Research Square

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Artificial neural network prediction and comparative evaluation of pharmaceutical important flavones and antioxidant compositions in Teucrium polium callus culture elicited with methyl jasmonate and TiO_2 nanoparticles

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Research Article

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Abstract

The present research study evaluated the effects of four concentrations of Benzylaminopurine (BAP) in combination with three concentrations of Naphthalene acetic acid (NAA), as well as methyl jasmonate and nano-sized TiO_2 elicitors on callus induction, pharmaceutical important flavones and antioxidant compositions in leaf-derived calli of *Teucrium polium*. The feed-forward Artificial Neural Network (ANN) model, as well, was applied to predict the effects of hormones and elicitors on phenolic compounds production. The best callus induction was obtained with basal media fortified with 1.5 mg L^{-1} BAP plus 0.5 mg L^{-1} NAA, followed by 1.5 mg L^{-1} BAP. Based on biochemical features (the total content of phenols, Ortho-diphenols, flavonoid, flavone, and rosmarinic acid), the extraction has substantially by elicitation raised in comparison to control, with the maximum ones in calli elicited with methyl jasmonate (50 µM). The exception was phenolic acids possessed much greater content in 10 mg L^{-1} nano TiO_2-treated calli. The high antioxidant activities (IC_{50} 2.066 ± 0.24 µg mL^{-1} and 383.79 ± 33.60 mg Fe^{+2} 100 g^{-1} DW) with approximate 2- and 1.5-fold increases compared to control, was observed in hydromethanolic extracts of calli elicited by 50 µM methyl jasmonate on an MS medium containing 1.5 mg L^{-1} BAP plus 0.5 mg L^{-1} NAA. Likewise, the highest contents of anti-genotoxic flavones apigenin and luteolin were measured at pretreatment of callus with 50 µM methyl jasmonate on the media supplemented with 1 mg L^{-1} BAP (180.67 ± 2.52 and 201.11 ± 3.63 µg 100 g^{-1} extract, respectively) and 1.5 mg L^{-1} BAP plus 0.5 mg L^{-1} NAA (206.01 ± 7 and 256.22 ± 2.1 µg 100 g^{-1} extract, respectively). ANN with hyperbolic-tangent (10-1-3) activation function, R^2 0.99, SSE 0.01, and RE 0.001 showed a strong correlation between ANN model outputs and the experimental amounts of polyphenols, Ortho-diphenols, and phenolic acids. The results support that methyl jasmonate elicitation could more trigger and increase the pharmaceutical phenolic compositions in *T. polium* callus cultures as compared with nano-sized TiO_2 treatment. Meanwhile, the ANN model could successfully interconnect the output parameters (phenolic metabolites) to input variables (hormone and elicitor).

Key Message

Methyl jasmonate and TiO_2 nanoparticle elicitors had a significantly improved the content of the phenolic compounds, specific flavonoids and antioxidant properties in vitro. The highest contents of anti-genotoxic flavones apigenin and luteolin in callus culture of *Teucrium polium* depends on the concentration of methyl jasmonate and TiO_2 nanoparticles in the media.

Introduction

Teucrium sp., with 434 species, is a widespread genus of the Lamiaceae family that grows on hills, deserts, and mountains in the Mediterranean area (Navarro et al. 2010). The species of *Teucrium polium* L., commonly Persian known as 'Maryam nokhodi' or 'Kalpoore', is used in 23 regions of the world and most frequently in Iran (Koocheki et al. 2008). The abundant secondary metabolites in *T. polium* are essential oils, phenolic acid, and the highest number of 30 distinct flavonoids. The predominant subgroup of flavonoids recorded in the genus *Teucrium* is flavones that are represented by apigenin and luteolin derivatives (Carreiras et al. 1990; Kawashty et al. 1999; Sharififar et al. 2009; Jaradat 2015), suggesting that these compounds may be chemotaxonomic markers for this genus (Mihailović et al. 2017)(Fig. 1). Previous studies reported that, due to the presence flavonoids, the extract of *T. polium* shows strong antioxidant properties and subsequently antimicrobial, antiproliferative, anti-inflammatory, and antihypertensive activities (Briskin 2000; Sharififar et al. 2009). Some of these pharmacological activities, in particular, anti-genotoxic, anti-cancer, and anti-inflammatory potentials, are attributed to apigenin and luteolin. These biologically active compounds have also been suggested as an agent for the treatment of several diseases, including diabetes, Parkinson's and Alzheimer's diseases, HIV, and autoimmune disorders (Ali et al. 2017). However, to date, little knowledge is available regarding quantitative analysis of flavonoid composition of *Teucrium* plants.

Plant tissue culture technology could be a helpful technique for stimulating the biological activity of cells to abundantly synthesize and accumulate specific metabolites (Chinnamadasamy et al. 2010). Many parameters can affect the tissue culture of plants particularly the whole plant regeneration. Among these variables, genotype, explant origin, culture medium, and plant hormones are the most effective (Minaei Chenar et al. 2016). Elicitors as chemical or biological agents from a variety

of sources can also cause physiological changes that lead to improvement of plant culture and the biosynthesis and accumulation of secondary metabolites (Ramawat and Mérillon 2008). From which, Methyl jasmonate (MeJA) and nanomaterials (NMs) have attracted considerable attention due to their unique properties (Scrinis and Lyons 2007; Namdeo 2007). Although MeJA plays an essential role as a signaling molecule and second messenger in the production of secondary metabolites through inducing the specific gene expression in plants (Xing et al. 2018a), the lucrative effects of TiO_2 nanoparticles vary depend on plant tissues, growth stages, plant species, functional concentrations and specific properties of nanoparticles (Castiglione et al. 2011). It has been confirmed that elicitors can stimulate oxidative stress in plants to induce the synthesis and accumulation of phenolic metabolites (Narayani and Srivastava 2017), most notably phenolic acids; and of which TiO_2 NM and MeJA treatments are the main (Xing et al. 2018a). Although there are some literature data about the profitable effect of plant hormones on callus production of *Teucrium* plant (Javidi Moghadam et al. 2016; Meguellati et al. 2019; Hashemyan et al. 2020), publications on the impact of elicitors on *Teucrium* callus induction and the improvement of bioactive compound synthesis in vitro are relatively rare. Hashemyan et al. (2020) found that elicitation with MeJA can enhance the content of total phenol of *T. polium* callus under 2,4-Dichlorophenoxyacetic acid and BAP. Despite these limited researches, no any comparison study on whether MeJA and nano-sized TiO_2 can influence the secondary metabolic potential from in vitro culture of *T. poliumn* was founded.

In recent years, intelligent models such as artificial neural networks have been widely used in biological sciences. The main idea of artificial neural networks (ANN), which is basically a mathematical model, is to some extent inspired by the way the biological nervous system works, to process data and information for the prediction and creating knowledge. The critical element of this idea is the creation of new structures for the information processing system. With ANN, it becomes possible to map input and output data to obtain an understanding of the complex relationships between them compared to traditional statistical techniques. The application of ANN in predicting the optimal in vitro plant culture environment has been reported in some literature (Zahid Rizvi et al. 2012; Arab et al. 2016)

In this study, (a) an in vitro culture was optimized for callogenesis induction and proliferation from leaf segments of *T. polium* by using various concentrations of BAP alone or in combination with NAA; (b) the improved production potential of desired secondary metabolites (including total phenolic, Ortho-diphenols, flavonoids, flavones, phenolic acids, and rosmarinic acid) and antioxidant properties of calli was evaluated after elicitation by MeJA and nano-sized TiO_2; and (c) the content changes of the major individual flavonoid (apigenin and luteolin) were also estimated in treated calli with corresponding elicitors. Our current study was also designed to determine the impact of the hormonal and elicitor factors on the callus accumulation and development of phenolic metabolites by using ANN.

Materials And Methods

Plant material and explant preparation

The seeds of *T. polium* were obtained from the Research Center of Agriculture (Jahad-e Keshavarzi) of Mashhad-Iran. After surface sterilization with aqueous ethanol (70% v/v, for 30 sec) and sodium hypochlorite solution (5% v/v, for 3 to 5 min), the seeds were kept in Petri dishes with sterilized filter paper and gibberellic acid (1000 ppm) under 10 ± 2 °C and dark condition. After the appearance of the radicle and the top of the cotyledon, the samples were exposed under standard condition (2000 lux light, 16 hr light/ 8 hr dark, 25 ± 2 °C, and 50% relative humidity) for one week. Then the little seedlings were transferred into pots containing Hoagland's complete nutrient solution (Hoagland and Arnon 1950) and kept in a growth chamber under the condition as mentioned earlier. Detached leaf explants (as the best explant reported in our previous research) were immersed in sodium hypochlorite (5% v/v) for 3 min and aqueous ethanol (70% v/v) for 30 sec and then rinsed three timeswith sterile distilled water.

Establishment of callus induction

The disinfected leaf pieces (approximately 5–5 mm^2) were placed on MS media supplemented with 12 treatments of various concentrations of BAP (0, 0.5, 1, and 1.5 mg L^{-1}) and NAA (0, 0.5, and 1 mg L^{-1}). Hormone-free MS medium was used as a

control. Each treatment had three replication with five leaf explants (for each jar) per replicate. The cultures were maintained in the darkness for two weeks and then transferred to a condition with a 16 hr light/ 8 hr dark for four weeks. The first subculture was carried out after six weeks on MS media augmented with the same hormones. At the end of the eight weeks, the percentage of callogenesis was counted as the number of callus / total number of explants × 100. The weight of fresh and dried calli [dried in an oven (Memmert, 5-1486) at 40 °C for 24 hr] was expressed in gram mass. According to callus induction with 100% of callogenesis and callus fresh weight, the green calli of the best sources were selected for further studies. The whole process was repeated twice.

Elicitors' preparation and treatment

The elicitation effect of different concentrations of MeJA *and nano-sized* TiO_2 was analyzed. Nano-sized TiO_2 was provided by the Nanomaterials Pioneers Co, NANOSANY (Mashhad, IRAN). Shows a transmission electron microscopy (TEM) of nano-sized TiO_2 powder with the particles exhibiting an approximate equi-axes shape and a size of fewer than 25 nm (Fig. 2). In brief, a certain amount of MeJA and *nano-sized* TiO_2 were dissolved in absolute ethanol (96% v/v) and sterile distilled water, respectively (Shoja et al. 2022). After using ultrasonic vibration by providing a 40 kHz wave at 40 °C for 25 min for *nano-sized* TiO_2 stock, both elicitors were filtered and sterilized through a membrane filter (0.22 µm) and used in the present study. In the continuation of the research and after selecting the best MS culture, the calli of media containing 1 mg L^{-1} BAP, 1.5 mg L^{-1} BAP, and 1.5 mg L^{-1} BAP plus*0.5*mg L^{-1} NAA were transferred to fresh MS media supplemented with MeJA (0, 50, 100, and 200 µM) and *nano-sized* TiO_2 (0, 10, 60, and 120 mg L^{-1}) for two weeks.

Extract preparation

To quantify the set of phytochemical compounds, calli cultures were extracted by ultrasound-assisted extraction according to the protocol of Annegowda et al. (2012), under the following conditions; methanol concentration 80% (v/v), extraction time and temperature of 30 °C, and 30 min, respectively. After filtration, with filter paper, the solvent of each extract was evaporated under air and dark condition for 24 hr. Eventually, the extract powders were prepared in 5 mL of methanol solvent (80% v/v) and used as hydromethanolic extracts for phytochemical analysis.

Secondary metabolite quantification

Total Polyphenols

An aliquot (100 µL) of methanolic extract was mixed with 2.5 mL of Folin Ciocalteu reagent 10% (v/v), and after 5 min of rest in the dark, 2 mL of sodium carbonate (7.5% v/v) was added. Before reading the absorbance at 765 nm, the samples were placed at 25 ± 2 °C for 1.5 hr. The content of phenolic compound was calculated based on the standard of gallic acid equivalents (GAE), and the result was calculated in terms of mg GAE per 100 g DW of the callus sample (Marinova et al. 2005).

Total *Ortho*-diphenols

The content of total *Ortho*-diphenol compound was based on the method of Carrasco-Pancorbo et al. (2005). Accurately, 100 µL of methanolic extract was mixed with 2 mL of methanol (50% v/v) and 0.5 mL of sodium molybdate (5% w/v) and subsequently placed in a dark room for 15 min. The absorbance was read at 370 nm, and the total content of compounds was calculated as a gallic acid equivalent, in terms of mg GAE per 100 g DW of the callus sample.

