Stimulation of Phenolic Compounds Accumulation and Antioxidant Activity in *In Vitro* Culture of *Salvia Tebesana* Bunge in Response to Nano-TiO₂ and Methyl Jasmonate Elicitors

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Abstract

In the present study, we first attempted to achieve an efficient procedure for optimizing callogenesis from apical meristem and leaf explants of *S. tebesana* on MS media containing different concentrations of BAP alone and in combination with 2,4-D. Then, the inducing effect of nano-TiO₂ (10, 60, and 120 mg L⁻¹) and methyl jasmonate (50, 100, and 200 µM), as abiotic elicitors were studied on the enhancement of phenolic compounds, rosmarinic acid, and some individual flavonoids as well as antioxidant capacity of callus extracts. According to the results, the highest callogenesis rate (100 and 93.33, respectively) and DW (0.55 ± 0.03 and 0.36 ± 0.02 g, respectively) per responsive explant were achieved from apical meristem on MS media containing "BAP₁ + 2,4-D₁" mg L⁻¹ and from leaf explant on the medium supplemented with "BAP_{0.5} + 2,4-D₁" mg L⁻¹. The elicitation with 10 and 60 mg L⁻¹ nano-TiO₂ (respectively for apical meristem and leaf), and 50µM MeJa could significantly promote the production of predominant phenolic derivatives in *S. tebesana* calli, where the highest content of total phenolics, *O*-diphenols, phenolic acid, flavonoid, flavone and flavonol, proanthocyanidin was recorded. Additionally, in increasing the amount of rosmarinic acid of callus, nano-TiO₂ treatment was more effective than the elicitation with MeJa. Also, the highest content of Apigenin (0.33 ± 0.02 µg g⁻¹ DW) was detected after MeJa-elicitation (50µM), while the maximum level of Quercetin (2.61 ± 0.09 µg g⁻¹ DW) and Rutin (13.79 ± 08 µg g⁻¹ DW) were obtained after exposure to 60 mg L⁻¹ nano-TiO₂, both from leaf-derived calli. While a significant positive correlation was recorded between antioxidant assays (DPPH and FRAP) and phenolic derivatives of treated calli; a very strong correlation occurred between the content of rosmarinic acid of apical meristem-derived calli and DPPH and FRAP values ($r^2 = -0.921$ and $r^2 = -0.913$, $P < 0.01$ respectively). Our results showed that the combination of *in vitro* culture and elicitation would be a good technique to successfully produce and enhance the content of pharmacologically valuable metabolites in *S. tebesana*.

Key Message

Nano-TiO₂ and methyl jasmonate elicitors in a concentration-dependent manner increased the phenolic compounds, specific flavonoids, and antioxidant properties in the callus culture of *Salvia tebesana* Bunge.

Introduction

Salvia tebesana Bunge is a small shrub plant, which its geographical distribution restricts to Iran, Afghanistan, and Pakistan. In Iran, it has only been reported in a limited area of South Khorasan province (Khatamzaz 2002). This endemic plant belongs to the genus *Salvia* genus (family Lamiaceae), with over 900 species that have been identified around the world, and about 17 species are endemic to Iran (Walker et al. 2004). *Salvia* L. contains a large assortment of secondary metabolites, including essential oils, phenolic acids, flavonoids, and terpenoids (Russo et al. 2013). Studies have also shown that phenolic compounds can exhibit a broad range of physiological-therapeutic properties, such as anti-inflammatory, anti-allergic, antioxidant, antimicrobial, anti-proliferative activities, anti-thrombotic, and cardiovascular protective effects. Because of high antioxidant activities, phenolic compounds function as the main bioactive compounds to determine the antioxidant power in foods (Ulubelen and Topcu, 1992; Taârit et al. 2012; Aleebrahim-Dehkordy et al. 2016). In addition to high levels of flavonoids (Đorđević et al. 2000; Kamatou et al. 2008), it has been proved that one of the most abundant phenolic acids present in *Salvia* species is rosmarinic acid (RA)(Bandoniene et al. 2005; Koşar et al. 2008; Jadeja et al. 2014). This compound has also been reported with antibacterial, antiviral, antioxidant, and anti-inflammatory effects (Petersen and Simmonds 2003).

In vitro production of callus-derived secondary metabolites is a suitable technique for continuous and mass production of these valuable compounds with high efficiency for a short time (Ourmazdi and Chalabian 2006). Callus induction from plant tissues is influenced by several factors, such as genotype, explant type, growth regulators, culture medium, type of nutrients, environmental conditions as well as elicitors (Hasanloo et al. 2009). Moreover, elicitors' roles in culture conditions for boost the production of a diversity of metabolites have been confirmed (Zhao et al. 2005). Recent studies have shown that these inductions are highly depend on the alteration of physiological responses and the accumulation of phytoalexins under stress conditions (Baskaran et al. 2012). Generally, the application of various elicitors, like nano-titanium dioxide (nano-TiO₂) and methyl jasmonate (MeJa) with a concentration-dependent manner, is remarkably advisable to promote the generation and accumulation of phenolic compounds during the culture process (Ge and Wu 2005; Bahreini et al. 2015). Based on the literature, the nano-TiO₂ effect on the induction and accumulation of phenolic compounds has been studied in different plant species, such as *Salvia officinalis* (Ghorbanpour 2015), *Papaver somniferum* (Khodayari et al. 2014), *Aloe vera* (Raei et al. 2014), and *Foeniculum vulgare* (Bahreini et al. 2015). It has also

been proved that the production of phenolic compounds, especially rosmarinic acid, can be promoted after MeJa elicitation in *Salvia virgata* (Dowom et al. 2017), *Salvia officinalis* (Grzegorzczak and Wysokińska 2009), *Salvia miltiorrhiza* (Ge and Wu 2005) and *Exacum affine* Balf. f. ex (Skrzypczak-Pietraszek et al. 2014).

Up to the present time, very little information is available about secondary metabolites of *S. tebesana* as a traditional medicinal plant (Goldansaz et al. 2017; Eghbaliferiz et al. 2018; Fotovvat et al. 2018). Although Hemmati et al. (2020) has studied the effect of some growth hormones (BAP, 2,4-D, and NAA) on tissue culture and phenolic compounds accumulation of *S. tebesana in vitro*, there still is no report available regarding cell and tissue culture or the effect of elicitors on secondary metabolites of *S. tebesana*. This study was done for the first time (a) to create an efficient system to optimize the culture condition and hormonal composition for the large-scale production of callus, (b) to find the optimal concentration of nano-TiO₂ and MeJa elicitors to enhance the level of phenolic secondary metabolites and antioxidant properties, and (c) to evaluate the content changes of RA and some individual flavonoids (Apigenin, Quercetin, and Rutin) in elicitor- treated calli.

Materials And Methods

Plant material

Plant collecting and *in vitro* growth condition was performed according to our previous research (Hemmati et al. 2020). The plant grown in Hoagland nutrient solution (Hoagland and Arnon 1950) was used as a source of apical meristem and leaf explants. For sterilization of explants, the samples rinsed 3 times with sterile distilled water, followed by immersing in 70% (v/v) methanol (for 30 sec and once). Leaf explants were also plunged in 5% (v/v) sodium hypochlorite (3 min and once). The final wash of both explants was done 3 times with sterile distilled water.

