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An Efficient and Effective *In-Vitro* Callus Production in *Bacopa monnieri* by Using Copper Nanoparticles.

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ABSTRACT

In the present article described an efficient effect of copper nanoparticles (NPs) on the induction of *in-vitro callus* from explants. However, the effects of autoclaving on copper nanoparticles were observed by UV-Vis spectroscopy that shows the wavelength of nanoparticles increases as the size of copper nanoparticles decrease. Further, it was characterized by powder x-ray diffraction stud and an average crystallite size of the powder was calculated 20 nm. *In-vitro callus* was induced from explants *of Bacopa monnieri* in MS media supplemented with different concentration of copper nanoparticles. The effect of copper nanoparticles on callus induction and autoclaving were studied within MS media and without MS media. The MS medium with the lower concentration (16×10^3 ppm) of copper nanoparticles capable for medium growth of callus as that control and simultaneously reducing the rate of contamination.

Keyword: Copper nanoparticles, *Bacopa monnieri,* Callus.

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INTRODUCTION

Bacopa monnieri commonly known as 'Brahmi' the member of family *Scrophulariaceae*, is placed second in the priority list of Indian medicinal plants has been used in the Ayurvedic system of medicine for centuries [1]. *Bacopa*'s antioxidant properties may offer protection from free radical damage in cardiovascular disease and certain types of cancer .It has been used for centuries in folklore and traditional system of medicine as a memory enhancer, anti-inflammatory, analgesic, sedative, antipyretic and anti-epileptic agent [2-6]. The memory enhancing effects of this plant have been attributed to the active constituent bacosides A and B [7]. In addition to unique medicinal use, it has also been linked to phytoremediation programmes for the removal of heavy metals such as Cadmium and Chromium [8]. With increasing demand for herbal drugs, the natural populations of these plants are threatened with over exploitation. The requirement of Brahmi is meeting solely from the natural populations, leading to their gradual depletion [9]. Therefore, development of a rapid clonal multiplication protocol of this medicinally important herb has become imperative in order to reduce the existing pressure on natural populations and constant supply of plant material for pharmaceutical industry [10].

On the other hand, metals nanoparticles attracted enormous interest in biological as well as pharmacological activities because of their unique physico-chemical properties. Their properties can be forbidden depending on the synthesis method. One of the main effects, which are enhanced by controlling particle size, is their antimicrobial action [11-13]. The antimicrobial activity of NPs is known to be a function of surface area in contact with microorganisms. For this cause, high surface area NPs assure a wide range of reactions on the surface of microorganisms, inhibiting the normal function of cells or causing cell death [14].

Notwithstanding, in our previous work on metal nanoparticles (NPs) [15-17] were reported an efficient and effective catalytical properties in solvent free synthesis of various organic compounds *via* one-pot, three-component reactions as well biological assay.

In the present work is highlighted biological investigation on an *in-vitro* callus production in *Bacopa monnieri* by using copper nanoparticles.

MATERIALS AND METHOD

Callus induction

All plant materials required for different experiments were selected from mother plant field station of Krishi Mitra Biotech, Arvi (Dist. Wardha) .Only elite disease free plants were selected for the experiments. All chemicals and plant growth regulators (PGR's) were purchased from Hi Media Laboratories, Mumbai. Glassware's used for all these experiments were of Borosilicate purchased from Alka Scientific Corporation, Nagpur.

The basal medium employed in this study for culture of *Bacopa. monnieri* was MS medium [18] The concentrated stock solutions of the major salts, minor salts and vitamins were prepared, for preparation of the media and stored under refrigeration. Auxins and Cytokinins were dissolved in 1N NaOH and 2, 4-D was dissolved in Ethyl alcohol before making up the final volume with distilled water. The medium was prepared by adding appropriate quantities of the stock solutions and correct volume was made up with the distilled water. The pH was adjusted in all cases to 5.8 by using 1N NaOH and 1N HCl and CleriGel™ (HiMedia: PT079). Agar 0.8% (w/v) was used as solidifying agent. Before autoclaving, the media was poured into washed culture bottles (30-35mL) which were capped and labeled properly. These were then autoclaved at 121^0C for 15 minutes at 15-psi pressure and transferred to the inoculation room where they were stored under aseptic conditions till their use.

