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Research Article

Silver nitrate and silver nanoparticles for enhancing the caffeic acid derivates production in cell suspension culture of *Lactuca undulata* Ledeb

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Abstract

***Lactuca undulata* Ledeb. is a medicinal plant belonging to the Asteraceae family. Chicoric acid is one of the main derivatives of caffeic acid, with various pharmacological and biological properties. This study was conducted to optimize cell suspension culture and enhance chicoric acid production in *L. undulata* by eliciting secondary metabolites using silver nitrate ($AgNO_3$) and silver nanoparticles (AgNPs). Seeds were cultured on ½ MS medium to produce sterile seedlings. In order to produce callus, leaf and root explants were obtained from 2 months old sterile seedlings and placed on ½ MS medium containing 2 mg/L 2,4-D/ plus 0.5 mg/L Kin and 2 mg/L 2,4-D/ plus 2 mg/L Kin, respectively. Cell suspension cultures from leaf and root-derived calluses were established and treated with different concentrations (0, 2, and 4 mg/L) of $AgNO_3$ and AgNPs during the logarithmic growth phase. Then cells were harvested after 24, 48, and 72 hours. Overall, elicitation by AgNPs was more effective on chicoric acid production compared to $AgNO_3$. The highest amounts of chicoric acid (9.7 ± 0.48 mg/g DW) and caffeic acid (15.3 ± 0.8 mg/L) were found in leaf and root cell suspension cultures after 48 hours of exposure to 4 and 2 mg/L AgNPs, respectively. In contrast, 4 mg/L of $AgNO_3$ stimulated the greatest accumulation of chlorogenic acid (4.56 ± 0.3 mg/g DW) in root cell culture at 72 h after elicitation. The current results revealed that the use of AgNPs can be an efficient strategy to improve cichoric acid accumulation in cell suspension culture.**

Keywords: $AgNO_3$, AgNPs, Cell culture, Chicoric acid, Elicitation, *Lactuca undulata*

Introduction

Plant secondary metabolites (SMs) play various important roles, such as involvement in defense mechanisms (Ryals *et al.*, 1996), response to biological and environmental stresses (Yang *et al.*, 1997), and pollination (Abrol, 2012). SMs are produced through pathways derived from primary metabolites. However, they are not considered essential for the growth and development of plants (Bruce, 2022).

Chicoric acid, as a phenolic acid compound, has various pharmacological and biological properties (Lee and Scagel, 2013; Azay-milhau *et al.*, 2013). This metabolite has been identified in different plant families (Lee and Scagel, 2013). Asteraceae is the most important family, containing high levels of chicoric acid. The amount of chicoric acid produced in plants depends on different factors such as plant varieties (Jaiswal *et al.*, 2011), harvest time, plant age, and plant drying temperature (Shekarchi *et al.*, 2012). Caffeic acid is a precursor for the biosynthesis of chicoric and chlorogenic acids. Caffeic acid possesses anti-tumor properties (Chung *et al.*, 2004) and stimulates immune system activity (Manayi *et al.*, 2015). Chlorogenic acid is made from caffeic acid and (−) quinic acid. It is found in all higher plants and is known as a powerful antioxidant (Johanson *et al.*, 2003). Chlorogenic acid also plays a role in the defense system against pathogens (Cle *et al.*, 2008).

Nowadays, Plant cell and tissue culture is applied as a useful technique to produce valuable secondary metabolites. On the other hand, enhancement of SMs using different kinds of elicitors is an attractive approach in plant cell and tissue culture techniques (Giri and Zaheer, 2016). Elicitors are low molecular weight compounds that can be introduced into living cells and stimulate SMs biosynthesis (Ebel and Cosio, 1994). In the last few decades, nanotechnology has made many advances in cell culture and the production of valuable pharmaceutical compounds. Nanoparticles (NPs) are particles with a size range between 1 to 100 nm that have the capacity to induce secondary metabolite production in different plant species. NPs are classified based on their size, morphology, and physical and chemical characteristics. One of the most common classifications of NPs is according to their physical and chemical properties (Laurent *et al.*, 2010). Silver

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nanoparticles (AgNPs) are one of the most widely used elicitors in plant cell culture. This NP can enter the plant cell and affect growth, development, physiology, and metabolism (Chung *et al.,* 2018; Anjum *et al.,* 2019; Amir *et al.,* 2019). It seems that the most common response of plants to NPs is ROS production and oxidative stress at the cellular level (Zia-ur-Rehman *et al.,* 2022).