Total flavonoids

Based on the method described by Zhishen et al. (1999), 3.3 mL of methanol (30% v/v), 0.5 µL of 0.5 M sodium nitrite, and 150 µL of 0.3 M aluminum chloride were added to 300 µL of methanolic extract. After incubating for 5 min in the dark, 1 mL of 1 M sodium hydroxide was added to the samples and the absorbance was measured at 510 nm. The flavonoid content in extracts was expressed in terms of catechin equivalents (mg CATE) in 100 g DW.

Total flavones

The flavone content was expressed as quercetin equivalent (QE)(Kosalec et al. 2004). A sample volume (*50 µL*) of methanolic extract was mixed with 50 µL of aluminum-chloride (10%, w/v), 50 µL of 1 M potassium acetate, 750 µL of methanol (80% v/v), and 1.4 mL of distilled water. After incubation for 30 min under darkness, the absorbance was measured at 415 nm. The flavone content was expressed as mg QE for 100 g DW.

Total phenolic acids

Using a test tube, 250 µL of methanolic extract, 1.25 mL of distilled water, 250 µL of HCL, and 250 µL of Arnow's reagent [sodium-molybdate 10% (w/v) and sodium nitrite 10% (w/v)] was mixed. After 30 min of incubation in the dark, 250 µL of sodium hydroxide and 250 µL of distilled water were added, and then, the absorbance was read at 490 nm using a spectrophotometer (Jasco7800, Germany). The contents of total phenolic acid content in the extracts were determined based on an equation attained from the standard caffeic acid curve (Matkowski et al. 2008).

Rosmarinic acid

By using the method of Öztürk et al. (2010), 920 µL of ethanol (96% v/v) and 40 µL of the extract (1 mg dry extract in 1 mL^{-1} absolute methanol) were introduced to 40 µL of 0.5 M zirconium oxide chloride. After 5 min, the absorbance was defined at 362 nm. The content of rosmarinic acid in the extract was determined based on the rosmarinic acid curve (mg RA for 100 g DW).

Antioxidant activity

DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging assay

To measure the antioxidant assay of the extracts, 100 µL of different concentrations of methanolic extract was mixed with 100 µL of methanolic DPPH (0.2 mM) solution as a sample. The methanolic extract (100 µL, 50:50 v/v%) and methanolic DPPH solution (100 µL, 50:50 v/v%) were used as blank and control samples, respectively. After 30 min of incubation in the dark, the absorbance was recorded by the ELISA Reader (Gentaur, ST2100) at 490 nm. In order to make a comparison, butylated hydroxytoluene (BHT) was tested under the same condition as a positive control. The total antioxidant activity in the extracts were determined based on the equation scavenging activity (%) = $[1- (A_S - A_B) / (A_S - A_C)] \times 100$

Here, A_S is mixture absorbance with the sample, A_B is mixture absorbance with blank, and A_C is mixture absorbance with control. The extract concentration providing 50% inhibition (IC_{50}) was from the graph of scavenging effect percentage against extract concentration (de Torre et al. 2019).

Ferric antioxidant power assay (FRAP)

To measure the antioxidant capacity, 20 µL of methanolic extract was added to 130 µL of FRAP solution. After 30 min of rest, the reduction of ferric iron (Fe^{3+}) to ferrous iron (Fe^{2+}) in the presence of antioxidants was recorded by ELISA Reader at 630 nm. The antioxidant content of the sample was expressed in mg of ferrous sulfate per 100 g DW of callus (Li et al. 2017).

LC-MS/MS analysis

Standards

The following standards apigenin (4',5,7-trihydroxyflavone) and luteolin (3',4',5,7-tetrahydroxyflavone) purchased from Sigma Co and prepared in HPLC grade methanol. The samples were recognized by comparing the standard calibration of apigenin and luteolin.

Sample preparation

Form the calli with the highest content of phenolic derivatives; approximately 0.5 g of dry matter was suspended in 5 mL of absolute methanol (96% v/v) and incubated in an ultrasonic bath for 2 hr at 30 ± 2 °C and 40 kHz frequency. *After filtration by a 0.45 µm filter*, the remaining solvent (methanol) was evaporated at room temperature. To extract flavones, the residue (0.5 mg of each extract) was dissolved in 1 mL of methanol 75% (v/v) and formic acid 0.1% (v/v) solvent *according* to *a procedure described* by Gómez et al. (2018).

LC-MS/MS

To provide a brief review of LC-MS/MS *system model* based on Shoja et al. (2022), the separation of flavones was accomplished *with* an *Atlantis T3* column, 100 Å, *3* µm, 2.1 mm X 150mm at a flow rate of 0.2 mL min^{-1}. The solvent system was a mixture of acetonitrile and water containing *formic acid 0.1*% (v/v) *that* were combined in a *gradient*. The mobile phase was sonicated and degassed before injection into HPLC. *The temperature of* 40 ± 2 °C was held for 5 µL *sample* injection. The chromatographic peaks of the flavones mentioned above, in the sample solutions were confirmed by comparing their retention time and UV spectrum with those of the reference standard.

Data analysis

Statistical analysis of data was evaluated by analysis of variance (ANOVA) followed by using the SPSS data analysis software package (version 25 for windows). All results presented are mean ± standard error and a *probability of* $P \leq 0.01$ *and* $P \leq 0.05$ *was considered significant.*

ANN modeling

ANN model with feed-forward multiplayer perceptron and K-fold cross-validation was designed by MATLAB software 2020a. The units of this model comprise input/output, weights, hidden layer, and activation function. The mathematical explanation of the fundamental ANN is as follows:

O (Outputs) = f (wx+bias); W (Weights) = w1, w2, . . . ,wn; X (inputs) = x1, x2, . . ., xn.

In the present study, the layouts of ANN were two inputs (with ten levels), one output, and a single hidden layer. Inputs consist of the selected concentrations of BAP and NAA, as well as MeJA and *nano-sized* TiO_2 and outputs comprise total phenolic, *Ortho*-diphenols, flavonoids, flavones, phenolic acids, rosmarinic acid, DPPH, and FRAP of leaf-derived calli. In order to simplify the analysis, each of the output variables was separately analyzed.

Presents the flow chart of the neural network optimized by K-fold cross-validation. Based on this, the data is randomly broken up into K groups, after which, for each group, the following operations are performed (Fig.3) (Lyu et al., 2022): 1) Select one of the training folds as the testing dataset, 2) The remaining K-1 groups are used as the training set, and 3) Use the selected training dataset to predict the model. Figure 4 shows the final network architecture of ANN used in this study for secondary metabolites prediction.

In a small sample dataset of the present work, the data was classified into two categories to develop the ANN model: training (70%) and testing (30%). Correlation coefficient (R^2), sum squared error (SSE), and relative error for training and testing (RE) were used as the main indicators to determine the best layout of ANN (Ramezannejad et al. 2022). The normal range of R^2 values is 0-1, and the closer it is to 1, there is a perfect correlation between inputs and outputs. In addition, the lower the values of SSE and RE, the better the perdition of data.

Results

Effect of BAP and NAA on callus regeneration and biomass accumulation

To select suitable culture media in the forthcoming study, 12 different treatments of BAP and NAA were conducted to evaluate their effects on percent callus induction and callus morphology (Table 1). Callus initiation of the leaf explants was observed two weeks after sample placement on MS media fortified with plant hormones. Exogenously applied BAP alone or the combination of BAP and NAA induced a 100% callus formation from leaf explant (Table 1). While the media containing NAA alone, in the concentration of 0.5 and 1 mg L^{-1} (S5 and S9), induced the lowest callus formation percentage of 33.3 and 6.67, respectively. Besides, hormone-free media did not stimulate explants to initiate and form callus as compared with other media. Similarly, and with a positive correlation with callus rate, the maximum biomass accumulation (fresh and dry weights) was obtained with MS medium containing 1.5 mg L^{-1} BAP plus 0.5 mg L^{-1} NAA, followed by media containing 1.5 and 1 mg L^{-1} BAP. The color of the callus varied from dark green to brown, with a compact appearance at the end of growth (Fig. 5).

Table 1
Effect of BAP alone or in combination with NAA on the characteristics of callus originated from leaf explant of *T. polium.*

Culture symbol	BAP [mg L^{-1}]	NAA [mg L^{-1}]	Callus induction frequency [%]	Callus fresh weight [g]	Callus dry weight [g]	Callus morphology
S1	0	0	0	0	0	compact
S2	0.5	0	100	3.23 ± 0.28 ^{c}	1.28 ± 0.05 ^{abc}	compact
S3	1	0	100	4.04 ± 0.38 ^{ab}	1.07 ± 0.13 ^{c}	compact
S4	1.5	0	100	4.19 ± 0.06 ^{a}	1.60 ± 0.28 ^{ab}	compact
S5	0	0.5	33.3	0.31 ± 0.21 ^{f}	0.13 ± 0.11 ^{d}	compact
S6	0.5	0.5	100	2.73 ± 0.23 ^{cd}	1.19 ± 0.08 ^{abc}	compact
S7	1	0.5	100	2.36 ± 0.28 ^{d}	1.01 ± 0.11 ^{c}	compact
S8	1.5	0.5	100	4.79 ± 0.27 ^{a}	1.63 ± 0.27 ^{a}	compact
S9	0	1	6.67	0.06 ± 0.62 ^{f}	0.02 ± 0.01 ^{d}	compact
S10	0.5	1	100	1.52 ± 0.49 ^{e}	0.31 ± 0.03 ^{d}	compact
S11	1	1	100	3.20 ± 0.19 ^{c}	1.17 ± 0.14 ^{bc}	compact
S12	1.5	1	100	3.30 ± 0.15 ^{bc}	1.30 ± 0.04 ^{abc}	compact

*Each value represents mean ± SE. Within a column, means followed by the same letter are not significantly different ($P \leq 0.05$) according to Duncan's multiple range test.

Impact of plant hormones and elicitors on the accumulation of phenolic compositions

The phytochemical analysis of variance showed that the accumulation of phenolic compositions was significantly affected by hormone type and different concentrations of studied elicitors. The analysis of calli on 'selected media' (1 mg L^{-1} BAP, 1.5 mg L^{-1} BAP, 1.5 mg L^{-1} BAP plus 0.5 mg L^{-1} NAA), under-examined elicitation, showed that the highest amount of all phenolic compositions observed in basal MS media supplemented with 1.5 mg L^{-1} BAP plus 0.5 mg L^{-1} NAA (Table 2). In comparison between two elicitors, MeJA and nano-sized TiO_2, although the most potent effects were observed after exposure to low levels of both, the elicitation with MeJA was more profitable than nano-sized TiO_2 pretreatment. The total phenolic compound of callus extracts ranged between 319.22 ± 4.30 and 2073 ± 11.27 mg per 100 g DW, with the highest concentration measured in the callus treated with the concentration of 50 µM MeJA on the MS medium with 1.5 mg L^{-1} BAP plus 0.5 mg L^{-1} NAA. Exception of phenolic acids possessed much greater content in a culture subjected to 10 mg L^{-1} nano-sized TiO_2, the most

increment in total *Ortho*-diphenols, flavonoid, flavone, and rosmarinic acid of calli (1514.93 ± 37.60, 2029.82 ± 36.83, 736.18 ± 11.38, and 33.41 ± 0.42 mg 100 g^{-1} DW, respectively) was evaluated following 50 µM MeJA elicitation on corresponding hormonal treatment. More broadly, the most promotive effect of 50 µM MeJA and 10 mg L^{-1} nano-sized TiO_2 in three hormonal callogenesis media was on the accumulation of flavonoid, phenolic acid, and rosmarinic acid (more than a 1.5-fold increase concerning non-elicited controls). In general, as shown in Table 3, the results of the change percentage of phytochemical traits indicated that the positive impact of all analyzed concentrations of both elicitors belonged to those calli induced on media fortified with the combination of BAP with NAA.

Table 2

The effect of plant hormone combination as well as MeJA and nano-sized TiO_2 elicitors on the concentration of phenolic constituents in *T. polium* calli.

