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Effect of nanoparticles on callus induction to produce secondary metabolites in *Salvia hispanica* L

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Abstract

Objective

The seeds of chia (*Salvia hispanica* L.) have a variety of polyphenolic compounds, which include flavonoids, phenolic acids, depsides and catechins. They are rich in nutrients and bioactive compounds and contain bioactive compounds; therefore, they are allowed as ingredients in food, dietary supplements, and cosmetic products. Moreover, nanoparticles are emerging as innovative elicitors in agriculture and plant biotechnology. Thus, the aim of this study was to examine the effectiveness of iron oxide nanoparticles (Fe_2O_3 NPs) as nano-elicitors for enhancing the biosynthesis of bioactive phenolic compounds found in callus cultures derived from *Salvia hispanica* L. (chia).

Materials and methods

Callus cultures were produced from shoot explants on Murashige and Skoog (MS) growth medium supplemented with 1 mg/L BA and 1 mg/L IAA. The cultures were subjected to various concentrations of Fe_2O_3 NPs (0, 5, 10, 15, 20, and 25 mg/L) and subsequently analyzed by high-performance liquid chromatography (HPLC) to determine the profile of the phenolic compounds. Data were analyzed using analysis of variance (ANOVA), and Duncan's multiple range test at $p \leq 0.05$.

Results

Strong concentration-dependent modulation of the phenolic profile was observed, with six phenolic acids (protocatechuic, ferulic, vanillic, syringic, chlorogenic, and p-coumaric) exhibiting maximum accumulation at the lowest dosage of 5 mg/L (increased by 282%) and the rosmarinic acid accumulating progressively with a peak level at 25 mg/L (increased by 123%). Gallic acid peaked at 15 mg/L, while rosmarinic acid increased progressively, reaching 1771 µg/mL (123%

higher than control) at 25 mg/L. Treatment with Fe_2O_3 NPs enhanced phenolic acid production in callus cultures. The 5 mg/L concentration led to the highest overall accumulation of phenolic acids, whereas higher concentrations preferentially stimulated the biosynthesis of specific compounds, including gallic acid and rosmarinic acid.

Conclusion

Results show that Fe_2O_3 NPs can act as potent, low-cost elicitors that selectively increase the biosynthesis of value-added phenolics in the callus cultures of chia for use as nutraceuticals. This study provides a basis for further optimization and scale-up of nanoparticle-mediated elicitation to enhance the production of bioactive metabolites in plant cell culture systems.

Key words: HPLC, iron oxide nanoparticles, polyphenolic compounds, rosmarinic acid

Paper Type: Research Paper.