Lactuca undulata is an annual herb belonging to the Asteraceae family that is known as a new source of chicoric acid. This species contains different valuable compounds, including caffeic acid, chicoric acid, and chlorogenic acid. The highest amount of chicoric acid (2.31 mg/g DW) was obtained from the stem of *L. undulata* during the reproductive stage (Ramezannejhad *et al.,* 2019a). This herbaceous species has a relatively short growth period and can be easily grown in the field or in *in vitro* conditions. For this reason, this plant was selected as a suitable model for the production of chicoric acid under tissue culture conditions.

So far, only a few reports have been published on chicoric acid production under *in vitro* conditions in various plant species, such as *Echinacea purpurea* (Ramezannejhad *et al.,* 2019b; Butiuc-Kuel *et al.,* 2012). However, there has been no report on optimizing the cell suspension culture of *L. undulata* to produce chicoric acid and other phenolic acids using AgNPs and $AgNO_3$ elicitors. The goal of this study was to assess the effects of different concentrations of $AgNO_3$ and AgNPs on the production of phenolic acids in cell suspension culture.

Material and methods

Plant material and seed *in vitro* culture: *L. undulata* seeds were collected from Binalood Mountains in northeastern Iran (N: 36°25 ′ and E: 36°33) during June and July 2018. The seeds were sterilized using a 7% (V/V) commercial bleach solution for 1 min, followed by 10 min with 5% commercial Clorox. They were then washed and rinsed four times with sterile water. Sterilized seeds were cultured on 1/2 MS (Murashige and Skoog, 1962) medium supplemented with sucrose (15 g/L) and agar-agar (8 g/L) at pH 5.7. Then, the medium culture was autoclaved at 121 °C for at least 20 minutes. Afterward, cultures were incubated in a growth chamber at 25 ± 2 °C with a 16 h light and 8 h dark photoperiod for 2 months. The sterile seedlings obtained were used as a source of root and leaf explants.

According to our previous experiments, we selected 2,4-D and kin, which induced the highest chicoric acid production in leaf and root-derived callus (Ramezannejhad *et al.,* 2022). Then, leaf and root explants were cultured on ½ MS medium cultures supplemented with 2 mg/L 2,4-D/ plus 0.5 mg/L Kin and 2 mg/L 2,4-D/ plus 2 mg/L Kin, respectively. Then, cultures were placed in a growth chamber at 25±2 °C with a 16-hour light and 8-hour dark photoperiod. Sub-culturing was performed every 14 days. After 2 months, the obtained callus was used to establish a cell suspension culture.

Establishment of cell suspension culture: In the first step, 0.5 g of callus was transferred into 20 mL of ½ MS medium supplemented with 30 g/L sucrose without agar, and homogenized at pH 5.8 on a rotary shaker (120 rpm, 25 °C). Also, 0.5 mg/L Kin + 2 mg/L 2,4-D and 2 mg/L Kin + 2 mg/L 2,4-D were added to cell suspension cultures from leaf and root-derived calluses, respectively. The cell suspension culture was subcultured in a fresh medium every 14 days. After a month, homogenized cells were harvested on whatman grade 40 filter paper.

Elicitor preparation and elicitation: AgNPs were purchased from Sigma-Aldrich Company (Anala R grade). The size of the spherical nanoparticles was 35 nm.

To obtain a fresh stock solution, 10 mg of $AgNO_3$ and AgNPs elicitor were dissolved in 100 mL of ½ MS medium culture. Two different concentrations (2 and 4 mg/L) of AgNPs and $AgNO_3$ were prepared from the stock solution, along with the control. The pH value used during cell suspension studies ranged from 6.5 to 7. Elicitors were added during the logarithmic phase (18 days) to the cell suspension culture. After harvesting the cells, chicoric acid, chlorogenic acid, and caffeic acid were measured after 24, 48, and 72 h.

