Elicitation of silver nanoparticles enhanced the secondary metabolites and pharmacological activities in cell suspension cultures of bitter gourd

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Abstract

This study describes the influence of bio-synthesized silver nanoparticles (AgNPs) on phytochemicals and their pharmacological activities in the cell suspension cultures (CSC) of bitter gourd. To standardize the effect of sucrose, plant growth regulators, medium, AgNPs and growth kinetics for the biomass and bioactive compounds accumulation in CSC of bitter gourd. The medium comprising MS salts, sucrose (30 g/L) with 2,4-D (1.0 mg/L) and TDZ (0.1 mg/L) at 28 days of CSC was appropriate for biomass and bioactive compound accumulation. The contents of silver, malondialdehyde and hydrogen peroxide were highly elevated in AgNPs (10 mg/L)-elicited CSC when compared with non-elicited CSC. AgNPs (5 mg/L) elicited CSC extracts had significantly enhanced the production of total phenolic (3.5 ± 0.2 mg/g), and flavonoid (2.5 ± 0.06 mg/g) contents than in the control CSC extracts (2.5 ± 0.1 and 1.6 ± 0.05 mg/g). AgNPs (5 mg/L) elicited CSC showed a higher amount of flavonols (1822.37 μ g/g), hydroxybenzoic (1713.40 μ g/g) and hydroxycinnamic (1080.10 μ g/g) acids than the control CSC (1199, 1394.42 and 944.52 μ g/g, respectively). Because of these metabolic changes, the pharmacological activities (antioxidant, antidiabetic, antibacterial, antifungal and anticancer) were high in the AgNPs (5 mg/L)-elicited CSC extracts in bitter gourd. The study suggested the effectiveness of elicitation process in enhancing the accumulation of phenolic compounds and pharmacological activities. AgNPs-elicited CSC offered an effective and favorable in vitro method to improve the production of bioactive compounds for potential uses in pharmaceutical industries.

Keywords Cell suspension culture · Pharmacological activity · Phenolic compounds · Reactive oxygen species · Silver nanoparticles

Abbreviations

2,4-D	2,4-Dichlorophenoxyacetic acid	DNSA	Dinitrosalicylic acid
AgNO ₃	Silver nitrate	DPPH	1,1-Diphenyl-2-picrylhydrazyl
AgNPs	Silver nanoparticles	H ₂ O ₂	Hydrogen peroxide
AlCl ₃	Aluminum chloride	H ₈ MoN ₂ O ₄	Ammonium molybdate
AOA	Antioxidant activity	IAA	Indole acetic acid
BAP	Benzyl amino purine	ICP-MS	Inductively coupled plasma-mass spectrometry
C ₆ N ₆ FeK ₃	Potassium ferricyanide	MDA	Malondialdehyde
CH ₃ CO ₂ K	Potassium acetate	MS	Murashige and Skoog
CSC	Cell suspension culture	MTT	Thiazolyl blue tetrazolium bromide
		NAA	Naphthalene acetic acid
		NaClO	Sodium hypochlorite
		NH ₄ OH	Ammonia solution
		PGRs	Plant growth regulators
		ROS	Reactive oxygen species
		TCA	Trichloroacetic acid
		TDZ	Thidiazuron

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TEM Transmission electron microscopy
UHPLC Ultra-high-performance liquid chromatography

Introduction

Bitter gourd or bitter melon (*Momordica charantia* L.) is a highly nutritive vegetable and also traditional medicinal plant belonging to the family of Cucurbitaceae. It is used as a natural remedy in Ayurveda systems due to its anti-diabetic, anti-inflammatory, antimicrobial, and insecticidal properties (Grover and Yadav 2004). Bitter gourd is a good source of carbohydrates, proteins, minerals such as iron, calcium, vitamins, especially vitamin C and dietary fibers. It also contains various bioactive molecules such as phenolic compounds, triterpenes, charantin, momorcharin, saponin, momordin, vicine, oleanolic acids, alkaloids, triterpene glycosides, and saponins (Grover and Yadav 2004). Phenolic compounds have been widely reported to have both high antioxidant, antidiabetic, antimicrobial, anticancer, and anti-inflammatory activities (Deshaware et al. 2017). Cell suspension cultures (CSC) offer an excellent alternative for the large-scale production of phytochemicals (Sarkate et al. 2017). Cell suspension culture techniques deliver uniform high yield of phytochemical synthesis short time by overwhelming the effect of unexpected climatic conditions and diseases in field grown plants. A significant amount of phytochemicals production via CSC would help to save natural phyto-communities. A cell suspension culture has been efficiently utilized in the production of many marketable significant medicines such as taxol, camptothecin, vinblastine, and vincristine (Wilson and Roberts 2012). Furthermore, CSC could potentially induce novel bioactive compounds with pharmacological activities, which cannot be manufactured by chemical synthesis (Siahsar et al. 2011).

