Elicitation of the Himalayan Toothache Relieving Tree (Zanthoxylum armatum DC .): Enhancing Secondary Metabolites in In vitro Shoot Cultures

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Abstract

This study investigates the impact of various elicitors on the production of secondary metabolites in the "Himalayan Toothache Relieving Tree" (*Zanthoxylum armatum* DC.) using *in vitro* shoot cultures. Five elicitors—Proline (50, 100, and 200 mg/L), Salicylic acid (50, 100, and 200 mg/L), salt (50, 100, and 200 mg/L), sucrose (0, 20, 40, and 60 g/L), and pH levels (4.0 and 8.0)—were tested on nodal segments. The MS medium fortified with 40 g/L sucrose yielded the best results, achieving the highest average shoot length (6.91 ± 0.50 cm) and leaf number (14.56 ± 1.69). Conversely, the least growth was observed with 100 mg/L salicylic acid. No rooting was detected in any treatment. *In vitro* plant materials were analyzed for total phenolics, flavonoids, tannin content, and antioxidant activity using DPPH, FRAP, and MCA assays. Additionally, GC-MS and HPLC analyses revealed that elicitor treatments significantly enhanced the accumulation of secondary metabolites and led to the synthesis of novel phytochemicals. Notably, elicited microshoots exhibited increased levels of compounds like Tetratetracontane, Phytol, Fargesin, and (+)-Sesamin compared to controls. These findings suggest that elicitation is a viable method to boost the production of valuable secondary metabolites in *Z. armatum*, potentially benefiting other medicinal plants as well.

Introduction

Zanthoxylum armatum (Family: Rutaceae), commonly known as the toothache tree, holds significant aromatic, cultural, and economic value in the Himalayan region. This species is a vital component in many commercial products such as Zuroor-e-Qula, Dabur Toothpaste, Dabur Lal Dantmanjan, MDH Dantmanjan, ZenthoDent, *Zanthoxylum* Organic Nepal, and Tomar seed oil (Agnihotri et al. 2022). *Z. armatum* is known to synthesize a wide array of bioactive compounds, including alkaloids, flavonoids, lignins, coumarins, phenols, and terpenoids, in response to biotic and abiotic stresses. These compounds have various pharmaceutical uses and are known to exhibit antimicrobial (Irshad et al. 2021), antifungal (Alam and Saquib 2017), larvicidal (Kumar et al. 2016), anti-inflammatory (Sati et al. 2011), antinociceptive (Ibrar et al. 2012), antioxidant (Batool et al. 2010), hepatoprotective (Talluri et al. 2019), antiplasmodial (Barkatullah et al. 2013), cytotoxic (Barkatullah et al. 2011), and anticonvulsant (Ibrar et al. 2012) activities.

The production of these valuable secondary metabolites is of great interest due to their pharmaceutical importance (Chetri et al. 2016). However, their production in plants is typically low and dependent on factors such as growth stage, season, stress, and nutrient availability (Ramirez-Estrada et al. 2016). Additionally, extracting these bioactive compounds is an expensive process with generally low yields. As an alternative, plant cell and organ cultures offer a sustainable, controllable, and environmentally friendly means of producing bioactive secondary metabolites (Ochoa-Villarreal et al. 2016). Among the various strategies for enhancing the production of these compounds, elicitation is one of the most effective approaches currently in use (Naik and Al-Khayri 2016). Elicitors can induce stress responses in plants, which in turn stimulates the synthesis and accumulation of secondary metabolites or the production of novel compounds. This strategy is widely utilized in pharmaceuticals (Mathur and Shekhawat 2013).

Proline, salicylic acid, sucrose, and salt are compounds known to mediate plant responses to biotic and abiotic stresses, and they have been shown to increase resistance to drought stress (Hassini et al. 2019). Proline acts as an osmolyte, antioxidant, energy source, and proteinogenic amino acid (Xu et al. 2009). Salicylic acid, a plant-derived elicitor, plays a key role in coordinating plant defense gene expression and is involved in signal transduction through the elicitor-receptor complex (Menke et al. 1999). It is also essential for establishing systemic acquired resistance in plants and enhancing the production of secondary metabolites like terpenes, alkaloids, flavonoids, phenolic compounds, and phytoalexins (Silva et al. 2017). Sucrose, commonly used as a carbon source in *in vitro* culture media, can also induce osmotic stress in plants (Reyes-Díaz et al. 2018). Plant growth, productivity, and the synthesis of secondary metabolites are significantly affected by various abiotic stresses, including pH extremes, salinity, and heavy metals (Rao and Ravishankar 2002).

To date, elicitation in *Z. armatum* has not been well-studied. Therefore, the response, growth, secondary metabolite production, and phytochemical potential of this species in *in vitro* conditions remain to be explored. Given the above, the present study aims to: i) investigate the effect of different elicitors on the growth response of nodal segments; ii) optimize elicitor

concentrations to enhance the accumulation of secondary metabolites; iii) quantify total polyphenol content and antioxidant potential; and iv) assess secondary metabolites using GC/MS and HPLC analysis.

Materials and Methodology

Plant Materials and Surface Sterilization

The explants (nodal segments) of *Zanthoxylum armatum* were collected from plants growing near Thandi Road and the DSB campus of Kumaun University, Nainital, during February to April 2021 (Fig. 1). The plant was identified by the Botanical Survey of India (BSI), Dehradun, with an accession number of 737. Nodal explants were excised from the plant and surface sterilized using 1% Tween 20 and 1.5% Bavistin fungicide for 20 minutes, followed by four rinses with distilled water. The nodal segments were then treated with 0.1% (w/v) mercuric chloride ($HgCl_2$) for 2 minutes, followed by four additional rinses with sterile distilled water under Laminar Air Flow. The sterilized nodal explants were subsequently used for *in vitro* plant material collection.

Culture Media and Conditions

Murashige and Skoog's (MS) medium (1962) was used to study the growth responses of the nodal explants. The MS medium was fortified with five elicitors at different concentrations, as follows: Proline: 50 mg/L (P1), 100 mg/L (P2), and 200 mg/L (P3); Salicylic acid: 50 mg/L (S1), 100 mg/L (S2), and 200 mg/L (S3); Salt: 50 mg/L (SA1), 100 mg/L (SA2), and 200 mg/L (SA3); Sucrose: 0 g/L (SU1), 20 g/L (SU2), 40 g/L (SU3), and 60 g/L (SU4); pH levels: 4.0 (PH1) and 8.0 (PH2).

The MS medium contained 3.0% (w/v) sucrose and 1.0% (w/v) agar. MS medium without any elicitor treatment served as the control. The pH of all media was adjusted to 5.7 using 0.1 N NaOH/HCl before adding agar, and 100 ml of the medium was poured into culture bottles and autoclaved at 121°C for 15–20 minutes. Following sterilization, nodal explants (ranging between 1.0–2.0 cm) were inoculated in the culture bottles. The cultures were maintained at 25 ± 2˚C, with a 16-hour light cycle under cool white fluorescent tubes (Philips; 40 W) and an 8-hour dark cycle. Sub culturing was performed in fresh medium as required for continuous growth of aseptic cultures. Visual data, including percent shoot formation, shoot color, shoot length, and number of leaves, were recorded after the 3rd week of inoculation for *in vitro* growth measurement.

Polyphenol Estimation

Determination of Total Phenolic Content (TPC)

The total phenolic content in plant extracts was determined using the Folin-Ciocalteu (FC) colorimetric method described by Singleton and Rossi (1965), with minor modifications. One ml of the sample dilution (1 mg/ml) was taken in a test tube and mixed with 9 ml of distilled water. One ml of FC reagent was added to the sample and mixed. After 7 minutes, 10 ml of 7% sodium carbonate solution was added, followed by 10 ml of deionized distilled water. The mixture was shaken vigorously and incubated in the dark at 23°C for 90 minutes. Absorbance was recorded at 750 nm using a UV-Vis spectrophotometer. Gallic acid was used to prepare a standard curve, with dilutions of 25–125 µg/ml in methanol. Results were expressed as mg gallic acid equivalents (GAE) per gram of dry weight (DW).