Cell viability: To evaluate the viability of cells, Evan's blue staining test (Baker and Mock, 1994) was used. At first, a 1 mL sample was mixed with 0.1% Evan's blue stain for 3–4 min and then at least 500 cells were counted under a light microscope at different exposure times (24, 48, and 72 h). Growth of the cell suspension and cell viability were measured at 3-day intervals up to 30 days. The number of cells was calculated by the following equation:

Cells/mL = [(cell count × diluents factor (mL))/number of counting squares] × 10^4.

Extraction of caffeic acid derivatives: The harvested cells were dried at 40 °C for 3 days. Then, dried samples (0.01 g) were dissolved in 20% (V/V) aqueous acetonitrile (10 ml). The prepared extractions were put in the shaker under full darkness for 24 hours. Then the extracts were filtered and centrifuged at 13000 g for 10 min. The supernatant was used to determine caffeic acid derivatives content (Luo *et al.,* 2003).

HPLC analysis: An HPLC method was applied to determine the amount of caffeic acid derivatives. To perform this measurement, we used an HPLC (Merck Hitachi) equipped with a eurospher C18 (250 x 4 mm) column and a UV/VIS detector. Acetonitrile and aqueous acetic acid (0.1%) were used as phases A and B, respectively. The flow rate was 1 mL/min, and caffeic acid derivatives were detected at 276 and 330 nm wavelengths. The amount of chicoric acid, caffeic acid, and chlorogenic acid was determined by the

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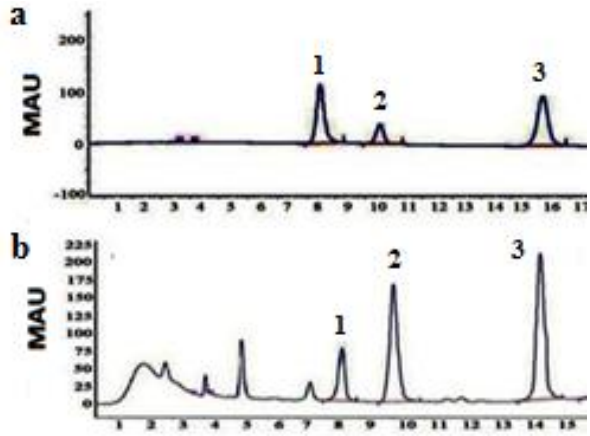


Figure 1. HPLC Chromatograms: a) Standard curve of chlorogenic acid (1), cichoric acid (2) and caffeic acid (3) and b) aqueous acetonitrile extract of cell suspension culture from root derived callus after elicitation by 2 mg/L AgNPs after 48 h. The absorption intensity is expressed in MAU (measurement of absorbance unit).

standard curve (Figure 1).

Statistical analysis: All experiments were performed in a completely randomized design with three replicates. The mean values were compared by Duncan's multiple range test at P=0.05 significant level using SAS software (version 9.1). All graphs were drawn by Excel software.

Results and discussion

Callus induction: In order to produce callus, leaf and root explants obtained from 2 months old sterile seedlings of *L. undulata* were cultured on ½ MS culture medium containing different concentrations of plant growth regulators (2,4-D and Kin) (Figure 2a). The first signs of callus production were observed about four to six days after transferring the leaf and root explants to the culture medium. The results showed that the callus induction rate of both leaf and root explants was 100%. Morphological properties showed that leaf derived callus on ½ MS medium supplemented with 0.5 mg/L Kin + 2 mg/L 2,4-D was light green (Figure 2b). Also, root derived callus on ½ MS medium supplemented with 2 mg/L Kin + 2 mg/L 2,4-D was cream light in color (Figure 2c). Callus induction depends on various factors, such as the type of explant and the type and levels of plant growth regulators. Despite the abundant applications of auxins and cytokinins in callus induction, there is still little information about their functional mechanisms at the molecular level (Ikeuchi *et al*., 2013). Dimitrova *et al*. (2010) reported that the effect of auxin on callus induction is through its effect on the cell cycle. In this way, following activation of the auxin signaling pathway by the ARF7 and ARF19 transcription factors and the LBD (Lateral Organ Boundaries Domain) transcription factor family, which is downstream of ARF, callus induction takes place in selected explants (Zhang *et al*., 2020). Although the mechanism of callus induction by cytokinins is less known, it seems cytokinins induce callus induction by activating cell cycle cyclins, especially CYCD3;1 (Bano *et al*., 2022).