Recently, there have been few reports on increased production of phytochemicals by addition of elicitors to the CSC (Cai et al. 2012; Yue et al. 2016; Chung et al. 2017). Elicitors are biotic or abiotic molecules that have efficiently stimulated the production of phytochemicals in CSC. Silver (Ag) was utilized as an elicitor to upsurge the phytochemicals production in CSC of several plants (Zhao et al. 2005). Silver nanoparticles (AgNPs) is most frequently used metallic NP in consumer products such as cosmetics, textiles, electronics, household products, biomedical and sanitization devices (Chung et al. 2016a). Nanoparticles are 1–100 nm size has a large surface area than their bulk states. Nanoparticles have the potential to be utilized as new effective elicitors in plant biotechnology for the elicitation of phytochemical production (Sharafi et al. 2013; Ghorbanpour and Hadian 2015; Hatami et al. 2016). Previously, Thiruvengadam et al. (2015) demonstrated that high concentrations of

NP-induced ROS and toxicity in plants depend on NP size, surface, structure, concentration, dissolution and exposure ways. To our knowledge the influence of NPs on CSC and their possible helpful or harmful effects on production of phytochemicals in bitter gourd has not been investigated. Therefore, this study was conducted to determine the potential effects of AgNPs in CSC on biomass (fresh and dry mass) accumulation and bioactive compounds (individual phenolic compounds, total phenolic and flavonoid contents) production as well as pharmaceutical activities (antioxidant, antidiabetic, antibacterial, antifungal, and anticancer) in bitter gourd.

Materials and methods

Biosynthesis and characterization of AgNPs

AgNPs synthesis was prepared using the following previously described method (Kalimuthu et al. 2008). Briefly, *Bacillus marisflavi* (KCCM 41588) was sub-cultured into nutrient agar plates and Luria broth (LB) agar slants. A loop full of 24-h-old cultures were taken and transferred into an Erlenmeyer flask containing 200 mL of LB broth. The flasks were incubated in an orbital incubator shaker at 37 °C and agitated at 200 rpm for 24 h. The cell-free extract was obtained by centrifugation for 12 min at 10,000 rpm and followed by decantation. The absolute concentration of 1 mM AgNO₃ was added into 100 mL of cell filtrate in a 250-mL Erlenmeyer flask and incubated at 37 °C for 4 h. The flask with cell filtrate alone was maintained as control (without AgNO₃) along with experimental flask. The AgNP samples were centrifuged at 10,000 rpm for 10 min, and then the supernatant was filtered through a sterilized filter (0.22 µm) to purify the bio-synthesized material. The bio-synthesized NPs were further characterized by UV visible spectroscopy (UV-2550, Shimadzu) in the range of 300–800 nm. The size and morphology of the AgNPs were characterized by TEM (JEM-1200EX, JEOL, Japan) that was operated at a voltage of 300 kV (Thiruvengadam et al. 2015).

Establishment of callus and cell suspension cultures (CSC)

Bitter gourd seeds were surface-sterilized with 2% NaClO for 1 min followed by rinsing with sterile H₂O. Sterilized seeds were grown in MS (Murashige and Skoog 1962) semi-solid medium (8.0 g/L agar and 30 g/L sucrose). Cultures were kept in growth chamber conditions 16/8-h (light/dark) of 30 µmol/m²/s irradiances at 26 ± 2 °C. Leaf tissue of 2-week-old bitter gourd seedlings were cultured on solid medium (MS, 8.0 g/L agar, 30 g/L sucrose with 1.0 mg/L 2,4-D and 0.1 mg/L BAP). Callus cultures were maintained

for 21 days under the culture settings above mentioned. For initiation of CSC, friable callus were transferred in 125-mL flasks containing 25 mL liquid medium (MS, 30 g/L sucrose with 1.0 mg/L 2,4-D and 0.1 mg/L TDZ). The cultures were kept on a rotary shaker at 110 rpm at 25 ± 2 °C, 16/8 h light/dark regime using fluorescent lamps (30 $\mu\text{mol}/\text{m}^2/\text{s}$). The chemicals of plant growth regulators, sucrose, and agar purchased from Sigma-Aldrich, USA.

Influence of medium, sucrose, plant growth regulators (PGRs) and growth kinetics on biomass accumulation in CSC

The effect of culture medium, sucrose, growth kinetics, auxins and cytokinins on cell development and biomass production in CSC was evaluated. The friable callus cells (500 mg fresh mass) were cultured in MS liquid medium with sucrose (30 g/L) containing auxins (2,4-D, NAA and IAA; 0, 0.5, 1.0, and 2.0 mg/L) with cytokinins (BAP and TDZ; 0, 0.1, and 0.5 mg/L). An MS suspension culture medium lacking PGRs was used as a control. The effects of using various culture medium such as MS, NN (Nitsch and Nitsch 1969), N6 (Chu 1978) and B5 (Gamborg et al. 1968) were evaluated. Various concentrations of sucrose (10, 20, 30, and 40 g/L) were determined. Cultures were collected at 7, 14, 21, 28 and 35 days post-cultivation, and then examined for biomass accumulation and growth kinetics. Cell suspension cultures (CSC) were kept on a rotary shaker set at 110 rpm and maintained under the culture settings above-mentioned. Fresh and dry mass of harvested cells were assessed after 4 weeks.