Treatment number	BAP [mg L^{-1}]	NAA [mg L^{-1}]	MeJA [µM]	Nano-TiO_2 [mgL^{-1}]	Polyphenol conc. [mg GAE 100 g^{-1} DW]	Ortho-diphenol conc. [mg GAE 100 g^{-1} DW]	Flavonoid conc. [mg CATE 100 g^{-1} DW]	Flavone conc. [mg QE 100 g^{-1} DW]	Phenolic acid conc. [mg CAE 100 g^{-1} DW]	Rosmarinic acid conc. [mg RA 100 g^{-1} DW]
T1	1	0	-	-	891.75 ± 39.93 ^{i}	531.21 ± 266.04 ^{fg}	518.39 ± 259.21 ^{hij}	198.15 ± 99.27 ^{ef}	288.55 ± 144.29 ^{g}	11.44 ± 0.12 ^{n}
T2	1	0	-	10	1248.66 ± 5.20 ^{g}	801.45 ± 0.64 ^{d}	898.65 ± 0.23 ^{ef}	283.04 ± 0.03 ^{e}	472.9 6 ± 2.82 ^{de}	13.96 ± 0.20 ^{ki}
T3	1	0	-	60	963.00 ± 3.60 ^{i}	436.59 ± 0.36 ^{fg}	706.32 ± 66.72 ^{fghi}	223.89 ± 0.37 ^{e}	326.54 ± 0.63 ^{fg}	13.49 ± 0.31 ^{ki}
T4	1	0	-	120	661.00 ± 4.93 ^{jk}	463.43 ± 3.32 ^{fg}	608.40 ± 0.20 ^{fghi}	216.74 ± 0.39 ^{e}	294.21 ± 0.97 ^{g}	10.51 ± 0.12 ^{o}
T5	1	0	50	-	1491.02 ± 42.48 ^{e}	1116.44 ± 7.04 ^{c}	1198.99 ± 8.53 ^{cd}	229.28 ± 149.88 ^{de}	635.24 ± 25.91 ^{abc}	20.11 ± 0.12 ^{f}
T6	1	0	100	-	319.22 ± 4.30 ^{m}	361.55 ± 6.14 ^{g}	299.55 ± 6.21 ^{j}	63.16 ± 31.64 ^{f}	89.24 ± 3.41 ^{h}	17.57 ± 0.20 ^{h}
T7	1	0	200	-	624.39 ± 4.91 ^{i}	389.31 ± 194.65 ^{g}	589.26 ± 294.64 ^{ghi}	240.54 ± 120.58 ^{e}	266.54 ± 133.30 ^{g}	16.21 ± 0.13 ^{i}
T8	1.5	0	-	-	907.11 ± 31.57 ^{j}	765.77 ± 3.20 ^{def}	690.40 ± 0.53 ^{fghi}	289.28 ± 17.28^{e}	441.79 ± 22.21 ^{def}	12.51 ± 0.29 ^{m}
T9	1.5	0	-	10	1288.33 ± 4.40 ^{g}	808.14 ± 1.02 ^{d}	835.49 ± 0.19 ^{efg}	265.26 ± 0.87 ^{bc}	507.49 ± 1.11^{cd}	20.43 ± 0.26 ^{f}
T10	1.5	0	-	60	1070.66 ± 2.90 ^{h}	657.12 ± 1.19 ^{def}	737.28 ± 0.68 ^{fghi}	320.23 ± 0.45 ^{cde}	455.45 ± 9.83 ^{def}	18.49 ± 0.22 ^{g}
T11	1.5	0	-	120	985.00 ± 1.73 ^{i}	652.94 ± 1.02 ^{def}	784.86 ± 10.03 ^{fgh}	311.59 ± 0.54 ^{de}	452.35 ± 0.87 ^{def}	15.27 ± 0.39 ^{ij}
T12	1.5	0	50	-	1397.32 ± 26.36 ^{f}	1229.46 ± 11.02 ^{bc}	1321.81 ± 1.62 ^{cd}	524.64 ± 21.80 ^{b}	531.45 ± 20.04 ^{cd}	25.19 ± 0.20 ^{c}
T13	1.5	0	100	-	696.86 ± 11.23 ^{k}	485.54 ± 4.74 ^{fg}	478.31 ± 2.92 ^{ij}	253.39 ± 8.62 ^{e}	301.47 ± 8.49 ^{g}	20.56 ± 0.28 ^{f}

*Each value represents mean ± SE. Within a column, means followed by the same letter are not significantly different ($P \leq 0.05$) according to Duncan's multiple range test.

Treatment number	BAP [mg L^{-1}]	NAA [mg L^{-1}]	MeJA [µM]	Nano-TiO_2 [mgL^{-1}]	Polyphenol conc. [mg GAE 100 g^{-1} DW]	Ortho-diphenol conc. [mg GAE 100 g^{-1} DW]	Flavonoid conc. [mg CATE 100 g^{-1} DW]	Flavone conc. [mg QE 100 g^{-1} DW]	Phenolic acid conc. [mg CAE 100 g^{-1} DW]	Rosmarinic acid conc. [mg RA 100 g^{-1} DW]
T14	1.5	0	200	-	682.69 ± 0.91 ^{k}	556.37 ± 4.34 ^{efg}	570.17 ± 6.00 ^{ghij}	253.66 ± 16.30 ^{e}	345.16 ± 2.69 ^{efg}	14.20 ± 0.21 ^{k}
T15	1.5	0.5	-	-	1241.81 ± 11.56 ^{g}	1028.54 ± 16.53 ^{c}	1103.53 ± 43.81 ^{de}	487.67 ± 30.34 ^{b}	650.52 ± 14.49 ^{abc}	13.34 ± 0.36 ^{ki}
T16	1.5	0.5	-	10	1881.00 ± 3.05 ^{a}	1233.84 ± 1.36 ^{bc}	1403.07 ± 0.72 ^{c}	501.96 ± 0.09 ^{a}	765.72 ± 0.76 ^{ab}	30.75 ± 0.09 ^{b}
T17	1.5	0.5	-	60	1632.33 ± 3.05 ^{d}	1126.89 ± 1.19 ^{c}	1318.24 ± 1.20 ^{cd}	497.04 ± 0.44 ^{b}	623.30 ± 3.29 ^{bc}	21.41 ± 0.43 ^{e}
T18	1.5	0.5	-	120	1516.33 ± 0.88 ^{e}	1082.08 ± 11.48 ^{c}	1277.54 ± 0.35^{cd}	449.47 ± 0.37 ^{b}	692.80 ± 2.60 ^{ab}	15.60 ± 0.15 ^{j}
T19	1.5	0.5	50	-	2073.48 ± 11.27 ^{a}	1514.93 ± 37.60 ^{a}	2029.82 ± 36.83 ^{a}	736.18 ± 11.380 ^{a}	762.53 ± 14.83 ^{ab}	33.41 ± 0.42 ^{a}
T20	1.5	0.5	100	-	1695.67 ± 14.10 ^{c}	1168.65 ± 20.44 ^{c}	1462.65 ± 34.53 ^{c}	609.95 ± 17.96 ^{ab}	707.68 ± 27.81 ^{ab}	23.11 ± 0.38 ^{d}
T21	1.5	0.5	200	-	1876.02 ± 7.73 ^{b}	1428.90 ± 22.54 ^{ab}	1766.30 ± 3.99 ^{b}	675.34 ± 11.38 ^{ab}	714.50 ± 28.07 ^{ab}	13.40 ± 0.21 ^{ki}

*Each value represents mean ± SE. Within a column, means followed by the same letter are not significantly different ($P \leq 0.05$) according to Duncan's multiple range test.

Table 3
The percentage of change in the content of phenolic compositions under different concentrations of plant hormone combination as well as MeJA and nano-sized TiO_2 elicitors, compared to their corresponding controls. The arrows show the change percentage of indexes.

Treatment number	Polyphenol conc.	Ortho-diphenol conc.	Flavonoid conc.	Flavone conc.	Phenolic acid conc.	Rosmarinic acid conc.
T2	40%↑	51% ↑	73% ↑	43% ↑	64% ↑	22% ↑
T3	7.9% ↑	18% ↓	36% ↑	12% ↑	13% ↑	18% ↑
T4	35 ↓	15% ↓	17% ↑	9.4% ↑	1.9% ↑	8.1% ↓
T5	67% ↑	110% ↑	131% ↑	16% ↑	120% ↑	75% ↑
T6	64% ↓	32% ↓	73% ↓	68% ↓	69% ↓	54% ↑
T7	30% ↓	37% ↓	12% ↑	21% ↑	7.6% ↓	42% ↑
T9	42% ↑	5.4% ↑	21% ↑	8.3% ↓	15% ↑	63% ↑
T10	18% ↑	14% ↓	22% ↑	11% ↑	33% ↑	48% ↑
T11	8.6% ↑	1.5% ↓	14% ↑	7.7% ↑	2.4% ↑	2.2% ↑
T12	54% ↑	60% ↑	91% ↑	81% ↑	20% ↑	101% ↑
T13	23% ↓	37% ↓	31% ↓	12% ↓	32% ↓	64% ↑
T14	25% ↓	27% ↓	17% ↓	12% ↓	21% ↓	14% ↑
T16	51% ↑	20% ↑	27% ↑	2.8% ↑	17% ↑	130% ↑
T17	32% ↑	8.7% ↑	16% ↑	1.9% ↑	4.2% ↓	60% ↑
T18	22% ↑	5.2% ↑	15% ↑	7.8% ↓	6.1% ↑	17% ↑
T19	67% ↑	42% ↑	83% ↑	51% ↑	17% ↑	150% ↑
T20	37% ↑	14% ↑	25% ↑	20% ↑	8.8% ↑	73% ↑
T21	51% ↑	38% ↑	60% ↑	39% ↑	9.8% ↑	0.45 ↑

Effect of plant hormones and elicitors on radical scavenging activity

The results obtained by analyzing radical scavenging data showed that there were significant differences among all the values under elicitation by MeJA and nano-sized TiO_2 ($p \leq 0.05$ for DPPH and FRAP) (Fig. 6). Based on the results, DPPH and FRAP radical scavenging assays decreased with excess MeJA and nano-sized TiO_2 concentrations, in such a way that the treatment with 50 µM MeJA and 10 mg L^{-1} nano-sized TiO_2 showed a significant maximum impact on antioxidant potentials of calli created on the culture medium with the single or combined hormones. Additionally, the elicitation with 50 µM MeJA in the hormonal condition of 1.5 mg L^{-1} BAP plus 0.5 mg L^{-1} NAA showed the maximum values for both antioxidant assays (IC_{50} 2.066 ± 0.24 µg mL^{-1} and 383.79 ± 33.60 mg Fe^{2+} 100 g^{-1} DW). Interestingly, and comparable to IC_{50} value of reference standard BHT, 50 µM MeJA-treated callus on MS media augmented with 1.5 mg L^{-1} BAP and 1.5 mg L^{-1} BAP plus 0.5 mg L^{-1} NAA possessed potent free radical scavenging activities.

To analyze the relationship between the total antioxidant capacities and the content of phenolic compositions, Pearson's correlation was used ($P \leq 0.01$ and $P \leq 0.05$). For two antioxidant DPPH and FRAP methods, although a significantly high correlation was recorded between them and Ortho-diphenol (r^2 = -0.530 and r^2 = 0.873, $P \leq 0.01$ respectively), the correlation between radicals scavenging assays and flavone and flavonoid contents was also noticeable (Table 4). An interesting matter

was that the FRAP assay could show a better correlation with the estimated polyphenolic constituents than DPPH (IC_{50} values).

Table 4
Pearson's correlation between radical scavenging activity and phenolic constituents in calli obtained from leaf explants on MS media supplemented with various plant hormones and elicitors.

Parameters	Polyphenols	Ortho-diphenol	Flavonoid	Flavone	Phenolic acid	Rosmarinic acid	DPPH (IC_{50})	FRAP
Polyphenols	1							
Ortho-diphenol	0.946 **	1						
Flavonoid	0.952 **	0.968 **	1					
Flavone	0.899 **	0.818 **	0.938 **	1				
Phenolic acid	0.955 **	0.936 **	0.918 **	0.891 **	1			
Rosmarinic acid	0.620 **	0.593 **	0.600 **	0.582 **	0.523 *	1		
DPPH (IC_{50})	-0.366 *	-0.530 **	-0.458 *	-0.532 *	-0.417 *	-0.369 *	1	
FRAP	0.781 **	0.873 **	0.810 **	0.830 **	0.791 **	0.644 **	-0.761 **	1

*and** are significant at $P \leq 0.05$, and significant at $P \leq 0.01$ probability levels, respectively

Flavones content by LC-MS/MS analysis

To our knowledge, no quantitative investigations on flavone contents of *T. polium* have been conducted, and we report here the effect of elicitation on the accumulation of two main flavones of this species for the first time. The highest contents of apigenin and luteolin were achieved in pretreatment of callus with a concentration of 50 µM MeJA on the media supplemented with 1 mg L^{-1} BAP (180.67 ± 2.52 and 201.11 ± 3.63 µg 100 g^{-1} extract, respectively) and media with 1.5 mg L^{-1} BAP plus 0.5 mg L^{-1} NAA (206.01 ± 7 and 256.22 ± 2.1 µg 100 g^{-1} extract, respectively) which were approximately 101, 68, 126, and 69 percentage increase more than that of the control group (Fig. 7). The production of apigenin was also raised in elicited calli on 1.5 mg L^{-1} BAP medium with the weaker the elevating effect of MeJA concerning nano-sized TiO_2.

