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# Green synthesis of silver nanoparticles of bio-active phytochemicals with anti-bacterial activity from callus cultures of bitter gourd, *Momordica charantia* L.

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**ABSTRACT**

*Momordica charantia (bitter gourd), an anti-diabetic plant species, is a rich source of several bioactive molecules. In the present study the callus cultures of M. charantia were established in vitro and the presence of therapeutic compounds including lignans was detected in them. Rapid synthesis of silver nanoparticles by such calli was achieved. These nanoparticles as well as the ethanolic extract of the callus cultures exhibited strong anti-bacterial activity against methicillin-resistant Staphylococcus aureus. In addition the silver nanoparticles were also found active against drug-resistant strains of Pseudomonas aeruginosa. This is the first report of synthesis of silver nanoparticles from callus cultures of Momordica charantia.*



## INTRODUCTION

Plant-derived natural products can be effective therapeutic molecules to counter multi-drug resistant microbes that pose a formidable challenge to human healthcare. *Momordica charantia* of the family Cucurbitaceae, commonly known as bitter gourd, is a renowned herbal species for its anti-diabetic effect [6]. It is also known to have several bio-active phytochemicals with anti-microbial [2], anti-HIV [6] and anti-cancer [7] actions. There is a need for application of tissue culture technology for the production of its bio-molecules to facilitate the synthesis of silver nanoparticles which are regarded as more powerful agents for the control of multidrug resistant bacteria [24]. Silver nanoparticles have also been documented to have more efficacious bio-medical applications in the treatment of HIV, HBV [17], cancer [12], plasmodial [23] and larvicidal infections [21]. Plant-mediated synthesis of nanoparticles is an eco-friendly, greener and a more effective strategy [10, 14, 28]. Use of plant extracts can facilitate production of nanoparticles of different size and geometry [8] with a high degree of stability and improved compatiblity for pharmaceutical applications. However there are only a few reports on the use of plant callus cultures for nanoparticle synthesis [1, 18, 20, 27]. Recent trends suggest that callus cultures are more effective in nanoparticle generation than field-grown plants and differentiated plant organs [11, 20]. The present study describes the use of callus cultures of *M. charantia* for the rapid synthesis of silver nanoparticles with anti-bacterial activity.

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## MATERIALS AND METHODS

### *Establishment of callus cultures*

Seeds removed from fresh fruits of *M. charantia* were surface-sterilized in a laminar flow cabinet by immersing in 0.1% $HgCl_2$ for 10 min followed by several rinses with sterile distilled water. The intact or broken zygotic embryos aseptically extracted from the seeds were cultured on a MS (Murashige and Skoog) media [19] supplemented with various combinations of growth regulators (BAP-6- benzylaminopurine; 2, 4-D-2, 4-dichloro phenoxy acetic acid; NAA- $\alpha$-naphthalene acetic acid) at pH 5.7. All the media were fortified with 3% sucrose and 1% agar. The cultures were incubated at 25±2°C in continuous light with a photosynthetic photon flux density of 25 ìmol $m^{-2}$ $s^{-1}$ provided by cool white fluorescent lamps (Philips, India). Subcultures were carried out at regular intervals. Cultures were examined regularly and data were recorded to document the number of days required to initiate callusing and other morphogenetic responses. Photomicrographs were taken using Euromex stereo-zoom microscope with CCD camera and image processing system.

### *Phytochemical analysis of the callus*

Phenolic compounds were extracted from 1.17 g of 8 week old callus using hot 80% methanol and qualitatively & quantitatively analyzed by a simple colour test [3]. Lignans were isolated from the callus cultures as described by Koulman *et al* [15]. Eight wk old callus (1.17 g) was extracted with 80% methanol and the mixture was centrifuged for 10 min at 1000 g. The supernatant was extracted with an equal volume of dichloromethane and water and centrifuged for 6 min at 1000 g. Thin Layer Chromatography (TLC) of the dichloromethane extract was carried out on silica gel 60 (5 x 10 cm) plates developed with chloroform: methanol (9:1). The compounds were visualized by spraying the plates with methanol: sulphuric acid (1:1), followed by heating at 150°C for 2 min in an iodine chamber. The $R_f$ values were recorded.

### *Biosynthesis of silver nanoparticles*

Ten weeks old callus cultures of *M.charantia* were used for preparation of silver nanoparticles. A weighed amount of callus was ground with 5 volumes of sterile double distilled water in a mortar and pestle. The extract was filtered with Whatman No.1 filter paper. To the filtrate, 9 volumes of 1 mM $AgNO_3$ was added. The mixture was incubated at room temperature for 24 hrs. Observations of colour change were recorded. The bioreduction of silver nitrate was analysed using Cary 100 Bio UV-VIS spectrophotometer.