Elicitations of AgNPs on biomass accumulation in CSC

Bioengineered AgNPs (0, 1, 5, and 10 mg/L) were added on CSC liquid medium (MS, 30 g/L sucrose with 2,4-D 1.0 mg/L and TDZ 0.1 mg/L) at 25 days culture. Cell suspension cultures (CSC) was induced with AgNPs for 48 h and then transferred to CSC liquid medium without AgNPs. Cell suspension cultures were incubated on a rotary shaker set at 110 rpm and maintained under the culture conditions mentioned above. The fresh and dry mass of cells was determined at 28 days. Cell suspension cultures isolated from the medium through sieve filters, washed with germ-free distilled H_2O , and blotted before fresh mass measurement. Filtered cells were frozen and lyophilized overnight then used for further experiments as dry mass.

Determination of silver (Ag) content in CSC

The Ag content in the AgNPs-elicited and non-elicited CSC was estimated by ICP-MS (Varian 820-MS). AgNPs-elicited and non-elicited CSC samples were dehydrated at 70 °C for

48 h in hot air oven. Cell suspension culture samples (50 mg dry mass) were digested with 3 mL of HNO_3 at 110 °C for 1 h in a dry bath. Then, 2 mL of H_2O_2 (30% w/w) was added to the cooled down samples and again heated at 110 °C on a hot plate for 25 min. The completely digested samples were diluted with deionized water and used for ICP-MS analysis.

Determination of MDA and H_2O_2 contents in CSC

The MDA content in AgNPs-elicited and non-elicited CSC samples were estimated by using the method of Heath and Packer 1968). One hundred milligrams of CSC samples were homogenized in 5 mL of TCA (0.1% w/v). The extracts were centrifuged at 6000 rpm for 15 min and the supernatant (2 mL) was mixed with 2 mL of TBA (0.68% w/v) and incubated at 100 °C for 25 min. The mixtures were cooled in an ice-bath and centrifuged at 5000 rpm for 10 min and optical density (OD) of the supernatant was recorded at 532 and 600 nm in spectrophotometer (UV-Vis). The MDA concentration was calculated using extinction coefficient 155/ mmol/cm .

The H_2O_2 content in AgNPs-elicited and non-elicited CSC samples was measured the protocol (Brennan and Frenkel 1977). One hundred milligrams of CSC samples in 3 mL cold acetone was centrifuged at 4000 rpm at 4 °C for 10 min. One milliliter of supernatant was gently mixed with 0.1 mL titanium tetrachloride (20%) in concentrated HCl (v/v). One microliter concentrated ammonia solution was added to the mixture to precipitate the titanium hydrogen peroxide complex, centrifuged at 5000 rpm for 30 min and the precipitate was dissolved in 2N H_2SO_4 . The content of H_2O_2 was determined OD at 410 nm in spectrophotometer (UV-Vis) and standard curve set with recognized concentrations of H_2O_2 .

Determination of total phenolic and flavonoid contents in CSC

Total phenolic and flavonoid contents in the AgNPs-elicited and non-elicited CSC samples were determined. Total phenolic content was estimated by using the calorimetric method rendering to the Folin–Ciocalteu (FC) assay (Singleton et al. 1999). Briefly, 200 μL (1 mg/mL) of CSC extracts was mixed with 200 μL FC reagents. After 5 min, 600 μL of sodium carbonate solution (20%) was added, and then the mixed solution was allowed to stand for 1 h at room temperature. The OD was noted at 765 nm in UV-visible spectrophotometer. Total phenolic content was calculated as mg of gallic acid equivalent by using an equation acquired from gallic acid calibration curve.

Total flavonoid content was evaluated by the aluminum chloride (AlCl_3) calorimetric method (Willet 2002). In brief, 200 μL (1 mg/mL) of CSC extracts was mixed with

4 mL of disinfected H₂O and then 0.1 mL AlCl₃ (10%) and 0.1 mL CH₃CO₂K (1 M) solution were added. The mixture was incubated at room temperature for 30 min. The OD was measured at 415 nm in spectrophotometer (UV–Vis). Quercetin was used to make the calibration curve.

Extraction and estimation of individual phenolic compounds in CSC

Lyophilized AgNPs-elicited and non-elicited CSC powder (1 g) was extracted in 10 mL of acetonitrile and 2 mL of 0.1 N hydrochloric acid. The mixture was stirred in rotary shaker (100 rpm) at room temperature for 2 h. The extract was filtered through Whatman filter paper (no. 42) and the filtrates were dissolved in 10 mL of MeOH (80%) and filtered through membrane filter (0.45 µm). The filtrate of AgNPs-elicited and non-elicited CSC was used for the UHPLC analysis. Individual phenolic compounds were analyzed by Ultra-HPLC (Thermo Accela, USA) and separation was achieved using a reverse phase column (C₁₈, 2.1 × 100 mm, 2.6 mm) and the absorbance was measured at 280 nm. The standard, solvent and gradient method was stated earlier (Thiruvengadam et al. 2014).