ANN modeling

In this study, the ANN model has been applied to predict the effects of different concentrations of BAP and NAA as well as MeJA and nano-sized TiO_2 elicitors on the production of phenolic derivatives in leaf-derived callus from *T. polium*. Figure 7 reveals the experimental outputs (total phenolic, *Ortho*-diphenols, flavonoids, flavones, phenolic acids, rosmarinic acid, DPPH, and FRAP) and prediction results obtained from leaf-derived calli, and Table 5 summarizes the performance of factors in the most effective models of ANN. Based on these data, the structure of 10-1-3 with hyperbolic-tangent activation function was determined as the best model for predicting the amount of total phenolic, *Ortho*-diphenols, and phenolic acids in leaf-derived callus with an R^2 value 0.99 (Fig. 8). Although the hyperbolic-tangent activation function had the most accurate prediction for the other analyzed outputs, different neurons (2, 3, 4, and 5) in a single hidden layer were determined (Table 5).

Table 5
ANN model performance applied to predict the experimental outputs (total phenolic, Ortho-diphenols, flavonoids, flavones, phenolic acids, rosmarinic acid, DPPH, and FRAP). Correlation coefficient (R^2), sum squared error (SSE), and relative error (RE).

Sample	Output	Activation function	Architecture	R^2	SSE	RE
Leaf-derived callus	Polyphenols	Hyperbolic tangent	10-1-3	0.999	0.014	0.001
	Ortho-diphenol	Hyperbolic tangent	10-1-3	0.999	0.019	0.002
	Flavonoid	Hyperbolic tangent	10-1-4	0.999	0.020	0.003
	Flavone	Hyperbolic tangent	10-1-5	0.999	0.015	0.002
	Phenolic acids	Hyperbolic tangent	10-1-3	0.999	0.017	0.001
	Rosmarinic acid	Hyperbolic tangent	10-1-3	0.989	0.268	0.018
	DPPH	Hyperbolic tangent	10-1-4	0.989	0.474	0.033
	FRAP	Hyperbolic tangent	10-1-2	0.984	0.193	0.026

Discussion

Very important roles of plant hormones in inducing callus have been affirmed in previous literature, with the emphasis that the combining of different growth regulators generated superior outcomes compared to using either of them alone (Mederos-Molina 2004; Antognoni et al. 2012; Parthibhan et al. 2018). Based on the result of the present study, the significant induction of callus was recorded in concomitant use of BAP and NAA, and the highest rate of callogenesis was achieved in a medium enhanced with 1.5 mg L^{-1} BAP plus 0.5 mg L^{-1} NAA. In agreement with our results, Bakhtiar et al. (2016) confirmed that the medium supplemented with 2 mg L^{-1} BAP plus 1 mg L^{-1} NAA gave the maximum callus production from leaf explant of *Thymus persicus* F. The more success in the combined pretreatment of various concentrations of BAP and NAA for callogenesis than the individual exposure of corresponding hormones has also been affirmed for the explants of *Ocimum basilicum* L. (Dode et al., 2003), *Salvia canariensis* L. (Mederos-Molina 2004), *Melissa officinalis* L. (Soltani Pool et al., 2011), *Satureja bachtiarica* L. (Elyasi et al. 2017), and *Salvia tebesana* (Hemmati et al., 2020). However and based on the present work, the stimulatory effect of BAP alone on callus induction of *T. polium* was in contrast with the results of Dronne et al. (1999), which stated that BA had no significant effect on callus production of *Lavandula intermedia* Emeric, definitely associated with the plant species or the source and age of different explant.

The solitary comparison of three types of hormonal conditions in the present study showed that basal MS media augmented with the combinations of BAP (1.5 mg L^{-1}) and NAA (0.5 mg L^{-1}) gave the maximum accumulation of all phenolic compositions in compared to the rest of media (BAP alone) used. These findings were in accordance with the finding of Shilpashree and Rai Vittal (2009), Karalija and Parić (2011), and Hemmati et al. (2020), who demonstrated a better effect of the combinations of plant hormones on phenolic compound production in *Hypericum mysorense* microshoots, *Thymus vulgaris* in vitro shoot, and *S. tebesana* calli, respectively. Similarly, El-Baz and Mohamed (2010) showed that the pretreatment of a higher concentration of BAP (more than 1 mg L^{-1}) to the culture medium with the lower NAA levels (1 mg L^{-1}) caused the highest contents of polyphenol and flavonoid in callus culture of *Citrullus colocynthis*. These observations were also supported by the findings of Maharik et al. (2009), who found that a high BAP to NAA ratio induced more accumulation of anthocyanin in *Crataegus sinaica* calli. Besides the inducing influence of plant hormones on the activity of the key enzyme of phenolic production, phenylalanine ammonia-lyase (PAL), outlined in the literature review showed that the level of this induction varies based on the type of hormone and its concentration (Cheng et al. 2005; Saleh Abadi and Mehraban Sang Atash 2015).

Application of the exogenous elicitors on the plant triggers the hypersensitivity reaction signals and subsequent activation of specific genes, leading to increased secondary metabolite activation. The increment in the transcription value of *PAL* (the main enzyme in the shikimate-phenylpropanoids-flavonoids pathway) has been reported after MeJA (Singh et al. 2002; Yousefian et

al. 2020) and TiO_2 nanoparticles (Karimzadeh et al. 2019) treatments. In the current study, the application of both elicitors not only enhanced the content of total phenolic compounds, but their promotive effects on the values of flavonoid, phenolic acid, rosmarinic acid, as well as main flavones apigenin and luteolin were more remarkable. Similarly, the enhanced accumulation of polyphenols in *T. polium* callus culture (Hashemyan et al. 2020) and *Salvia officinalis* shoot cultures (Grzegorczyk and Wysokińska 2010), the elevated value of phenolic acids in *S. leriifolia* Benth callus culture (Ghasemihagh et al. 2018), S. *miltiorrhiza* hairy root cultures (Xing et al. 2018b) and *Exacum affine* Balf. shoot culture (Skrzypczak-Pietraszek et al. 2014), and the increased level of flavonoids in *Pueraria lobata* Ohwi tissue culture (Thiem and Krawczyk 2010) and *Pueraria tuberosa* suspension culture (Goyal and Ramawat 2008) have been indicated after MeJA pretreatment. A review of the current literature on NMs indicated that the treatment with nano-sized TiO_2 greatly enhanced the phenolic compositions of *S. officinalis* and *Cicer arietinum* L. in vitro as compared with the control samples (Mazarie et al. 2019; AL-Oubaidi and Kashid 2015). More broadly, several studies have shown that the corresponding elicitors trigger the production of secondary metabolites in a dose-dependent manner (Hashemyan et al. 2020, Dovom et al. 2017, Samadi et al. 2014, Xing et al. 2018b), which coincides with the present results, with an emphasis on the highest stimulation of phenolic accumulation under the optimal concentration of MeJA (50 µM) and nano-sized TiO_2 (10 mg L^{-1}) treatments. This might be attributed to the overproduction of reactive oxygen species (ROS) as inductive dues for modulating secondary metabolism directly or indirectly. Elicitors as signaling molecules regulate a complex array of oxidative defense machinery in plant cells by initial increasing ROS production, elevating ionic currents across the plasma membrane, activation of downstream transcription factors that ultimately leads to activating defense-related genes, and the synthesis and accumulation of phytoalexins (Zhao et al. 2005; Wang and Weller 2006). However, sometimes and with overstimulation, it lead to disruption of ROS homeostasis and subsequent explosion of oxidative stress. This can thereby decrease the production of phenolic metabolites, via the modifying and reducing effect on enzyme activity or expression of the relevant genes (Samadi et al. 2014; Mir et al. 2019). Besides, multiple studies have shown that the effects of methyl esters of jasmonic acid may vary depending on the plant species, the type of specific secondary metabolite, and the duration of exposure to the elicitor (Vasconsuelo and Boland 2007; Verma et al., 2016). Although elicitors are believed to lead to the signaling of jasmonic acid (Doares et al. 1995), fewer effects of TiO_2 than MeJA on in vitro phenolic production of *T. polium* may be explained by over-accumulation of MeJA and its subsequent negative feedback.

The balance between oxidants and antioxidants plays an important role in neutralizing the harmful effects caused by the increase of free radicals in plants (Saleh Abadi and Mehraban Sang Atash 2015). The antioxidant defense system in plants includes the enzymatic antioxidants (namely peroxidase, catalase, superoxide dismutase, etc.), and non-enzymatic antioxidants (such as vitamins, carotenoids, polyphenol, etc.)(Shen et al. 2009). Natural antioxidants of many plants are related to polyphenolic compounds, and any factor that increases the composition of phenolic and flavonoid compounds would improve the antioxidant properties (Karabal et al. 2003). Our data indicate that the extracts originating from calli grown on MS media containing a concentration of MeJA and nano-sized TiO_2, respectively, showed an intense antioxidant activity (lower IC_{50} value) as compared to the reference control reagent, BHT. Strong radical scavenging activity of plant extracts was due to resultant increment of phenolic constituents that were in agreement with that has been investigated previously on *S. tebesana* (Hemmati et al. 2020), T. *polium* (Hashemyan et al. 2020), S. *virgata* (Dovom et al. 2017), *Lavandula angustifolia* (Kiarostami and Mosafa 2015), *Ziziphora teniur* (Dakah et al. 2014), *Citrullus colocynthis* (El-Baz et al., 2010), *Star fruits* (Annegowda et al. 2012) ,and *Basil* (Kim et al. 2006). In other words, the level of phenolic compounds and antioxidant reactivity of elicitor subjected samples depend on the source of elicitor, its concentration, and plant growth stage.

There are complex interactions between medium constituents (Nas et al. 2013), so it is difficult to determine the optimum levels of medium compounds for a culture medium. Based on the present result, the application of ANN provides this system the ability to model complex non-linear relationships between the input and output of bioprocesses, such as in vitro production of phenolic compounds. These data confirmed that the prediction of ANN for those culture conditions, in which maximum phenolic derivatives can be produced, is coincide with the behavior of our experimental in vitro culture confirming that the selected concentrations of hormone and elicitors of the MS medium were adequate for optimal secondary metabolite accumulation.

Conclusion

This study noticeably suggested that the hormonal combinations of BAP plus NAA in selected concentrations (1.5 and 0.5 mg L^{-1}, respectively) are noticeable effective in inducing callus from leaf explants of *T. polium*. Compared to other concentrations, 50 µM MeJA as a more potent elicitor can induce the production of various phenolic derivatives, most notably apigenin, luteolin, and rosmarinic acid; and consequently, enhance the antioxidant capacity in *Teucrium* callus as compared with 10 mg L^{-1} nano-sized TiO_2. However, there is no linear relation in the elevating of phenolic composition contents proportional to elicitor exposure concentration, in a way the lower concentrations of elicitors favored more callus biomass and secondary metabolites production. Based on a strong correlation between the hyperbolic-tangent activation function of outputs and present experimental data (R^2 more than 0.98), ANN could successfully model and optimize a new culture media for sufficient phenolic derivatives production.

Abbreviations

ANN	Artificial Neural Network
BAP	6-Benzylaminopurine
DPPH	2,2-Diphenyl-1-picrylhydrazyl
DW	Dry weight
FRAP	Ferric reducing antioxidant power
FW	Fresh weight
MeJA	Methyl jasmonate
MS	Murashige and Skoog medium
NAA	Naphthalene acetic acid
RA	Rosmarinic acid
ROS	Reactive oxygen species

Declarations

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Author contributions Monireh Cheniany conceived and designed the experiments. Mahshid Tabarifard performed the experiments. Monireh Cheniany and Mohammad Khalilian-movahhed analyzed the data. Monireh Cheniany and Mohammad Khalilian-movahhed contributed reagents/materials/ analysis tools. Monireh Cheniany and Mahshid Tabarifard performed wrote the manuscript. Monireh Cheniany checked and validated the results. All authors read and approved the final manuscript.

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

Data availability All data generated or analysed during this study are included in this article.