### *Anti-bacterial activity of the ethanolic extracts of callus and their silver nanoparticles by disc diffusion assay*

The ethanolic extract of the callus tissue was prepared by grinding 1g of 5-weeks old callus of *M. charantia* in 80% ethanol. The extract was centrifuged for 10 min. The supernatant was evaporated to dryness and dissolved in 1 ml water. The anti-bacterial activity of the ethanolic extract of the callus and the corresponding silver nanoparticle was assayed against selected gram-positive and gram-negative organisms by the disc diffusion method [13]. Different volumes (5,10 or 15 µL) of the ethanolic extract or the silver nanoparticle suspension were adsorbed on the surface of sterile Whatman No.1 filter paper disks (6 mm in diameter) and placed in Mueller Hinton agar swabbed with pure cultures of gram positive strains of *Staphylococcus aureus* and gram negative strain of *E.coli* and *Pseudomonas aeruginosa*. The plates were incubated at 37°C overnight and the zone of inhibition was measured.

## RESULTS AND DISCUSSION

### *In vitro response of intact and fragmented zygotic embryos*

Intact zygotic embryos of *M. charantia,* cultured in MS based media germinated *in vitro* and formed several roots (Fig.1a). When fragmented zygotic embryos were cultured *in vitro* on media without charcoal (M3-M5), the cotyledons turned green and callus was formed at their edges within 2 weeks (Table 1; Fig. 1b, c). The callus was whitish (Fig.1 c) or pale green ( Fig.1 d) and exhibited rapid growth and protiferation (Fig. 1e). Long term callus

**Table 1. Morphogenic responses from fragmented zygotic embryos on different MS based media with various combinations of growth regulators**

| Medium code | NAA (mg/L) | BA (mg/L) | 2,4D (mg/L) | Acti-vated Charcoal (%) | Coconut milk (%) | Responses | % cultures showing callusing |
|---|---|---|---|---|---|---|---|
| M1 | 1 | - | 5 | 0.25 | 15 | Greening but no callusing | - |
| M2 | 5 | 1 | - | 0.25 | 15 | Greening but no callusing | - |
| M3 | 5 | 1 | - | - | 15 | Callusing | 100 |
| M4 | 1 | - | 5 | - | 15 | Callusing | 80 |
| M5 | - | 4 | - | - | - | Callusing | 87.5 |

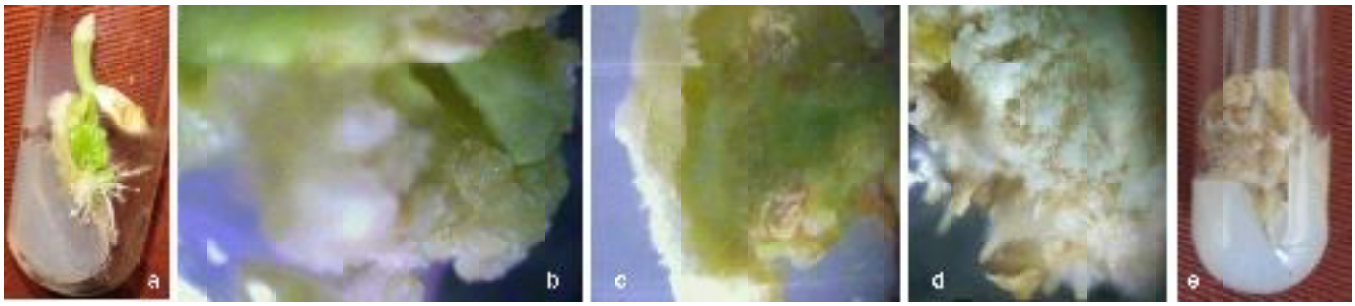



**Fig. 1.** ***In vitro*** **responses of cultured intact and fragmented zygotic embryos of** ***M. charantia*** **in M4 medium** (a) **plantlet formation from intact zygotic embryos;** (b) **greening and callusing on the surface of fragmented zygotic embryo explants after two weeks (X8);** (c) **initiation of callusing on the edge of cotyledon explants ( X8);** (d) **formation of greenish callus from fragmented zygotic embryo explants (X8);** (e) **massive callusing from fragmented somatic embryos**