Preparation of CSC extract for pharmacological activities

AgNPs-elicited and non-elicited cell suspension powder (1 g DM) were subjected to extraction with 50 mL of methanol (95%) and kept at room temperature for 24 h with repeated shaking used rotary shaker at 100 rpm. Subsequently, the solution was passed through Whatman no. 1 filter paper and evaporated to dry the filtrate using a rotary evaporator. Dried methanolic extract was then dissolved in the minimum amount of methanol necessary and stored at 4 °C until needed for subsequent analyses on pharmacological activities.

Determination of antioxidant activity (AOA)

Brand-Williams et al. (1995) method was adopted for the analysis of radical-scavenging activity (RSA) using the DPPH. Precisely, 100 µL of CSC extracts were added to 1.4 mL DPPH solution and incubated in the dark environments at room temperature for 1 h. The OD was measured at 517 nm in UV–visible spectrophotometer.

$$\text{RSA (\%)} = [(\text{OD}_{\text{control}} - \text{OD}_{\text{sample}}) / \text{OD}_{\text{control}}] \times 100.$$

Ferric reducing power was determined as described earlier method of Oyaizu (1986). In brief, 100 µL CSC extracts were mixed with 2.5 mL sodium phosphate buffer (Na₃PO₄, 200 mM pH 6.6) and 2.5 mL C₆N₆FeK₃ (1% w/v in de-ionized H₂O) and the mixture was incubated in a water bath

for 20 min at 50 °C. Then, 2.5 mL of TCA (10% w/v in de-ionized H₂O) was added to the mixture and centrifuged at 750 g for 10 min. The supernatant (2.5 mL) with de-ionized H₂O (2.5 mL) and 0.5 mL FeCl₃ (0.1% w/v in de-ionized H₂O) was vortexed well. The OD was recorded at 700 nm in UV–visible spectrophotometer.

This assay is based on the reduction of Mo (VI) to Mo (V) by the CSC extracts, which produces phosphomolybdenum (V) complex under acidic conditions (Prieto et al. 1999). One hundred microliter of CSC extracts (1 mg/mL) was mixed with 1 mL of phosphomolybdate reagent (0.6 M H₂SO₄, 28 mM Na₃PO₄, and 4 mM H₈MoN₂O₄). The tubes were incubated at 95 °C for 90 min in a thermal block (Thermo Fisher Scientific, USA). The mixture was then allowed to reach room temperature when its OD was recorded at 695 nm in UV–visible spectrophotometer.

Determination of antidiabetic activity (α-amylase inhibitory activity)

The inhibition activity was achieved using the dinitrosalicylic acid (DNSA) procedure (Poovitha and Parani 2016). The test mixture contained 500 µL of 0.02 M Na₃PO₄ buffer having α-amylase solution (1 U/mL) and AgNPs-elicited and non-elicited CSC extracts at 20–100 µg/mL. Then, 100 mL of 1% starch solution was added and incubated for 20 min at 37 °C. The reaction was ended by adding 500 µL of DNSA reagent and then incubated in a boiling water bath for 10 min. This reaction OD was measured at 540 nm in UV–vis spectrophotometer (Mecasys, Korea).

$$\text{Inhibition activity (\%)} = [(\text{OD}_{\text{control}} - \text{OD}_{\text{extract}}) / \text{OD}_{\text{control}}] \times 100.$$

Determination of antimicrobial activity

The pathogenic microorganisms of Gram-positive bacteria [*Staphylococcus aureus* (KACC 13257) and *Bacillus subtilis* (KTCC 1021)], Gram-negative bacteria [*Pseudomonas aeruginosa*, (KACC 10259) and *Escherichia coli* (KACC 13821)] and fungal strains [*Candida albicans*, *Aspergillus niger* and *Fusarium oxysporum*] were used to test for antimicrobial activities using the disc diffusion method. The pure bacterial strains were obtained from the Korean Agricultural Culture Collection (KACC), Suwon, South Korea. The fungal strains were received from Prof. S.C. Chun, Department of Molecular Biotechnology, Konkuk University, Seoul, South Korea. Concisely, 100 mL of culture bacterial cells (10⁸ CFU/mL) and fungi (10⁴ spores/mL) were spread onto medium of nutrient agar and potato dextrose agar, respectively. For the antibacterial and antifungal tests, paper discs (10 mm in diameter) were independently soaked with 50 µL (100 mg/mL) CSC extract. Paper discs were soaked with 50 µL of antibiotics (chloramphenicol or thymol) for the positive control and solvent is used for the negative control. For bacterial plates were allowed

to stay at 37 °C for 18–24 h and fungal plates for 24–48 h (Rawat et al. 2016). Relative inhibition of the test extract (%) = $[(A - B)/(C - B)] \times 100$, where A is a total area of inhibition of the test extract, B is a total area of inhibition of the solvent and C is a total area of inhibition of the standard drug.

Determination of anticancer activity

Cell lines

Two human cancer cell lines, namely, colon HT-29 (human colorectal adenocarcinoma) and estrogen-dependent breast MCF-7 (human breast adenocarcinoma) cancer cell lines were purchased from the KCLB (Korean Cell Line Bank, Seoul, South Korea). All cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS), penicillin–streptomycin 1% (v/v) (Gibco, Grand Island, NY). The cells were cultured in 5% CO₂ incubator at 37 °C in a humidified atmosphere.