Culture conditions and callus induction

Because one of the aims of the present study was to assess the effect of elicitors on the best callus-inducing hormonal medium, some hormonal conditions related to Hemmati's study were nominated to evaluate the best conditions (Hemmati et al. 2020). Apical meristem and leaf explants were cultured on MS medium with different concentrations of BAP (0, 0.5, and 1 mg L⁻¹) and 2,4-D (0, 0.5, 1, and 1.5 mg L⁻¹). Three replications were considered for each treatment, and five explants were cultured in each jar. After 3 weeks of dark treatment, the samples were exposed to 16 h of light and 8 h of darkness at 25 ± 2 °C for 4 weeks. Subculturing was performed in the 7th week with the same hormone concentrations. 90-day-old calli were harvested, and some morphological characteristics, including the percentage of callus induction and fresh and dry weight (FW and DW), were recorded. The percentage of callogenesis was calculated based on the following equation:

$$\text{Rate of callogenesis} = \text{number of explants with callus} / \text{total number of explants used} \times 100$$

The data obtained from the best growth rates of callus (the highest percentage of callogenesis and callus DW) was used to determine the best culture media.

Preparation of elicitors

Two elicitors, including nano-TiO₂ (10, 60, and 120 mg L⁻¹) and MeJa (50, 100, and 200 µM), were used. The stock of nano-TiO₂ (stock #: US3490, US Research Nanomaterials, Inc.) was prepared by dissolving the specified amounts (1.2 mg) of nano-TiO₂ powder in 10 mL of sterile distilled water and dispersed using ultrasonic vibration by providing 40 kHz wave signals at the temperature of 40 °C for 25 min (Ghorbanpour 2015). To prepare MeJa stock, a certain amount of it was dissolved in 96% ethanol (Wang et al. 2015). Both elicitors were filtered and sterilized using 0.22 µm filters.

Elicitation

The culture of selected media was performed in a like manner to the first experiment till the 7th week. At the end of the 7th week and during the subculturing, elicitor solutions were added to basal culture media. The calluses were harvested after 10 days of elicitors' treatment, and the callus biomass (FW and DW) was estimated.

Extraction for phytochemical analysis

The 60-day-old calli were dried in an oven at 40 °C for 48 h, and then, 80% (v/v) methanol was added to the callus powder in a ratio of 1 to 100 (w/v). The obtained extracts were placed in an ultrasonic device (Parsonic 2600s, Japan) for 30 min at a temperature of 30 °C and a frequency of 40 kHz. Then the extracts were filtered with Whatman paper (No. 1), and the solvent was evaporated under air condition. To prepare the final extract, the extract powder (0.0015 g) was dissolved in 5 mL of 80% (v/v) methanol (Annegowda et al. 2012). This solution was used as a "methanolic extract" for all the phytochemical assays.

Determination of total phenolic compounds

The content of phenolics in elicitor-treated calli was assessed by the Folin-Ciocalteu method (Marinova et al. 2005). For this purpose, 2.5 mL of 10% (v/v) folin was added to 100 µL of "methanolic extract". After 5 min in the dark condition and room temperature, 2 mL of 7.5% (w/v) sodium carbonate was added, and after 1.5 h, the absorption was read by a spectrophotometer (Jasco7800, Germany) at 765 nm. The content of phenolic compounds of any sample was calculated based on the standard curve of gallic acid equivalent (GAE) ($y = 0.0032x + 0.0313$, $r^2 = 0.987$), and the result was presented in terms of mg GAE per 100 g DW of the callus sample.

Determination of total *O*-diphenols content

The reaction mixture was prepared by adding 2 mL of 50% (v/v) aqueous methanol and 0.5 mL of 5% (w/v) sodium molybdate dihydrate to 100 µL of "methanolic extract". The samples were shaken for 15 min and exposed to darkness at room temperature. Finally, the light absorption of the samples was recorded at 370 nm (Carrasco-Pancorbo et al. 2005). The basis for calculating of total *O*-diphenols content was the gallic acid graph ($y = 0.0042x + 0.0674$, $r^2 = 0.998$), which was expressed as mg of GAE per 100 g DW of callus.

Determination of phenolic acids content

The content of phenolic acids was determined based on the Matkowski method (Matkowski 2008). Distilled water (1.25 mL), hydrogen chloride (250 µL), Arnow's reagent [10% (w/v) aqueous sodium molybdate and 10% (w/v) aqueous sodium nitrite] (250 µL) were added, respectively, to 250 µL of "methanolic extract". After being kept in total darkness for 30 min (25 ± 2 °C), 250 µL of sodium hydroxide with 250 µL of distilled water were added to the sample, and the absorbance was read at 490 nm. The phenolic acid content was calculated based on the standard caffeic acid equivalent (CAE) graph ($y = 0.0041x + 0.0258$, $r^2 = 0.983$), and the result was presented in terms of mg of CAE per 100 g DW of callus.

Determination of flavonoids content

A volume of 300 µL of "methanolic extract" was mixed to 3.4 mL of 30% (v/v) methanol. Then 150 µL of 0.5 M sodium nitrite and 150 µL of 0.3 M aluminum chloride were added, respectively. After keeping the samples in the dark at room temperature for 5 min, 1 mL of 1 M sodium hydroxide was added, and the absorbance was recorded at 510 nm (Zhishen et al. 1999). Catechin (CAT) equivalent was used to prepare the standard curve ($y = 0.0018x + 0.0106$, $r^2 = 0.989$), and the result was calculated in mg of CATE per 100 g DW of callus sample.

Determination of flavone and flavonol content

Quercetin equivalent (QE) was used to make the calibration curve for this assay ($y = 0.0022x - 0.007$, $r^2 = 0.982$). It was added 250 µL of "methanolic extract" to 50 µL of 10% (w/v) aluminum chloride and 50 µL of 1 M potassium acetate. Then, 750 µL of 80% methanol (v/v) and 1.4 mL of distilled water were added till the final volume reached 2.5 mL. After 30 min at room temperature, the absorbance was read at 415 nm (Kosalec et al. 2004). Using the standard curve line equation obtained from quercetin, the amount of flavone and flavonol in the extract was calculated and expressed as mg QE per 100 g DW of callus sample.

Determination of total proanthocyanidins content

In this step, 250 µL of the diluted extract was added to pure methanol (1 mL) and then, to 3 mL of 1% (w/v) vanillin. After vortex (WiseMix, Korea) for 30 sec, 2.5 mL of 9 N methanolic sulfuric acid was added. The samples were placed in Ben-Marie (Kavoosh Mega, Iran) for 15 min at 38 °C (in the dark). Finally, the optical absorption of the reaction mixture was recorded at 500 nm (Sun et al. 1998). Total proanthocyanidin content was defined with the standard curve of catechin solution ($y = 0.001x + 0.0653$, $r^2 = 0.939$) and expressed as mg of CATE per 100 g DW of callus sample.

Determination of rosmarinic acid content

According to the method described by Öztürk et al. (2010), the concentration of rosmarinic acid in the samples was calculated based on the rosmarinic acid complex with zirconium ions (Zr^{4+}) by spectrophotometer assay. 920 μ L of pure ethanol was added to 40 μ L of the extract, and the final volume was increased to 1 mL by adding 40 μ L of 0.5 M zirconium chloride. About 5 min following the addition of the zirconium salt solution, the absorbance of the sample was measured at 362 nm. Using the obtained equation ($y = 0.014x + 0.024$, $r^2 = 0.991$) from the standard curve of RA, the amount of rosmarinic acid was calculated as mg of RA per 100 g of DW of callus sample.