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Introduction

Chia, or *Salvia hispanica* L., is a plant that grows annually and is herbaceous in nature. It is native to southern Mexico and northern Guatemala. It belongs to the order Lamiales, family Lamiaceae (the mint family), subfamily Nepetoideae, and genus Salvia, which has approximately 900 species distributed throughout southern Africa, central and southern America, North America, and Southeast Asia (Ranjana & Akan, 2017; Grancieri et al., 2019). Chia is grown in many places around the world, including Egypt and Iraq (Motyka et al., 2022). Chia seeds are rich in nutrients and bioactive compounds and contain bioactive compounds; therefore, they are allowed as ingredients in food, dietary supplements, and cosmetic products (Motyka et al., 2022; Gabal, 2025). The seeds of chia have a variety of polyphenolic compounds (either free or bound via glycosidic bond), which include flavonoids (e.g., apigenin, quercetin, kaempferol and myricetin), phenolic acids (e.g., gallic, ferulic, caffeic, p-coumaric, vanillic, and syringic), depsides (rosmarinic acid and chlorogenic acid) and catechins (e.g., epicatechin) (Motyka et al., 2022). Some minor carotenoids are present (Grancieri et al., 2021). These compounds offer antioxidant, anti-inflammatory, anticancer, and cardioprotective effects. Specifically, in a murine model, a diet high in ω-3 fatty acids from chia was found to decrease tumor growth (Espada et al., 2007). Though some advantages exist, more research is needed to enhance these bioactive compounds for potential pharmaceutical and nutraceutical applications (Gabal, 2025). Nanoparticles (NPs, 1–100 nm) are emerging as innovative elicitors in agriculture and plant biotechnology (Mohammadabadi et al., 2009; Lala, 2021). Advances in nanoscience and nanotechnology have routinely enabled the fabrication and identification of submicron bioactive carriers. The delivery of bioactive substances to target sites in the body and their release behavior are directly affected by particle size (Mortazavi et al., 2005; Zarrabi et al., 2020). Compared to micrometer-sized carriers, nanocarriers provide more surface area and have the potential to increase solubility, increase bioavailability, improve controlled release, and enable precise targeting of entrapped substances (Heidarpour et al. 2011; Mohammadabadi and Mozafari, 2019). The concentration of NPs affects their effect on the plant: higher doses may exert a toxic effect and inhibit growth, while lower doses of NPs would elicit abiotic stress to the plant; therefore, promoting secondary metabolite biosynthesis due to their defense mechanisms (Heidarpour et al. 2011; Miralles et al., 2012; Romanovski, 2024). Use of lower concentrations of NPs may also promote growth, enhance uptake of nutrients and bioactive compounds (Mohammadabadi and Mozafari, 2018; Cao et al., 2022). Iron oxide nanoparticles (Fe_2O_3 NPs) represent one of the most effective NPs for plants because of the important function iron plays in chlorophyll biosynthesis, photosynthesis, respiration, the activity of enzymes, and DNA synthesis (Merinero et al., 2022). Interaction with Fe_2O_3 NPs increases plants' stress tolerance as well as improves nutrient utilization, resulting in increased yield (Cao et al., 2022). In Lamiaceae medicinal plants, Fe_2O_3 NPs act as elicitors of the secondary metabolites. There are examples of increased levels of secondary metabolite production in *Dracocephalum kotschyi* (hairy root cultures) where application of 75 mg L^{-1} Fe_2O_3 NPs increased phenolic compound production via the upregulation of phenylalanine ammonia lyase (PAL) and rosmarinic acid synthetase (RAS) genes, resulting in the production of more rosmarinic acid and flavonoids (Nourozi et al., 2019). Previous studies demonstrate that treatment with various substrates produced an increase in the concentration of hyoscyamine and scopolamine in *Hyoscyamus reticulatus* (Moharrami et al., 2017) and enhanced essential oils, flavonoid content, and antioxidant activity in *D. kotschyi* (Khanizadeh et al., 2024). In addition, silver nanoparticles have been shown to act as nanoelicitors to stimulate the production of rosmarinic, caffeic, chlorogenic, and p-coumaric acids in *Melissa officinalis* callus tissue at their optimal dose (Coskun & Kapdan, 2025) while they stimulated withanolide production in *Withania somnifera* callus tissue despite a decrease in biomass production rate (Abed et al., 2025). Thus, the aim of the present study was to evaluate the effects of iron oxide (Fe_2O_3) nanoparticles on the induction of callus and phenolic secondary metabolites present in the *Salvia hispanica* L. callus. Phenolic secondary metabolite concentrations will also be quantified using HPLC as part of an investigation into the potential of Fe_2O_3 NPs as nano-inducers of enhanced production of bioactive compounds.

Materials and methods

Plant material and sterilization: Seeds from the plant Salvia hispanica L (Figure 1) were purchased from a local market located in Baghdad, Iraq. They were sterilized by putting them into a solution of sodium hypochlorite (NaOCl) water at different concentrations: 1%, 2%, 4%

and 6% (v/v). The seeds were placed in the solution for either 10 or 15 minutes (min.). The seeds then underwent rinsing three times with distilled water to remove all traces of sodium hypochlorite that may remain from surface sterilization. Following this process, the seeds were then placed on a Murashige and Skoog agar medium (Murashige & Skoog, 1962) containing 3% sucrose and solidified using 0.8% agar. Prior to autoclaving the medium, the pH was adjusted to 5.7+/-0.1. The medium was then autoclaved at 121°C for 20 minutes. The resulting cultures were grown for a minimum of four weeks in a temperature of 25+/-2°C with a photoperiod of 16/8 hr. continuous light/dark derived from fluorescent bulbs.

Figure 1. Seeds of *Salvia hispanica* L

Callus induction and plant regeneration: For the induction of callus, two different types of explant materials were used: shoot tips and stem pieces (about 1.0 cm long) obtained from aseptic seedlings that had been growing for 4 weeks. The explants were then placed on Murashige and Skoog medium containing different (PGR) concentrations; Auxins and Cytokinin(s) (including IAA, 2,4-D, BA, Kin, TDZ) in several combinations. For the first week after placing explants on Murashige and Skoog medium, explants were incubated in total darkness (no light), with the intent of encouraging callus development before being moved to controlled light conditions. After incubating the explants on MS medium for a period of 4 weeks, the % of explants producing callus and the fresh weight of callus produced will both be recorded. For shoot regeneration, the proliferated callus masses were placed onto new regeneration media with differing ratios of PGRs, and the rate at which regeneration occurred was assessed after six weeks.

$$\text{Callus induction (\%)} = \frac{\text{Number of explants producing callus}}{\text{Total number of cultured explants}} \times 100$$

Effect of Fe_2O_3 NPs on metabolites content in the callus of *Salvia hispanica* L.: A uniform, fine adventitious root culture was developed on MS root induction medium containing 1.0 mg/L BA and 1.0 mg/L IAA and selected to perform elicitation studies. After subculturing this callus into fresh limiting nutrient (PGR) media using the same PGR concentrations on solid (i.e., filter sterilized) iron oxide (Fe_2O_3) nanoparticles in the following concentrations: (0; control;

5; 10; 15; 20 and 25 mg/L) for a 4-week elicitation period, and maintained under standard growth conditions.