MTT cytotoxicity assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) colorimetric assay was performed to evaluate the cytotoxicity of the CSC extracts (AgNPs-elicited and control) in bitter melon (Chung et al. 2016b). Briefly, the cells were seeded in a flat-bottomed 96-well plate and incubated for 24 h at 37 °C in 5% CO₂. Both cell lines were exposed to the CSC extract samples (12.5, 25, 50, 100, and 200 µg/mL per AgNPs-elicited and control). The solvent for the dimethyl sulfoxide (DMSO)-treated cells was used as the control. The cells were treated with the MTT reagent (20 µL/well) for 4 h at 37 °C, and DMSO (200 µL) was added to each well to dissolve the formazan crystals. Optical density (OD) was recorded at 492 nm using a microplate reader. Cell viability percentage of the residual was determined as $[1 - (\text{OD of treated cells}/\text{OD of control cells})] \times 100$.

Statistical analysis

The experimental results were expressed as mean $\pm$ standard deviation of three replicates. Where applicable, the data were subjected to one-way analysis of variance (ANOVA) and the differences among samples were determined by Duncan's multiple range test (DMRT) was used to determine significant differences at $P \leq 0.05$ (SPSS Ver. 20 statistical software package).

Results and discussion

Biological synthesis of AgNPs and their characterization

AgNPs were synthesized through reduction of Ag⁺ into Ag⁰ in the presence of *B. marisflavi* extracts. The colorless AgNO₃ solution changed to dark yellowish brown color within 2 h. The color change and UV absorption analysis thus confirms the reduction of AgNO₃ to AgNPs through *B. marisflavi* culture supernatant. Similar with our results, AgNPs synthesized from *B. licheniformis* (Kalimuthu et al. 2008). Figure 1a shows the UV–visible spectra, a strong, broad peak, located at 420 nm, was observed for NPs synthesis using the *B. marisflavi* culture supernatant. Figure 1b depicts the TEM of the Ag synthesized by *B. marisflavi* showed distributed spherical shaped particles, with an average size of approximately 8 nm with numerous sizes ranging from 2 to 12 nm. The particle size distribution is shown in Fig. 1c.

Effects of media, sucrose, plant growth regulators (PGRs), and growth kinetics on biomass accumulation in cell suspension cultures (CSC)

Bitter melon leaf discs were cultured on MS solid medium with 1.0 mg/L 2,4-D and 0.1 mg/L BAP induced yellowish friable callus (81.0%) after 3 weeks. The callus were white or green and compact occurred to BAP with IAA or NAA, both of which induced less callogenesis than the combination of 2,4-D and BAP. Previously, it has been reported that 2,4-D is necessary for inducing yellowish friable callus in *M. charantia* (Thiruvengadam et al. 2006). Various concentrations of auxins (NAA, IAA and 2,4-D) were studied for their effects on CSC. Among them, 1.0 mg/L 2,4-D in MS led to biomass accumulation 3.95 FM; 0.94 DM than NAA and IAA 2.65 FM; 0.67 DM and 2.42 FM; 0.58 DM, respectively (Fig. 2a–c), whereas combination with 0.1 mg/L BAP and 1.0 mg/L 2,4-D induced biomass accumulation 6.25 FM; 1.52 DM (Fig. 2d). However, the high quantity of biomass accumulation was induced by 1.0 mg/L 2,4-D and 0.1 mg/L TDZ 7.75 FM; 1.82 DM (Fig. 2e). Similarly, 2,4-D with TDZ combination was highly induced phytochemical production and biomass accumulation in the CSC of *Polygonum multiflorum* (Thiruvengadam et al. 2016). The addition of cytokinins (TDZ, BAP, and KN) has been revealed to increase the production of phytochemicals in the CSC of *Panax ginseng* and *Artemisia absinthium* (Ali et al. 2013; Murthy et al. 2014). The influence of sucrose (10–40 g/L in MS) on biomass accumulation (Fig. 2f) was then explored.

Fig. 1 Characterization of AgNPs prepared from *B. marisflavi* culture supernatant using various analytical techniques. **a** UV-vis absorption spectrum of AgNPs exhibited a strong broad peak at 420 nm. **b**

Transmission electron microscopy (TEM) images of AgNPs. **c** Particle size distribution of AgNPs

Sucrose (30 g/L) was appropriate for biomass accumulation. Our observation was supported by one more investigation that also exposed higher biomass and phytochemical accumulation after treatment with 30 g/L of sucrose (Ali et al. 2013). Various growth medium confirmed (MS, B5, NN, and N6); MS was greater and produced high quantity of biomass accumulation 7.75 FM; 1.82 DM

(Fig. 2g). Numerous prior investigations have also found MS suitable for biomass and phytochemical accumulation in CSC (Ali et al. 2013; Thiruvengadam et al. 2016). Tracking biomass (fresh and dry mass) accumulation showed that quantity was at high on 28-day 7.75 FM; 1.82 DM (Fig. 2h). In agreement with our investigations, the 28-day-old CSC of *P. multiflorum* (Thiruvengadam et al.