Explants for the initiation of callus induction were largely detected by the method adopted for *in vitro* clonal propagation [19]. According to the procedure vigorously growing leaves of "*Bacopa monnieri*" were excised from proliferating shoots and their leaflets were used as explants for callus induction studies [20].

The selected explants were washed thoroughly with constant shaking with tap water for 5 minutes for the removal of external dust and contaminants. Thereafter, they were washed twice with distilled water and

then with 10% Dettol, a liquid disinfectant used for 3 minutes to avoid chance of any internal microbial contamination and again rewashed with distilled water for two times. Finally explants were kept in sterile distilled water bottle.

These explants were taken inside the laminar air flow for further sterilization. Firstly, they were washed with sterile distilled water by continuous shaking for 5 minutes and this process was repeated two times. Then they were treated with 0.1% mercury chloride for approximately 3-4 minutes for the removal of contaminants. After that, explants were washed thoroughly two times with sterile distilled water so as to remove all traces of mercury chloride. Thereafter, surface sterilization was done with Sodium hypochloride for approximately 3-4 minutes. Then they were removed from the sterilizing solution and rinsed twice in sterilized distilled water so as to remove all traces of Sodium hypochloride. Finally the extra water was removed with the help of sterile tissue paper from the explants and then they were used for the inoculation.

Methods of inoculation and culture condition

Sterilized explants were transferred aseptically from the bottles to sterile glass plates for giving a fresh cut on both sides. The forceps were rinsed in the 70% ethanol, flamed them and allowed to cool for a while. The explants were placed inside the bottle on semisolid (MS) basal medium supplemented with different concentrations of PGRs using long forceps without touching the rim of the bottle. At least three explants were placed in each bottle. Cap of the bottle was carefully placed, tighten and then sealed with clean film. The same procedure was repeated for all available explants and finally bottles were kept in the rack of growth room, which maintained at $25 \pm 2^{o}C$, with 16 h photoperiod under cool, white fluorescent lamps and 8 h night break with (2000 Lux) light intensity.

Synthesis and characterization of copper nanoparticles

In the present study emphasized on the callus production in *Bacopa monnieri* at different concentration of previously reported copper nanoparticles [21]. Diethylene glycol (DEG) is non-aqueous solvent as a reaction medium allows us to minimize the copper surface oxidation. Furthermore, poly (vinylpyrrolidone) was added as a dispersing agent also effectively prevents the oxidation process. In the case of Cu-nanoparticles synthesis, the reducing ability of DEG is insufficient to reduce the copper ions because copper is easily oxidized to either CuO or Cu_2O in air atmosphere. Here, $NaH_2.PO_2.H_2O$ used as reducing agent for the preparation of Cu nanoparticles. Copper nanoparticles were prepared through chemical reduction method, using PVP as capping agent and sodium phosphinate monohydrate in DEG used as reducing agent. The aqueous solution of copper sulphate pentahydrate (20 mmol) was injected into hot reaction medium via syringed. The synthesized copper nanoparticles were separated by centrifuged and washed with double distilled water and acetone respectively. Copper nanoparticles obtained after centrifugation was kept in vacuum oven at $40^{o}C$ for drying. The crystal structure of the sample was characterized by Bruker AXS D8 Advance X-ray diffractometer using CuKα radiation. UV-Vis measurements were made by a Varian, Cary 5000 UV-Vis-NIR Spectrophotometer.

Inoculation of callus in medium supplemented with nanoparticles

Early developed callus of *Bacopa monnieri* with initiating stages was placed in MS medium with different concentrations of Copper nanoparticles i.e. 10^{10}, 10^{5} and 10^{3} ppm. For each concentration four tubes were prepared for experimental set and four tubes for control set.

Characterization of nanoparticles

The UV spectra showed that copper nanoparticles have absorbed maximum wavelength at 273 nm (Figure 1). The effect of autoclaving on Copper nanoparticles by UV-Vis spectra shows that the wavelength of nanoparticles increases as the size of copper nanoparticles decrease.