Antioxidant activity

DPPH scavenging activity assay

As described by de Torre et al. (2019), 100 μ L of "methanolic extract" and 100 μ L of methanolic DPPH solution (0.2 mM) were poured into each well of the 96-well plate, respectively. The plate was incubated in the dark at 25 ± 2 °C for half an hour, and then, its absorbance was read at 490 nm by the Elisa Reader (Gentaur ST2100, America). The antioxidant capacity was calculated in terms of Inhibitory concentration (IC_{50} ; effective concentration of sample with 50% DPPH inhibitory capacity), and in comparison with the antioxidant capacity of ascorbic acid (as positive control) at the same concentrations of the sample. To calculate IC, the following equation was used:

$$IC \% = [1 - (AS - AB) / (AC - AB)] \times 100$$

In the above equation, AS represents the adsorption of the extracted sample, AB represents the adsorption of the blank sample (100 μ L of extract and 100 μ L of pure methanol), and AC represents the adsorption of the control sample (100 μ L of pure methanol and 100 μ L of DPPH solution).

Ferric Reducing Antioxidant Power (FRAP) assay

The reduction ability of callus extract was analyzed using FRAP assay according to Li et al. (2017) with a minor modification. For this purpose, FRAP reagent was prepared by adding acetate buffer (300 mM; pH=3.6), 10 mM 2,4,6-tri (2-pyridyl)-1,3,5-triazine (TPTZ) solution in 40 mM HCL, and 20 Mm $FeCl_3 \cdot 6H_2O$ solution in a volume ratio of 10:1:1. The "methanolic extract" of callus (20 μ L) was mixed with 130 μ L of FRAP solution and incubated in the dark for 30 min. The absorbance of reaction mixture was assayed through the ELISA system at 630 nm. Ferrous sulfate II ($FeSO_4$) was applied to produce a standard curve ($y = 0.0165x + 1.323$, $r^2 = 0.908$). The results were expressed in mg of Fe^{+2} per 100 g DW of callus sample.

LC-MS analysis

Callus extraction

For LC-MC analysis, both explant-derived calli with the highest content of flavonoids, flavone and flavonols, were selected. 0.25 gr of dry callus powder was dissolved in 2.5 mL of HPLC grade methanol. After 2 h using an ultrasonic bath at a temperature of 30 °C and a frequency of 40 kHz, the solution was filtered through a 45 μ m filter, and the solvent was evaporated under air condition (Skerget et al. 2005). For flavonoid extraction of each dry sample, the extractive solution (75% methanol/0,1% formic acid) and continuing process were done according to the method described by Gómez et al. (2018).

LC-MS analysis of specific individual flavonoids

HPLC separation was carried out using the mobile phase containing solvent A and B in gradient. Mobile phase A consisted of 0.1% formic acid in acetonitrile (v/v), and mobile phase B consisted of 0.1% formic acid in H₂O (v/v). The volume of injection was 5 μ L in all samples. Chromatographic separation was performed on an Atlantis T3-C18 3 μ , 2.1 $\times$ 150 mm column at a flow rate of 0.15 mL/min. The column oven temperature was set at 40 °C. Mass spectra were acquired in the positive ion mode (ESI+). The following parameters were used in all experiments: cone voltage, 30 V; capillary voltage, 4kV; extractor, 2 V; RF lens, 0.2 V; collision energy, 30 eV; gas nebulizer, N₂ (grade 5); flow gas, 200 L/h; source temperature, 120 °C; desolation temperature, 300 °C. A Waters Alliance 2695 HPLC-Micromass Quattro micro API Mass Spectrometer was used for the analysis. The reference substances of flavone (Apigenin) and flavonols (Quercetin and Rutin) were obtained from Sigma-Aldrich (St. Luis, MO, USA).

Statistical analysis

Statistical analysis of data was performed using SPSS software (version 25). Using Duncan's Multiple Range Test, the mean values were compared with the probabilities of $P \leq 0.01$ and $P \leq 0.05$. Pearson's correlation coefficient analysis was used to assess the associations between different parameters. The graphs were drawn by EXCEL software (Office 2016).

Results

Callus induction of apical meristem and leaf explants

The results showed that internal hormones significantly affected the callus induction at $p \leq 0.01$ (Duncan's multiple range test). The individually adding of BAP and 2,4-D are shown that they were not successful for callogenesis. Results obtained for 2,4-D alone were zero, or near-zero for callus formation of both explants, while apical meristem in the media supplemented with BAP alone was more desired to branch. However, the simultaneous use of these hormones and their interaction significantly increased the callus induction (Table 1). For apical meristem explant, the best callus induction rate with the highest fresh and dry weight was obtained from the media with "BAP_{0.5} + 2,4-D_{0.5}" and "BAP₁ + 2,4-D₁" mg L⁻¹, while in the case of the leaf explant, the highest callogenesis rate, fresh and dry weight was recorded for the media supplemented with "BAP_{0.5} + 2.4 -D₁", "BAP_{0.5} + 2.4 -D_{1.5}" and "BAP₁ + 2.4 -D_{0.5}" mg L⁻¹ (Fig. 1). In terms of appearance, all the calli were also observed in friable form with yellow, light, or dark green colors.

Table 1

Callus induction of apical meristem and leaf explants of *S. tebesana* on MS medium supplemented with different concentrations of BAP and 2,4-D.

Explant type	Hormone treatment (mg L ⁻¹)	% of Callus induction	Fresh weight (g)	Dry weight (g)
Apical meristem	Control 1: "BAP ₀ + 2,4-D ₀ "	0 ^d	0.00 ± 0.00 ^e	0.00 ± 0.00 ^e
	"BAP _{0.5} + 2,4-D ₀ "	6.66 ^{de}	0.63 ± 0.62 ^e	0.05 ± 0.05 ^{de}
	"BAP ₁ + 2,4-D ₀ "	6.66 ^{de}	0.64 ± 0.64 ^e	0.05 ± 0.05 ^{de}
	"BAP ₀ + 2,4-D _{0.5} "	6.66 ^{de}	0.62 ± 0.62 ^e	0.04 ± 0.04 ^{de}
	"BAP _{0.5} + 2,4-D _{0.5} "	93.33 ^a	6.32 ± 0.26 ^a	0.44 ± 0.04 ^a
	"BAP ₁ + 2,4-D _{0.5} "	73.33 ^{ab}	3.20 ± 0.42 ^c	0.19 ± 0.01 ^{bcd}
	"BAP ₀ + 2,4-D ₁ "	13.33 ^{de}	1.19 ± 0.59 ^{de}	0.10 ± 0.05 ^{cde}
	"BAP _{0.5} + 2,4-D ₁ "	53.33 ^{bc}	4.66 ± 0.39 ^b	0.28 ± 0.03 ^b
	"BAP ₁ + 2,4-D ₁ "	100 ^a	5.95 ± 0.08 ^a	0.55 ± 0.03 ^a
	"BAP ₀ + 2,4-D _{1.5} "	6.66 ^{de}	0.47 ± 0.47 ^e	0.04 ± 0.04 ^{de}
	"BAP _{0.5} + 2,4-D _{1.5} "	40 ^{cd}	2.30 ± 0.13 ^{cd}	0.14 ± 0.01 ^{bcde}
	"BAP ₁ + 2,4-D _{1.5} "	50 ^{bc}	3.24 ± 0.53 ^c	0.22 ± 0.05 ^{bc}
Leaf	Control 2: "BAP ₀ + 2,4-D ₀ "	0 ^d	0.00 ± 0.00 ^e	0.00 ± 0.00 ^c
	"BAP _{0.5} + 2,4-D ₀ "	6.66 ^d	0.25 ± 0.25 ^{de}	0.01 ± 0.01 ^c
	"BAP ₁ + 2,4-D ₀ "	6.66 ^d	0.21 ± 0.21 ^{de}	0.01 ± 0.01 ^c
	"BAP ₀ + 2,4-D _{0.5} "	13.33 ^d	0.37 ± 0.18 ^{de}	0.02 ± 0.01 ^c
	"BAP _{0.5} + 2,4-D _{0.5} "	40 ^c	1.73 ± 0.66 ^{cd}	0.11 ± 0.04 ^b
	"BAP ₁ + 2,4-D _{0.5} "	86.66 ^{ab}	2.92 ± 0.31 ^c	0.16 ± 0.03 ^b
	"BAP ₀ + 2,4-D ₁ "	6.66 ^d	0.20 ± 0.20 ^{de}	0.01 ± 0.01 ^c
	"BAP _{0.5} + 2,4-D ₁ "	93.33 ^a	6.39 ± 1.02 ^b	0.36 ± 0.02 ^a
	"BAP ₁ + 2,4-D ₁ "	66.66 ^b	2.51 ± 0.18 ^c	0.15 ± 0.01 ^b
	"BAP ₀ + 2,4-D _{1.5} "	6.66 ^d	0.24 ± 0.24 ^{de}	0.01 ± 0.01 ^c
	"BAP _{0.5} + 2,4-D _{1.5} "	93.33 ^a	8.20 ± 0.83 ^a	0.33 ± 0.03 ^a
	"BAP ₁ + 2,4-D _{1.5} "	66.66 ^b	2.40 ± 0.31 ^c	0.17 ± 0.03 ^b