Fig. 2 Effect of plant growth regulators, sucrose, media, and growth kinetics on biomass accumulation in cell suspension culture of bitter melon. **a** 2,4-Dichlorophenoxyacetic acid (2,4-D). **b** Naphthalene acetic acid (NAA). **c** Indole acetic acid (IAA). **d** 2,4-D 1.0 mg/L with benzyl amino purine (BAP) 0.1 mg/L. **e** 2,4-D 1.0 mg/L with

thidiazuron (TDZ) 0.1 mg/L. **f** Sucrose. **g** Media. **h** Growth kinetics. Means $\pm$ standard deviation of triplicates followed by the same letters are not significantly different according to Duncan's multiple range test was used to determine significant differences at $P \leq 0.05$

2016) and *A. absinthium* (Ali et al. 2013) exhibited high metabolite production and biomass accumulation.

Effect of AgNPs on biomass accumulation in CSC

The effect of AgNPs on biomass accumulation was tested in CSC. In this study, fresh and dry mass was increased (8.15 FM; 1.87 DM) with AgNPs (1 mg/L), whereas treatment with higher concentrations (10 mg/L) caused decrease (2.85 FM; 0.69 DM) in fresh and dry mass (Fig. 3a). In agreement with our observations, plant biomass (fresh and dry mass) decreases with increasing concentration of AgNPs in *Brassica rapa* (Thiruvengadam et al. 2015) and *Arabidopsis thaliana* (Kaveh et al. 2013). Our observation exhibited that higher concentrations of AgNPs (5 and 10 mg/L) were caused by the severe decline of biomass accumulation in CSC.

Effect of AgNPs on silver, MDA and H_2O_2 contents in CSC

The Ag content analysis of AgNPs-elicited (1, 5, and 10 mg/L), and non-elicited CSC was determined using ICP-MS. The results showed the Ag content was higher in

AgNPs (10 mg/L)-elicited CSC. The increase in Ag accumulation was found when the CSC was treated with higher concentration of AgNPs (Fig. 3b). Similarly, Ag accumulation was increased at higher concentrations of AgNPs in *B. rapa* (Thiruvengadam et al. 2015). Lipid peroxidation is oxidative injury that disturbs cellular membranes, lipoproteins, and other molecules that have lipids in oxidative stress conditions. The exposure of AgNPs at higher concentration could increase the ROS formation which causes peroxidative damage to membrane lipids (Dietz et al. 1999). The toxicity of NPs is due to the production of ROS in the cells (Thiruvengadam et al. 2015). Quantitative measurement of the MDA and H_2O_2 contents of CSC elicited with AgNPs (0, 1, 5 and 10 mg/L) showed significant differences (Fig. 3c, d). However, the MDA and H_2O_2 contents significantly increased when treated with 1, 5 and 10 mg/L of AgNPs. A gradual, dose-dependent increase of MDA and H_2O_2 concentrations was observed in AgNPs treatment which indicates the NP-mediated oxidative stress induction. Under stresses, oxygen in peroxisomes acquires the hydrogens resulted from the dehydrogenation of the fatty acids produced from the beta-oxidation process and ultimately changes to H_2O_2 . High levels of the H_2O_2 , in addition to direct mediation in lipid

Fig. 3 Effect of AgNPs on biomass accumulation and contents of silver (Ag), malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) in cell suspension culture extracts of bitter gourd. **a** Biomass accumulation. **b** Ag content. **c** MDA content. **d** H_2O_2 content. Means $\pm$ standard deviation of triplicates followed by the same letters are not significantly different according to Duncan's multiple range test and was used to determine significant differences at $P \leq 0.05$

peroxidation, can increase the rate of this process by accelerating the Haber–Weiss reaction and forming the hydroxyl radical (Valizadeh-Kamran et al. 2018). An enhancement of MDA and H_2O_2 levels in plant tissues under the effect of AgNPs in turnip (Thiruvengadam et al. 2015) and mung bean (Nair and Chung 2015).

Effect of AgNPs on phenolic compounds production in CSC

Phenolic biosynthetic pathways are induced by the adding of elicitors in CSC (Thiruvengadam et al. 2016). In this study, the AgNPs-elicited CSC induced the accumulation of total phenolic and flavonoid contents in bitter gourd. A gradual increase in total phenolic and flavonoid contents concentration was noticed in the AgNPs treatment, and the CSC treated with 5 mg/L of AgNPs accumulates elevated total phenolic and flavonoid contents (Fig. 4a, b). Meanwhile, CSC treated with 10 mg/L of AgNPs decrease the contents of total phenolic and flavonoid (Fig. 4a, b). Similarly, the total phenolic and flavonoid contents enhanced the elicitation Ag and gold NPs in callus culture of *Prunella vulgaris* (Fazal et al. 2016). In vitro-regenerated shoots of *Vanilla planifolia* cultured in MS medium supplemented with 25, and 50 mg/L AgNPs showed a substantial upsurge

