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Original Article

Enhancement of total phenolics, antioxidant capacity, and esculentoside A content in pokeweed (*Phytolacca americana* L.) callus under salinity and hormonal elicitation

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Abstract

Phytolacca americana L. (pokeweed) Root contain valuable pharmaceutical compounds but their slow growth and invasive spread make large-scale cultivation difficult. The purpose of this study was to optimize conditions for the enhancement of secondary metabolites while examining the effects of NaCl, MeJA, and SA on the amount of phenolics, flavonoids, and saponins in pokeweed callus cultures. Significant interactions between elicitor concentration and duration were observed for all phytochemical parameters ($p < 0.05$). MeJA at 100 µM for 3 days and SA at 150 µM for 9 days produced the highest phenolic contents (14.26 and 15.82 µg/mg DW, respectively; $p < 0.001$) and enhanced antioxidant activities (DPPH, ABTS, FRAP). NaCl at 200 mM for 21 days maximized EsA content (16.73 µg/mg DW), surpassing natural roots and all other treatments ($p < 0.001$). Phenolic content correlated positively with antioxidant capacity, indicating phenolic compounds as major contributors to antioxidant activity. In contrast, EsA showed negative correlations with other parameters, suggesting its accumulation was independent of phenolic biosynthesis and antioxidant properties. All elicitors upregulated biosynthetic genes via signaling and transcription factor activation. Overall, NaCl, MeJA, and SA effectively enhanced bioactive compounds in pokeweed callus cultures, offering a promising alternative for metabolite production without relying on natural plant resources.

Keywords: Abiotic elicitor, Antioxidant activity, Elicitation, Esculentoside A, Saponin

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Introduction

Pokeweed belongs to the family Phytolaccaceae and is native to North America (Abaye, 2019) but has been introduced and domesticated in many places of the world (Balogh and Juhasz, 2008). It has been used historically for a number of purposes such as dying textiles and treating rheumatism, edema and skin infection (Alshamar et al., 2022; Aweke, 2008; Bailly, 2021). The species is considered as invasive weed in several areas due to its ease of establishment, quick germination and occurrence in a variety of habitats (Balogh and Juhasz, 2008; Liu et al., 2024; Strgulc Krajšek et al., 2023). Its bioactive components have garnered attention in more recent times, with interest focused on its potential for pharmacological uses. Strong antioxidant, antibacterial, and anti-tyrosinase properties are caused by the high concentration of phenolic compounds and flavonoids found in berries, seeds, and aerial parts (Hadipour et al., 2020; Marinaş, 2021; Shin et al., 2016). Conversely, the roots contain triterpenes that have a variety of therapeutic uses. Esculentoside A (EsA), triterpenoid saponin with anti-inflammation (Bailly and Vergoten, 2020), cancer inhibitory (Liu et al., 2018), viral suppression (Zeng et al., 2021) and neuroprotective properties (He et al., 2022), is one such example. These findings demonstrate that pokeweed is a valuable resource for both pharmaceutical and medicinal uses. One significant technique for producing large quantities of high-quality metabolites from material grown *in vitro* is plant tissue culture. Additionally, by using this method, the dangers of extensive field cultivation and the unintended spread of this invasive species are avoided.

A common technique in plant tissue culture is *in vitro* elicitation, which applies elicitors in a sterile environment to promote the production of secondary metabolites (Thakur et al., 2018). Among the chemical elicitors, methyl jasmonate (MeJA) and salicylic acid (SA) are crucial because they act as signaling molecules, which coordinate plant responses to stress and promoting the biosynthesis of specialized metabolites (Jeyasri et al., 2023). According to earlier reports, both compounds have been effectively used in a variety of species, including *Thevetia peruviana* and *Phyllanthus acuminatus* (Benavides et al., 2024; Mendoza et al., 2018). More physical abiotic stress has also been used as an elicitor. One of the intriguing elicitors is salinity stress, which is typically brought on by sodium chloride (NaCl). This elicitor is used to initiate signaling cascades that result in increased production of metabolites (Patel et al., 2020). According to earlier research, stress caused by NaCl has been shown to enhance the levels of phytochemicals like phenolics in *Melissa officinale* (Hawrylak-Nowak et al., 2021), flavonoids in *Vigna unguiculata* (Rajendra et al., 2019), and terpenoids in *Mentha spicata* (Choi et al., 2014).