Each value represents mean ± SE. Within a column, means followed by the same letter are not significantly different ($P \leq 0.01$) according to Duncan's Multiple Range Test. BAP: 6-benzylaminopurine, 2,4-D: 2,4-dichlorophenoxyacetic acid.

Effect of elicitors on polyphenols content of apical meristem-derived callus

The content of different phenolic compounds in apical meristem-derived calli was significantly affected ($P \leq 0.01$) by elicitor type and hormonal treatment. As a general result, it can be said that most polyphenolic compounds were observed in the samples on basal media with "BAP_{0.5} + 2,4-D_{0.5}" mg L⁻¹. The highest content of total phenols (1074.07 ± 47.59 mg 100 g⁻¹ DW) and rosmarinic acid (87.22 ± 0.61 mg 100 g⁻¹ DW) was found in calli treated with 10 mg L⁻¹ nano-TiO₂ and "BAP_{0.5} + 2,4-D_{0.5}" mg L⁻¹. In such a manner that the phenol content of callus on this medium was more than double that of control in the same hormonal treatment. Regarding total

O-diphenol, flavone, and flavonol contents, the treatment with 10 mg L⁻¹ nano-TiO₂ and 50 µM MeJa on the media containing "BAP_{0.5} + 2,4-D_{0.5}" mg L⁻¹ led to the highest levels of these compounds in comparison with other samples. Additionally, 50 µM MeJa-treated callus in the hormonal concentration of "BAP_{0.5} + 2,4-D_{0.5}" mg L⁻¹ showed the highest flavonoids (398.41 ± 4.56 mg.100 g⁻¹ DW). Concerning the content of phenolic acids and proanthocyanidins, the result showed that 10 mg L⁻¹ nano-TiO₂ and 50 µM MeJa treatments on calli generated on "BAP_{0.5} + 2,4-D_{0.5}" mg L⁻¹ caused the highest content of these compounds (Table 2).

Table 2

Effect of nano-TiO₂ and MeJa elicitors as well as hormonal combination on phenolic derivatives contents in apical meristem-derived calli of *S. tebesana*.

Explant type	Hormone treatment	Elicitor type	Total phenolics mg GAE 100 g ⁻¹ DW	Total <i>O</i> - di phenols mg GAE 100 g ⁻¹ DW	Total phenolic acids mg CAE 100 g ⁻¹ DW	Total flavonoids mg CATE 100 g ⁻¹ DW	Total flavone & flavonol mg QE 100 g ⁻¹ DW	Proanthocyanidins mg CATE 100 g ⁻¹ DW	Rosmarinic acid mg 100 g ⁻¹ DW
Apical meristem	BAP _{0.5} + 2,4-D _{0.5} mg L ⁻¹	Control 1 (0)	427.20 ± 22.11 ^g	123.01 ± 1.70 ^g	118.84 ± 1.14 ^{ef}	207.43 ± 7.20 ^e	117.17 ± 5.79 ^{gh}	80.95 ± 1.65 ^e	37.27 ± 0.52 ⁱ
		10 mg L ⁻¹ nano- TiO ₂	1074.07 ± 47.59 ^a	228.96 ± 4.88 ^a	211.74 ± 1.67 ^a	341.63 ± 10.59 ^c	226.15 ± 3.32 ^a	123.86 ± 1.59 ^a	87.22 ± 0.61 ^a
		60 mg L ⁻¹ nano- TiO ₂	762.72 ± 36.82 ^c	194.78 ± 9.38 ^c	173.31 ± 2.98 ^c	304.25 ± 1.48 ^d	181.18 ± 3.46 ^c	106.16 ± 4.25 ^c	66.23 ± 0.55 ^d
		120 mg L ⁻¹ nano- TiO ₂	690.40 ± 15.04 ^d	179.51 ± 1.06 ^d	142.67 ± 0.97 ^d	280.77 ± 8.20 ^{de}	143.07 ± 4.57 ^{de}	104.44 ± 3.97 ^c	58.30 ± 0.56 ^e
		50 µM MeJa	643.30 ± 8.91 ^{de}	230.95 ± 3.39 ^a	213.44 ± 2.24 ^a	472.41 ± 15.91 ^a	200.30 ± 3.70 ^b	126.94 ± 0.47 ^a	65.17 ± 0.54 ^d
		100 µM MeJa	40803 ± 4.89 ^g	194.99 ± 2.07 ^c	178.87 ± 5.00 ^{bc}	304.97 ± 8.80 ^d	148.10 ± 3.66 ^d	112.32 ± 1.16 ^b	55.19 ± 0.63 ^f
		200 µM MeJa	330.07 ± 7.48 ^h	209.33 ± 3.00 ^b	186.85 ± 2.21 ^b	258.28 ± 3.84 ^e	115.11 ± 5.86 ^{gh}	105.14 ± 0.71 ^c	37.29 ± 0.57 ⁱ
	BAP ₁ + 2,4-D ₁ mg L ⁻¹	Control 2 (0)	401.1 ± 9.99 ^g	105.25 ± 3.43 ^h	107.81 ± 6.96 ^{fg}	183.56 ± 7.36 ^g	105.77 ± 2.80 ^h	48.59 ± 1.55 ^g	35.82 ± 0.34 ⁱ
		10 mg L ⁻¹ nano- TiO ₂	867.76 ± 6.81 ^b	206.10 ± 2.96 ^{bc}	181.95 ± 2.54 ^{bc}	298.13 ± 2.88 ^d	203.70 ± 2.92 ^b	89.94 ± 2.21 ^d	79.09 ± 0.53 ^b
		60 mg L ⁻¹ nano- TiO ₂	630.89 ± 10.74 ^e	141.52 ± 4.84 ^f	138.43 ± 4.03 ^d	226.67 ± 11.59 ^f	132.09 ± 5.42 ^{ef}	55.95 ± 1.52 ^f	55.14 ± 0.63 ^f
		120 mg L ⁻¹ nano- TiO ₂	554.92 ± 12.33 ^f	130.82 ± 4.41 ^{fg}	129.79 ± 4.80 ^{de}	265.06 ± 3.41 ^e	122.56 ± 2.65 ^{fg}	48.90 ± 1.75 ^g	53.24 ± 0.64 ^{fg}
		50 µM MeJa	686.95 ± 3.33 ^{de}	207.56 ± 4.45 ^b	203.60 ± 2.73 ^a	398.41 ± 4.56 ^b	221.11 ± 2.47 ^a	110.31 ± 1.05 ^{bc}	76.59 ± 0.86 ^c