in total phenolic contents (Spinoso-Castillo et al. 2017). Quantitative and qualitative assessments were achieved with ultra-HPLC on individual phenolic compounds from non-elicited and AgNPs-elicited CSC extracts (Table 1). Twenty individual phenolic compounds were detected, namely: eight hydroxybenzoic acids (protocatechuic, gallic, *p*-hydroxybenzoic, syringic, gentisic, salicylic, vanillic and β -resorcylic acids), five hydroxycinnamic acids (caffeic, *p*-coumaric, *t*-cinnamic, chlorogenic, and ferulic acids) and seven flavonols (quercetin, myricetin, rutin, catechin, kaempferol, naringenin and biochanin A). AgNPs-elicited CSC contained a significantly increased hydroxybenzoic acid (1713.40 μ g/g), hydroxycinnamic acid (1080.10 μ g/g) and flavonols (1822.37 μ g/g) than non-elicited CSC at 1394.42, 944.52 and 1199 μ g/g, respectively. This increase may be due to the influence that silver ions have on metabolic flux among various phenolic acids. Similarly, silver induced productions of rosmarinic, ferulic and caffeic acids, but decreased the contents of salvianolic and cinnamic acids (Xing et al. 2015). AgNPs can enhance the activation of the enzymatic pathway that contributes to the accumulation of phytochemicals (Zhang et al. 2004). Similarly, the elicitation Ag– SiO_2 core-shell nanoparticles improved the content of artemisinin in *A. annua* hairy root cultures (Zhang et al. 2013). Consistently, elicitation of cobalt NPs increased the

Fig. 4 Effect of AgNPs on phytochemicals in cell suspension culture extracts of bitter gourd. **a** Total phenolic content. **b** Total flavonoid content. Means $\pm$ standard deviation of triplicates followed by the same letters are not significantly different according to Duncan's multiple range test and was used to determine significant differences at $P \leq 0.05$

content of artemisinin in CSC of *A. annua* (Jamshidi et al. 2016). This suggests that AgNPs-elicitation improved the amount of individual phenolic compounds, total phenolic and flavonoid contents in CSC of bitter gourd.

Effect of AgNPs on pharmacological activities in CSC

DPPH is a suitable indicator for studying the free radical-scavenging activities of phenolic compounds (Esmailzadeh Kenari et al. 2014). Figure 5 shows the antioxidant activity of AgNPs-elicited CSC in comparison to non-elicited CSC. The phenolic levels were significantly higher

Table 1 Ultra-high performance liquid chromatography (UHPLC) analysis of phenolic compounds in AgNPs-elicited and non-elicited cell suspension culture (CSC) extracts of bitter gourd

No.	Phenolic compounds	Concentration (µg/g dry mass)	
		Non-elicited CSC	Elicited CSC (AgNPs 5.0 mg/L)
Hydroxybenzoic acid			
1	<i>p</i> -Hydroxybenzoic acid	129.12 ± 1.0 ⁱ	144.15 ± 1.2 ⁱ
2	Gallic acid	175.15 ± 2.0 ^g	181.10 ± 2.0 ^g
3	Protocatechuic acid	35.25 ± 1.0 ⁿ	51.50 ± 1.0 ⁿ
4	Syringic acid	61.15 ± 1.0 ^l	78.25 ± 1.0 ^l
5	Gentisic acid	553.00 ± 1.5 ^a	695.50 ± 2.0 ^a
6	Salicylic acid	255.50 ± 2.1 ^e	350.20 ± 2.0 ^d
7	Vanillic acid	130.00 ± 1.5 ⁱ	140.50 ± 1.0 ^j
8	β-Resorcylic acid	55.25 ± 1.0 ^m	72.20 ± 1.0 ^m
	Total	1394.42	1713.40
Hydroxycinnamic acid			
9	Caffeic acid	131.50 ± 1.5 ⁱ	154.50 ± 2.0 ^h
10	<i>p</i> -Coumaric acid	117.12 ± 1.0 ^j	140.25 ± 1.0 ^j
11	Ferulic acid	191.55 ± 1.0 ^f	220.15 ± 1.5 ^f
12	Chlorogenic acid	501.10 ± 1.0 ^b	555.10 ± 1.0 ^b
13	<i>t</i> -Cinnamic acid	3.25 ± 0.2 ^p	10.10 ± 0.5 ^p
	Total	944.52	1080.10
Flavonols			
14	Myricetin	174.25 ± 2.0 ^g	242.10 ± 2.0 ^e
15	Quercetin	151.12 ± 1.0 ^h	221.12 ± 2.0 ^f
16	Kaempferol	88.25 ± 0.5 ^k	145.00 ± 1.5 ⁱ
17	Catechin	361.12 ± 2.0 ^c	455.00 ± 2.0 ^c
18	Rutin	285.47 ± 1.5 ^d	352.00 ± 2.0 ^d
19	Naringenin	118.32 ± 1.2 ^j	130.00 ± 1.0 ^k
20	Biochanin A	20.47 ± 0.5 ^o	27.15 ± 1.5 ^o
	Total	1199	1822.37