References

1. Ali F, Rahul Naz F, Jyoti S, Siddique YH (2017) Health functionality of apigenin: A review. Int J Food Prop 20: 6, 1197-1238. https://doi.org/10.1080/10942912.2016.1207188
2. AL-Oubaidi HKM, Kashid NM (2015) Increasing phenolic and flavonoids compounds of *Cicer arietinum* L. from embryo explant using titanium dioxide nanoparticle in vitro. World J Pharm Res 2015, 4, 1791
3. Annegowda HV, Bhat R, Min-Tze L; Karim AA, Mansor SM (2012) Influence of sonication treatments and extraction solvents on the phenolics and antioxidants in star fruits. J Food Sci Technol 49: 510-514. https://doi.org/10.1007/s13197-011-0435-8
4. Antognoni F, Iannello C; Mandrone M, Scognamiglio M, Fiorentino A, Giovannini PP, Poli F (2012) Elicited *Teucrium chamaedrys* cell cultures produce high amounts of teucrioside, but not the hepatotoxic neo-clerodane diterpenoids. Phytochem 81: 50–59. https://doi.org/10.1016/j.phytochem.2012.05.027
5. Arab MM, Yadollahi A, Shojaeiyan A, Ahmadi H (2016) Artificial neural network genetic algorithm as powerful tool to predict and optimize In vitro proliferation mineral medium for G × N15 rootstock. Front Plant Scie 19: 1526. https://doi.org/10.3389/fpls.2016.01526
6. Bakhtiar Z, Mirjalili M, Sonboli A (2016) In vitro callus induction and micropropagation of *Thymus persicus* (Lamiaceae), an endangered medicinal plant. Crop Breed Appl Biotechnol 16: 48-54. https://doi.org/10.1590/1984-70332016v16n1a8
7. Briskin DP (2000) Medicinal plants and phytomedicines. Linking plant biochemistry and physiology to human health. Plant Physiol 124:507–514. https://doi.org/10.1104/pp.124.2.507
8. Carrasco-Pancorbo A, Cerretani L, Bendini A, Segura-Carretero A, Gallina-Toschi T, Fernandez-Gutiérrez A (2005) Analytical determination of polyphenols in olive oils. J Sep Sci 28, 837–858. https://doi.org/10.1002/jssc.200500032
9. Carreiras MC, Rodríguez B, de la Terre MC, Perales A, Torres MR, Savona G, Piozzi F (1990) Rearranged abietane diterpenoids from the root of *Teucrium polium* subsp. Vincetinum. Tetrahedron 46: 847–860. https://doi.org/10.1016/S0040-4020(01)81367-3
10. Castiglione MR, Giorgetti L, Geri C, Cremonini R (2011) The effects of nano-TiO_2 on seed germination, development and mitosis of root tip cells of *Vicia narbonensis* L. and *Zea mays* L. J Nanoparticle Res 13: 2443-2449. https://doi.org/10.1007/s11051-010-0135-8
11. Cheng S, Wang Y, Liu W, Chen K (2005) Effects of plant growth regulators on phenylalanine ammonia-lyase (PAL) activities in leaves of *Ginkgo biloba* in vitro. *J Plant Resour Environ* 14: 20-22
12. Chinnamadasamy K, Arjunan D, Ramasamy MV (2010) Rapid micropropagation of *Plumbago zeylanica* L. An important medicinal plant. J Am Sci 6: 1027-1031
13. Dakah A, Zaid S, Suleiman M, Abbas S, Wink M (2014) In vitro propagation of the medicinal plant *Ziziphora tenuior* L. and evaluation of its antioxidant activity. Saudi J Biol Sci 21: 317-323. https://doi.org/10.1016/j.sjbs.2013.12.002
14. de Torre MP, Cavero RY, Calvo MI, Vizmanos JL (2019) A simple and a reliable method to quantify antioxidant activity in vivo. Antioxidants 8: 142. https://doi.org/10.3390/antiox8050142
15. Doares SH, Syrovets T, Weiler EW, Ryan CA (1995) Oligogalacturonides and chitosan activate plant defensive genes through the octadecanoid pathway. Proc Natl Acad Sci 92: 4095- 4098. https://doi.org/10.1073/pnas.92.10.4095
16. Dode L, Bobrowski VL, Braga EJB, Seixas FK, Schuch MW (2003) In vitro propagation of *Ocimum basilicum* L. (Lamiaceae). Acta Sci Biol Sci 25(2): 435-437
17. Dowom SA, Abrishamchi P, Rajabian T, Salami SA (2017) Enhanced phenolic acids production in regenerated shoot cultures of *Salvia virgata* Jacq. after elicitation with Ag^+ ions, methyl jasmonate and yeast extract. Ind Crops Prod 103: 81-88. *https://doi.org/*10.1016/j.indcrop.2017.03.043
18. Dronne S, Jullien F, Caissard JC, Faure O (1999) A simple and efficient method for in vitro shoot regeneration from leaves of *lavandin* (*Lavandula intermedia* Emeric ex Loiseleur). Plant Cell Rep 18: 429–433. https://doi.org/10.1007/s002990050598
19. El-Baz FK, Mohamed AA, Ali SI (2010) Callus formation, phenolics content and related antioxidant activities in tissue culture of a medicinal plant colocynth (*Citrullus colosynthis*). *Nova Biotechnol et Chim* 10(2):79–94.

https://doi.org/10.36547/nbc.1118

20. Elyasi L, Mehrabi AA, Seyedi M, Safari Z (2017) Effect of explant origin and different concentrations of growth regulators on optimization of cell suspension in *Satureja bachtiarica* L. Iran J Med Aromat Plants 32: 998-1009. https://doi.org/10.22092/IJMAPR.2017.109318
21. Ghasemihagh Z, Jokar S, Bodaghi H, Modarres M (2018) Effect of salicylic acid and methyl jasmonate on the production of rosmarinic acid and caffeic acid in callus culture of *Salvia lerrifolia* Benth. Iran J Plant Biol 10: 67-80. https://doi.org/10.22108/ijpb.2018.108819.1071
22. Gómez JD, Vital CE, Oliveira MG, Ramos HJ (2018) Broad range flavonoid profiling by LC/MS of soybean genotypes contrasting for resistance to *Anticarsia gemmatalis* (Lepidoptera: Noctuidae). Plos One 13. https://doi.org/10.1371/journal.pone.0205010
23. Goyal S, Ramawat KG (2008) Ethrel treatment enhanced isoflavonoids accumulation in cell suspension cultures of *Pueraria tuberosa*, a woody legume. Acta Physiol Plant 30: 849 – 853. https://doi.org/10.1007/s11738-008-0190-2
24. Grzegorczyk I, Wysokińska H (2009) The effect of methyl jasmonate on production of antioxidant compounds in shoot cultures of *Salvia officinalis* L. Herba Pol 55: 238–43
25. Hashemyan M, Ganjeali A, Cheniany M (2020) Effect of methyl jasmonate and salicylic acid elicitors on the production of secondary metabolites and antioxidant capacity of *Teucrium polium* L. in-vitro. Iran J Plant Biol 12: 61-76. https://doi.org/10.22108/IJPB.2020.118410.1164
26. Hemmati N, Cheniany M, Ganjeali A (2020) Effect of plant growth regulators and explants on callus induction and study of antioxidant potentials and phenolic metabolites in *Salvia tebesana* Bunge. Bot Serbica 44: 163-173. https://doi.org/10.2298/BOTSERB2002163H
27. Hoagland DR, Arnon DI (1950) The water-culture method for growing plants without soil. Circ Calif Agric Exp Stn 347: 32. https://doi.org/10.3390/ijms21030716
28. Jaradat NA (2015) Review of the taxonomy, ethnobotany, phytochemistry, phytotherapy and phytotoxicity of Gramander plant (*Teucrium polium*). Asian J Pharm Clin Res 8: 13–19
29. Javidi Moghadam M, Cheniany M, Ganjeali A, Lahouti M (2016) An investigation on callogenesis and antioxidant capacity of different explants of *Teucrium polium.* Iran J Plant Biol 8(29): 37-52
30. Karabal E, Yücel M, Öktem HA (2003) Antioxidant responses of tolerant and sensitive barley cultivars to boron toxicity. Plant Sci 164: 925-933. https://doi.org/10.1016/S0168-9452(03)00067-0
31. Karalija E, Parić A (2011) The effect of BA and IBA on the secondary metabolite production by shoot culture of *Thymus vulgaris* L. Biol Nyssana 2: 29-35
32. Karimzadeh F, Haddad R, Garoosi Gh (2019) The effects of nano-ZnO and nano-TiO_2 on expression of Phenylalanine ammonia lyase (PAL) and Cinnamyl alcohol dehydrogenase (CAD) genes in cell culture of *Linum usitatissimum* L. Mod Genet J 14(2): 171-177
33. Kawashty SA, Gamal El-Din EM, Saleh NAM (1999) The flavonoid chemosystematic of two *Teucrium* species from Southern Sinai, Egypt. Biochem Syst Ecol 27: 657-660. https://doi.org/10.1016/S0305-1978(97)00109-9
34. Kiarostami K, Mosafa N (2015) The study of antioxidant properties of *Lavandula angustifolia* in tissue culture. J Plant Res 28: 835-843
35. Kim HJ, Chen F, Wang X, Rajapakse NC (2006) Effect of methyl jasmonate on secondary metabolites of sweet basil (*Ocimum basilicum* L.). J Agr Food Chem 54: 2327-2332. https://doi.org/ 10.1021/jf051979g
36. Koocheki A, Nassiri-Mahallati M, Azizi G (2008) Effect of drought, salinity, and defoliation on growth characteristics of some medicinal plants of Iran. J Herbs Spices Med Plants 14: 37-53. https://doi.org/10.1080/10496470802341201
37. Kosalec I, Bakmaz M, Pepeljnjak S, Vladimir-Knezević S (2004) Quantitative analysis of the flavonoids in raw propolis from northern Croatia. *Acta Pharm* 54: 65-72
38. Li T, Elhadi D, Chen GQ (2017) Co-production of microbial polyhydroxy alkanoates with other chemicals. Metab Eng 43: 29-36. https://doi.org/10.1016/j.ymben.2017.07.007

39. Lyu Z, Yu Y, Samali B, Rashidi M, Mohammadi M, Nguyen TN, Nguyen A (2022) Back-propagation neural network optimized by K-fold cross-validation for prediction of torsional strength of reinforced concrete bean. Mater 15: 1477. https://doi.org/10.3390/ma15041477
40. Maharik N, Elgengaihi S, Taha H (2009) Anthocyanin production in callus cultures of *Crataegus sinaica* Boiss.*Int J Acad* Res 1(1):30–34. https://doi.org/10.7813/2075-4124.2009/1-1/A.3.
41. Marinova D, Ribarova F, Atanassova M (2005) Total phenolics and total flavonoids in Bulgarian fruits and vegetables. J Univ Chem Technol Metallurgy 40(3): 255-260
42. Matkowski A, Zielińska S, Oszmiański J, Lamer-Zarawska E (2008) Antioxidant activity of extracts from leaves and roots of *Salvia miltiorrhiza* Bunge, *S. przewalskii* Maxim. and *S. verticillata* L. Bioresour Technol 99: 7892-7896. https://doi.org/10.1016/j.biortech.2008.02.013
43. Mazarie A, Mousavi nik SM, Ghanbari A, Fahmideh L (2019) Effect of titanium dioxide spraying on physiological characteristics of sage (*Salvia officinalis* L.) under water stress. Environ Stress Crop Sci 12: 539-553. https://doi.org/10.22077/ESCS.2019.1369.1304
44. Mederos-Molina S (2004) In vitro callus induction and plants from stem and petiole explants of *Salvia canariensis* L. Plant Tissue Cult, 14: 167-172
45. Meguellati H, Ouafi S, Saad S, Djemouai N (2019) Evaluation of acute, subacute oral toxicity and wound healing activity of mother plant and callus of *Teucrium polium* L. subsp. *geyrii* Maire from Algeria. S Afr J Bot 127, 25-34. https://doi.org/10.1016/j.sajb.2019.08.023
46. Mihailović V, Stanković JSK, Mihailović N (2017) Phenolic compounds diversity of *Teucrium* Species. *Teucrium* species: biology and applications: Springer Nature: Cham, Switzerland 143-177. https://doi.org/10.1007/978-3-030-52159-2-6
47. Minaei Chenar H, Kahrizi D, Molsaghi M (2016) Callus induction is affected by explant type and plant growth regulators in chickpea (*Cicer arietinum* L.). Biharean Biol 10(1): 28-32
48. Mir MY, Hamid S, Kamili AN, Hassan QP (2019) Sneak peek of *Hypericum perforatum* L.: phytochemistry, phytochemical efficacy and biotechnological interventions. J Plant Biochem Biotechnol 28: 357-373. https://doi.org/10.1007/s13562-019-00490-7
49. Namdeo AG (2007) Plant cell elicitation for production of secondary metabolites: a review. Pharmacogn Rev 1(1): 69-79
50. Narayani M, Srivastava S (2017) Elicitation: a stimulation of stress in in vitro plant cell/tissue cultures for enhancement of secondary metabolite production. Pharmacogn Rev 16: 1227-1252. https://doi.org/10.1007/s11101-017-9534-0
51. Nas MN, Bolek Y, Sevgin N (2013) Shortcut to long-distance developing of a tissue culture medium: micropropagation of mature almond cultivars as a case study. Turk J Bot 37: 1134–1144. https://doi.org/10.3906/bot-1302-49
52. Navarro T (2010) Syetematics and biogeography of genus *Teucrium* (Lamiaceae). *Teucrium* Species: Biology and Applications: Springer Nature: Cham, Switzerland 1-38. https://doi.org/10.1007/978-3-030-52159-2-1
53. Öztürk M, Duru ME, İnce B, Harmandar M, Topçu G (2010) A new rapid spectrophotometric method to determine the rosmarinic acid level in plant extracts. Food Chem 123: 1352-1356. https://doi.org/10.1016/j.foodchem.2010.06.021
54. Parthibhan S, Venkateswara Rao MV, Teixeira da Silva JA, Senthil Kumar T (2018) Somatic embryogenesis from stem thin cell layers of *Dendrobium aqueum*. Biol Plant 62: 439-450. https://doi.org/10.1007/s10535-018-0769-4
55. Ramawat KG, Mérillon JM (2008) Bioactive molecules and medicinal plants. Berlin: Springer 18-22
56. Ramezannejad R, Mofid Bojnoordi M, Armin M, Aghdasi M (2022) Artificial neural network modeling to predict and optimize phenolic acids production from callus culture of Lavtuca undulate Ledeb. In vitro Cell Dev Biol 58(4): 653-663. https://doi.org/10.21203/rs.3.rs-750673/v1
57. Saleh Abadi S, Mehraban Sang Atash M (2015) Evaluation of the antioxidant activity and total phenols, flavonoids in methanolic, dichloromethane and ethyl acetate extracts of aerial parts of *Rubia florida*. J North Khorasan Univ Med Sci 7: 101-112
58. Samadi S, Ghasemnajad A, Alizadeh M (2014) Investigation on phenylalanine ammonia-lyase activity of artichoke (*Cynara scolymus* L.) affected by methyl jasmonate and salicylic acid in in-vitro conditions. J Plant Prod (J Agric Sci Nat