Plant growth regulators (PGRs) are important factors for pokeweed *in vitro* propagation. From the previous reports of pokeweed cultures, cytokinins such as benzylaminopurine (BAP) (El-Minisy et al., 2017) and kinetin (Trunjaruen et al., 2022) were effectively stimulated shoot proliferation, while auxins such as 2,4-dichlorophenoxyacetic acid (2,4-D) are suitable for inducing the betalain-rich callus (Sakuta et al., 1991; Trunjaruen et al., 2022). However, the combinations of BAP with naphthaleneacetic acid (NAA) have also been reported to generate the chlorophyll-rich calli (Luecha et al., 2024). Phytochemicals such flavonoids and phenolics are frequently accumulated by these calli (Luecha et al., 2024; Trunjaruen et al., 2022). Although callus and cell suspension cultures of pokeweed have been studied, data on salinity stress and chemical elicitors on phytochemicals accumulation in these cultures is also rare. Therefore, aim of this study was to investigate the effects of NaCl, MeJA, and SA on phenolics, flavonoids, and saponins content in pokeweed callus cultures, with optimizing conditions for secondary metabolite enhancement.

Material and Methods

Seed germination and callus induction

Pokeweed seeds were collected from ripened berries grown in Sakon Nakhon Province, Thailand. Seed coats were softened by treating with concentrated sulfuric acid (H_2SO_4) under agitation for 15 min, followed by rinsing with running tap water for 5 min. Seeds were then surface sterilized using 10% (v/v) sodium hypochlorite for 45 min and rinsed three times with sterile distilled water (Luecha et al., 2024). Sterilized seeds were germinated on Murashige and Skoog medium (1962) supplemented with 3% (w/v) sucrose, pH adjusted to 5.8 and solidified with 0.8% (w/v) agar. The medium was autoclaved at 121 °C for 15 min. Cultures were maintained at 25 °C under a 16/8 h light/dark photoperiod with a light intensity of 40 $\mu mol \cdot m^{-2} \cdot s^{-1}$ under sterile conditions. Callus induction followed the protocol of Trunjaruen et al.

(2022). Mature leaves from 75-day-old aseptically grown pokeweed seedlings were cut into 1 × 1 cm^2 segments and cultured on callus induction medium (CIM) consisting of basal MS salts supplemented with 2 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D). Cultures were maintained under sterile conditions for six weeks.

Pokeweed callus elicitation

Pokeweed calli induced from CIM after six weeks were used as explants and subcultured in elicitation media containing chemical and physical elicitors. Methyl jasmonate (MeJA) and salicylic acid (SA) were prepared as a stock solution with 50% (v/v) ethanol and sterilized by filtration through a 0.45 µm syringe filter for use as chemical elicitors. Elicitation media were prepared with MS medium fortified with different concentrations of MeJA (0, 100, 200, 300 and 400 µM) and SA (0, 50, 100, 150 and 200 µM). Pokeweed calli were elicited with chemical elicitors for 9 days and then harvested at 3-day intervals.

Elicitation with salinity stress was performed using sodium chloride (NaCl) as the physical elicitor. The pokeweed calli were subcultured in elicitation media supplemented with various concentrations of NaCl (0, 50, 100, 150, and 200 mM). The cultures were maintained under aseptic conditions and the elicited calli were harvested at weekly intervals for three weeks. Six-week-old pokeweed calli without elicitation were used as the control treatment.

Crude extraction

Crude extracts have been prepared according to Luecha et al. (2024) with minor adjustments. First, treated calli were dried at 50 ± 2 °C for 7 days before subsequently ground into a fine powder. After that, the powdered material was soaked in methanol (MeOH; RCI Labscan, Thailand) at a ratio of 1:20 (w/v) prior to exposed to sonication for 30 min. This step was repeated three times, each time using a new portion of the solvent. The mixed methanolic extracts were concentrated under reduced pressure at 50 ± 2 °C and 130 mbar for 10–12 h using a vacuum concentrator (Thermo Scientific, USA). The remaining water was then removed by the freeze-drying (Labconco, USA) for 24 h. The resulting extracts were stored at -20 °C until further phytochemical analyses.

Total phenolic content (TPC)

The total phenolic content (TPC) of the elicited pokeweed callus was determined based on the procedure of Mwamatope et al. (2020) with slight modifications. After dissolving the extract in MeOH, 20 µL aliquots were put into a 96-well plate. Next, 100 µL of 0.2 M Folin-Ciocalteu's reagent (Loba, India) and 80 µL of a 7% (w/v) sodium carbonate solution (Na_2CO_3, Ajax Finechem, Australia) were added to each well. After 30 min of dark incubation, the mixtures were measured for absorbance at 760 nm using a microplate spectrophotometer (Ensight® Multimode Plate Reader, PerkinElmer, USA) with gallic acid (Sigma-Aldrich, USA) as the calibration standard. The TPC content was expressed as µg GAE/mg DW, or gallic acid equivalent per milligram of dry weight.