Cell suspension culture and growth curve: Cell suspension culture is known as a powerful technique that can be used for the production of plant SMs. In this regard, cell suspension culture establishment was considered. In the present study, leaf and root cell cultures were harvested at different time intervals to count the number of cells (Figure 3a). The obtained results revealed that the cell number was significantly affected by culture duration (Figure 3b). A cell growth curve shows the lag, logarithmic, stationary, and death phases of cell growth. The obtained data showed that the lag phase was observed for the first nine days, followed by a logarithmic phase for 12 days in both cell suspension cultures. The highest number of leaf and root cells was observed during the logarithmic phase in 20-day-old suspension cultures. As the number of days increased, the percentage viability of cells decreased. The lowest percentage of cell viability reached 62% and 52% at 30-day-old leaf and root cell suspension cultures, respectively (Figure 3c).

Effects of AgNPs and $AgNO_3$ on cell viability: After elicitation by various concentrations of AgNPs and $AgNO_3$ (0, 2, and 4 mg/L), the viability of leaf and root cell suspensions was measured. The results indicated that cell viability was reduced after elicitation by both elicitors compared to the control in the leaf and root cell suspension cultures. By adding 2 and 4 mg/L $AgNO_3$, cell viability was significantly decreased both in leaf and root cell suspension cultures compared to AgNPs treatment. Meanwhile, applying 4 mg/L $AgNO_3$ caused the lowest percentage of cell viability in leaf (46%) and root (53%) cell suspension cultures (Figure 4a, b). Figures 4c to f show the microscopic images of viable (v) and non-viable (nv) leaf and root cell suspensions after elicitation by AgNPs and $AgNO_3$. Microscopic images showed that the cells were round or oval. According to Evans blue staining (staining the dead cells) results, the viability of cell suspension culture of leaf and root-derived callus decreased by

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Figure 2. a) *Lactuca undulata* plant in nature; b) fifteen-days old leaf derived callus on ½ MS medium supplemented with 0.5 mg/L Kin + 2 mg/L 2,4-D; c) fifteen-days old root derived callus on ½ MS medium supplemented with 2 mg/L Kin + 2 mg/L 2,4-D.

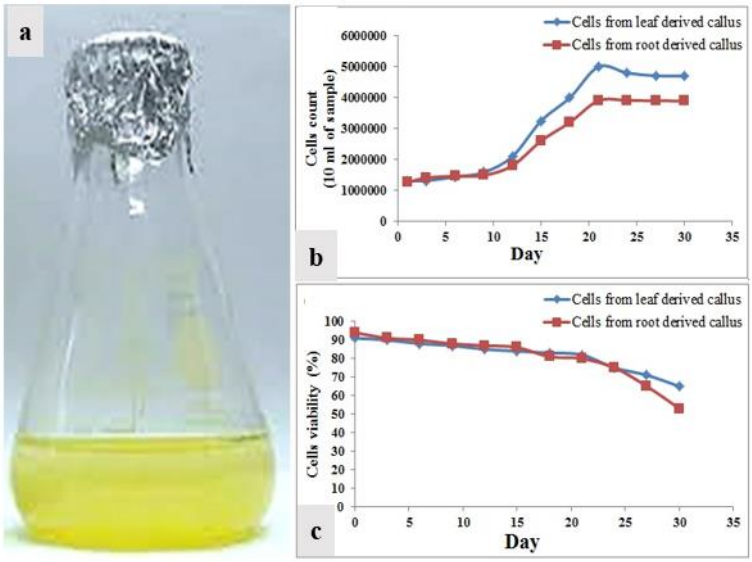


Figure 3. a) Cell suspension culture obtained from root derived callus on ½ MS containing 2 mg/L Kin + 2 mg/L 2,4-D; b) growth curve of cell suspension culture from leaf and root-derived callus of *Lactuca undulata* and c) cell viability during cell suspension culture establishment (n=3).