Resour) 21(4): 135-148

59. Scrinis G, Lyons K (2007) The emerging nano-corporate paradigm: nanotechnology and the transformation of nature, food and agri-food systems. Int J Sociol Agric Food 15: 22-44. https://doi.org/10.48416/ijsaf.v15i2.293
60. Sharififar F, Dehghn-Nudeh G, Mirtajaldini M (2009) Major flavonoids with antioxidant activity from *Teucrium polium* L. Food Chem 112: 885-888. https://doi.org/10.1016/j.foodchem.2008.06.064
61. Shen Y, Jin L, Xiao P, Lu Y, Bao J (2009) Total phenolics, flavonoids, antioxidant capacity in rice grain and their relations to grain color, size and weight. J Cereal Sci 49(1): 106-111. https://doi.org/10.1016/j.jcs.2008.07.010
62. Shilpashree HP, Rai Vittal R (2009) In vitro plant regeneration and accumulation of flavonoids in *Hypericum mysorense*. Int J Integr Biol 8(1): 43-49
63. Shoja AA, Çirak C, Ganjeali A, Cheniany M (2022) Stimulation of phenolic compounds accumulation and antioxidant activity in in vitro culture of *Salvia tebesana* Bunge in response to nano-TiO_2 and methyl jasmonate elicitors. Plant Cell Tissue Organ Cult 149: 423–440. https://doi.org/10.1007/s11240-022-02251-2
64. Singh RP, Chidambara Murthy KN, Jayaprakasha GK (2002) Studies on the antioxidant activity of pomegranate (*Punica granatum*) peel and seed extracts using in vitro models. J Agr Food Chem 50: 81-86. https://doi.org/10.1021/jf010865b
65. Skrzypczak-Pietraszek E, Słota J, Pietraszek J (2014) The influence of L-phenylalanine, methyl jasmonate and sucrose concentration on the accumulation of phenolic acids in *Exacum affine* Balf. f. ex Regel shoot culture. Acta Biochim Pol 61(1): 47-53. https://doi.org/10.18388/abp.2014-1922
66. Soltani pool MM, Mohamadi A, Rahnama H, Abbaszadeh B (2011) Callus genesis investigation of lemon balm (*Melissa officinalis* L.). J Agron Plant Breed 7: 45-54
67. Thiem B, Krawczyk A (2010) Enhanced isoflavones accumulation in methyl jasmonate-treated *in vitro* cultures of kudzu (*Pueraria lobata* Ohwi). Herba Pol 56(1): 48-56
68. Vasconsuelo A, Boland R (2007) Molecular aspects of the early stages of elicitation of secondary metabolites in plants. Plant Sci 172(5): 861-875. https://doi.org/10.1016/j.plantsci.2007.01.006
69. Verma S (2016) Medicinal plants with anti-inflammatory activity. The Journal of Phytopharmacology, 5 (4): 157–159
70. Wang L, Weller CL (2006) Recent advances in extraction of nutraceuticals from plants. Trends Food Sci Tech 17: 300-312. https://doi.org/10.1016/j.tifs.2005.12.004
71. Xing B, Yang D, Liu L, Han R, Sun Y, Liang Z (2018a) Phenolic acid production is more effectively enhanced than tanshinone production by methyl jasmonate in *Salvia miltiorrhiza* hairy roots. Plant Cell Tissue Organ Cult 134: 119-129. https://doi.org/10.1007/s11240-018-1405-x
72. Xing B, Yang D, Yu H, Zhang B, Yan K, Zhang X, Han R, Liang Z (2018b) Overexpression of SmbHLH10 enhances tanshinones biosynthesis in *Salvia miltiorrhiza* hairy roots. Plant Sci 276: 229-238. https://doi.org/10.1016/j.plantsci.2018.07.016
73. Yousefian S, Lohrasebi T, Farhadpour M, Haghbeen K (2020) Effect of methyl jasmonate on phenolic acids accumulation and the expression profile of their biosynthesis-related genes in *Mentha spicata* hairy root cultures. Plant Cell Tissue Organ Cult 142: 285–297. https:// doi. org/ 10. 1007/ s11240- 020- 01856-9
74. Zhao J, Davis LC, Verpoorte R (2005) Elicitor signal transduction leading to production of plant secondary metabolites. Biotech Adv 23(4): 283-333. https://doi.org/10.1016/j.biotechadv.2005.01.003
75. Zhishen J, Mengcheng T, Jianming W (1999) The determination of flavonoid contents in mulberry and their scavenging effects on superoxide radicals. Food Chem 64: 555–559. https://doi.org/10.1016/S0308-8146(98)00102-2h

Figures

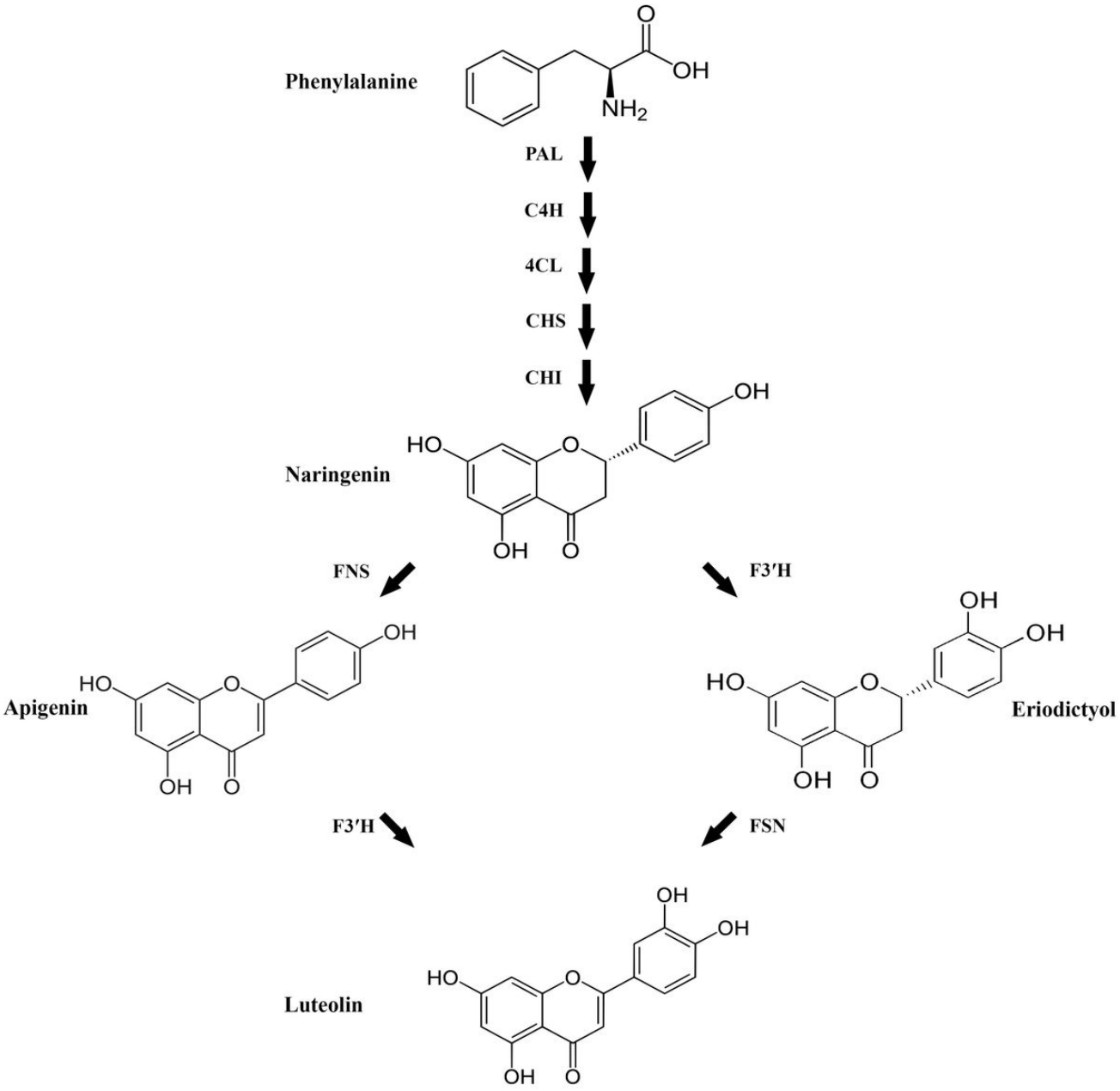


Figure 1

A brief biosynthetic pathway of the flavones apigenin and luteolin in plants. Abbreviations: PAL, phenylalanine ammonia-lyase; C4H, cinnamic acid 4-hydroxylase; 4CL, 4-coumaric acid:CoA ligase; CHS, chalcone synthase; CHI, chalcone isomerase, FNS, flavone synthase; F3'H, flavonoid 3' hydroxylae.