Each value represents mean ± SE. Within a column, means followed by the same letter are not significantly different ($P \leq 0.01$) according to Duncan's Multiple Range Test.

Explant type	Hormone treatment	Elicitor type	Total phenolics mg GAE 100 g ⁻¹ DW	Total <i>O</i> - di phenols mg GAE 100 g ⁻¹ DW	Total phenolic acids mg CAE 100 g ⁻¹ DW	Total flavonoids mg CATE 100 g ⁻¹ DW	Total flavone & flavonol mg QE 100 g ⁻¹ DW	Proanthocyanidins mg CATE 100 g ⁻¹ DW	Rosmarinic acid mg 100 g ⁻¹ DW
		100 μM MeJa	522.28 ± 5.54 ^f	180.43 ± 2.24 ^d	98.42 ± 4.15 ^g	336.98 ± 5.16 ^c	130.25 ± 1.81 ^f	92.77 ± 1.42 ^d	52.56 ± 1.24 ^g
		200 μM MeJa	377.23 ± 6.54 ^{gh}	164.10 ± 1.30 ^e	134.02 ± 9.08 ^d	265.69 ± 5.46 ^e	148.45 ± 1.33 ^d	83.61 ± 0.66 ^e	39.96 ± 0.86 ^h
Each value represents mean ± SE. Within a column, means followed by the same letter are not significantly different (P ≤ 0.01) according to Duncan's Multiple Range Test.									

Effect of elicitors on polyphenols content of leaf derived callus

Taking into account the changes of all analyzed compounds in leaf-derived calli, the highest amount of these compounds was observed in media with MS salts and "BAP_{0.5} + 2,4-D₁" mg L⁻¹ hormones. Total phenolic compound in calluses was found to range between 1392.14 ± 37.77 and 292.56 ± 9.42 mg100 g⁻¹ DW. The highest concentration of phenolics was measured in the callus treated with 60 mg L⁻¹ nano-TiO₂ on the medium with "BAP_{0.5} + 2,4-D₁" mg L⁻¹ hormonal treatment, which was about 3 times more than the amount (504.53 ± 42.47 mg 100 g⁻¹ DW) recorded in the control sample. Exposure to 60 mg L⁻¹ nano-TiO₂ in the medium supplemented with "BAP_{0.5} + 2,4-D₁" mg L⁻¹ induced the most increment in total *O*-diphenols, phenolic acids, proanthocyanidins, and rosmarinic acid contents, which were 236.74 ± 9.58, 341.74 ± 9.71, 244.50 ± 4.35, and 113.39 ± 2.08 mg.100 g⁻¹ DW callus, respectively. However, almost double the content of flavonoids than the control was observed when the lowest concentration of MeJa treatment, 50 μM, was applied to the medium with "BAP_{0.5} + 2,4-D₁" mg L⁻¹. For all media, effective concentrations of treatments to increase the amounts of flavone and flavonol in calli were 60 mg L⁻¹ nano-TiO₂ and 50 μM MeJa (Table 3).

Table 3
Effect of nano-TiO₂ and MeJa elicitors as well as hormonal combination on phenolic derivatives contents in leaf-derived calli of *S. tebesana*.

Explant type	Hormone treatment	Elicitor type	Phenolics content mg GAE 100 g ⁻¹ DW	Total <i>O</i> - di phenols mg GAE 100 g ⁻¹ DW	Total phenolic acids mg CAE 100 g ⁻¹ DW	Total flavonoids mg CATE 100 g ⁻¹ DW	Total flavone & flavonol mg QE 100 g ⁻¹ DW	Proanthocyanidins mg CATE 100 g ⁻¹ DW	Rosmarinic acid mg 100 g ⁻¹ DW
Leaf	BAP _{0.5} + 2,4-D ₁ mg L ⁻¹	Control 1 (0)	504.53 ± 42.47 ^{ghi}	127.35 ± 9.38 ^{efg}	160.03 ± 11.39 ^e	195.22 ± 10.50 ^{gh}	112.19 ± 2.79 ^{ij}	117.62 ± 5.49 ^k	43.15 ± 0.56 ^k
		10 mg L ⁻¹ nano- TiO ₂	763.01 ± 30.69 ^{bcd}	163.70 ± 5.31 ^{cd}	211.50 ± 16.58 ^d	241.62 ± 6.08 ^f	130.86 ± 1.07 ^{gh}	187.11 ± 3.52 ^{de}	86.35 ± 0.52 ^c
		60 mg L ⁻¹ nano- TiO ₂	1392.14 ± 37.77 ^a	236.74 ± 9.58 ^a	341.74 ± 9.71 ^a	376.87 ± 17.83 ^b	235.15 ± 5.11 ^a	244.50 ± 4.35 ^a	113.39 ± 2.08 ^a
		120 mg L ⁻¹ nano- TiO ₂	785.39 ± 53.58 ^{bc}	139.58 ± 5.99 ^{ef}	257.13 ± 5.57 ^c	278.18 ± 7.96 ^e	133.36 ± 7.25 ^{gh}	159.79 ± 1.56 ^{gh}	59.29 ± 0.53 ^{ghi}
		50 µM MeJa	711.91 ± 7.11 ^{cd}	202.47 ± 13.82 ^b	263.41 ± 2.07 ^c	402.56 ± 4.79 ^a	232.93 ± 5.21 ^a	202.40 ± 4.31 ^c	87.28 ± 0.60 ^c
		100 µM MeJa	439.15 ± 9.23 ^{ij}	129.75 ± 0.89 ^{efg}	216.17 ± 5.06 ^d	224.66 ± 3.85 ^f	143.64 ± 4.16 ^{fg}	179.78 ± 1.44 ^e	66.61 ± 0.99 ^f
		200 µM MeJa	436.10 ± 10.34 ^k	119.68 ± 1.43 ^{fg}	157.04 ± 1.14 ^e	179.06 ± 3.71 ^{hi}	164.09 ± 3.41 ^e	159.37 ± 1.48 ^{gh}	59.37 ± 0.44 ^{ghi}
	BAP _{0.5} + 2,4-D _{1.5} mg L ⁻¹	Control 2 (0)	319.04 ± 26.20 ^k	109.75 ± 2.87 ^g	151.58 ± 14.13 ^e	179.83 ± 8.41 ^{hi}	113.46 ± 4.39 ^{ij}	100.74 ± 6.51 ⁱ	49.29 ± 0.54 ^j
		10 mg L ⁻¹ nano- TiO ₂	493.69 ± 55.96 ^{hi}	146.18 ± 8.29 ^{de}	202.70 ± 6.10 ^d	238.86 ± 6.70 ^f	122.53 ± 3.65 ^{hij}	142.94 ± 4.24 ^{ij}	77.06 ± 0.87 ^e
		60 mg L ⁻¹ nano- TiO ₂	839.74 ± 36.01 ^b	201.17 ± 7.55 ^b	258.18 ± 9.44 ^c	282.68 ± 11.12 ^e	233.77 ± 6.03 ^a	199.32 ± 4.07 ^c	90.71 ± 0.81 ^b
		120 mg L ⁻¹ nano- TiO ₂	609.68 ± 62.73 ^{ef}	125.31 ± 12.67 ^{efg}	305.75 ± 22.96 ^b	224.87 ± 16.84 ^f	166.74 ± 3.91 ^e	142.42 ± 5.63 ^{ij}	68.26 ± 0.51 ^f
		50 µM MeJa	585.07 ± 5.78 ^{fg}	205.44 ± 3.47 ^b	282.13 ± 8.19 ^{bc}	310.75 ± 5.46 ^d	220.38 ± 2.67 ^b	202.80 ± 1.18 ^c	78.28 ± 0.60 ^{de}
		100 µM MeJa	473.13 ± 7.37 ⁱ	171.88 ± 3.92 ^c	153.12 ± 1.45 ^e	215.00 ± 2.91 ^{fg}	150.43 ± 5.29 ^f	167.77 ± 2.28 ^{fg}	62.18 ± 0.57 ^g