adding $AgNO_3$ and AgNPs elicitors. Although the elicitors have important effects on plant cell growth and metabolism, specific attention must be paid to the concentration and duration used. It seems that specific concentrations of AgNPs and $AgNO_3$ ions can be toxic and suppress gene expression and enzyme activities. Depending on the size of the surface, AgNPs can bind to biomolecules and reduce those (Singh *et al.*, 2009). By increasing exposure time to the elicitors, negative effects were induced in cells, and the expression of genes involved in growth and metabolism (Naguib *et al.*, 2012).

Effects of AgNPs and $AgNO_3$ on phenolic acids production: The effects of various concentrations of applied elicitors on the accumulation of chicoric acid were recorded in the cell suspension culture of *L. undulata* (Table 1). The obtained results showed that AgNPs were more effective than $AgNO_3$ on chicoric acid accumulation in leaf-derived cell suspension culture. But in root-derived cell suspension culture, $AgNO_3$ was more effective than AgNPs. The present study found that application of both elicitors at 2 and 4 mg/L did not significantly affect the production of chicoric acid in the cell suspension culture from leaf and root-derived callus at 24 h after elicitation when compared to the control. However, applying 2 mg/L AgNPs to leaf cell cultures had a significant effect on chicoric acid content (7.95±0.7 mg/g DW) after 72 h. On the other hand, the greatest amount of chicoric acid (9.7±0.48 mg/g DW) was recorded in the cell suspension culture from leaf-derived callus 48 h after elicitation with 4 mg/L AgNPs. Whereas, it has been

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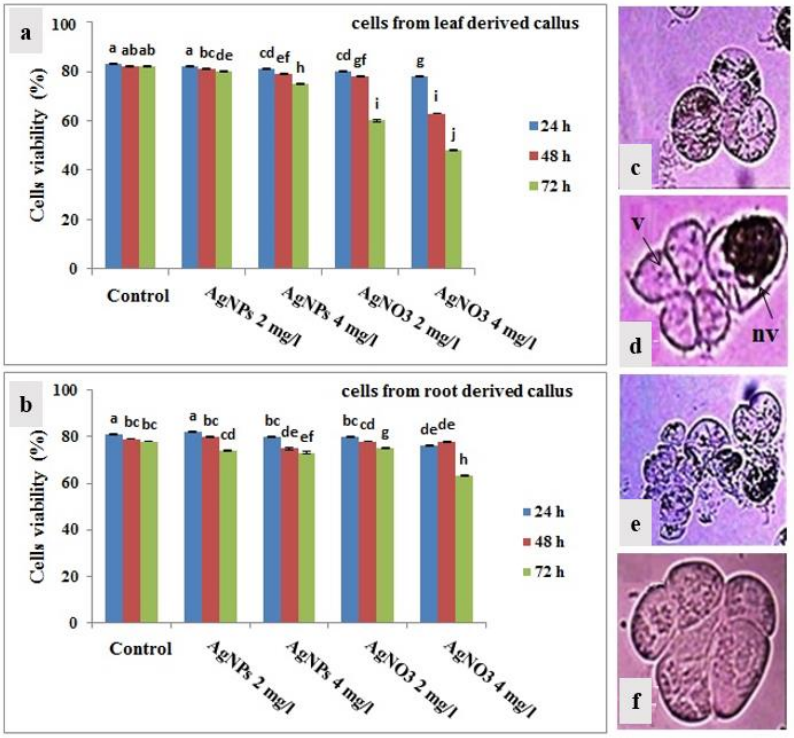


Figure 4. Cell viability during cell suspension culture of the leaf (a) and root (b) derived callus of *L. undulata* (n=3). Evans Blue staining of cell suspension cultures of leaf derived callus treated with 4 mg/L $AgNO_3$ after 48 h (c), 4 mg/L $AgNO_3$ after 72 h (d), 4 mg/L AgNPs after 48 h (e) and 4 mg/L AgNPs after 72 h (f). Different letters within each column show significance at P = 0.05 by the least significant difference (LSD) test. Viable (v) and non-viable (nv) cells.