Sample preparation for HPLC analysis: Elicited samples of callus were harvested by removing the callus from the flask culture and washing with distilled water. The callus was then dried in an oven at 40 °C until a constant weight was obtained, as specified in the following procedures.

Milling & defatting:

1. Grind sample 100g to fine powder (<500 µm); store at −20 °C, dark.
2. Defat the sample with n-hexane (3 × 2 mL, vortex/sonicate 5 min), centrifuge, discard oily layer. (Defatting improves chromatographic cleanliness reported for chia and other oils.) europepmc.org.
3. The clear supernatant of each sample was subjected to charcoal treatment to remove pigments before evaporation under vacuum (Buchi Rotavapor Re Type). Dried samples were resuspended in 1.0 mL HPLC-grade methanol by vortexing, the mixture was passed through a 2.5 um disposable filter, and stored at 4°C for further analysis. 20 µL of the sample was injected into the HPLC system according to the optimum condition. (Rahman et al., 2017).

- **Milled:** Approximately 100 g of dry callus was finely ground (<500 µm) using a sterile ceramic mortar and pestle.
- **Defatted:** The ground callus was defatted by vortex mixing or sonicating with 2.0 mL of n-hexane for 5 min, followed by centrifugation (5000 rpm) for 5 min. The supernatant (oily layer) was discarded. This defatting process was repeated three times to ensure complete removal of lipids, which can cause artefacts during chromatographic analysis.
- **Extraction:** Defatted residue was extracted using 120 mL of an 80:20 (v/v) methanol:water solution by ultrasonication (Branson Sonifier, USA) at 60% duty cycle for 25 min at 25 °C.
- **Clarification & Purification:** Extract was centrifuged at 7500 rpm for 15 minutes. The resulting clear supernatant was treated with activated charcoal, vortexed, and filtered to remove pigment and interfering compounds.

Extraction: 100 gm of plant leaf was dried, dissolved in 20 mL of hexane to remove fat, 120 mL of 80:20 (methanol: water). The extract was subjected to ultrasonication (Branson Sonifier, USA) at 60 % duty cycles for 25 min at 25°C, followed by centrifugation at 7,500 rpm for 15 min. The clear supernatant of each sample was subjected to charcoal treatment to remove pigments before evaporation under vacuum (Buchi Rotavapor Re Type). Dried samples were re-suspended in 1.0 mL HPLC-grade methanol by vortexing, the mixture was passed through a 2.5 um disposable filter, and stored at 4°C for further analysis. 20 µL of the sample was injected into the HPLC system according to the optimum condition.

HPLC analysis of phenolic compounds: The phenolic compounds were quantified by using a High-Performance Liquid Chromatography (Shimadzu 10AV-LC) system with a binary pump (LC-10A), degasser, autosampler, and UV-Vis (SPD-10A) detector. A Phenomenex C18 reversed-phase column (50 x 4.6 mm, 3 µm particle size) was used to separate the compounds.

The column was operated at 30 °C. The mobile phase was (A) water with 0.1% formic acid and (B) acetonitrile with 0.1% formic acid. A constant flow rate of 1.2 mL/min was employed during the gradient elution method. Detection occurred at 280 nm. The external standards (gallic acid, protocatechuic acid, chlorogenic acid, vanillic acid, syringic acid, p-coumaric acid, ferulic acid, and rosmarinic acid) were used to identify and quantify compounds by retention time and area under the curve. Each sample's concentration was derived from the below equation:

$$C_{sample} = \frac{A_{sample}}{A_{standard}} \times C_{standard} \times DF$$

Where, A=peak area, C= concentration, m=slope, b= intercept, and DF = dilution factor. The eluted peaks were monitored by UV-Vis detector (SPD-10A).

Statistical analysis: GenStat 12th edition software was used to conduct the statistical analyses of data obtained from the present study. Data were analyzed using analysis of variance (ANOVA), and Duncan's multiple range test was used to compare means at the a priori significance level of 0.05 ($p \leq 0.05$). All letters in each of the tables indicate that there are statistically significant differences between values, thus providing strong verification of the concentration-dependent effects of treatment across all experimental methods.