Explant type	Hormone treatment	Elicitor type	Phenolics content mg GAE 100 g ⁻¹ DW	Total <i>O</i> -di phenols mg GAE 100 g ⁻¹ DW	Total phenolic acids mg CAE 100 g ⁻¹ DW	Total flavonoids mg CATE 100 g ⁻¹ DW	Total flavone & flavonol mg QE 100 g ⁻¹ DW	Proanthocyanidins mg CATE 100 g ⁻¹ DW	Rosmarinic acid mg 100 g ⁻¹ DW
		200 μ M MeJa	426.43 $\pm$ 5.64 ^{ij}	128.21 $\pm$ 7.06 ^{efg}	152.53 $\pm$ 4.86 ^e	162.98 $\pm$ 5.82 ⁱ	125.17 $\pm$ 3.67 ^{hi}	196.27 $\pm$ 1.15 ^{cd}	56.28 $\pm$ 0.60 ⁱ
	BAP₁ + 2,4-D_{0.5} mg L⁻¹	Control 3 (0)	292.56 $\pm$ 9.42 ^k	125.71 $\pm$ 1.84 ^{efg}	118.26 $\pm$ 4.20 ^f	193.57 $\pm$ 6.98 ^{gh}	110.32 $\pm$ 1.69 ^j	99.48 $\pm$ 6.52 ⁱ	43.28 $\pm$ 0.72 ^k
		10 mg L ⁻¹ nano-TiO ₂	453.96 $\pm$ 28.00 ^{ij}	161.66 $\pm$ 5.14 ^{efg}	120.20 $\pm$ 7.59 ^f	224.02 $\pm$ 4.73 ^f	140.90 $\pm$ 5.62 ^{fg}	151.83 $\pm$ 1.89 ^{hi}	58.94 $\pm$ 2.13 ^{hi}
		60 mg L ⁻¹ nano-TiO ₂	620.00 $\pm$ 9.31 ^{ef}	210.62 $\pm$ 6.14 ^b	204.53 $\pm$ 13.61 ^d	349.11 $\pm$ 14.13 ^c	205.23 $\pm$ 3.35 ^c	221.25 $\pm$ 2.90 ^b	88.31 $\pm$ 1.05 ^{bc}
		120 mg L ⁻¹ nano-TiO ₂	567.12 $\pm$ 3.71 ^{fgh}	214.82 $\pm$ 2.99 ^b	163.08 $\pm$ 4.73 ^e	240.79 $\pm$ 1.10 ^f	122.15 $\pm$ 4.73 ^{hij}	136.49 $\pm$ 7.33 ^j	61.55 $\pm$ 1.71 ^{gh}
		50 μ M MeJa	687.70 $\pm$ 7.65 ^{de}	125.71 $\pm$ 1.84 ^{efg}	201.88 $\pm$ 1.19 ^d	331.26 $\pm$ 6.06 ^{cd}	210.18 $\pm$ 2.08 ^{bc}	221.22 $\pm$ 1.49 ^b	80.39 $\pm$ 1.47 ^d
		100 μ M MeJa	426.31 $\pm$ 7.94 ^{ij}	195.62 $\pm$ 3.10 ^{bc}	79 $\pm$ 1.13 ^f	230.99 $\pm$ 9.38 ^f	129.83 $\pm$ 2.66 ^{gh}	157.42 $\pm$ 1.70 ^{gh}	51.93 $\pm$ 0.33 ^j
		200 μ M MeJa	374.06 $\pm$ 3.88 ^{jk}	120.96 $\pm$ 7.96 ^{fg}	153.05 $\pm$ 2.97 ^e	158.50 $\pm$ 5.98 ⁱ	187.97 $\pm$ 6.64 ^d	177.06 $\pm$ 2.55 ^{ef}	49.17 $\pm$ 0.42 ^j

Assessment Of Antioxidant Activity

Following the determination of the biophenolic contents, the antioxidant activities of all calli derived from apical meristem and leaf were evaluated by DPPH and FRAP radical scavenging assays. As can be seen in Figs. 2A and 2B, DPPH ($P \leq 0.01$) and FRAP ($P \leq 0.05$) result illustrated that the highest antioxidant activity of apical meristem-derived callus (IC_{50} 23.33 $\pm$ 2.27 μ g mL⁻¹ and 475.77 $\pm$ 22.39 mg Fe⁺² 100 g⁻¹ DW, respectively) was found after treatment with 10 mg L⁻¹ nano-TiO₂ on the media containing "BAP_{0.5} + 2,4-D_{0.5}" mg L⁻¹. Moreover, the IC_{50} values of elicitor-treated extracts were near to ascorbic acid (IC_{50} 12.52 $\pm$ 0.97 μ g mL⁻¹).

The DPPH antioxidant activity in leaf-derived calli varied from (IC_{50} 88.31 $\pm$ 5.14 μ g mL⁻¹) to (IC_{50} 25.71 $\pm$ 6.91 μ g mL⁻¹). The low- IC_{50} value (as the highest antioxidant capacity) was observed in the presence of "BAP_{0.5} + 2,4-D_{1.5}" mg L⁻¹ and 60 mg L⁻¹ nano-TiO₂ (Fig. 3A) followed by methanol extract of callus on media with 50 μ M MeJa and "BAP_{0.5} + 2,4-D₁" mg L⁻¹. The results from FRAP antioxidant assay revealed that all the extracts showed considerable antioxidant potency compared to the controls and the application of 60 mg L⁻¹ nano-TiO₂ and 50 μ M MeJa caused the highest amount of antioxidant activities (Fig. 3B).