Antioxidant assays

For the antioxidant activity tests, extracts from the elicited pokeweed calli were dissolved in MeOH. 50 µL of the extract and 50 µL of 0.2 mM 2,2-diphenyl-1-picrylhydrazyl (DPPH; Sigma-Aldrich, USA) prepared in MeOH were added to each well of a 96-well plate for the DPPH assay. The reaction mixtures were incubated for 30 min in the dark prior to absorbance measured at 517 nm using a microplate spectrophotometer (Mwamatope et al., 2020).

With some modification, the ABTS assay was performed based on González-Palma et al. (2016). The ABTS reagent was prepared using combination of 7 mM ABTS (Sigma-Aldrich, USA) and 2.45 mM potassium persulfate ($K_2S_2O_8$, Loba, India) in a 1:1 (v/v) ratio, which was then incubated in the dark for 16 h. After that, distilled water was added to the solution for dilution until the absorbance reached 0.70 ± 0.03 at 734 nm. A microplate spectrophotometer was used to measure the absorbance at 734 nm following a 10-min incubation period in the dark. The following formula was used to determine the radical scavenging activity (%).

$$\textbf{Radical scavenging (\%)} = [(A_{control} - A_{Trolox})/A_{control}] \times 100$$

where the absorbance of the blank and the Trolox reaction are denoted by $A_{control}$ and A_{Trolox}, respectively. The findings were expressed as ng Trolox equivalent per mg dry weight (ng TE/mg DW) and compared to a Trolox (Sigma-Aldrich, USA) standard curve.

For the FRAP assay, the working reagent was freshly prepared by mixing of 300 mM sodium acetate buffer (pH 3.6), 40 mM 2,4,6-tri (2-pyridyl)-s-triazine (TPTZ, Sigma-Aldrich, USA) and 20 mM ferric (III) chloride hexahydrate ($FeCl_3.6H_2O$, Ajax Finechem, Australia) in a 10:1:1 (v/v/v) ratio. Ten µL of the extract were added to each well followed by 190 µL of the FRAP reagent. After incubation in the dark for 15 min, the absorbance at 593 nm was measured using a microplate spectrophotometer (Kang et al., 2021). Trolox was used as the calibration standard. The results were reported as ng TE/mg DW.

Optimization of EsA content conditions using high-performance liquid chromatography (HPLC)

Crude extracts from the elicited calli were dissolved in MeOH and filtered through a 0.45 µm syringe filter. The optimal condition to characterize and evaluate esculentoside A contents of pokeweed samples was determined using an HPLC Agilent 1220 Infinity II LC system (Agilent Technologies, USA), which connected with an Agilent 1200 Series Autosampler (Agilent Technologies, USA). The samples were eluted in TSKgel ODS-100V, 5 µm, 4.6 mm diameter x 250 mm length (Tosoh Asia Pte. Ltd., Singapore). The mobile phases of the gradient elution system were (A) acetonitrile (RCI Labscan, Thailand) and (B) 0.1% (v/v) formic acid (Sigma-Aldrich, USA) in deionized (DI) water. The gradient system was conducted as 0-30 min, 29-31% (A); 30-35 min, 31-95% (A); 35-40 min, 95% (A); 40-45 min, 95-29% (A), and 45-55 min, 29% (A). The samples were injected at 10 µL and the flow rate was 1 mL/min, with the wavelength set at 203 nm. The concentration of EsA in pokeweed calli was calculated using the linear regression equation derived from the calibration curve (Section 2.7), which exhibited R^2 =0.9994:

$$\mathbf{Y=3.1358X-43.151}$$

Validation and determination of EsA content conditions using high-performance liquid chromatography (HPLC)

System suitability

A standard solution of EsA was prepared from the EsA standard compound (MedChemExpress, USA) with MeOH at 1 mg/mL, and 10 µL of the solution was injected and eluted using the gradient system described in section 2.6. Retention time (R_t), peak area, asymmetric factor, and theoretical plate were considered for the system suitability test of EsA. These parameters were conducted for six replicates. Theoretical plate values were calculated using the equation:

$$\mathbf{N = 16(R_t/W)^2}$$

where N represents the theoretical plate of EsA in the system, and R_t and W are retention time (min) and the width of the esculentoside A peak (min), respectively. The test was performed with six replicates, with data expressed as mean, standard deviation, and relative standard deviation percentage (%RSD).