Determination of Total Flavonoid Content (TFC)

Total flavonoid content was estimated using the Aluminum Chloride ($AlCl_3$) colorimetric method described by Chang et al. (2002), with some modifications. A stock solution of quercetin was prepared by dissolving 10 mg of quercetin in 80% methanol. Various concentrations (25, 50, 75, 100, and 125 µg/ml) were prepared by adding the appropriate amount of solvent to 1 ml in an Eppendorf tube. One ml of standard solution was mixed with 1.5 ml of 95% ethanol. After 5 minutes, 0.3 ml of 10% $AlCl_3$ solution was added, followed by 2 ml of 1M potassium acetate at 6 minutes. The final volume was adjusted to 10 ml with

distilled water. The procedure was repeated for plant extracts in the respective solvents. The reagent mixture, except for $AlCl_3$, served as a blank. Absorbance was measured at 415 nm using a UV-Visible spectrophotometer. Results were expressed as mg quercetin equivalents (QE) per gram of DW.

Determination of Total Tannin Content

Total tannin content was measured using the Folin-Ciocalteu colorimetric method as described by Chandran and Indira (2016), with minor modifications. Tannic acid was used as the standard, with concentrations ranging from 20 to 100 µg/ml. A 100 µl aliquot of each tannic acid concentration was added to a test tube containing 7.5 ml of distilled water, 0.5 ml of FC phenol reagent, and 1.0 ml of 35% sodium carbonate solution. The mixture was diluted to 10 ml with distilled water, shaken well, and incubated at room temperature for 30 minutes. Plant extract solutions were prepared similarly. The blank contained all reagents except tannic acid and plant extract. Absorbance was measured at 700 nm using a UV/Visible spectrophotometer. Results were expressed as mg tannic acid equivalents (TAE) per gram of DW.

Antioxidant Potential

DPPH (2,2-Diphenyl-1-picrylhydrazyl) Assay

Antioxidant activity was determined spectrophotometrically using the DPPH free radical assay as described by Brand-Williams et al. (1995), with slight modifications. Plant extracts (1 mg/ml) were prepared by dissolving 0.001 g of semi-solid extract in 1 ml of methanol. A 1 mM DPPH solution was prepared by dissolving 0.0039 g of DPPH powder in 100 ml of methanol. DPPH cation (100 µM, 1000 µl) was mixed with 200 µl of sample extract and diluted with 800 µl of 80% solvent to make a 2 ml solution. The mixture was shaken vigorously and incubated in the dark at room temperature for 20–25 minutes. Absorbance was measured at 517 nm using a UV-Visible spectrophotometer, with ascorbic acid as the standard. A blank sample was prepared using the same amount of methanol and DPPH solution without the plant extract. The DPPH radical scavenging activity was calculated as the inhibition percentage using the following formula:

$$\%inhibition = \frac{Absorbance\ of\ Control\ \ (Ac) - Absorbance\ of\ Sample\ \ (As)}{Absorbance\ of\ Control\ \ (Ac)} \times\ 100$$

Metal Chelating Assay (MCA)

The chelation of ferrous ions (Fe^{2+}) by plant extracts was determined using a modified version of the method by Dinis et al. (1994). Different fractions of plant extracts (20–100 µg/ml) were diluted in methanol to prepare 3 ml of solution. Aliquots (200 µl) from these dilutions were mixed with 20 µl of 2 mM ferric chloride and 800 µl of distilled water. The reaction was initiated by adding 1000 µl of 2.5 mM ferrozine. The mixture was shaken vigorously and incubated at room temperature for 20 minutes. Absorbance was measured at 562 nm using a UV-Vis spectrophotometer. Di-sodium EDTA (1 mg/ml) was used as the standard, and a blank was prepared using all reagents except the plant extract. The percentage of metal chelation activity was calculated using the following equation:

$$\%metal\ chelating\ ativity = \frac{Absorbance\ of\ Control\ \ (Ac) - Absorbance\ of\ Sample\ \ (As)}{Absorbance\ of\ Control\ \ (Ac)} \times\ 100$$

Ferric Reducing Antioxidant Power (FRAP) Assay

The Ferric Reducing Antioxidant Power (FRAP) of the plant extracts was determined using a modified version of the method by Faria et al. (2005). The FRAP reagent was composed of: i) 150 mM acetate buffer (10 ml); ii) 5 mM TPTZ (2,4,6-tripyridyl 1,3,5-triazine) (1 ml); and iii) 10 mM ferric chloride (1 ml).

A 100-µg extract was mixed with 80% methanol to make 1 ml of solution and then combined with 1.5 ml of pre-warmed (37°C) FRAP reagent. The mixture was incubated at room temperature for 8–10 minutes. Absorbance was recorded at 593 nm using a UV-Vis spectrophotometer, and results were compared against an ascorbic acid standard (1 mg/ml). The FRAP reagent without plant extract was used as a blank. The reducing capacity of the extracts was expressed in mg of ascorbic acid equivalent (AAE)/g dry weight (DW). A standard curve was prepared, and the regression equation (y = mx + c) was derived from ascorbic acid measurements.

GC-MS Profiling of Secondary Metabolites

GC-MS analysis was performed to screen for secondary metabolites in the methanol extracts of *in vitro*-raised microshoots (with and without elicitor treatments). The analysis was carried out at the Advanced Instrumentation Research Facility (AIRF), Jawaharlal Nehru University (JNU), Delhi. The prepared extracts of *Z. armatum* were analyzed using a GCMS-QP2010 Ultra system, equipped with an AOC-20i auto-sampler and a Gas Chromatograph interfaced to a Mass Spectrometer (GC-MS). The column used was an RT×-5 MS fused silica capillary column (30 m × 0.25 µm ID, Film thickness: 0.25 µm). The oven temperature was programmed from 100°C to 280°C, beginning at 100°C for the first two minutes, increasing by 10°C to 250°C over five minutes, and then further increasing by 15°C to 280°C, where it was maintained isothermally for 18 minutes. Nitrogen was used as a carrier gas at a flow rate of 113.0 ml/min. The injector temperature was set to 260°C, with an injection volume of 0.5 µl. The split ratio was 1:90, and the mass spectra were recorded at 70 eV. The total runtime for each GC-MS sample was approximately 50 minutes.

HPLC Analysis

Phytochemicals were identified and characterized using a reversed-phase HPLC system (Alliance Waters e2695, Waters, Milford, USA) equipped with a photodiode array detector (PDA, Waters 2998) and Empower 3 software (Waters). The mobile phase consisted of methanol (Merck HPLC grade) and water in a 40:60 ratio, mixed with 0.1% ortho-phosphoric acid at a flow rate of 0.8 mL/min over a 50-minute runtime. Individual compounds were separated using an isocratic system on a Waters SPHERISORB C18 column (5 µm particle size, 4.6 × 250 mm i.d.) at 30°C. Standard solutions of phenolic compounds, including ascorbic acid, gallic acid, chlorogenic acid, epigallocatechin gallate, vanillic acid, p-hydroxybenzoic acid, and caffeic acid, were prepared in methanol. The PDA detector was set to record at wavelengths between 190–400 nm, and results were expressed in milligrams per gram of dry weight (mg/g DW). Methanol extracts of *in vitro*-raised microshoots (with and without elicitor treatments) were analyzed by HPLC-PDA. Chromatograms were extracted at the following wavelengths for different compounds: 254 nm for ascorbic acid and p-hydroxybenzoic acid, 220 nm for gallic acid, epigallocatechin gallate, vanillic acid, phloridzin, rutin hydrate, p-coumaric acid, and salicylic acid, 310 nm for p-coumaric acid and caffeic acid, 320 nm for chlorogenic acid, and 272 nm for o-coumaric acid.