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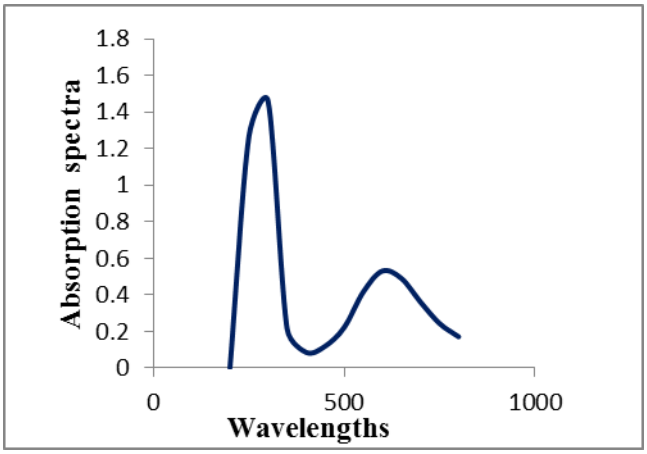

Figure 1: UV-Vis absorption spectra of Copper NPs at various wavelengths

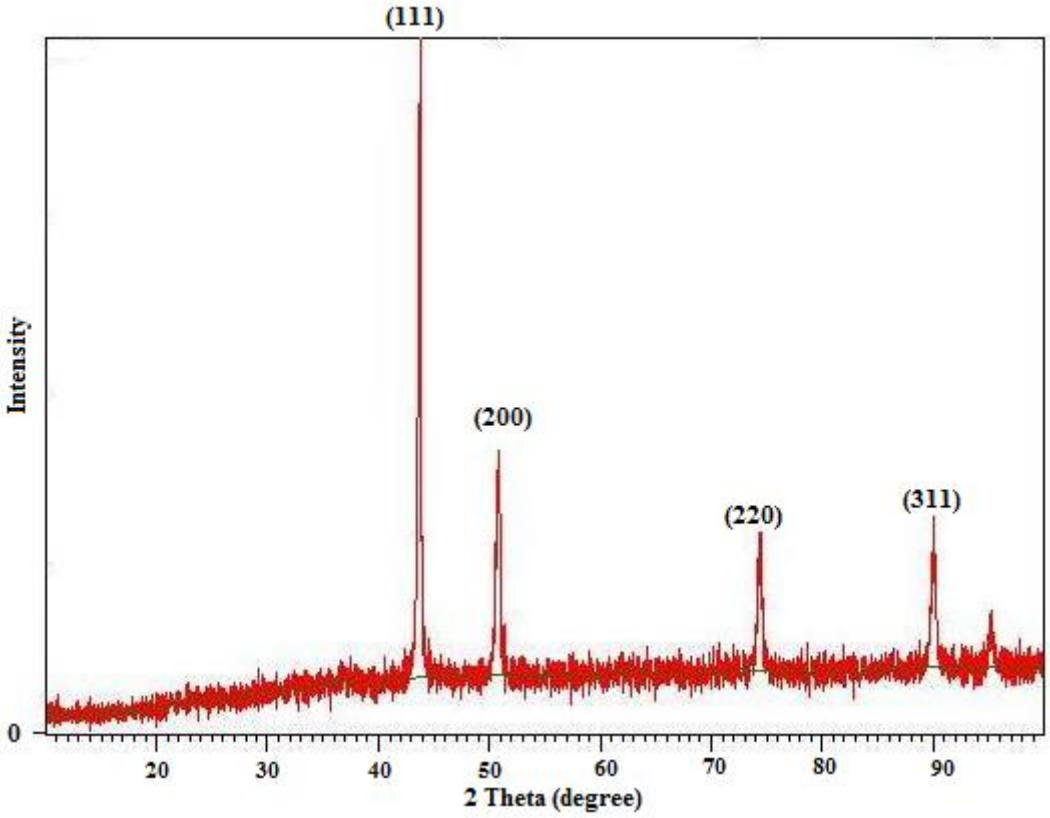

Figure 2: X-ray diffraction analysis of Copper NPs

The XRD spectra were recorded by using CuK_{α} and XRD pattern of copper nanoparticles as shown in Figure 1. Three peaks at 2θ values of 43.274, 50.427 and 74.123 deg corresponding to (111), (200) and (220) planes of copper were observed and compared with the standard powder diffraction card of JCPDS, Copper file No. 04-0838. The XRD study indicates that the resultant particles are (fcc) Copper Nanoparticles. The average crystalite size of the powder was calculated to be 20 nm according to half width of the (1 1 1) diffraction peak using Debye Scherrer formula.