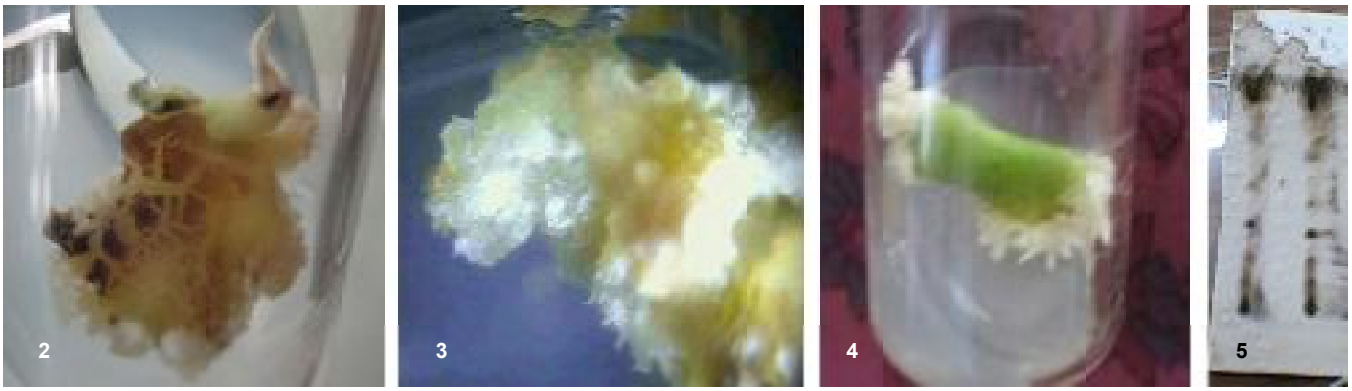
cultures which could be subcultured repeatedly were established. In case of fragmented zygotic embryos cultured on media with charcoal (M1 & M2), there was no callus formation, indicating that activated charcoal inhibited callusing from such explants. However, massive callusing occurred from the explants that were initially cultured in a charcoal supplemented media and subsequently shifted to non-charcoal M5 media after one week (Fig. 2). Somatic embryo-like structures also appeared from greened cotyledons of zygotic embryo explants two weeks after transfer to M4 medium (Fig. 3 & 4). It way be mentioned here that no reports of induction of somatic embryogenesis from zygotic embryo explants of *M. charantia* exist in literature, though somatic embryogenesis from leaf explants has been reported earlier [22] in this plant system.

### *Phytochemical analysis of induced callus*

Development of blue coloration in the ethanolic extract of 8 week old callus with Folin-Phenol reagent indicated the potential of the callus for synthesis of phenolics which is significant since plant phenolics have a wide variety of medicinal uses including anti-oxidant [9] and anti-microbial effects [25]. Lignans were also extracted from 8 week old callus. TLC analysis of the dichloromethane extract of the callus revealed four violet spots with $R_f$ values of 0.23, 0.36, 0.93 and 0.97 indicating the presence of lignans (Fig. 5). This observation assumas significance becasue lignans are known to have strong antimicrobial properties and other beneficial effects on health [5]. Presence of liguanes in *M. charantia* callus also expands the scope of their *in vitro* manipulations by the use of elicitors to enhance their yield [26].

### *Preparation of silver nanoparticles with callus extracts*

2.74 g of 10 week old callus cultures of *M. charantia* was used in this study for the preparation of silver nanoparticles. To 3 ml extract (equivalent to 1.37 g callus), 1 mM $AgNO_3$ (27 ml) was added.