Statistical Analysis

All experiments were conducted in a completely randomized block design with 9 explants per treatment, in triplicate. The results are expressed as mean values ± standard error. Data were analyzed using SPSS (Statistical Package for Social Science, version 20), and ANOVA was performed to assess the significance of differences between groups. Duncan's multiple range Posthoc test ($P \leq 0.05$) was used to determine statistical significance. The IC50 values were calculated from concentration-effect regression lines to indicate antioxidant capacity. All statistical analyses and graphs were prepared using Microsoft Excel 2019.

Results and Discussion

Effect of Elicitors on Shoot Induction from Nodal Explants

Elicitors play a crucial role in influencing the growth and development of plant tissue under *in vitro* conditions, especially at controlled concentrations. In this study, nodal segments of *Z. armatum* showed significantly enhanced responses when cultured on Murashige and Skoog (MS) medium supplemented with elicitors compared to the control medium (MS without elicitors). The results are presented in Table 1.

Table 1
Effect of different elicitors on aseptic shoot initiation from nodal explant.

Treatments			% callus ± SE	Color of Callus	(%) shoot formation ± SE	Color of shoot	Days required for induction	Average shoot length ASL (cm)	Average leaf number (ALN)
Elicitors	Code	Concentration							
Control	C	0.00	-	-	59.25 ± 0.17^{b}	Green	13.78 ± 0.53^{e}	3.45 ± 0.08^{cde}	10.78 ± 0.28^{f}
Proline	P1	50.00 mg/l	-	-	100.00 ± 0.00^{c}	Green	9.19 ± 0.55^{bc}	4.98 ± 0.27^{g}	12.67 ± 0.41^{g}
	P2	100.00 mg/l	18.52 ± 0.27^{a}	Crystal White	81.48 ± 0.13^{c}	Green	8.67 ± 0.33^{b}	3.71 ± 0.24^{ef}	8.22 ± 0.32^{d}
	P3	200.00 mg/l	70.37 ± 0.15^{b}	Crustal White	29.63 ± 0.16^{a}	Light Green	10.30 ± 1.05^{c}	3.41 ± 0.10^{cd}	6.33 ± 0.24^{b}
Salicylic Acid	S1	50.00 mg/l	-	-	96.30 ± 0.07^{c}	Green	12.37 ± 1.06^{d}	3.18 ± 0.15^{bc}	13.33 ± 0.41^{h}
	S2	100.00 mg/l	-	-	92.59 ± 0.09^{c}	Green	13.81 ± 1.19^{e}	2.32 ± 0.11^{a}	7.33 ± 0.29^{c}
	S3	200.00 mg/l	-	-	40.74 ± 0.17^{ab}	Dark Green	13.89 ± 0.49^{e}	3.59 ± 0.16^{def}	5.11 ± 0.20^{a}
Sucrose	SU2	20.00 g/l	-	-	81.48 ± 0.13^{c}	Yellow	15.48 ± 1.02^{f}	3.76 ± 0.17^{f}	9.11 ± 0.20^{e}
	SU3	40.00 g/l	-	-	100.00 ± 00^{c}	Green	6.85 ± 0.58^{a}	5.42 ± 0.15^{h}	14.22 ± 0.32^{i}
	SU4	60.00 g/l	-	-	51.85 ± 0.17^{b}	Green	18.56 ± 0.93^{g}	3.10 ± 0.22^{b}	6.89 ± 0.11^{c}

Among the treatments, MS medium with SU2 elicitor yielded the best results, with 100% shoot development observed in an average of 6.85 ± 0.58 days. The highest average shoot length (ASL) of 6.91 ± 0.50 cm and average leaf number (ALN) of 14.56 ± 1.69 were recorded in the SU2 treatment group. In contrast, the lowest ASL (2.31 ± 0.79 cm) and ALN (7.11 ± 3.29) were observed in the S2 treatment group (Fig. 2).

Both low and high concentrations of sucrose in the medium led to yellowing of the leaves, which is indicative of chlorosis. This phenomenon has been linked to imbalanced sugar concentrations causing either chlorosis or explant deterioration in *in vitro* cultures. Similar findings of growth inhibition at high sugar concentrations (45–60 g/L) have been reported in species like *Billbergia zebrina* (Martins et al. 2015) and *Stevia rebaudiana* (Ghorbani et al. 2017).

At lower concentrations of proline and salicylic acid (50 mg/L), the nodal segments demonstrated successful shoot development. However, higher concentrations of both compounds led to a reduction in shoot induction ability. Lower concentrations of proline facilitated 100% shoot bud induction in *Z. armatum*, likely due to osmotic adjustments provided by the exogenous supply of proline (Samie et al. 2021). The positive influence of salicylic acid was further validated in other plants such as *Ziziphus spinachristi* (Abdelnasser 2012) *Hibiscus acetosella*, and *Hibiscus moscheutos* (Sakhanokho and Kelley 2009).

However, as the concentration of proline increased (P2 and P3 treatments), morphological abnormalities, such as crystal-like structures, were observed in *Z. armatum* shoots (Table 1). Similar abnormalities have been documented in *Oryza sativa* (Roy et al. 1993), and *Solanum lycopersicum* (Heuer 2003). Additionally, higher salicylic acid concentrations were detrimental to shoot development such as reported in *Coleus aromaticus* (Govindaraju and Arulselvi 2018) and *Artemisia annua* (Putalun et al.

2007). High concentrations of both proline and salicylic acid may cause toxicity in plant cell cultures, affecting growth and development (Koo et al. 2020).

Moreover, when MS medium was modified with altered pH levels or salt concentrations, no shoot development was observed (data not shown). While some plants can tolerate pH fluctuations, *Z. armatum* exhibited an inability to thrive in media with altered pH. Changes in pH can have deleterious effects on plant growth and development, as reported by Kidd and Proctor (2001) and Pavlovkin et al. (2009). Elevated salt concentrations also hindered shoot induction, likely by increasing abscisic acid levels and reducing gibberellic acid levels, which together inhibit plant growth under stress conditions (Montero et al. 1997).

These findings were statistically validated by ANOVA analysis, showing that the differences between treatments were significant ($p \leq 0.05$).

Estimation of Secondary Metabolites

Polyphenol Estimation

Abiotic factors, including chemicals such as calcium, abscisic acid, salicylic acid, polyamines, jasmonates, and nitric oxide, are known to trigger stress responses in plants (Tuteja and Sopory 2008). These stress responses often lead to an increased yield of secondary metabolites in plant tissues as part of the plant's defense mechanism, which is activated by elicitors (Patel and Krishnamurthy 2013). In this study, elicitor application at certain levels significantly enhanced polyphenol content in the microshoots of *Z. armatum*. The results showed a marked increase in total polyphenol content (TPC) across the treated samples, as presented in Table 2.

Table 2
Estimation of total polyphenol contents (TPC, TFC, TTC) and antioxidant activity (DPPH, MCA and FRAP assay) of *in vitro* microshoots of *Z. armatum* (Elicitor treatment and control) in methanol solvent.