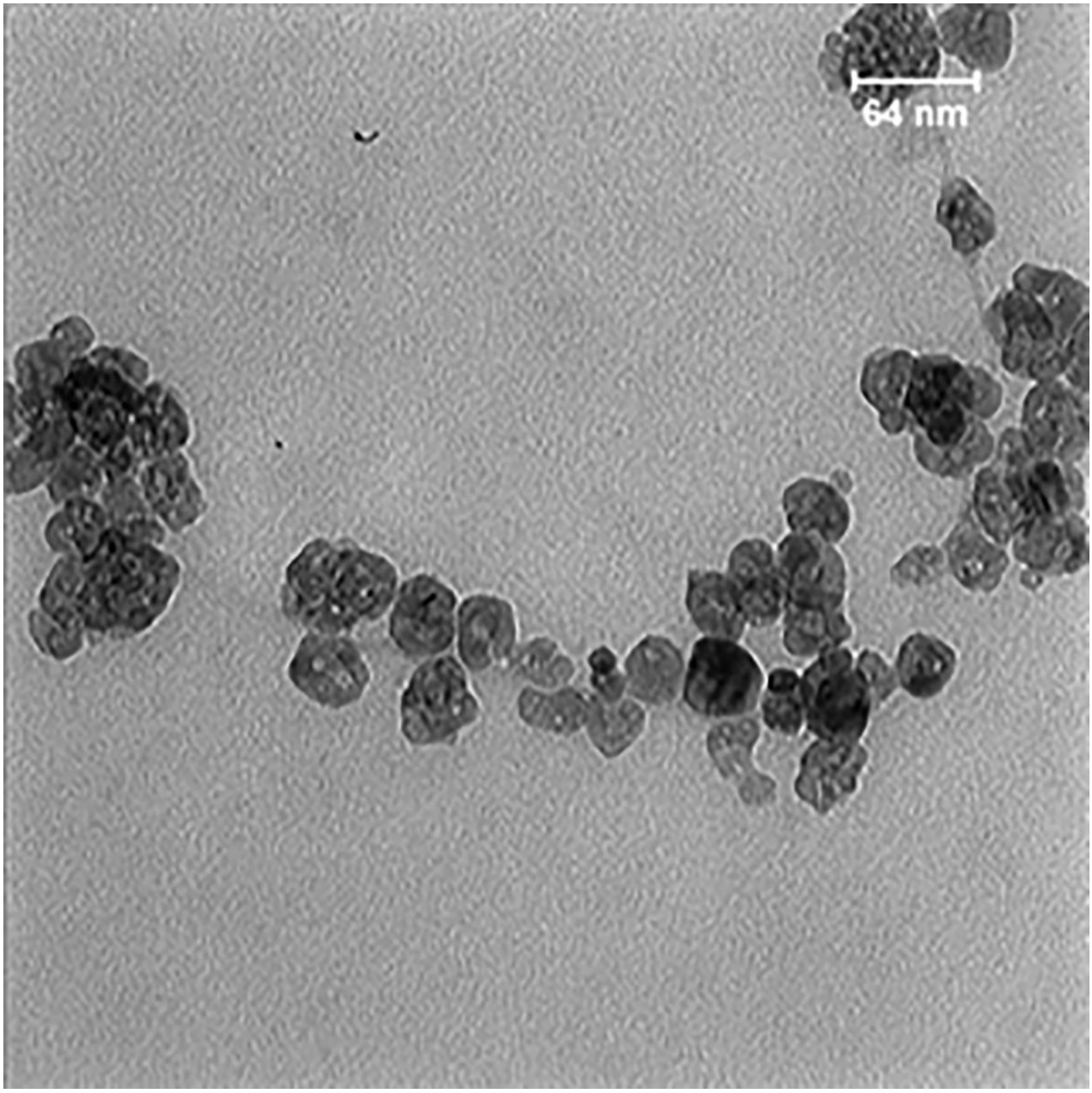


Figure 2

Transmission electron microscopy (TEM) image of TiO_2 nanoparticles. Distribution of particles size was estimated to be 20–25 nm, scale bar = 64 nm.

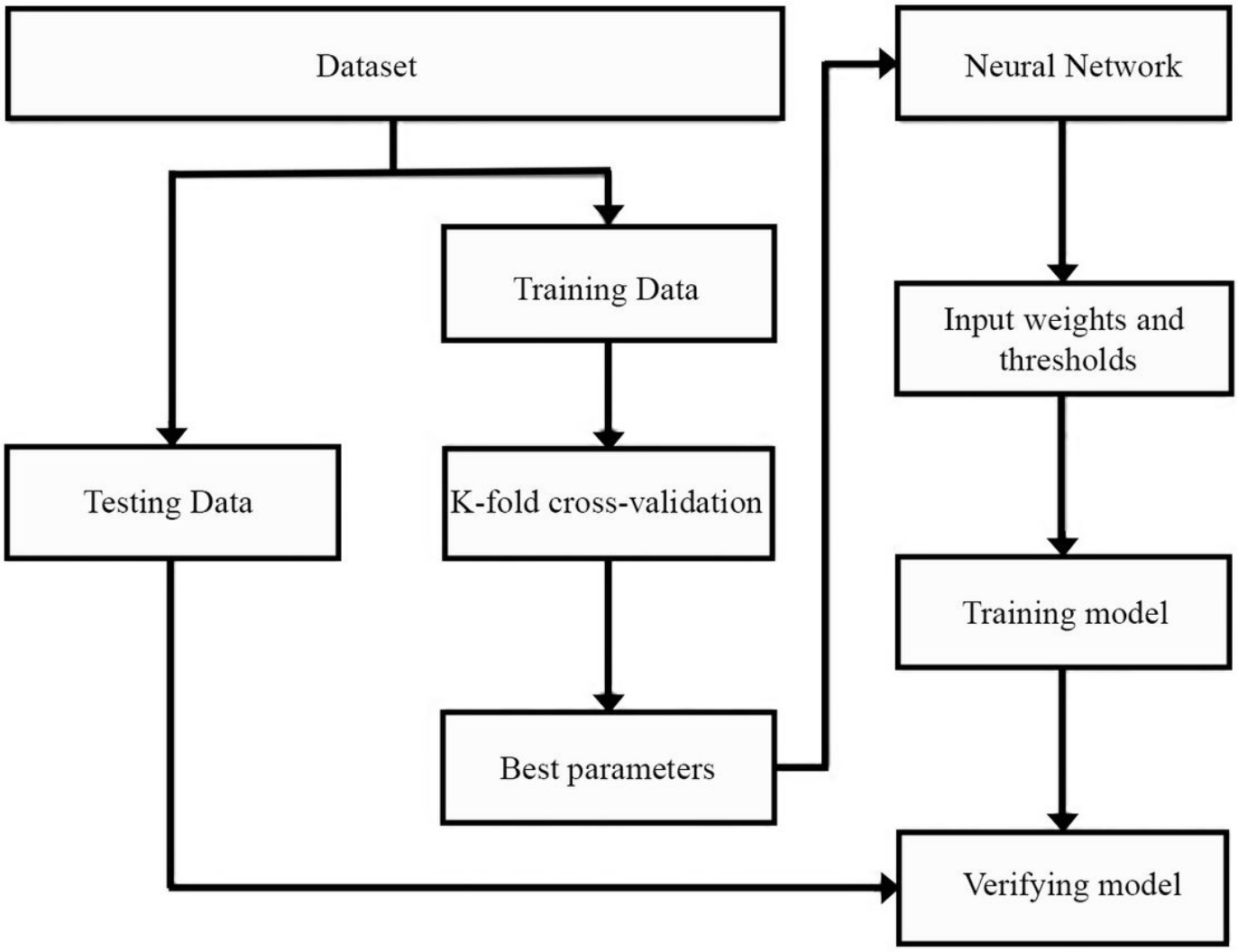


Figure 3

Flow chart of neural network optimized by K-fold cross-validation

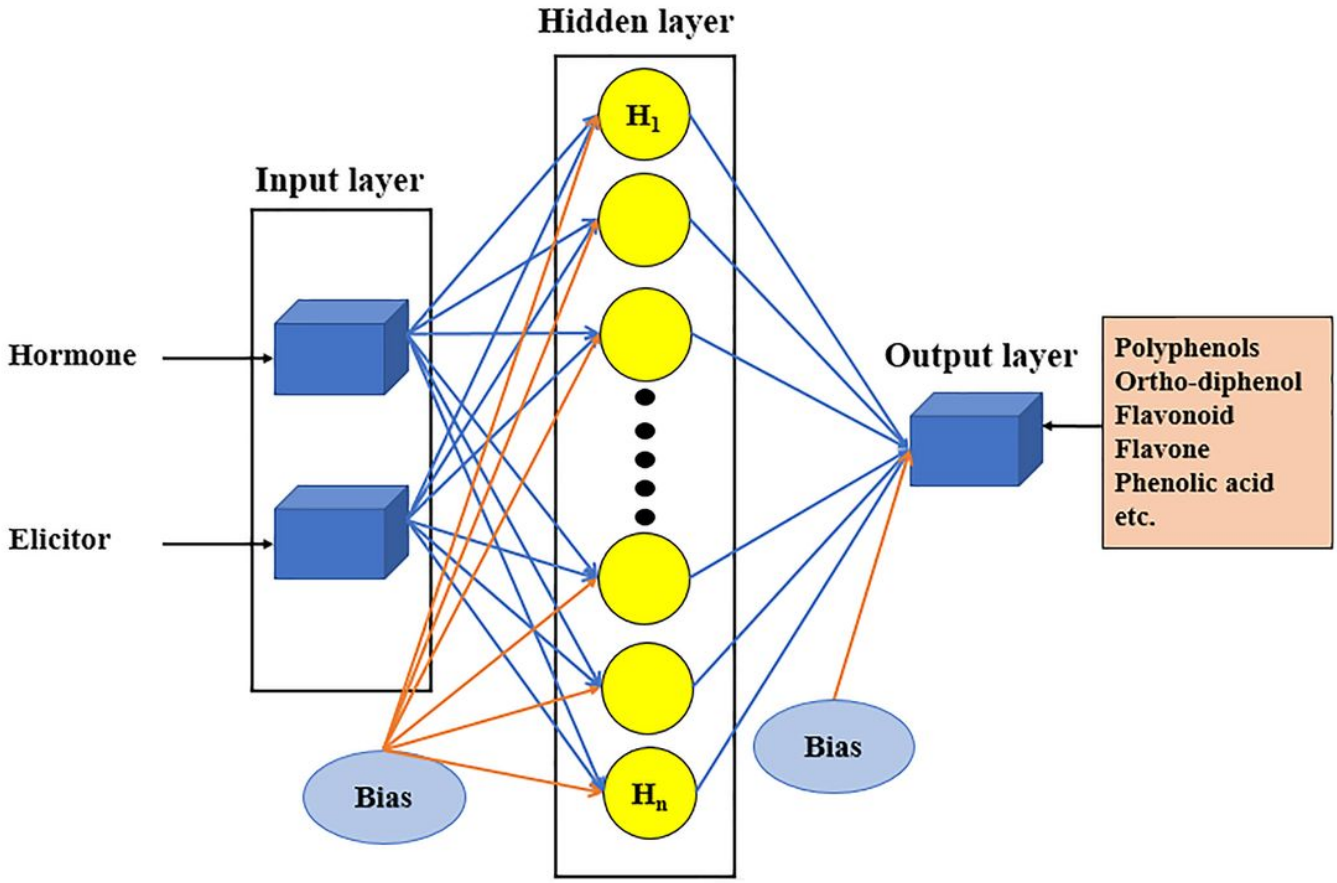


Figure 4

Back-propagation neural network architecture for the prediction of secondary metabolites under elicitors

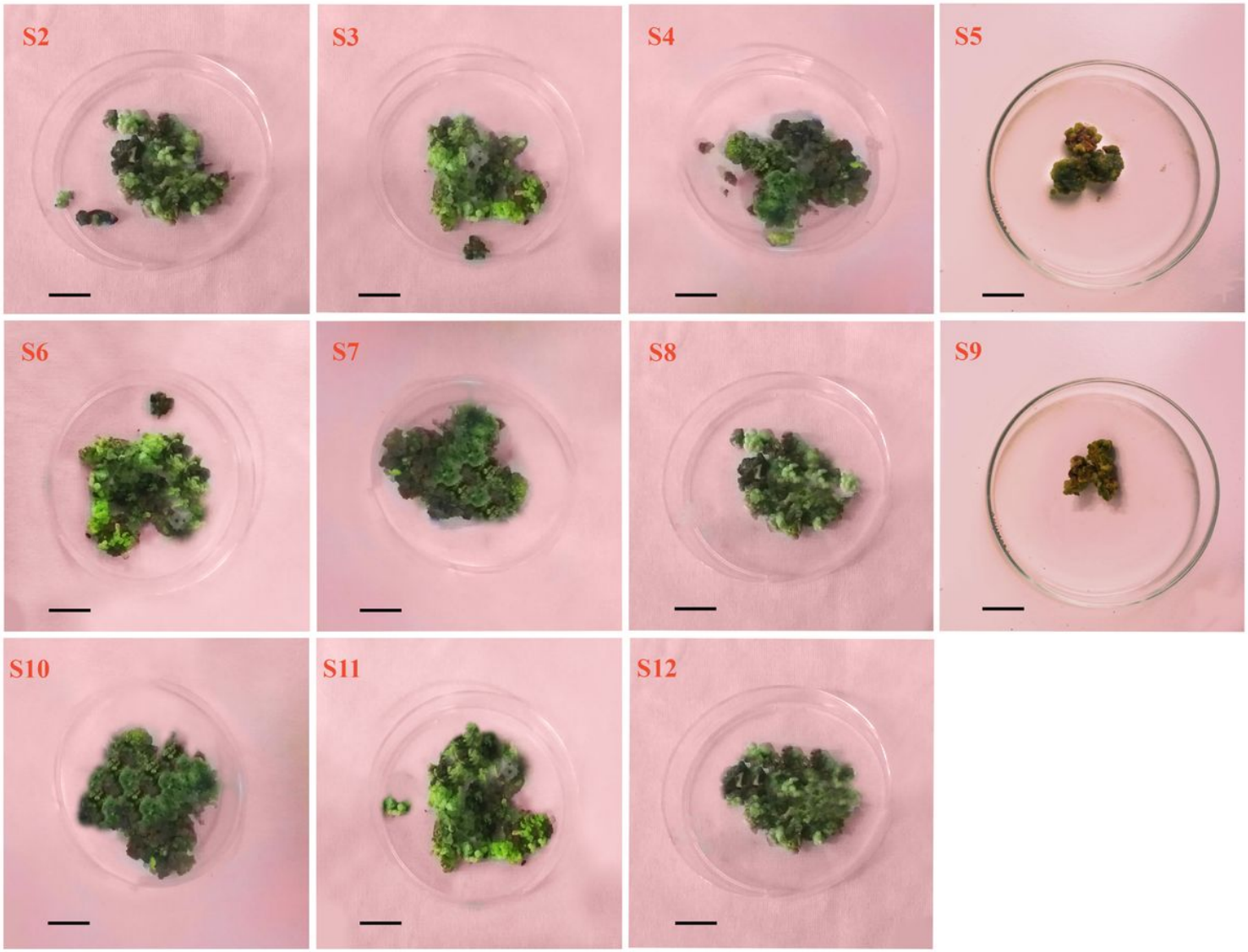


Figure 5

Appearance of callus *originated from* leaf explant of *T. polium,* on basal media supplemented with BAP or in combination with NAA, scale bar = 1 cm.