Table 1. Chicoric acid (mg/g dry weight) contents recorded in cell suspension culture from leaf and root-derived callus of *Lactuca undulata* after different exposure times to 2 and 4 mg/L of $AgNO_3$ and AgNPs elicitors. Data are means ±SE.

	treatment	2 mg/L			4 mg/L		
leaf derived callus		24(h)	48(h)	72(h)	24(h)	48(h)	72(h)
	Control	2.5±0.65^{c}	2.53±0.66^{c}	2.69±0.75^{c}	2.5±0.65^{cd}	2.53±0.66^{cd}	2.69±0.75^{cd}
	AgNPs	4.6±0.44^{c}	5.2±0.85^{c}	7.95±0.7^{a}	3.44±0.48^{d}	9.7±0.48^{a}	6.45±0.25^{b}
	$AgNO_3$	4.23±0.12^{c}	5.12±0.58^{c}	6.2±0.55^{b}	3.61±0.32^{d}	5.14±0.1^{c}	3.56±0.4^{d}
		2 mg/L			4 mg/L		
root derived callus	Control	4.35±0.09^{c}	4.37±0.26^{bc}	4.36±0.25^{c}	4.35±0.09^{ab}	4.37±0.26^{ab}	4.36±0.25^{ab}
	AgNPs	5.11±0.48^{bc}	6.3±0.46^{a}	5.69±0.45^{b}	4.5±0.13^{ab}	4.44±0.07^{b}	3.01±0.45 ^{d}
	$AgNO_3$	4.98±0.48^{bc}	5.23±0.68^{b}	5.87±0.25^{ab}	5.65±0.77^{a}	3.56±0.43^{c}	3.45±0.55^{cd}

Different letters within each treatment indicate significance at P=0.05 by the least significant difference (LSD) test (n=3).

reported that chicoric acid reached 9.54 mg/g DW in root cell suspension culture of *Echinacea purpurea* after 48 h of exposure to 2 mg/L (Ramezannejhad *et al.*, 2019b). *Echinacea purpurea* is the main source of chicoric acid in the world (Lee and Scagel, 2013). The effects of NPs on the production of secondary metabolites depend on the synthetic origin, exposure time, and concentration of elicitors along with plant parameters such as the type of tissue, cellular cycle, plant species, nutritional conditions, and developmental age, among others (Chung *et al.*, 2018; Patel and Krishnamurthy, 2013). In agreement with the current study, another study has reported that the effects of AgNPs on the growth of rice were more prominent than those of $AgNO_3$ under biotic stress (Ejaz *et al.*, 2018). Chung *et al.* (2018) demonstrated that elicitation of *Cucumis anguria* L. root hairs by 2 mg/L AgNPs for phenolic acids production was more effective than 2 mg/L $AgNO_3$ (Chung *et al.*, 2018). AgNPs can trigger stresses in plant cells through different mechanisms, including the stimulation of ROS production (Sharma *et al.*, 2016), mechanical damage and blockage of cell pores (Asli and Neuman, 2009), catalytic effects on oxidation and reduction reactions (Hossain *et al.*, 2013), damaging macromolecules, and changing chemical properties of the cell environment (Sund *et al.*, 2011). The phenolic compounds produced by plants serve as defenses against stress (Shalaby and Horwitz, 2015). AgNPs can activate the signal transduction cascades that can induce the biosynthesis of phenolic compounds (Yousefi *et al.*, 2016).

