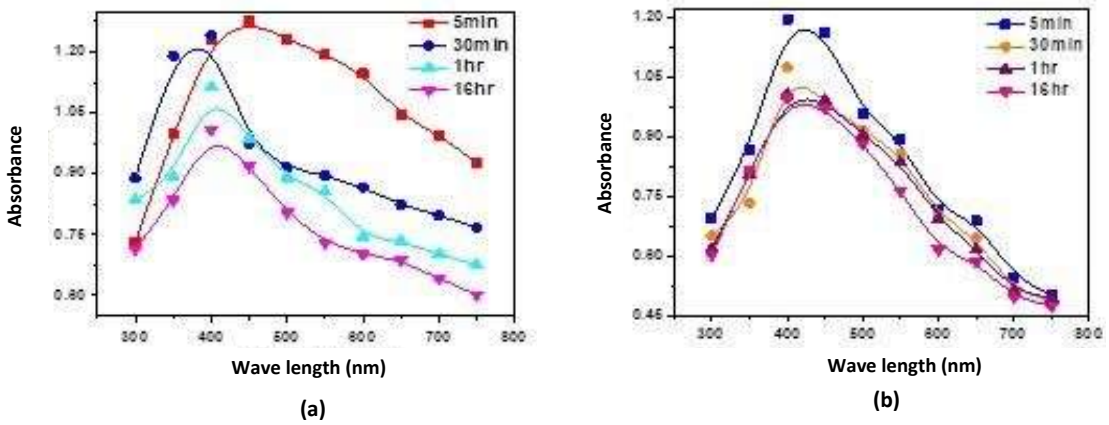


Figure 3: UV-Visible absorbance spectra of silver nanoparticles with *Centella asiatica* (a) leaf extract and (b) callus extract.

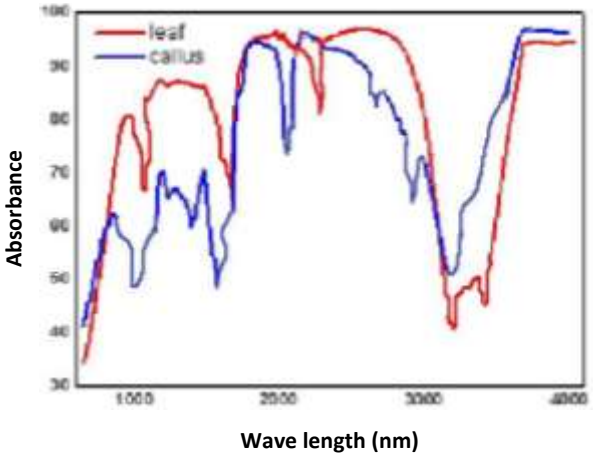


Figure 4: FTIR images of silver nanoparticles synthesized from *Centella asiatica* leaf and callus extracts.

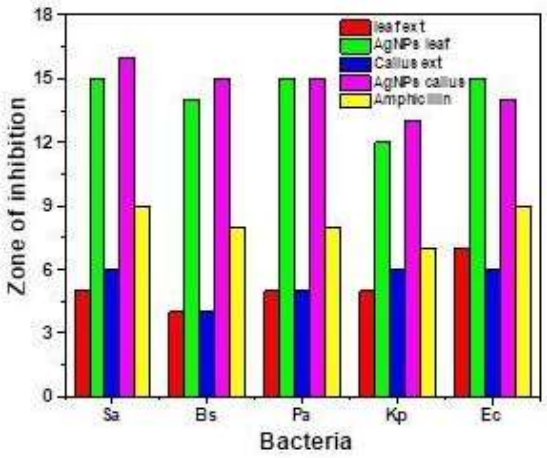


Figure 5: Zone of Inhibition of silver nanoparticles synthesized from leaf and callus extracts of *Centella asiatica.* **Sa** *Staphylococcus aureus*, **Bs** *Bacillus subtilis*, **Pa** *Pseudomonas aeruginosa*, **Kp** *Klebsella pneumonia*, **Ec** *Escherichia coli*.

bands at 3480, 2075, 1656, 1560 and 1065 cm^{-1}.The intense absorbance at 3480 cm^{-1} assigned to the N-H stretch of amide group and the band at 2075 cm^{-1} show the C≡C stretch of alkynes. The band at 1656 cm^{-1} assigned to the amide I band and band at 1560 cm^{-1} demonstrate C-H stretch of primary alcohols, and the band at 1065 cm^{-1} assigned to the C-O stretch present in the biomass of the callus of *C. asiatica*. Thus, it is obvious that bands assigned to the carbonyl groups and secondary amines are surrounded by some proteins and metabolites.

Antibacterial activity

The silver nanoparticles synthesized from leaf extract of *C. asiatica* showed antibacterial activity against all tested pathogenic microorganism. Maximum activity was found against *S. aureus*, *P. aeruginosa*, and *E. coli* at 15 mm and least activity was found against *Klebsella pneumonia* at 12 mm. Whereas silver nanoparticles synthesized from callus extract showed maximum activity against *S. aureus* at 16 mm and least against *Klebsella pneumonia* at 13 mm (Table 3 and Figure 5). Since nanoparticles have large surface area and small size, they bind to the surface of cell membrane and interacts with the cell more than the large particle and resist the function of bacteria.

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