Results

Surface sterilization optimization and In Vitro establishment: Effective surface sterilization is essential for the successful establishment of in vitro (IV) cultures. The degree of contamination directly relates to the volume and length of exposure of tissues to sodium hypochlorite (NaOCl), as shown in Table 1. Contamination was highest (58.3%) with 1% NaOCl, and lowest (11.7%) with 6% NaOCl. All treatments had their highest observed reduction (or elimination) of contamination level when tissues were exposed to NaOCl at 2, 4, and 6% for a time period of 15 minutes rather than 10 minutes. In contrast, the average surviving tissue presents the inverse relationship to the NaOCl treatment as represented in Table 2. The average surviving tissue was highest (70.4%) for all lower concentrations when exposed to 15 minutes of the 2, 4, and 6% NaOCl. The highest levels of survival for 2 and 4% treatments occurred with 100 and 75%, respectively. The longest exposure at 6% drastically reduced the survival percentage (6.7%). Therefore, 2% for 15 minutes is the recommended protocol for surface sterilization because it provides 0% contamination and 100% survivorship. Seed germinated well on basal (Murashige & Skoog, 1982) MS medium after being processed through sterilization. Germination of seeds with radicles is shown in Figure 2, and it was documented during the initial phase of germination. Developed plants with primary leaves and root systems can be seen in Figure 3; these plants were photographed two weeks after germination and demonstrate that successful germination may occur relative to the in vitro regeneration process that resulted from seed sterilization and the suitability of MS as a medium for chia seed germination.

Table 1. Effect of NaOCl concentration and exposure time on contamination percentage of *Salvia hispanica* L. seeds cultured on MS medium

NaOCl (%)	Duration Time		Mean
	10min	15min	
1	95^{a}	21.7^{d}	58.3^{a}
2	80^{b}	0^{e}	40^{b}
4	50^{c}	0^{e}	25^{c}
6	23.3^{d}	0^{e}	11.7^{d}
Mean	62.1^{a}	5.4^{b}	

* Means followed by the different letters within single or two variants or two variants' interactions are significantly different from each other based on 5% level according to Duncan's multiple range test

Table 2. Effect of NaOCl concentration and exposure time on survival percentage of *Salvia hispanica* L. seeds cultured on MS medium

NaOCl (%)	Duration Time		Mean
	10min	15min	
1	3.3^{e}	100^{a}	51.7^{a}
2	20^{d}	100^{a}	60^{a}
4	46.7^{c}	75^{b}	60.8^{a}
6	63.3^{b}	6.7^{e}	35^{b}
Mean	33.3^{b}	70.4^{a}	

* Means followed by the different letters within single or two variants or two variants' interactions are significantly different from each other based on 5% level according to Duncan's multiple range test

Figure 2. Seeds germination of the chia plant on MS medium

Figure 3. Seeds grow after two weeks

Callus Formation and Plant Regeneration: As indicated by the values listed in Table 3, the percentage of callus formation from shoot and stem explants, respectively, varied significantly depending on the type of plant growth regulator (PGR) medium used to induce callus formation. Callus induction was 100% effective (highest rate of formation) when using media containing either a combination of 1 mg L^{-1} 2,4-D plus 2 mg L^{-1} Kinetin or 2 mg L^{-1} TDZ plus 2 mg L^{-1} IAA; in contrast, media that incorporated only cytokinin, such as 0.5 mg L^{-1} BAP or 0.5 mg L^{-1} Kinetin, were ineffective.

Table 3. Callus induction (%) from shoot and stem explants of *Salvia hispanica* L. on MS medium supplemented with different plant growth regulators

PGR medium	Shoot (%)	Stem segments (%)	Mean induction (%)
0.5 BA (6-benzyladenine)	10^{de}	6.67^{e}	8.33^{d}
0.5 Kinetin	6.67^{e}	6.67^{e}	6.67^{d}
1 2,4-D + 2 Kinetin	100^{a}	100^{a}	100^{a}
1 BA (6-benzyladenine) + 1 IAA	16.67^{d}	35^{c}	25.83^{c}
2 BA (6-benzyladenine) + 2 Kinetin	88.33^{b}	91.67^{ab}	90^{b}
2 TDZ + 2 IAA	100^{a}	93.33^{ab}	96.67^{a}
Mean	53.61^{b}	55.56^{a}	

* Means followed by different letters indicate significant differences at $p \leq 0.05$ according to Duncan's multiple range test

The ability of the callus to accumulate fresh weight (FW) was also tested using the same media combinations (Table 4). Although the maximum percentage of callus formation from the media combinations of 1 mg L^{-1} 2,4-D + 2 mg L^{-1} Kinetin or 2 mg L^{-1} TDZ + 2 mg L^{-1} IAA equaled 100% for both combinations, FW levels of callus formed using these same combinations were very low, with average FW values of 0.2 mg and 0.1 mg, respectively. The amount of callus formed was relatively small and non-growing in size; therefore, the biomass (FW) produced would be minimal. Callus was induced but remained very small or non-proliferative.