Treatments	TPC (mg of GAE / g of extract) ± SE	TFC (mg of QE / g of extract) ± SE	TTC (mg of TAE/g of extract) ± SE	DPPH (IC 50 ± SE)	MCA (IC50 ± SE)	FRAP (AAE ± SE)
C	98.78 ± 2.31^{ab}	65.47 ± 1.58^{a}	89.47 ± 0.52^{a}	16.34 ± 0.06^{d}	11.41 ± 0.11^{d}	91.27 ± 0.36^{b}
P1	100.44 ± 0.27^{ab}	97.69 ± 0.91^{e}	102.92 ± 0.34^{e}	12.33 ± 0.06^{b}	8.56 ± 0.03^{bc}	77.02 ± 1.35^{a}
P2	108.22 ± 1.34^{de}	102.06 ± 1.44^{f}	109.29 ± 0.33^{g}	8.55 ± 0.23^{a}	7.59 ± 0.58^{b}	116.00 ± 3.58^{e}
P3	96.93 ± 1.25^{a}	75.79 ± 2.21^{b}	98.17 ± 0.36^{d}	13.36 ± 0.21^{c}	11.81 ± 0.08^{d}	123.20 ± 0.17^{f}
S1	112.19 ± 0.52^{e}	90.87 ± 1.34^{d}	105.25 ± 0.67^{f}	30.35 ± 0.08^{g}	4.94 ± 0.68^{a}	97.27 ± 0.64^{c}
S2	99.63 ± 0.96^{ab}	81.42 ± 1.07^{c}	109.38 ± 0.74^{g}	34.72 ± 0.13^{h}	5.88 ± 0.06^{a}	101.99 ± 1.13^{d}
S3	106.11 ± 2.01^{cd}	99.6 ± 1.85^{ef}	95.96 ± 0.57^{c}	28.25 ± 0.12^{f}	7.7 ± 0.58^{b}	90.63 ± 1.34^{b}
SU	102.74 ± 2.95^{bc}	89.6 ± 1.16^{d}	91.14 ± 0.28^{b}	20.71 ± 0.19^{e}	9.51 ± 0.15^{c}	89.34 ± 0.85^{b}

Values shown are means ± SE (standard error). Means with different superscript letters in the same column are significantly different at $p \leq 0.05$ (ANOVA, followed by Duncan test). **(C: control, P1: Proline 50 mg/l, P2: Proline 100 mg/l, P3: Proline 200 mg/l, S1: Salicylic acid 50 mg/l, S2: Salicylic acid 100 mg/l, S3: Salicylic acid 200 mg/l, SU: Sucrose 40 g/l treatments extract of Z. armatum in methanol solvent)**

The TPC of the extracts ranged from 96.93 ± 1.25 to 112.19 ± 0.52 mg of GAE/g of DW. Among the treatments, S1 yielded the highest TPC (112.19 ± 0.52 mg of GAE/g of DW), followed by other treatments. Salicylic acid (SA) application has been shown to mimic pathogen interactions and induce the expression of genes and enzymes involved in the biosynthesis of phenylpropanoid metabolites (Loc et al. 2016). In this study, S1 treatment enhanced the TPC, and S2 boosted the total tannin content (TTC) due to the stimulation of phenylalanine ammonia-lyase (PAL) activities and related metabolic pathways (Loc et al. 2016).

The total flavonoid content (TFC) ranged between 65.47 ± 1.58 to 102.06 ± 1.44 mg of QE/g of DW, with the highest content (102.06 ± 1.44 mg of QE/g of DW) observed in the P2 treatment. The increase in TFC upon the addition of proline to the MS medium is likely linked to the role of proline in enhancing phenol metabolism. Proline potentially facilitates the replenishment of NADP+, fueling the oxidative pentose phosphate pathway, which provides NADPH and carbon skeletons for phenylpropanoid biosynthesis, a crucial pathway for flavonoid, coumarin, and lignan production (Fahrendorf et al. 1995; Maggio et al. 1997).

These results are consistent with studies on other species, such as *Zanthoxylum stenophyllum* (Chueh et al. 2001) and *Salacia chinensis* (Chavan et al. 2013). Tissue-cultured microshoots often produce higher levels of secondary metabolites due to the controlled environment with optimized nutrient and elicitor supplies (Govindaraju and Indra Arulselvi 2018).

Antioxidant Activity

The antioxidant potential of elicitor-treated microshoots was evaluated based on the IC50 values for radical scavenging activity. Among the treatments, the P2 treatment exhibited the lowest IC50 value (8.55 ± 0.23), indicating the highest radical scavenging activity (Table 2). In contrast, the S2 treatment showed the lowest radical scavenging activity, with the highest IC50 value (34.72 ± 0.13).

The reducing power assay revealed that the P3 treatment had the highest reducing ability, with 123.20 ± 0.17 mg of AAE/g DW, whereas the SU treatment had the lowest (89.34 ± 0.85 mg of AAE/g DW). The metal ion chelating capacity was also evaluated, with the S1 treatment showing the highest chelating effect (IC50 = 4.94 ± 0.68), followed by the S2 treatment (IC50 = 5.88 ± 0.06).

This study confirms that elicitor treatments can enhance the antioxidant potential of *in vitro* cultures, which aligns with previous findings in species such as *Berberis asiatica* (Dobhal et al. 2022), *Ajuga bracteosa* (Saeed et al. 2017), *Nicotiana benthamiana* (Cai et al. 2020), and *Salacia chinensis* (Chavan et al. 2021). Elicitors applied at optimal concentrations can enhance polyphenol content in *Z. armatum*, thereby boosting its antioxidant potential and free radical scavenging activities (Rice-Evans et al. 1997).

Although a general trend of enhanced phytochemical content and antioxidant activity was observed, no significant relationship was found between the specific elicitors and phytochemical parameters ($p < 0.05$).

GC-MS Analysis

A total of 57, 37, 48, 40, 46, 41, 51, and 46 compounds were identified from control, P1, P2, P3, S1, S2, S3, and SU2 treated *Z. armatum* microshoots, respectively. The number of compounds detected in the *in vitro* raised microshoots, with and without elicitor treatments, was lower compared to those identified in wild *Z. armatum* leaves extracted with methanol solvents. Among the treatments, P3 yielded the highest number of phytochemicals, while the S2 treatment exhibited the fewest.

Based on the area percentage threshold (< 2), twenty-five major compounds were identified (Table 3). Of these, Tetratetracontane, Phytol, Fargesin, (+)-Sesamin, n-Hexadecanoic acid, Squalene, and 1H,3H-Furo(3,4-C) Furan, 1,4-Bis(3,4-Dimethoxy) were consistently present across all treatments. Notably, the P2 treatment revealed four novel compounds that were absent in other treatments: 2-Tridecanone, Diethyl Phthalate, 1,3,4,5-Tetrahydroxy-cyclohexanecarboxy, and Hexatriacontyl penta-fluoropropionate.

Table 3
Major Compounds isolated from methanol extract of elicitor treated microshoots of *Z. armatum*.