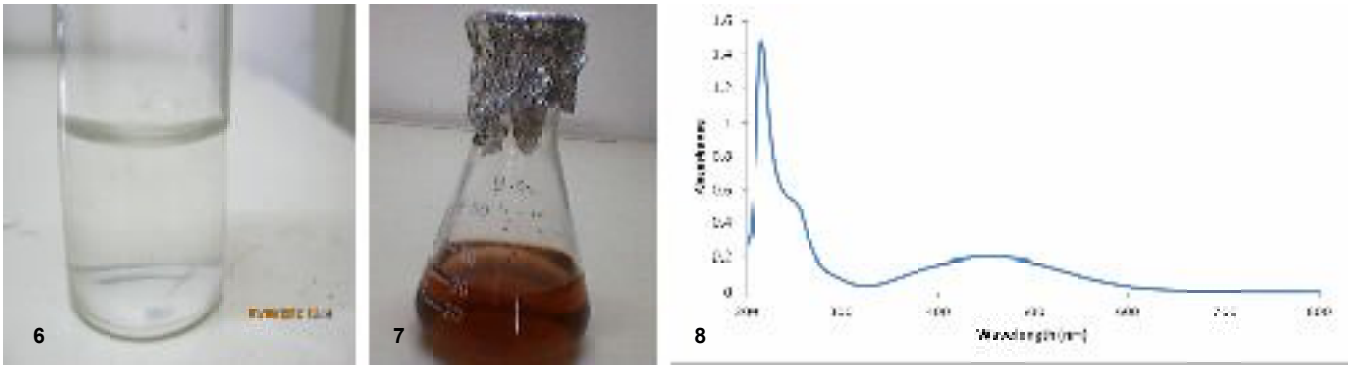


**Figs 2-5. Induction of massive callusing from fragmented zygotic embryo explants initially cultured on M2 and then shifted to non-charcoal M5 media (X8);** 3. **Formation of massive embryogenic callus (x8);** 4. **Formation of somatic embryo-like structures from cotyledon of zygotic embryo explants upon transfer to M4 medium callus (X8);** 5. **Detection of lignans in the dichloromethane extract of the callus after TLC in Chloroform: methanol and spraying with methanol : sulphuric acid and heating 2 min at 150°C**

The reaction started in about half an hour. The solution turned reddish-brown after 2 hours while the control did not show any color change (Fig. 6, 7). Using Cary 100 Bio UV-VIS spectrophotometer, the absorbance value of the sample was measured. From these values, a graph - wavelength (nm) vs absorbance was plotted. There was an absorbance peak at 450 nm which indicated the presence of silver nanoparticles (Fig.7). The synthesis of silver nanoparticles by the callus cultures is fairly rapid and could be attributed to the presence of phytochemicals including phenolics which may play a role in both reduction and stabilization [16]. There is no previous report on production of silver nanoparticles by *M. charantia*. Though silver nanoparticle synthesis by plant extracts has been reported widely there are only a few reports of production of silver nanoparticles by callus cultures of plants [1, 18, 20, 27]. The use of axenic cultures renders the nanoparticles more suitable for biomedical applications.

### *Antibacterial activity of ethanolic extract and callus nanoparticles*

15 µl of the ethanolic extract (equivalent to 15 mg of the callus) and 15 µl of nanoparticle suspension (prepared as aqueous extract equivalent to 0.68 mg of callus) were used for assaying thier antibacterial activity. The ethanolic callus extract exhibited significant antimicrobial activity against *Staphylococcus aureus* including the methicillin-resistant strain (Table 2). Though activity of ethanolic extract of fruits of *M. charantia* against *Staphylococcus* has been reported [4] this is the first report of antimicrobial activity of its callus cultures. The antimicrobial activity is presumably



**Figs 6&7. Synthesis of silver nanoparticles by callus :** (6) **control** (7) **The extract of callus incubated with $AgNO_3$ turns reddish brown after 2 h incubation;**

**Fig 8. Absorbance peak at 450 nm indicates the presence of silver nanoparticles of callus cultures of *M. charantia***

**Table 2. Inhibition zones (diameter in mm) obtained for various organisms with 15 µl of extracts of callus and their silver nanoparticles**

| Organism tested | Ethanolic extract | Silver nanoparticle suspension |
|---|---|---|
| ATCC *E.coli* | - | 10 |
| *Pseudomonas aeruginosa** | - | 10 |
| ATCC *Staphylococcus aureus* | 14 | - |
| *Staphylococcus aureus*** | 14 | 10 |

*-metallo-β-lactamase producing clinical isolate
**-methicillin-resistant standard strain

due to the phenolics [25] present in the callus. Silver nanoparticles obtained with *M. charantia* also exhibited strong antibacterial activity against gram negative and gram positive isolates. It was found to be active against drug resistant strains of both metallo-beta-lactamase producing strains *Pseudomonas aeruginosa* and methicillin-resistant strains of *Staphylococcus aureus* (Table 2). The results indicated the potential of the silver nanoparticles prepared using the callus of this species to control drug-resistant bacteria.

## CONCLUSION

Callus cultures together with corresponding silver nanoparticles of *M. charantia* with the potential of synthesizing bioactive phenolics and lignans were extablished *in vitro*. This synthesis of silver nanoparticles by the callus cultures was rapid. The silver nanoparticles as well as the ethanolic extracts of the cultured callus tissues showed anti-microbial activity against drug-resistant microbes indicating their enormous therapeutic potential. These results may be utilised in the large scale production of biomass to serve as sources of medicinal compounds of this species as well as for rapid synthesis of non-toxic silver nanoparticles which can combat the growth of several drug resistant bacteria.

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