Table 4. Fresh weight (mg) of callus induced from shoot and stem explants of *Salvia hispanica* L. cultured on MS medium supplemented with different plant growth regulators

PGR medium	Shoot (mg)	Stem segments (mg)	Mean (mg)
0.5 BA	23.3^{bc}	19.7^{bc}	21.5^{b}
0.5 Kinetin	6.7^{de}	13^{cd}	9.8^{c}
1 2,4-D + 2 Kinetin	0.2^{e}	0.1^{e}	0.2^{d}
1 BA + 1 IAA	38.3^{a}	28.3^{ab}	33.3^{a}
2 BA + 2 Kinetin	0.1^{e}	0.1^{e}	0.1^{d}
2 TDZ + 2 IAA	0.1^{e}	0.1^{e}	0.1^{d}
Mean	11.5^{a}	10.2^{b}	

* Means followed by different letters within each column are significantly different at $p \leq 0.05$ according to Duncan's multiple range test

Successful shoot regeneration from callus (Table 5) occurred most frequently on media that provided both auxin and cytokinin in relatively balanced levels, while a high percentage of shoot regeneration occurred on media that contained 2 mg L^{-1} TDZ + 2 mg L^{-1} IAA (overall average percentage of shoot regeneration = 47.17%), followed by 1 mg L^{-1} BAP + 1 mg L^{-1} IAA (overall average percentage of shoot regeneration = 37.33%). However, media that contained only cytokinin and media that contained high concentrations of strong auxins, such as 2, 4-D, produced very poor to no shoot regeneration.

Table 5. Shoot regeneration (%) from callus cultures of *Salvia hispanica* L. on MS medium supplemented with different plant growth regulators

PGR medium	Shoot (%)	Stem segments (%)	Mean (%)
0.5 BA	0^{d}	0^{d}	0^{d}
0.5 Kinetin	13.33^{c}	20^{c}	16.67^{c}
1 2,4-D + 2 Kinetin	0^{d}	0^{d}	0^{d}
1 BA + 1 IAA	45.33^{a}	29.33^{b}	37.33^{b}
2 BA + 2 Kinetin	13.33^{c}	13.33^{c}	13.33^{c}
2 TDZ + 2 IAA	44.33^{a}	50^{a}	47.17^{a}
Mean	19.39^{a}	18.78^{b}	

* Means followed by different letters within each column are significantly different at $p \leq 0.05$ according to Duncan's multiple range test

Figure 4 shows the weight of a fully developed chia plantlet that was regenerated in vitro using the same method mentioned above and grew outdoors after being acclimatized in a greenhouse to produce lush, healthy, green, broad-leafed chia plants.

Figure 4. Chia plant regenerated in vitro and grows in plastic house conditions

Effects of Fe_2O_3 nanoparticles on elicitation of phenolic compounds: Callus was grown on Murashige and Skoog (MS) medium supplemented with 1 mg L^{-1} of BA and 1 mg L^{-1} of IAA containing graded concentrations of Fe_2O_3 NPs (0, 5, 10, 15, 20, or 25 mg L^{-1}). HPLC quantitation at 280 nm indicated that the respective phenolic profiles were significantly and dependently modified by Fe_2O_3 NPs treatment compared with the control (0 mg L^{-1}) (Table 6; Duncan's test, $p < 0.05$ within each column). The highest level of gallic acid was observed at 15 mg L^{-1} of Fe_2O_3 NPs (3755 µg/mL, 62% increase vs. the control at 2318 µg/mL); a secondary peak was observed at 25 mg L^{-1} (3682 µg/mL). The lowest level of gallic acid occurred at 5 mg L^{-1} (1390 µg/mL, significantly lower vs. control). All other phenolic acids (protocatechuic, ferulic, vanillic, syringic, chlorogenic, and p-coumaric) had maximal accumulation at the lowest concentration of elicitor (5 mg L^{-1} Fe_2O_3 NPs) with the following values, respectively: 3691; 3385; 2916; 1856; 1881; and 1835 µg/mL. These concentrations represent a significant level of elicitation with increases of 60% (p-coumaric) to 282% (chlorogenic) over the controls (1708; 1213; 1037; 543; 492; and 1149 µg/mL). Most higher concentrations (10-25 mg L^{-1}) had lower concentrations than with the 5 mg L^{-1} elicitors; however, several remained above control for certain compounds. The amount of rosmarinic acid increased progressively with each dose of Fe_2O_3 NPs until the maximum concentration of 1771 µg/mL (123% more than control: 794 µg/mL) was reached at 25 mg/L (Fe_2O_3), while large amounts were observed at lower dosages (1654 µg/mL at 5 mg/L). The chromatograms of HPLC confirmed the peaks were separated and identified for the standard phenolic mixture and samples (Sample-1 = 0 mg/L; Sample-2 = 5 mg/L; Sample-3 = 10 mg/L; Sample-4 = 15 mg/L; Sample-5 = 20 mg/L; Sample-6= 25 mg/L). Each given concentration of phenolic acid had a unique retention time corresponding to that of authentic standards of gallic acid, protocatechuic acid, ferulic acid, vanillic acid, syringic acid, chlorogenic acid, p-coumaric acid, and rosmarinic acid. These findings provide evidence that the Fe_2O_3 NPs acted as effective nanoelicitors, whereby low concentrations (5 mg/L) promoted almost all of the phenolic acids broadly, and high concentrations selectively promoted gallic and rosmarinic acids in *Salvia hispanica* L. callus.