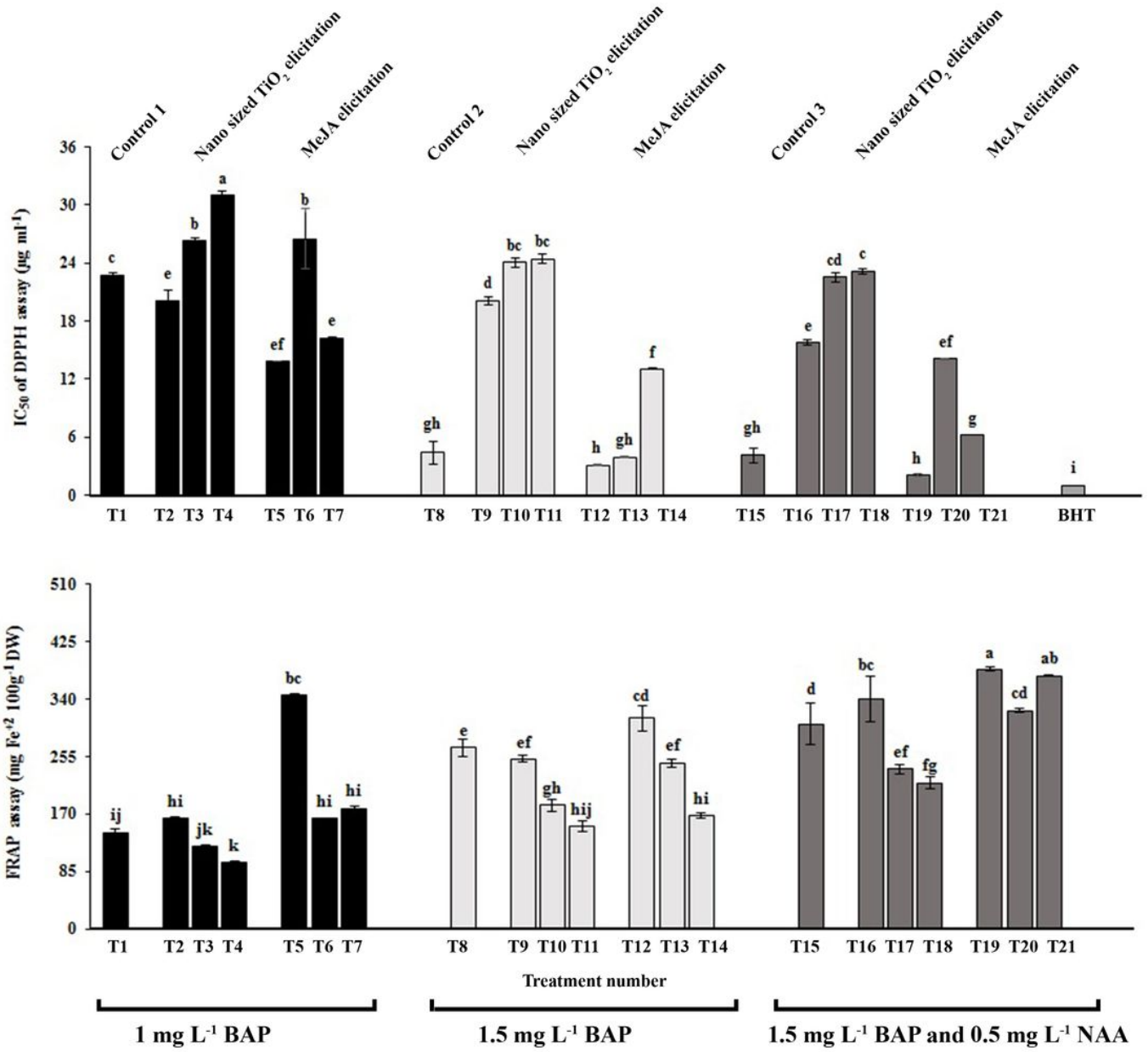


Figure 6

Comparison of the average data obtained from radical scavenging assays in elicitor-treated calluses created on the culture media with single or combined hormones: a) DPPH assay, and b) FRAP assay.

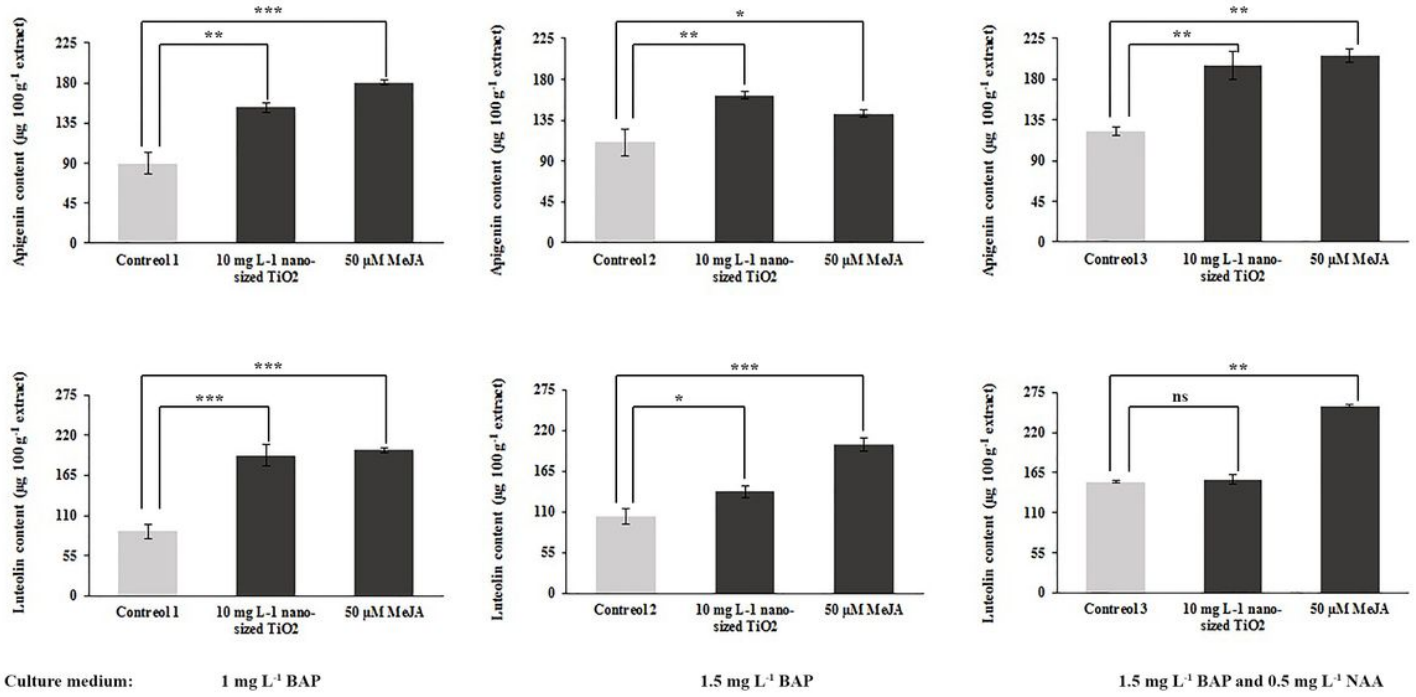


Figure 7

Changes in relative amounts of two main flavones (apigenin and luteolin) in leaf-derived callus of *T. polium,* elicited with 50 µM MeJA and 10 mg L^{-1} nano-sized TiO_2 elicitors. Data are expressed as mean ± standard deviation (SE), significant differences are indicated as *: $P \leq 0.05$, **: $P \leq 0.01$, ***: $P \leq 0.001$, and ns: non significant; n = 3.

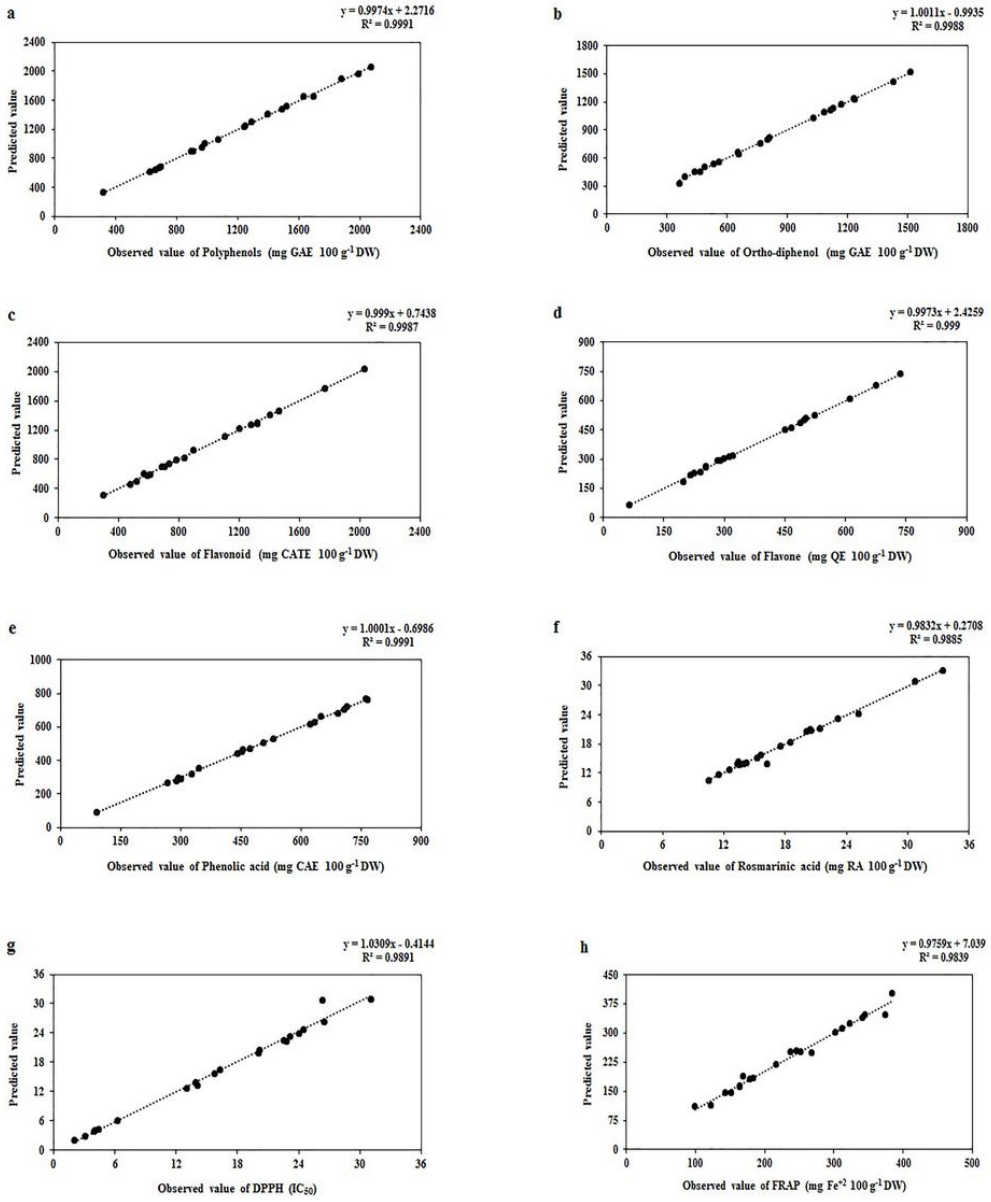


Figure 8

Scatter plot of observed experimental data: a) total phenolic, b) Ortho-diphenols, c) flavonoids, d) flavones, e) phenolic acids, f) rosmarinic acid, g) DPPH, and h) FRAP versus ANN model-predicted values.