Mean $\pm$ SD within a row followed by the same letters are not significantly different according to Duncan's multiple range test (DMRT) and was used to determine significant differences at $P \leq 0.05$

in the AgNPs-elicited CSC, which directly impact their antioxidant activity (Fig. 5a). Figure 5b exhibits the reducing capability of CSC proposed that AgNPs-elicited have the more antioxidant activity (AOA) than non-elicited CSC. The AOA of the AgNPs-elicited CSC was higher than non-elicited CSC (Fig. 5c). Consistently, AgNPs-elicitation stimulated the antioxidant defense system and also a significant amount of taxol production in cells of *Corylus avellana* (Jamshidi et al. 2016). Correspondingly, the higher enzymatic activity of POD was demonstrated in biologically synthesized AgNPs-treated seedlings of *Bacopa monnieri* (Krishnaraj et al. 2012). Thiruvengadam et al. (2015) demonstrated that bio-synthesized AgNPs significantly up-regulated the gene expression of three antioxidant enzymes (*CAT*, *POD*, and *GST*) in turnip

Fig. 5 Effect of AgNPs on antioxidant activities in cell suspension culture extracts of bitter gourd. **a** Free radical-scavenging activity by DPPH method. **b** Total Fe^{3+} – Fe^{2+} reductive potential reference antioxidants (butylated hydroxytoluene). **c** Total antioxidant capacity (TAC) by phosphomolybdenum method [TAC was expressed as

equivalents of α -tocopherol ($\mu\text{g/g}$ of extract)]. Means $\pm$ standard deviation of triplicates followed by the same letters are not significantly different according to Duncan's multiple range test and was used to determine significant differences at $P \leq 0.05$

seedlings. The α -amylase digests the carbohydrates and increases postprandial glucose levels in diabetic patients. Inhibiting the activity of α -amylase can control postprandial hyperglycemia, and reduce the risk of developing diabetes (Kar et al. 2003). Similarly, bitter gourd plant extract was used for controlling postprandial hyperglycemia in diabetes patients (Poovitha and Parani 2016). In this study, AgNPs-elicited CSC extracts significantly inhibited the activity of α -amylase enzyme (Fig. 6a). The results showed that α -amylase was significantly inhibited in a concentration-dependent manner following incubation with numerous concentrations of AgNPs-elicited and non-elicited CSC extracts. AgNPs-elicited and non-elicited CSC extracts at the concentrations of 100 $\mu\text{g/mL}$ caused 75.50 and 61.00% of α -amylase enzyme inhibition, respectively (Fig. 6a). Meanwhile, the standard compound acarbose showed 80% of inhibition at the same concentration used in the test (Fig. 6a). Similarly, bio-synthesized AgNPs were revealed to inhibit the carbohydrate digestive

enzymes, α -amylase and α -glucosidase (Balan et al. 2016). AgNPs-elicited CSC extracts displayed a significant increase in antimicrobial activity when compared to non-elicited CSC (Fig. 6b). Previously, AgNPs-treated plants enhanced the anti-bacterial and anti-fungal activities in *Achillea millefolium* (Ghanati et al. 2014). The inhibition of CSC extracts in MCF-7 and HT-29 cell lines were compared with tamoxifen and 5-fluorouracil (Fig. 7a, b), respectively. AgNPs-elicited CSC extracts (200 $\mu\text{g/mL}$) exhibited the highest anticancer activity in HT-29 and MCF-7, while non-elicited CSC extracts showed weak inhibition. Additionally, MCF-7 cells displayed higher percent inhibition values of 58.98% than HT-29 cells, 49.09% (Fig. 7a, b). In agreement with this data, Zakaria et al. (2011) demonstrated that bioactive compounds inhibited MCF-7 and HT-29 cells propagation. Ghanati et al. (2014) reported that AgNPs-treated *Achillea millefolium* plants were increased the inhibitory effects of HeLa cells.

Fig. 6 Effect of AgNPs on antidiabetic and antimicrobial potential in cell suspension culture (CSC) extracts of bitter gourd. **a** In vitro α -amylase activity. **b** Antimicrobial activity using disc diffusion method. Means $\pm$ standard deviation of triplicates followed by the same letters are not significantly different according to Duncan's multiple range test and was used to determine significant differences at $P \leq 0.05$

Fig. 7 Effect of AgNPs on cell viability of MCF-7 and HT-29 cell lines in cell suspension culture (CSC) extracts of bitter melon. **a** MCF-7. **b** HT-29. Means $\pm$ standard deviation of triplicates followed by the same letters are not significantly different according to Duncan's multiple range test and was used to determine significant differences at $P \leq 0.05$

Conclusions

The Ag content was enhanced in CSC as a result of AgNPs treatments. AgNPs-elicitation has resulted in the higher amount of MDA and H_2O_2 contents in the CSC. Phenolic constituents of flavonols, hydroxybenzoic and hydroxycinnamic acids were higher in AgNPs-elicited than in non-elicited CSC. The phenolic compounds and pharmacological activities (antioxidant, antidiabetic, antimicrobial and anticancer) were higher in the AgNPs-elicited in comparison to the non-elicited CSC. Our method will be utilized for bioprocess engineering and the large-scale production of bioactive compounds through cell suspension cultures.

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Author contributions MT designed and performed the experiment and also wrote the manuscript. IMC, KR and GR analyzed the experiments

and helped to write the manuscript. All authors read and approved the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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