Compounds	% Area								Molecular weight (g/mol)	Molecular formula
	C	P1	P2	P3	S1	S2	S3	SU		
Heneicosane	17.01	0.80	1.33	3.36	-	-	-	0.19	296.6	$C_{21}H_{44}$
Tetratetracontane	19.91	31.12	9.49	26.78	2.07	0.77	0.24	3.42	619.2	$C_{44}H_{90}$
Phytol	4.12	1.50	2.81	3.37	6.72	5.90	5.98	5.30	296.5	$C_{20}H_{40}O$
Fargesin	6.06	14.57	8.81	1\7.42	15.13	27.71	19.89	2.21	370.4	$C_{21}H_{22}O_6$
(+)-Sesamin	3.31	2.65	1.76	4.61	7.3	11.34	12.05	12.13	354.4	$C_{20}H_{18}O_6$
Paulownin	4.16	-	-	-	9.43	1.87	13.23	24.59	370.4	$C_{20}H_{18}O_7$
n-Hexadecanoic acid	3.98	3.78	7.17	6.91	9.42	7.69	8.67	8.57	256.42	$C_{16}H_{32}O_2$
Tetracontane	3.22	7.51	4.69	6.71	-	-	-	4.62	563.1	$C_{40}H_{82}$
9,12,15-Octadecatrienoic acid, methyl ester, (Z, Z, Z)	2.09	-	-	-	-	-	-	0.10	392.6	$C_{24}H_{40}O_4$
2-(1-Hydroxy-1-Methyl-Ethyl)-2,3-Dihydro- Furo [3,2-G] Chromen-7-One	-	0.99	7.35		8.07	6.09	4.33	6.72	314.4	$C_{19}H_{22}O_4$
Docosane	1.23	7.61	6.3	6.93	-	-	-	-	310.6	$C_{22}H_{46}$
Squalene	0.63	4.29	5.65	3.57	5.71	7.45	4.91	4.69	410.7	$C_{30}H_{50}$
1H,3H-Furo (3,4-C) Furan, 1, 4-Bis (3, 4-Dimethoxy)	1.89	3.79	1.72	2.20	4.59	6.12	6.34	4.47	114.14	$C_6H_{10}O_2$
1-Octadecene	-	-	-	0.21	-	-	1.23	-	252.5	$C_{18}H_{36}$
9,12-Octadecadienoic acid (Z, Z)	-	4.02	3.94	2.25	8.29	6.41	4.8	6.56	621	$C_{38}H_{72}N_2O_4$
Gamma-sitosterol	-	2.25	1.79	1.42	-	-	-	-	414.7	$C_{29}H_{50}O$
Tetratriacontyl hepta fluorobutyrate	-	2.47	-	0.34	-	-	2.61	-	634.8	$C_{34}H_{61}F_7O_2$
2-Tridecanone	-	-	2.49	-	-	-	-	-	198.34	$C_{13}H_{26}O$
Diethyl Phthalate	-	-	2.29	-	-	-	-	-	222.24	$C_{12}H_{14}O_4$
1,3,4,5-Tetrahydroxy-cyclohexanecarboxy	-	-	2.63	-	-	-	-	-	192.17	$C_7H_{12}O_6$
Hexatriacontyl pentafluoropropionate	-	-	7.58	-	-	-	-	-	669.0	$C_{39}H_{73}F_5O_2$
Hexatriacontane	-	-	4.00	-	2.57	-	-	-	507.0	$C_{36}H_{74}$
9,12-Octadecadienoyl chloride (Z,Z)	-	-	-	2.83	1.41	-	-	-	298.9	$C_{18}H_{31}ClO$
Stigmast-5-EN-3OL	-	-	-	-	2.00	2.67	-	-	638.9	$C_{37}H_{55}F_5OSi$

Compounds	% Area								Molecular weight (g/mol)	Molecular formula
	C	P1	P2	P3	S1	S2	S3	SU		

(C: Control, Microshoots treated with P1: Proline 50mg/l, P2: Proline 100mg/l, P3: Proline 200mg/l, S1: Salicylic acid 50 mg/l, S2: Salicylic acid 100 mg/l, S3: Salicylic acid 200 mg/l, SU: Sucrose 40 g/l)

Figure 3 illustrates the graphical representation of the major identified compounds from *in vitro* microshoots with and without elicitor treatments. Among all phytochemicals, Fargesin was the most abundant in all extracts, followed by Tetratetracontane, (+)-Sesamin, n-Hexadecanoic acid, Paulownin, Phytol, and others. These major compounds have been associated with various biological activities, as shown in Table 4.

Table 4
Biological Activities of identified compounds with their chemical structure.

Compounds	Biological Activity	References
Heneicosane	Antimicrobial, Bio Pesticidal Property, Anti-asthmatics, Urine Acidifiers	Vanitha et al., 2020
Phytol	Antimicrobial and Cytotoxic Activities, Anticonvulsant Effect	Islam et al., 2018; Costa et al., 2012
Fargesin	Cardioprotective Effect, Antihypertensive	Wang et al., 2015; Sha et al., 2016
(+)-Sesamin	Antihypertensive Effects and Antioxidant Activity	Matsumura et al., 1995; Osawa et al., 1985
Celidoniol, Deoxy-	Antibacterial and Anti inflammatory	Bulent Kose et al., 2016; Zakaria et al., 2014
Paulownin	Insecticide Activity and Antifungal Activity	Devi et al., 2015
n-Hexadecanoic acid	Anti-Inflammatory	Aparna et al., 2012
9,12,15-Octadecatrienoic acid, methyl ester, (Z, Z, Z)	Antimicrobial	Al-Gara et al., 2019
1-H-3A, 7-Methanoazulen-6-ol	Anti-perspirants and anti-pruritic	Ansarali, 2018
Neophytadiene	Antibacterial activity	Inoue et al., 2004
2-(1-Hydroxy-1-Methyl-Ethyl)-2,3-Dihydro-Furo [3,2-G] Chromen-7-One/ Rutamarin alcohol	Antiviral Activity	Xu et al., 2014
Docosane	Antifungal activity	Bierer et al., 1995
Squalene	Antioxidant activity	Ko et al., 2002
Phenol, 2,4-Bis (1,1- Dimethyl Ethyl)	Antioxidant activity and Antibacterial activity	Teresa et al., 2014
1-Octadecene	Antimicrobial activity	Kumar et al., 2011
1-Heneicosanol	Antibacterial activity	Nautiyal and Dubey, 2021
9,12-Octadecadienoic acid (Z, Z)	Antimicrobial	Al-Gara et al., 2019
Gamma-sitosterol	Cytotoxic effect on breast and lung cancer cell	Sundarraj et al. 2012
Tetratriacontyl hepta fluorobutyrate	Antimicrobial property	Sahu and Singh, 2022
2-Tridecanone	Insecticidal activity	Braga et al., 2007
Diethyl Phthalate	Antimicrobial activity, insecticide	Page and Lacroix, 1995
1,3,4,5-Tetrahydroxy-cyclohexanecarboxy	Antioxidant activity	Baratto et al. 2003; Hung et al. 2006
Hexatriacontyl pentafluoropropionate	Insecticidal activity	Sahu and Singh, 2022
Hexatriacontane	Antimicrobial activity, anti-inflammatory activity	Esmat et al., 2022
9,12-Octadecadienoyl chloride (Z, Z)	Nematicide Antiarthritic, Hepatoprotective, Antiandrogenic, Nematicide, Hypocholesterolemic, 5- Alpha-reductase	Vijayabaskar and Elango, 2018

Compounds	Biological Activity	References
	inhibitor, Antihistaminic, Anticoronary, Insectifuge, Antieczemic, Anticancer	
Stigmast-5-EN-3OL	Anti-diabetic, anti-proliferative	Sujatha et al., 2010; Moon et al., 2008

The comparative evaluation of elicitor treatments highlighted their unique regulatory roles, exhibiting both synergistic and antagonistic effects on secondary metabolite production. To the best of our knowledge, no prior studies have explored the effect of elicitors on the production of secondary metabolites in *Z. armatum in vitro* cultures. Although fewer major compounds were identified in the *in vitro* microshoots compared to wild leaves extracted with methanol, the relative abundance (% area) of the compounds was higher in the elicitor-treated microshoots. This might be attributed to the response of wild leaves to environmental stress and their maturity level, which results in a more heterogeneous mixture of secondary metabolites and a more robust structure (Radic et al. 2016).

These findings underscore the potential of elicitors to modulate the secondary metabolite profile in *Z. armatum* microshoots, thereby offering a more controlled approach to enhancing the production of bioactive compounds for potential use in pharmaceutical and industrial applications.