The results of Pearson's correlation showed a significant and positive correlation ($P \leq 0.01$) between estimated polyphenol derivatives and assayed antioxidant capacities. A very strong correlation was recorded between the content of rosmarinic acid of apical meristem-derived calli and DPPH and FRAP values ($r^2 = -0.921$ and $r^2 = -0.913$, $P < 0.01$ respectively). While there were highly statistically significant correlations between total phenolic content of all extracts and two radicals scavenging assays, correlations of these assays

with phenolic acids, flavonoids, flavones, and proanthocyanidins were somewhat weaker (Fig. 4). The weakest correlation occurred between the *O*-diphenols content of leaf-derived calli and DPPH scavenging activity and FRAP assay ($r^2 = 0.501$ and $r^2 = 0.491$, respectively).

Effect Of Elicitors On Specific Flavonoids Content

Based on the results of flavonoids, flavone, and flavonols contents, two explant-derived calli with the highest ones were selected for quantitative analysis of Apigenin, Quercetin, and Rutin. For apical meristem-derived callus on the medium containing "BAP_{0.5} + 2,4-D_{0.5}" mg L⁻¹, the highest content of Apigenin ($0.134 \pm 0.007 \mu\text{g g}^{-1}$ DW), Quercetin ($0.491 \pm 0.03 \mu\text{g g}^{-1}$ DW) and Rutin ($2.941 \pm 0.13 \mu\text{g g}^{-1}$ DW) were found after MeJa-elicitation (50 μ M). In callus of leaf explant on basal MS supplemented with "BAP_{0.5} + 2,4-D₁" mg L⁻¹, the maximum level of Apigenin ($0.330 \pm 0.02 \mu\text{g g}^{-1}$ DW) were measured after exposure to 50 μ M MeJa, while the application of 60 mg L⁻¹ nano-TiO₂ led to the highest concentration of Quercetin ($2.619 \pm 0.09 \mu\text{g g}^{-1}$ DW) and Rutin ($13.792 \pm 0.87 \mu\text{g g}^{-1}$ DW)(Fig. 5).

Discussion

The combination of plant hormones in the medium has been reported necessary to stimulate a high frequency of callus induction (Hong et al. 2008; Giri et al. 2012). In this work, the simultaneous use of BAP and 2,4-D significantly increased callus induction rate so that the highest callogenesis rate was recorded for apical meristem explant on the medium augmented with "BAP₁ + 2,4-D₁" mg L⁻¹ and for leaf explant on the medium containing "BAP_{0.5} + 2,4-D₁" mg L⁻¹. Hemmati et al. (2020) studies on *S. tebesana* also showed a similar result of the promotive effect of BAP and 2,4-D in callus production of different explants. The results of Hussein's research on the callus induction potential of *S. officinalis* hypocotyl indicated the enhancing effects of BAP and 2,4-D (Hussein et al. 2014). The positive influence of simultaneous use of BAP and 2,4-D for callogenesis was also reported earlier for several *Salvia* species (Lemraski et al. 2014; Modarres et al. 2018) and other plant species (Wang and Bao 2007; El-shafey et al. 2019). A remarkable point is a balance between auxin and cytokinin in the culture medium as a key regulator of *in vitro* callogenesis, leading to increased cell division as an important process for callus induction (Johri and Mitra 2001; Roy and Banerjee 2003).

The results of the present study showed that regardless of the type of elicitor used, the calluses of both apical meristem and leaf explants on MS medium supplemented with the lower concentrations of auxin (2,4-D) and cytokinin (BAP) can produce more phenolic compounds compared with other combination ratios of 2,4-D and BAP. Similarly, the research of Hemmati et al. (2020) on *Salvia tebesana* showed that the low combinations of 2,4-D (0.5 mg L⁻¹) and BAP (0.5 and 1 mg L⁻¹) caused the maximum phenolic accumulation in callus obtained from shoot apical meristem and leaf explants, but Chaâbani et al. (2015) confirmed MS medium supplemented with 2.0 mg L⁻¹ 2,4-D and 1.0 mg L⁻¹ BAP seemed to be the most suitable medium for the highest production of total phenols in leaf-derived callus of *Crataegus azarolus* L. var. *aronia* in comparison to lower 2,4-D/BAP ratio. In agreement with our results, Maharik et al. (2009) and El-Baz et al. (2010) suggested that the addition of a lower level of auxin (NAA) to the culture medium than BAP yielded the most phenolic derivatives contents in *in vitro* cultures. The type and concentration of plant hormones in the culture medium influence the pathways involved producing secondary metabolites (Adie et al. 2007). The biosynthesis of most phenolics dominantly takes place through the shikimate/arogenate pathway producing three amino acids of tryptophan, tyrosine, and phenylalanine. These amino acids are also involved in the synthesis of some hormones, in particular auxins (Ruiz and Romero 2001). Phenylalanine ammonia-lyase (PAL) and tyrosine ammonia-lyase (TAL) are the key enzymes of this pathway (Acamovic and Brooker 2005). Due to the confirmed effect of auxin (IAA and 2,4-D) on gene expression of these enzymes, as well the activation or the inhibition of their activities, the variation in the biosynthesis rate of phenolic compounds is normally happening (Tanaka and Uritani 1979; Chaâbani et al. 2015).

Based on the obtained results, both nano-TiO₂ and MeJa was stimulating the production of polyphenols compared to controls. In treatment with nanoparticles, the application of 10 mg L⁻¹ nano-TiO₂ and 60 mg L⁻¹ nano-TiO₂ led to the most increase of phenolic derivatives in calluses derived from apical meristem and leaf, respectively; which were about 1.5 times higher than the amounts observed in other nano-TiO₂ treated samples. The promotive effect of nano-TiO₂ elicitor on phenolic compounds accumulation has also been recorded in seedling of *S. officinalis* (Ghorbanpour 2015), seedling of *S. officinalis* (Mazarie et al. 2019), cell culture of *Aloe vera* (Raei et al. 2014), and *in vitro* cultures of other species such as *Papaver somniferum* (Khodayari et al. 2014) and *Lallemantia iberica* (Sattari et al. 2020). It has been suggested that the use of nanoparticles in plant cell culture is highly important to produce secondary metabolites (Asl et al. 2019). Due to cause oxidative stress in target cells (Aghdam et al. 2016; Mohammadi et al. 2014), non-

bio-elicitors including nanoparticles stimulate the plant to increase immune responses and increase *PAL* gene expression, leading to an increase in secondary metabolites, especially phenolic compounds, which are a part of the systemic acquired resistance process (Rajendran et al. 1994, Feizi et al. 2012; Singh et al. 2012). Studies have also shown that elicitors, including nano elicitors, have a positive effect on increasing the absorption of some nutrients, especially nitrates, leading to increased expression of genes involved in the synthesis of phenolic compounds (Bais et al. 2004; Lemraski et al. 2014; Baenas et al. 2014; Kavianifar et al. 2018; Rivero-Montejo et al. 2021). However, studies have shown that the effects of TiO₂ nanoparticles are highly concentration-dependent; so that if the concentration exceeded the particular threshold, the adverse and cytotoxic effects will appear in plants (Ghosh et al. 2010; Rafique et al. 2014; Mohammadi et al. 2016). Similar results regarding a decreased promotive effect of nano-TiO₂ at higher concentrations have been obtained by Raei et al. (2014), who showed that the treatment with 120 mg L⁻¹ nano-TiO₂ reduced the amount of Aloin in the tissue culture of *Aloe vera*. It is stated that treatment with non-optimal concentrations of nano elicitors probably can cause the overproduction amount of oxidative materials and disturbance in the cellular redox balance leading to many severe damages to plant cell structures, proteins, and DNA (Afaq et al. 1998; Raei et al. 2014; Rafique et al. 2014). The destructive effect on DNA and negative consequence on gene expression eventually lead to a decrease in the production of active substances (Lok et al. 2006; Raei et al. 2016).