RESULT AND DISCUSSION

The lower concentration (16×10^{3} ppm) of Copper nanoparticles showed contamination on callus culture. However, higher concentration (16×10^{5} ppm and 16×10^{10} ppm) have not shown any contamination (Table 1).

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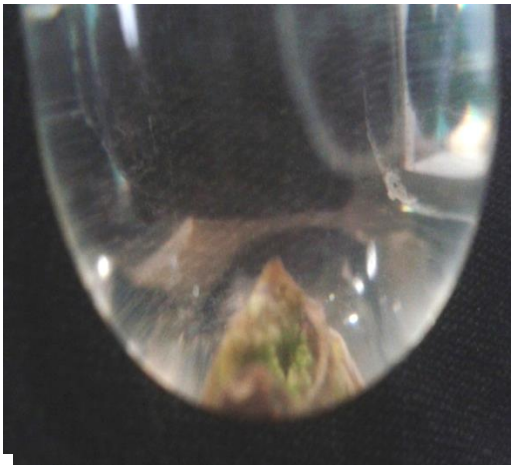
Table 1: Effect of Copper NPs within MS media on growth, colour and contamination of callus

Sr. No.	Concentration (ppm)	Days after inoculation	Response of callus		
			Growth	Color Change	Contamination
1.	Control	14	Medium growth (++)	No change Green to Green	More Contamination
2.	$16X10^{3}$	14	Medium growth (++)	No change Green to Green	Less Contamination
3.	$16X10^{5}$	14	Minimum growth (+)	Change Green to Light Brown	No contamination
4.	$16X10^{10}$	14	No growth	Change Green to Brown	No contamination

(a) Callus growth after 14 days (without Copper NPs)



(b) Callus growth after 14 days (with Copper NPs, dilution 103).

(c) Callus growth after 14 days (with Copper NPs, dilution 105)

(d) Callus growth after 14 days (with Copper NPs, dilution 1010)

The callus grown on the MS medium containing 16×10^{3} ppm and 16×10^{5} ppm concentrations of Copper nanoparticles showed the medium and minimum growth, respectively. However, no growth was observed in 16×10^{10} ppm concentration. The color of callus was not changed in 16×10^{3} ppm concentrations. However, it was changed from green to light brown in 16×10^{5} ppm concentration and from green to dark brown in 16×10^{10} ppm concentration. The lower concentration (16×10^{3} ppm) of Copper nanoparticles incorporated in MS medium was capable for medium growth of callus as that of control and simultaneously reducing the rate of contamination. However, their higher concentrations (16×10^{5} ppm and 16×10^{10} ppm) have prevented the contamination and inhibited growth completely [22, 23].

CONCLUSION

The UV spectra showed that Copper NPs have absorbed maximum wavelength at 273 nm. The average crystallite size of the powder was calculated to be 22 nm according to half width of the (111) diffraction peak using Debye Scherrer formula and the resultant particles are Copper Nanoparticles.

The callus grown on the MS medium containing 16×10^{3} ppm and 16×10^{5} ppm concentrations of Copper NPs showed the medium and minimum growth, respectively. However, no growth was observed in 16×10^{10} ppm concentration. The color of callus was not changed in 16×10^{3} ppm concentrations. However, it was changed from green to light brown in 16×10^{5} ppm concentration and from green to dark brown in 16×10^{10} ppm concentration. The lower concentration (16×10^{3} ppm) of NPs incorporated in MS medium was capable for medium growth of callus as that of control and simultaneously reducing the rate of contamination. However, their higher concentrations (16×10^{5} ppm and 16×10^{10} ppm) have prevented the contamination and inhibited growth completely.

The present investigator has observed the effect of Copper NPs incorporated in the MS medium for callus induction in *Bacopa monnieri*. The growth of callus was seen at the lowest concentration of Copper NPs *i.e.* 16×10^{3} ppm. whereas in 16×10^{5} ppm and 16×10^{10} ppm concentrations the callus has not showed any positive response. Initially the color of shoot was green. However, it was changed subsequently to brown in 16×10^{10} ppm dilution and remained same for longer duration.

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