HPLC Analysis

The HPLC analysis of methanol extracts from *Z. armatum* microshoots, both control and elicitor-treated, revealed the presence of various bioactive compounds. Control, P1, P3, S2, and SU treatments showed eight bioactive compounds, while P2 and S3 treatments revealed nine, and S1 treatment had six compounds (Table 5). Among these, several compounds were dominant in specific treatments:

Table 5
Bioactive compound of *Z. armatum* (*In vitro* plant material) quantified by HPLC-PDA.

Compound name	λ max	RT							
			C	P1	P2	P3	S1	S2	S3	SU
Ascorbic acid	254	3.07	11.14	3.90	10.70	3.63	0.76	5.78	4.45	4.06
Gallic acid	220	3.55	8.35	5.51	2.19	6.03	0.69	9.96	7.61	7.54
Chlorogenic acid	320	4.81	11.75	8.55	9.28	4.97	1.41	8.54	5.16	4.79
Epigallocatechin gallate	220	4.41	5.80	4.99	10.45	7.16	1.17	6.22	5.48	3.96
Vanillic acid	220	6.34	4.61	0.68	1.96	3.43	2.32	1.04	0.96	0.96
p-hydroxybenzoic acid	254	5.84	1.15	1.18	1.33	-	-	0.81	0.81	0.98
Caffeic acid	310	6.20	1.49	0.32	0.44	-	-	0.35	0.43	-
Salicylic acid	220	14.3	0.57	-	-	-	7.16	-	-	-
Phloridzin	220	13.0	-	-	-	5.26	-	3.38	4.13	3.07
Rutin hydrate	220	16.8	-	-	-	0.02	-	-	-	-
p-coumaric acid	310	9.12	-	-	8.63	1.91	-	-	0.88	3.27
O-coumaric acid	272	12.7	-	8.84	2.23	-	-	-	-	-

- Ascorbic acid was most abundant in the control group (11.14 mg/g DW).
- Gallic acid was highly present in the S2 treatment (9.96 mg/g DW).
- Chlorogenic acid peaked in the control (11.75 mg/g DW).

- Epigallocatechin gallate was significant in P2 treatment (10.45 mg/g DW).
- Vanillic acid dominated in the control (4.61 mg/g DW).
- *p*-Hydroxybenzoic acid was most abundant in P2 treatment (1.33 mg/g DW).
- Caffeic acid was detected in the control (1.49 mg/g DW).
- Phloridzin was highest in P3 treatment (5.26 mg/g DW).
- *p*-Coumaric acid was abundant in P2 (8.63 mg/g DW), as well as in other proline and salicylic acid-treated microshoots.
- Salicylic acid was dominant in the S1 treatment (7.15 mg/g DW).
- *o*-Coumaric acid was most abundant in P1 treatment (8.84 mg/g DW).

The elicitor treatments significantly enhanced the polyphenol content in the microshoots compared to the control. Notably, p-Coumaric acid was prevalent in proline and salicylic acid-treated microshoots, being present in concentrations of 8.63 mg/g DW (P2), 1.91 mg/g DW (P3), 0.88 mg/g DW (S2), and 3.27 mg/g DW (S3). o-Coumaric acid was also detected in proline-treated microshoots at concentrations of 8.84 mg/g DW (P1) and 2.23 mg/g DW (P3). Additionally, phloridzin was identified in P3, S2, S3, and SU treatments at varying concentrations (5.26, 3.38, 4.13, 3.07 mg/g DW, respectively).

Several of these bioactive compounds were absent in the control but were isolated following elicitor treatments. For instance, Phloridzin, Rutin hydrate, *p*-Coumaric acid, and *o*-Coumaric acid were detected exclusively in elicitor-treated microshoots.

These identified compounds are known for their significant biological activities:

- Ascorbic acid is an important antioxidant that functions as a redox cofactor, scavenging reactive oxygen and nitrogen species, and curing scurvy (Johnston et al. 2007).
- Gallic acid has anti-inflammatory, antioxidant, anticancer, and antiangiogenic properties (Ji et al. 2006; Choubey et al. 2015).
- Chlorogenic acid is known for its antispasmodic, antioxidant, anti-HIV, and anti-carcinogenic effects (Farah et al. 2005; Sato et al. 2011).
- Epigallocatechin gallate is recognized for its anticancer, antioxidant, cardiovascular, and neuroprotective benefits (Granja et al. 2017).
- Vanillic acid has demonstrated anticancer, anti-obesity, antidiabetic, and antibacterial properties (Kaur et al. 2022).
- *p*-Hydroxybenzoic acid has antifungal, antibacterial, antioxidant activities, and is used in cosmetics (Aalto et al. 1953).
- Caffeic acid is known for its anti-inflammatory, antidepressant, antioxidant, and antiviral effects (Birková et al. 2020).
- Phloridzin from apples has antidiabetic, antioxidant, and anti-inflammatory properties (Niederberger et al. 2020).
- Rutin hydrate offers neuroprotective and antioxidant effects (Mostafa et al. 2019).
- *p*-Coumaric acid has anti-inflammatory, antioxidant, and anti-cancer properties (Boo 2019).
- *o*-Coumaric acid is noted for its cytotoxicity and anticancer properties (Sen et al. 2015).
- Salicylic acid is widely recognized for its analgesic, anti-inflammatory, and antioxidant activities (Randjelović et al. 2015).

The results of this study are graphically represented in Fig. 4, showing the bioactive compounds present in various extracts, both with and without elicitor treatments, of *Z. armatum*. These findings emphasize the role of elicitors in modulating the production of bioactive compounds *in vitro*, which could have important implications for enhancing the therapeutic potential of *Z. armatum*.

Conclusion

A comprehensive study was conducted on the effect of different elicitors on the *in vitro* growth and secondary metabolite production in *Z. armatum*. The results indicated that elicitors significantly influence various *in vitro* parameters at specific concentrations. Comparing polyphenol content and antioxidant activities revealed that the application of elicitors enhanced both polyphenol levels and antioxidant activities in *Z. armatum*. Among the tested elicitors, proline, salicylic acid, and sucrose

were identified as the most effective in enhancing polyphenol content and antioxidant activities. Additionally, GC-MS and HPLC analyses of the elicited extracts of *Z. armatum* demonstrated the development of new industrially important phytochemicals, along with an increase in the overall phytochemical content. Further refinement of the conditions for each elicitor is recommended to optimize the elicitation process.

Declarations

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

SA: Investigation, Data collection, experiment design, Software, Writing – original draft preparation, **PD**: Investigation, Data curation, Validation, Reviewing and editing **SM**: Supervision, Validation, acknowledged for figures and formatting of the MS **ISR:** Investigation and Editing **DA**: Investigation, Data curation, Validation; **ST:** Conceptualization, Methodology, Supervision, Validation, Writing – reviewing and editing.

Data availability statements

All the data concerning the current work were included in the manuscript. Further inquiries can be directed to the first and corresponding author.

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Figures

Figure 1.