Table 6. Concentration of phenolic compounds in callus of *Salvia hispanica* L. treated with different concentrations of Fe_2O_3 nanoparticles

Phenolic compound	0	5	10	15	20	25
Gallic acid	2318^{e}	1390^{f}	2798^{c}	3755^{a}	2588^{d}	3682^{b}
Protocatechuic acid	1708^{e}	3691^{a}	1722^{e}	1877^{d}	2206^{b}	2042^{c}
Ferulic acid	1213^{e}	3385^{a}	1195^{f}	1658^{d}	1919^{b}	1705^{c}
Vanillic acid	1037^{f}	2916^{a}	1303^{e}	1703^{d}	1774^{c}	1958^{b}
Syringic acid	543^{e}	1856^{a}	785^{d}	1093^{c}	1111^{c}	1423^{b}
Chlorogenic acid	492^{f}	1881^{a}	732^{e}	952^{d}	962^{c}	1037^{b}
p-Coumaric acid	1149^{f}	1835^{a}	1179^{e}	1501^{c}	1470^{d}	1706^{b}
Rosmarinic acid	794^{f}	1654^{b}	1513^{c}	1437^{d}	1380^{e}	1771^{a}

* Means followed by different letters within the same row are significantly different at $p \leq 0.05$ according to Duncan's multiple range test