Our previous studies have shown that the amount of chicoric acid in leaf and root explants of *Lactuca undulata* is 1.11 and 1.76 mg/g DW, respectively (Ramezannejhad *et al.*, 2022). However, the amount of chicoric acid in leaf and root-derived callus was 4.9 and

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Table 2. Chlorogenic acid content (mg/g dry weight) recorded in cell suspension culture from leaf and root-derived callus of *Lactuca undulata* after different exposure times to 2 and 4 mg/L of $AgNO_3$ and AgNPs elicitors. Data are means ±SE.

	treatment	2 mg/L			4 mg/L		
		24(h)	48(h)	72(h)	24(h)	48(h)	72(h)
leaf derived callus	Control	2.34±0.19^{ab}	2.54±0.26^{a}	2.59±0.25^{a}	2.34±0.19^{d}	2.54±0.26^{cd}	2.59±0.25^{cd}
	AgNPs	2.32±0.23^{ab}	2.45±0.27^{ab}	2.23±0.3 ^{b}	2.96±0.19^{c}	4.35±0.26^{a}	3.45±0.25^{b}
	$AgNO_3$	2.12±0.4 ^{ab}	2.23±0.31^{ab}	2.34±0.3^{ab}	2.84±0.2^{c}	2.76±0.24^{c}	2.65±0.25^{c}
		2 mg/L			4 mg/L		
root derived callus	Control	2.5±0.29^{cd}	2.6±0.36^{cd}	2.65±0.25^{cd}	2.5±0.29^{c}	2.6±0.36^{c}	2.65±0.25^{c}
	AgNPs	2.78±0.23^{cd}	3.25±0.27^{b}	4.48±0.3^{a}	2.69±0.4^{c}	2.45±0.06^{c}	2.04±0.3^{d}
	$AgNO_3$	2.56±0.55^{cd}	2.28±0.55^{d}	2.94±0.55^{c}	2.78±0.33^{c}	3.45±0.37^{b}	4.56±0.3^{a}

Different letters within each treatment indicate significance at P=0.05 by the least significant difference (LSD) test (n=3)

Table 3. Caffeic acid (mg/g dry weight) content recorded in the cell suspension cultures from leaf and root-derived callus of *Lactuca undulata* after different exposure times to 2 and 4 mg/L of $AgNO_3$ and AgNPs elicitors. Data are means ±SE.

	treatment	2 mg/L			4 mg/L		
		24(h)	48(h)	72(h)	24(h)	48(h)	72(h)
leaf derived callus	Control	16.34±0.6^{a}	16.5±0.68^{a}	16.87±0.75^{a}	16.34±0.68^{ab}	16.5±0.68^{ab}	16.87±0.75^{ab}
	AgNPs	15.6±0.48^{ab}	15.98±0.8^{ab}	15.78±0.75^{ab}	17.35±0.48^{a}	15.52±0.48^{b}	15.34±0.25^{b}
	$AgNO_3$	10.34±0.63^{c}	13.23±0.7^{b}	9.45±0.45^{a}	13.2±0.39^{c}	12.5±0.1^{d}	10.23±0.45^{e}
		2 mg/L			4 mg/L		
root derived callus	Control	13.2±0.63^{b}	13.5±0.68^{b}	13.52±0.72^{b}	13.2±0.63^{a}	13.5±0.68^{a}	13.52±0.72^{a}
	AgNPs	14.2±0.45^{ab}	15.3±0.8^{a}	11.09±0.71^{d}	6.2±0.63^{c}	7.5±0.27^{c}	6.78±0.45^{c}
	$AgNO_3$	13.5±0.48^{b}	14.3±0.68^{ab}	12.2±0.25^{c}	8.32±0.77^{b}	5.45±0.46^{d}	6.32±0.55^{cd}

Different letters within each treatment indicate significance at P=0.05 by the least significant difference (LSD) test (n=3).

Table 4. Data from correlation analysis between the amount of chicoric acid, caffeic acid, and chlorogenic acid in cell suspension culture from leaf and root-derived callus of *Lactuca undulata* after different exposure times to 2 and 4 mg/L of $AgNO_3$ and AgNPs elicitors.