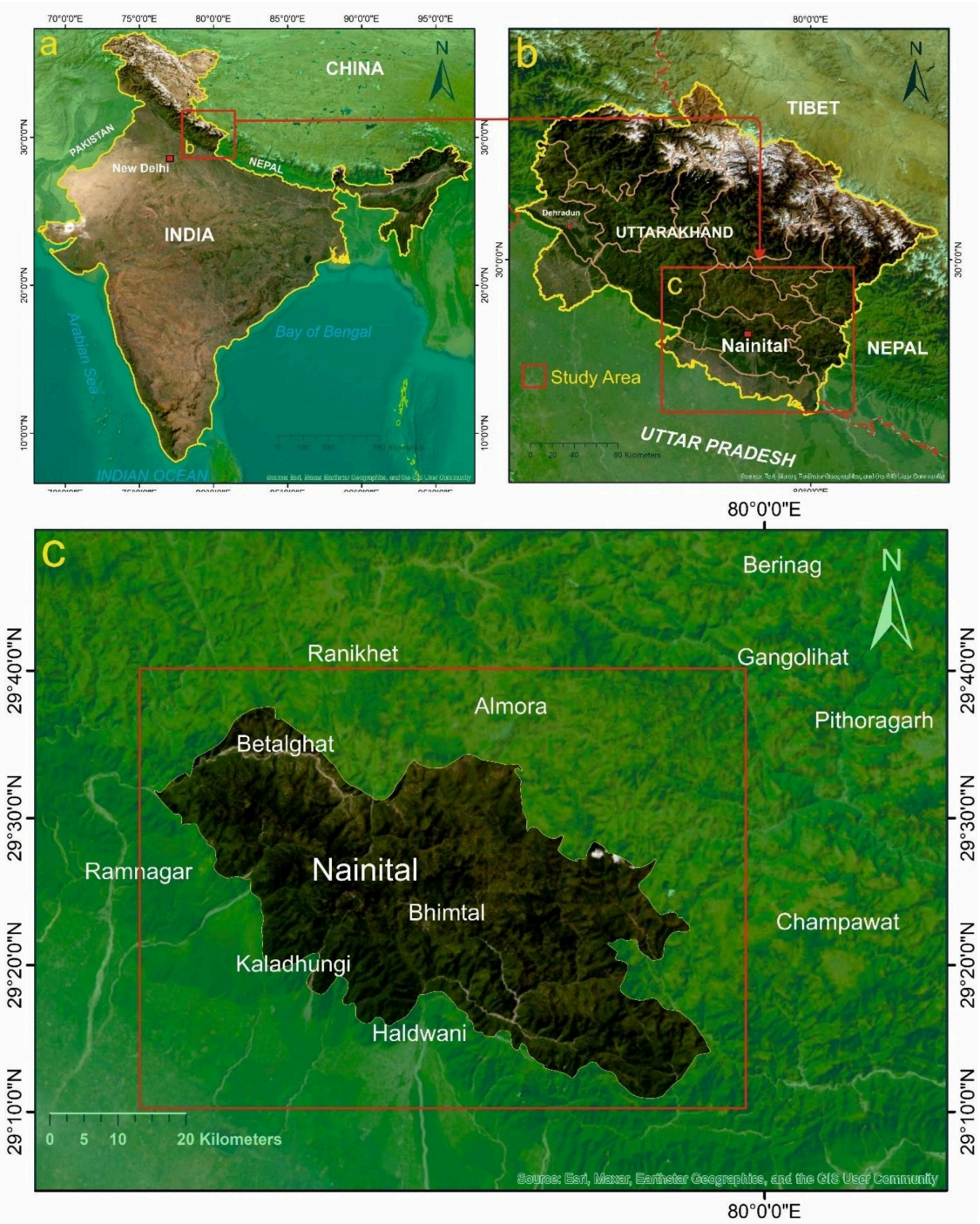

Figure 1

Site for plant material collection of *Z. armatum*

Figure 2.

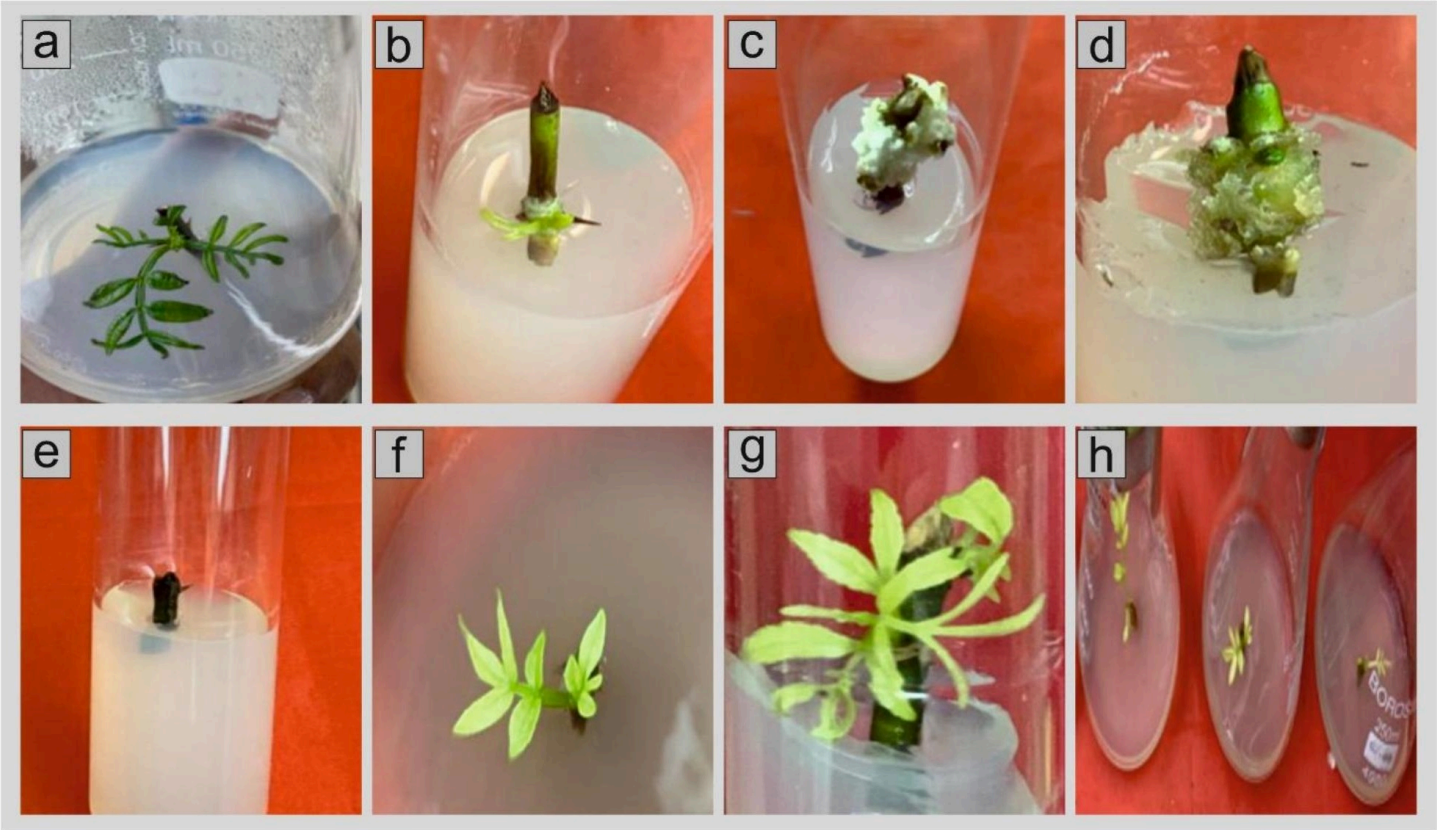

Figure 2

Influence of different elicitors on nodal segment A: shoot bud induction in MS medium, B: callus initiation in MS medium supplemented with proline 100 mg/l, C&D: Crystal like callus formation in MS medium supplemented with 200 mg/l of proline, E: no growth in high and low pH, F&G: Shoot bud induction in sucrose 60 mg/l, H: shoot bud induction in MS medium supplemented with sucrose 20 g/l.

Figure 3.