Discussion

According to the current study, Fe_2O_3 nanoparticles (Fe_2O_3 NPs) regulated callus formation and phenolic secondary metabolite production from *Salvia hispanica* L., or chia, under in vitro conditions. When reviewing the results obtained, we note that there is evidence of concentration-dependent effects of nanoelicitors on the majority of medicinal plants. Low to moderate concentrations of nanoelicitors have been shown to stimulate defense mechanisms that increase the biosynthesis of phenolic compounds; whereas, high concentrations of nanoelicitors appear to change the metabolic flux in cells and/or create stress at mild concentrations without producing significant toxic effects (Lala, 2021; Romanovski, 2024). Findings for seed sterilization (Table 1 and Table 2) demonstrate that a treatment of 2% NaOCl for 15 minutes provided the optimum balance of 0% contamination with 100% survival compared with all other combinations, enabling robust germination as depicted in Figure 2. Other combinations have shown contamination levels as high as 95% (1% NaOCl/10 minutes) or survival rates as low as 6.7% (6% NaOCl/15 minutes). These findings agree with the standard procedures of sterilization of other members of the Lamiaceae family, whereby excessive concentrations of NaOCl produce tissue damage, while suboptimal concentrations lead to excessive growth of microorganisms. Induction of callus (Table 3) was successful, with 100% occurring on MS media with 1 mg/L 2,4-D and 2 mg/L Kinetin. This was achieved from both the shoot and stem explants and was significantly higher than the other treatments (the lowest callus formation of just 8.33% occurred with 0.5 mg/L Benzyl adenine). The callus from the stem segment had a slightly higher callus formation than the shoot segment overall (mean = 55.56% for the stem segments, and 53.61% for the shoot segments), likely due to the higher levels of endogenous auxin or increased vascular system response, progressing to healthy plantlets as shown in Figure 3 (seedlings after two weeks). The highest fresh weight of the callus (33.3 mg) was produced where 1 mg/L of Benzyl adenine and 1 mg/L of Indole-3-Acetic Acid were used together (Table 4), with the shoots forming significantly higher biomass (38.3 mg) than the stems (28.3 mg), indicating that there is a synergetic relationship between the cytokinin and auxin for shoot proliferation without suppressing regeneration of the shoot. The highest percentage of regeneration (Table 5) of the shoots occurred when there were 2 mg/L of Thidiazuron and 2 mg/L of Indole-3-Acetic Acid (47.17%) and 2 mg/L of Benzyl adenine and 2 mg/L of Indole-3-Acetic Acid (37.33%), with no regeneration possible in the media containing predominately 2,4-D, which suggests that a balance between the auxins and cytokinins is critical to achieving organogenesis in chia callus, culminating in acclimatized plants (Figure 4). When looking at the quantified effects of core elicitation on the types and amount of phenolic compounds (Table 6), there were unique concentration-dependent patterns that were dissimilar from the controls using zero mg/L Fe_2O_3 NPs. When considering only the phenolic acids (protocatechuic acid, ferulic acid, vanillic acid, syringic acid, chlorogenic acid, and p-coumaric acid) produced the greatest amounts at the lowest concentration of the elicitors (5 mg/L), having maximum amounts of 3691 µg/mL of protocatechuic acid, 3385 µg/mL of ferulic acid, 2916 µg/mL of vanillic acid, 1856 µg/mL of syringic acid, 1881 µg/mL of chlorogenic acid, and 1835 µg/mL of p-coumaric acid, which are respectively 116– 282% greater than the controls which were 1708 µg/mL, 1213 µg/mL, 1037 µg/mL, 543 µg/mL, 492 µg/mL, and 1149 µg/mL. Maxima reached statistical significance (based upon Duncan's test) at $p < 0.05$, while mid-range doses (10 - 20 mg L^{-1}) usually yielded intermediate to lower values (e.g., ferulic acid = 1195 µg/mL at 10 mg L^{-1}). Thus, suggesting that at low concentrations, Fe_2O_3 NPs are mild abiotic elicitors that elicit phenylpropanoid pathway enzyme (e.g., PAL) activation via ROS signaling without overtaxing cellular resources (Nourozi et al. 2019; Khanizadeh et al. 2024). Gallic acid showed a distinct pattern and reached a maximum at a concentration of 15 mg L^{-1} (3755 µg/mL, ~62% greater than the control of 2318 µg/mL), then again at a secondary maximum at 25 mg L^{-1} (3682 µg/mL), and then the lowest level at 5 mg L^{-1} (1390 µg/mL). This response may represent a redistribution of flux toward the precursors of gallic acid due to moderate levels of stress. Rosmarinic acid improved continuously with greater concentrations. The maximum concentration of rosmarinic acid (25 mg L^{-1}; 1771 µg/mL, ~123% greater than the control of 794 µg/mL) coincided with moderate amounts at lower concentrations (e.g., 1654 µg/mL at 5 mg L^{-1}). This increase in concentration of rosmarinic acid has also been reported in the family Lamiaceae, where increased nanoelicitor doses of AgNPs yield higher accumulations of depsides (e.g., the maximum concentration of rosmarinic acid in *Melissa officinalis* callus tissue was reported at 50 µg L^{-1} of AgNPs) (Coskun & Kapdan, 2025). The analysis of the HPLC chromatograms showed prominent separation between peaks and assisted in the identification of those peaks at a wavelength of 280 nm. The quantitative changes in sample peaks were substantiated visually (Table 6). A majority of the elicited sample contain either more intense or uniquely appearing peaks compared to the control sample. The patterns mentioned above are also similar to those that were observed in previous studies of nanoelicitation: in *Dracocephalum kotschyi* (Lamiaceae), the application of Fe_2O_3 nanoparticles resulted in the upregulation of PAL/RAS genes, increasing the amount of rosmarinic acid and flavonoids at optimal concentrations (Nourozi et al. 2019; Khanizadeh et al. 2024). In *Melissa officinalis*, treatment with AgNp resulted in a significant increase in the levels of rosmarinic, caffeic, chlorogenic, and pcoumaric acids at a concentration of 50–75 µg L^{-1}; the essential oil components (neral and geranial) peaked at lower concentrations (Coskun & Kapdan, 2025). In *Withania somnifera*, treatment with AgNp decreased biomass but led to a considerable increase in withanolides (up to 277% higher at 3 mg/L) and increased antioxidant activity (Abed et al. 2025). In *Artemisia annua* callus treated with multi-NP (ZnO, CuO, CoO) treatments, phenolics and antioxidants were maximized at a low concentration (0.05–0.1 mg/L), but there were differences in response to each NP (Fatima et al. 2021). Overall, treatment with Fe_2O_3 NPs, particularly at a concentration of 5 mg L^{-1}, was able to elicit the largest number of phenolic acids; however, higher concentrations were able to stimulate the production of specific types of phenolic acids such as gallic acid and rosmarinic acid. It is likely that the increased concentrations of phenolic acids at higher doses were the result of continuous stress on the plant and would activate specific branches of the phenylpropanoid pathway. Therefore, Fe_2O_3 NPs are a good candidate nano-inducer for the large-scale production of chia bioactive; they have advantages compared to the production of chia bioactives through field production, such as productive production in controlled environments and year-round harvests. Future research should focus on molecular-level research related to gene expression (e.g., PAL/RAS) and testing of antioxidants to better understand the mechanisms and application of these materials.

Conclusion: The present study demonstrates that Fe_2O_3 nanoparticles can act as effective and versatile nanoelicitors to enhance the production of phenolic compounds via biotechnological methods in *Salvia hispanica* L. callus cultures. The results clearly show a concentration-dependent relationship between the addition of nanoparticulate Fe_2O_3 and the resulting phenolic compound yields, with different types of phenolic compounds (classes) showing different response patterns at the given Fe_2O_3 nanoparticle concentration levels. The best concentration for eliciting the broad spectrum of phenolic acids was 5 mg L^{-1}, while rosmarinic acid, which is a valuable phenolic compound, was elicited at 25 mg L^{-1}. Therefore, the results provide a means by which optimal concentrations can be applied to maximize the production of specific phenolic compounds in the callus cultures. Overall, the findings support the potential for nanotechnology to facilitate the precise modulation of plant secondary metabolism, presenting a sustainable and controllable approach to increasing the nutraceutical value of medicine-producing plants. This study lays the groundwork for continued optimization and scaling of nanoparticle-mediated elicitation as a viable strategy for enhancing the production of bioactive metabolites in plant cell culture systems.