	AgNPs				AgNO3		
	Chicoric acid	Chlorogenic acid	Caffeic acid		Chicoric acid	Chlorogenic acid	Caffeic acid
Caffeic acid	0.12^{ns}	0.72**	1	Caffeic acid	0.75**	0.73**	1
Chlorogenic acid	0.81**	1	0.72**	Chlorogenic acid	0.13 ^{ns}	1	0.73**
Chicoric acid	1	0.81**	0.12 ^{ns}	Chicoric acid	1	0.13 ^{ns}	0.75**

* and ** correlation is significant at the 5% and 1% level. Ns means non-significant

4.59 mg/g DW, respectively. In contrast, when cells derived from leaf callus were treated with AgNPs at a concentration of 4 mg/L, chicoric acid accumulated almost nine times more than in leaf explants.

Table 2 shows the effects of AgNPs and $AgNO_3$ on chlorogenic acid contents in leaf and root cell suspension cultures. The results showed that adding 2 mg/L of both elicitors to leaf cell suspension culture did not cause a significant change in the content of chlorogenic acid compared to the control. In addition, a notable increase in chlorogenic acid content (4.35±0.26 mg/g DW) was observed 48 h after treatment with 4 mg/L AgNPs in the leaf cell suspension. On the other hand, the results showed that the application of 2 mg/L AgNPs to root cell suspension cultures at 72 h after treatment increased the content of chlorogenic acid (4.48±0.3 mg/g DW). Meanwhile, applying 4 mg/L $AgNO_3$ significantly increased chlorogenic acid content (4.56±0.3 mg/g DW) in root cell suspension 72 h after elicitation (Figure 3d).

The current results showed that adding 2 mg/L AgNPs to leaf cell suspension culture did not alter the amount of caffeic acid after 24, 48, and 72 h after treatment. Caffeic acid was detected at the highest level in leaf-derived cell suspension cultures when exposed to 4 mg/L AgNPs after 24 hours (17.35±0.48 mg/g DW). Additionally, the application of 4 mg/L $AgNO_3$ significantly decreased the accumulation of caffeic acid in root cell suspension cultures at 48 h after treatment compared to the control. On the other hand, 4 mg/L AgNPs showed inhibitory effects on caffeic acid accumulation in root cell culture at all tested incubation times compared with the control (Table 3). After exposure to various concentrations of both elicitors, we found that caffeic acid was almost eliminated. Caffeic acid is known as a precursor of chicoric acid and chlorogenic acid. In the presence of cinnamoyl CoA ligase, it is converted to caffeoyl CoA. The produced caffeoyl CoA can be converted to chlorogenic acid or chicoric acid (Petersen *et al.*, 2009). According to current results, it seems that caffeic acid (a precursor) is converted more to chicoric acid) than chlorogenic acid. Similar observations were reported earlier in different plant species. Chung *et al*. (2017) showed that different concentrations of AgNPs and $AgNO_3$ (0.5, 1, and 2 mg/L) induce caffeic acid accumulation in hairy root of *Cucumis anguria*. To determine whether $AgNO_3$ or AgNPs stimulate gene expression or enzyme activities involved in this pathway, more experiments are needed.

Data from correlation analysis revealed that chicoric

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acid content was strongly correlated to chlorogenic acid production in cell suspension culture exposed to different concentrations of AgNPs (Table 4). It was also shown that the amount of caffeic acid was closely correlated with the amount of chlorogenic acid after AgNPs elicitation. The production and accumulation of chicoric acid and chlorogenic acid were not correlated following elicitation by $AgNO_3$.

Conclusion

In current research, we have successfully established cell suspension cultures of *L. undulata* to improve the production of valuable secondary metabolites production. In this way, we found that the application of AgNPs is more effective than $AgNO_3$ to enhance chicoric acid and chlorogenic acid production in cell culture medium. Most remarkably, the application of 4 mg/L of AgNPs increased the production of cichoric acid, up to 9.7 mg/g dry weight after 48 h, significantly. It seems that *L. undulata* can be a suitable model to produce cichoric acid via cell culture medium.

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