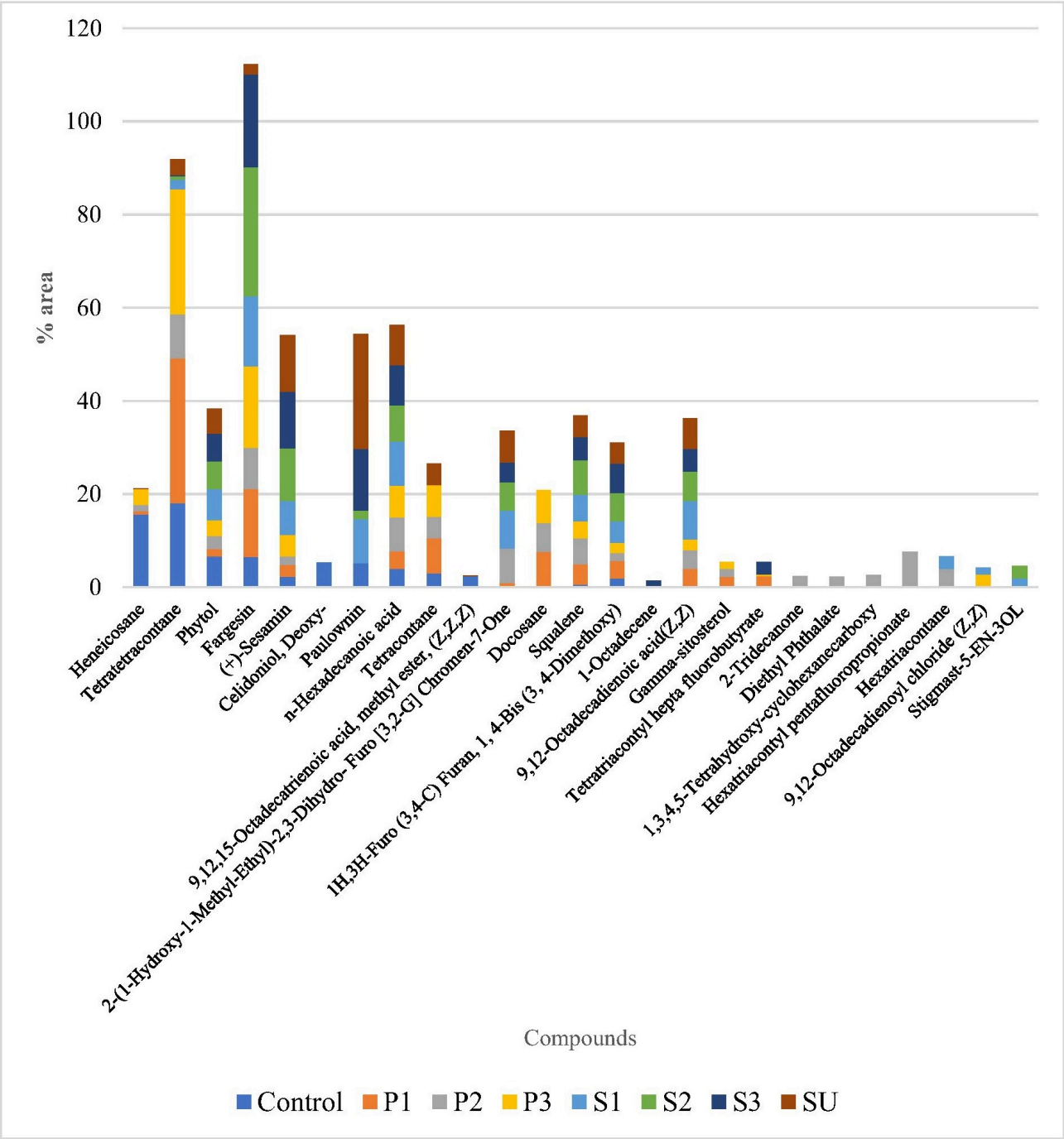

Figure 3

Major identified compounds of *in vitro* plant material (with and without elicitor treatment) of *Z. armatum.* (Microshoots treated with P1: Proline 50mg/l, P2: Proline 100mg/l, P3: Proline 200mg/l, S1: Salicylic acid 50 mg/l, S2: Salicylic acid 100 mg/l, S3: Salicylic acid 200 mg/l, SU: Sucrose 40 g/l).

Figure 4.

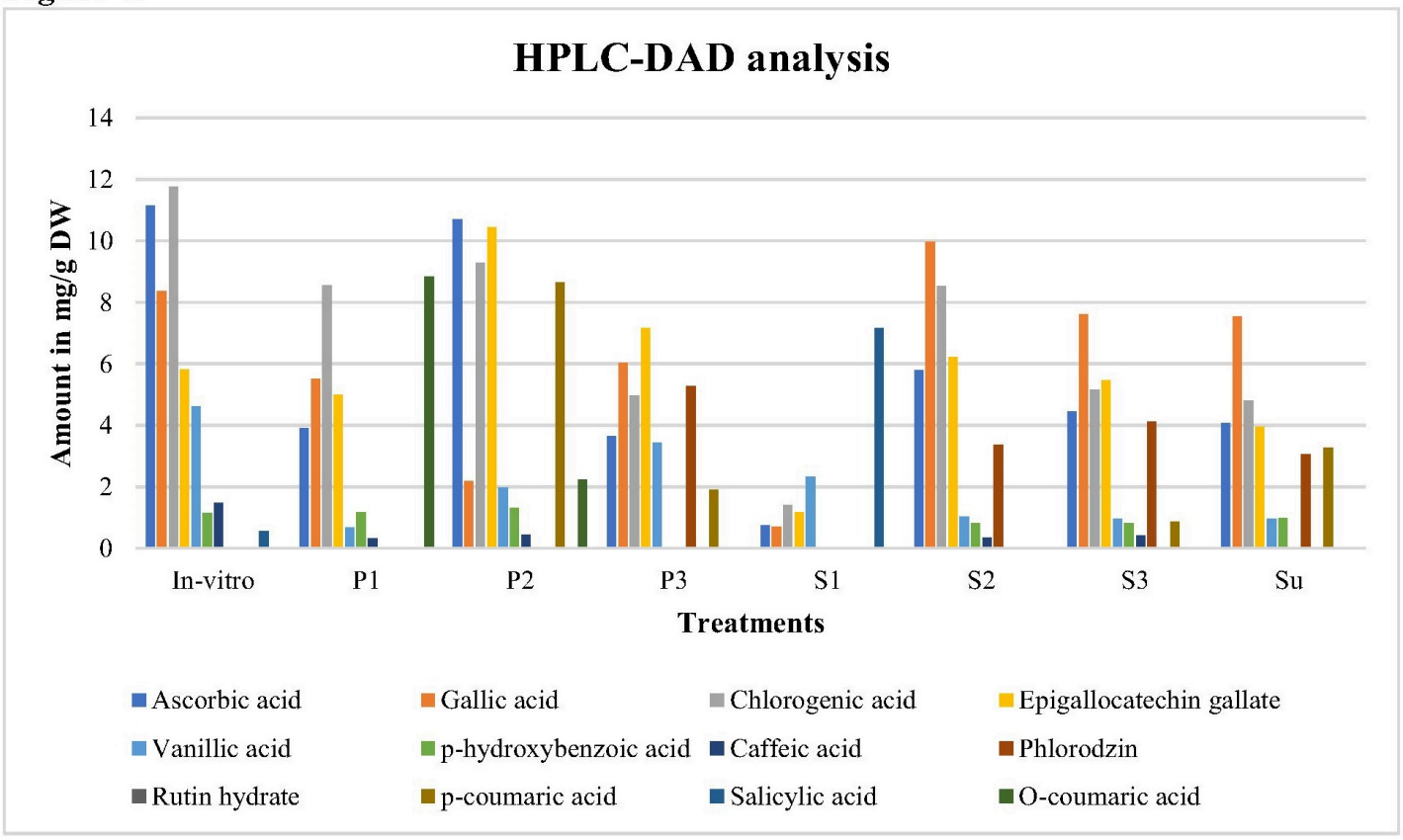

Figure 4

Bioactive compounds in different extracts of *Z. armatum*