Author contributions

S. N. M. and E. A. J. E. contributed to the study methodology and experimental design and performed data collection, L. A. M. A. and A. A. A. A. assisted with data analysis and contributed to writing the initial draft and statistical analysis, and S. N. M., E. A. J. E., L. A. M. A. and A. A. A. A. drafted the original manuscript and reviewed, discussed, and approved the final version of the manuscript.

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Data Availability

The data that contributes to the results of this study is provided by the researcher responsible for the request.

Ethical Considerations

No human or animal participants were used in this study. All laboratory procedures were performed according to standard guidelines.

Conflict of Interest

The researchers declare that there is no conflict of interest regarding the publication of this paper.

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تأثیر نانوذرات بر القای کالوس و تولید متابولیت‌های ثانویه در *Salvia hispanica* L

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چکیده

هدف: دانه‌های چیا (*Salvia hispanica* L.) دارای ترکیبات پلی‌فنولی متنوع شامل فلاونوئیدها، اسیدهای فنولی، دپسیدها و کاتچین‌ها هستند. این دانه‌ها غنی از مواد مغذی و ترکیبات زیست‌فعال بوده و به‌عنوان مؤلفه در صنایع غذایی، مکمل‌های رژیمی و محصولات آرایشی کاربرد دارند. نانوذرات به‌عنوان محرک‌های نوآورانه در کشاورزی و بیوتکنولوژی گیاهی مطرح شده‌اند. هدف این مطالعه بررسی اثربخشی نانوذرات اکسید آهن (Fe_2O_3 NPs) به‌عنوان نانو-الیسیتور در افزایش بیوسنتز ترکیبات فنولی زیست‌فعال در کشت کالوس‌های مشتق‌شده از *Salvia hispanica* L بود.

مواد و روش‌ها: کالوس‌ها از قطعات رویشی روی محیط کشت Murashige و Skoog (MS) حاوی ۱ mg/L BA و ۱ mg/L IAA تولید شدند. سپس کشت‌ها در معرض غلظت‌های مختلف Fe_2O_3 NPs (۰، ۵، ۱۰، ۱۵، ۲۰ و ۲۵ میلی گرم در لیتر) قرار گرفتند و ترکیب فنولی با استفاده از HPLC تحلیل شد. داده‌ها با آنالیز واریانس (ANOVA) و آزمون چندگانه دانکن ($p \le 0.05$) تجزیه و تحلیل شدند.

نتایج: افزایش غلظت نانوذرات باعث تغییرات وابسته به دوز در پروفیل فنولی شد. شش اسید فنولی شامل پروتکاتچوئیک، فیرولیک، وانیلیک، سیرینگیک، کلروژنیک و p-کوماریک بیشترین تجمع را در کمترین دوز ۵ mg/L نشان دادند (افزایش

۲۸۲٪)، در حالی که اسید رزمارینیک به‌طور پیوسته افزایش یافته و در ۲۵ mg/L به حداکثر رسید (افزایش ۱۲۳٪). اسید گالیک بیشترین میزان خود را در ۱۵ mg/L نشان داد، در حالی که اسید رزمارینیک به ۱۷۷۱ µg/mL (۱۲۳٪ بالاتر از کنترل) در ۲۵ mg/L رسید. استفاده از Fe_2O_3 NPs تولید اسیدهای فنولی را در کشت کالوس افزایش داد؛ غلظت ۵ mg/L بیشترین تجمع کلی فنول‌ها را ایجاد کرد، در حالی که غلظت‌های بالاتر به‌طور انتخابی سنتز ترکیبات خاصی مانند اسید گالیک و رزمارینیک را تحریک کردند.

نتیجه‌گیری: نتایج نشان می‌دهد که Fe_2O_3 NPs می‌توانند به‌عنوان الیسیتورهای مؤثر و کم‌هزینه عمل کنند و بیوسنتز فنول‌های ارزشمند را در کالوس چیا افزایش دهند. این مطالعه پایه‌ای برای بهینه‌سازی و توسعه مقیاس بزرگ استفاده از نانوذرات جهت افزایش تولید متابولیت‌های زیست‌فعال در سیستم‌های کشت سلول گیاهی فراهم می‌کند.

کلمات کلیدی: اسید رزمارینیک، ترکیبات پلی‌فنولی، نانوذرات اکسید آهن، HPLC

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