In this work, the pattern of phenolic derivatives accumulations in MeJa-elicited calli varied in an elicitor concentration-dependent manner. In a way that 50 µM MeJa had a dramatically stimulating effect on the analyzed compounds, and with increasing the levels of MeJa to 200 µM, the concentration of all phenolics decreased by almost half. Remarkably, the MeJa at 50 µM was also more effective at enhancing the total flavonoids and its individuals' content in calli obtained from both explants in comparison with the best nano-TiO₂ treatments (10 and 60 mg L⁻¹). This result is in agreement with the findings reported by Hashemyan et al. (2020) for *Teucrium polium*, with emphasis on the lower MeJa-elicitation (50 µM) for the highest contents of polyphenols. More promotive effects of the lower dose of exogenously applied MeJa on phenolic, flavonoid and/or phenolic acid contents were informed in shoot culture of *S. virgata* (11.2 ppm) (Dowom et al. 2017), cell culture of *S. miltiorrhiza* (10 µM) (Zhao et al. 2010), cell culture of *Cynara scolymus* (100 µM) (Samadi et al. 2019), cell suspension culture of *Thevetia peruviana* (3 µM) (Mendoza et al. 2018), callus suspension culture of *Habenaria edgeworthii* (10 µM) (Giri et al. 2012), shoot culture of *Centella asiatica* (50 µM) (Skrzypczak-Pietraszek et al. 2019) and root culture of *Polygonum multiflorum* (50 µM) (Ho et al. 2018). Induced production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) has been suggested as one of the potent signaling processes in the MeJa-treated elicitation leading to the overproduction of secondary metabolites, including polyphenols (Walker et al. 2002; Kim et al. 2005). Over-activation or activation of the phenylpropanoid pathway genes, *PAL* (Wang et al. 2005; Sun et al. 2013), *CHS* (Suzuki et al. 2005; Sánchez-Sampedro et al. 2005), *C4H* (Wang et al. 2014; Yousefian et al. 2020), and *4CL* (Yu et al. 2019; Yousefian et al. 2020) under conditions of MeJa treatment would be the reasons for these influence. Taking into account the results of many researches, the burst of oxidative stress and subsequent toxicity damage has been observed in plant samples exposed to a high level of MeJa (Scandalios et al. 1993; Di-Qiu et al. 1999; Mir et al. 2019; Ho et al. 2020). So it is important to optimize the MeJa concentration for improving secondary metabolite synthesis in *in vitro* culture system (Ho et al. 2020).

Phenolic and flavonoid compounds can protect cells from oxidation by electrolyzing and purifying the ROS produced by the electron transfer system in optical synthesis (Shen et al. 2009). The significant positive influence of increasing phenolic derivatives contents on antioxidant activities, due to the elicitation of nanoparticles and methyljasmonate (summarized in Fig. 4), show that the callus of *S. tebesana* is a potent scavenger of free radicals. The present data were in line with the results of experiments on *S. verticillata* (Tepe et al. 2007), *S. officinalis* (Grzegorzczak and Wysokłńska 2009; Ghorbanpour 2015; Vosoughi et al. 2018), *S. miltiorrhiza* (Dong et al. 2010), *S. tebesana* (Hemmati et al. 2020), *S. nemorosa* (Heydari et al. 2020), *S. multicaulis* (Salehi et al. 2018), *Nepeta binaloudensis* (Sagharyan et al. 2019), *Graecoanatolica dinarica* (Krstić-Milošević et al. 2017), *Orthosiphon stamineus* (Lim et al. 2013), indicating the relationship between the concentration of phenolic compounds and antioxidant properties. Meanwhile, some differences in the order of the samples between the DPPH and FRAP assays of "methanolic extracts" of both explants-derived callus may be ascribed to the different solvents used in each assay (Kouka et al. 2017).

Conclusion

In conclusion, it was successfully determined that *S. tebesana* has good callogenesis potential, with the highest callogenesis rate and callus dry weight for explants on MS media supplemented with the combined combination of BAP (0.5 and 1 mg L⁻¹) and 2,4-D (1 mg L⁻¹). Also, the present study showed that elicitation with nano-TiO₂ and MeJa at low concentrations, significantly promote the production of predominant phenolic derivatives in *S. tebesana* calli. Additionally, in increasing of rosmarinic acid, Quercetin, and Rutin, nano-TiO₂ treatment was more effective than MeJa elicitation. Our results showed that the combination of *in vitro* culture and

elicitation would be a good technique to successfully produce and enhance the content of pharmacologically valuable metabolites in *S. tebesana*.

Abbreviations

MS Murashige and Skoog medium

2,4-D 2,4-dichlorophenoxyacetic acid

BAP 6-Benzylaminopurine

DW Dry weight

FW Fresh weight

Nano-TiO₂ Titanium dioxide nanoparticles

MeJa Methyl jasmonate

FRAP Ferric Reducing Antioxidant Power

DPPH 2,2-Diphenyl-1-picrylhydrazyl

IC Inhibitory concentration

RA Rosmarinic acid

Declarations

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

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Figures

Figure 1

View of calluses with the highest percentage of callogenesis and dry weight of (a): apical meristem explant on medium with "BAP 1 + 2,4-D 1" mg L⁻¹, (b): apical meristem explant on medium with "BAP 0.5 + 2,4-D 0.5" mg L⁻¹ (c): leaf explant on medium with " BAP 0.5 + 2,4-D 1.5" mg L⁻¹ (d): leaf explant on medium with "BAP 0.5 + 2.4 -D 1" mg L⁻¹ and (e): leaf explant on medium with "BAP 1 + 2.4 -D 0.5" mg L⁻¹. Scale bar = 1 cm.

Figure 2

Antioxidant activity was assessed using DPPH (a) and FRAP (b) assays in apical meristem-derived callus. IC₅₀ represents the levels of biophenols required to scavenge 50% of DPPH radicals. All data are expressed as the mean ± SE. AA, Ascorbic acid.

Figure 3

Antioxidant activity was assessed using DPPH (a) and FRAP (b) assays in leaf-derived callus. IC₅₀ represents the levels of biophenols required to scavenge 50% of DPPH radicals. All data are expressed as the mean $\pm$ SE. AA, Ascorbic acid.

Figure 4

Linear correlation coefficients between phenolic composition and antioxidant capacity: in apical meristem-derived callus (a and b), and in leaf-derived callus (c and d). * Correlation is significant at the 0.01 level.

Figure 5

Chromatogram of standard samples of (1) Quercetin, (2) Rutin, and (3) Apigenin (a); Chromatogram of leaf-derived callus extract on MS medium supplemented with "BAP 0.5 + 2,4-D 1" mg L⁻¹ and 60 mg L⁻¹ TiO₂ (b); LC/MS analysis of Apigenin, Quercetin and Rutin content in treated and non-treated samples of apical meristem-derived callus on the medium containing "BAP 0.5 + 2,4-D 0.5" mg L⁻¹ (c, d, e); and leaf-derived callus on the medium containing "BAP 0.5 + 2,4-D 1" mg L⁻¹ (f, g, h). Each value represents mean $\pm$ SE.