Linearity, limit of detection (LOD) and limit of quantification (LOQ)

Before analysis, the stock solution was filtered through a 0.45 µm nylon membrane to yield five standard concentrations (200-1000 µg/mL). Five replicates of each concentration were measured, and the EsA peak areas were plotted against the concentration to create a calibration curve. Excellent linearity was indicated by the coefficient of determination ($R^2 > 0.9990$) obtained from the resulting linear regression. The following formulas were used to determine the limits of quantification (LOQ) and detection (LOD).

$$\mathbf{LOD = 3.3(\sigma/S)}$$
$$\mathbf{LOQ = 10(\sigma/S)}$$

where σ is the standard deviation of the response and S is the calibration curve's slope.

Precision

Repeatability (intra-day) and intermediate precision (inter-day) were used to evaluate precision. Five replicates of each of the five EsA concentrations (200-1000 µg/mL) were examined. The degree of precision was calculated using following equation and displayed as the relative standard deviation percentage (%RSD).

RSD (%) = (standard deviation/mean) x 100

Accuracy

The accuracy represents the closeness between the measured results or observed concentrations and the reference values or the calculated concentrations from the linearity curve. The accuracy of EsA was measured using four concentrations ranging from 300 to 900 µg/mL, with five replicates in each concentration. The accuracy of the samples was expressed as the recovery percentage and calculated using the following equation:

$$\text{Recovery percentage (\%)} = (\text{observed concentration}/\text{exact concentration}) \times 100$$

Data collection and statistical analysis

A completely randomized design (CRD) with five replicates was used to simultaneously assess multiple factors. Fresh and dry weights (FW and DW) of elicited calli were recorded prior to crude extraction, and extraction yields were calculated. Elicitation efficiency was evaluated based on total phenolic content (TPC), antioxidant activities (DPPH, ABTS, and FRAP), and EsA content. For comparison, extracts from natural roots and non-elicited calli were used as controls, with samples collected at 3-day intervals for chemical elicitors (MeJA and SA) and weekly for the physical elicitor (NaCl).

Factorial design experiments were conducted for each elicitor, with analysis of variance (ANOVA) applied to determine the interactions between different factors and identify the optimal conditions for pokeweed callus elicitation. Multiple comparisons were performed using Duncan's multiple range test to compare between treatments. All the parameters were analyzed using Pearson's correlation to clarify relationships and responses to elicitor effects. Statistical results were analyzed using R version 4.4.1 and version RStudio 2024.04.2+764 for Windows.

Results

Effects of elicitors and duration of elicitation on pokeweed callus growth and morphological characters

Elicited pokeweed calli exhibited a friable texture with sparsely distributed purple pigments. Calli treated with 0 and 50 mM NaCl showed increased size with prolonged elicitation (7 to 21 days), accompanied by sparse pigmentation and a watery texture (Figure 1A–B). In contrast, higher NaCl concentrations (up to 200 mM) led to a noticeable reduction in both callus size and pigment accumulation, particularly at 21 days (Figure 1C–D). Similarly, callus size increased over time in the absence of methyl jasmonate (MeJA; Figure 1E) and salicylic acid (SA; Figure 1G). However, increasing concentrations of MeJA and SA inhibited growth, as reflected by reduced callus size (Figure 1F, 1H).

Analysis of variance (ANOVA) showed that interactions between elicitor concentration (NaCl, MeJA, and SA) and elicitation duration significantly affected fresh weight (FW) and dry weight (DW) of calli ($p < 0.05$; Table 1). Calli treated with 0 and 50 mM NaCl exhibited increased biomass with longer elicitation periods, reaching optimal FW (2.61–2.64 g) and DW (138.38–145.14 mg) at 21 days ($p < 0.001$). In contrast, biomass significantly decreased under 100–200 mM NaCl treatments at 21 days, although values remained higher than those observed at 7 and 14 days ($p < 0.001$; Table 1). Similarly, MeJA and SA elicitation resulted in increased biomass over time, but higher concentrations applied for 9 days led to progressive reductions in biomass, approaching levels observed after 3 and 6 days at the same concentrations. The highest FW and DW were recorded in calli elicited with 0 mM MeJA and SA for 9 days ($p < 0.001$). These findings highlight the significant interactive effects of elicitor concentration and duration on pokeweed callus biomass.

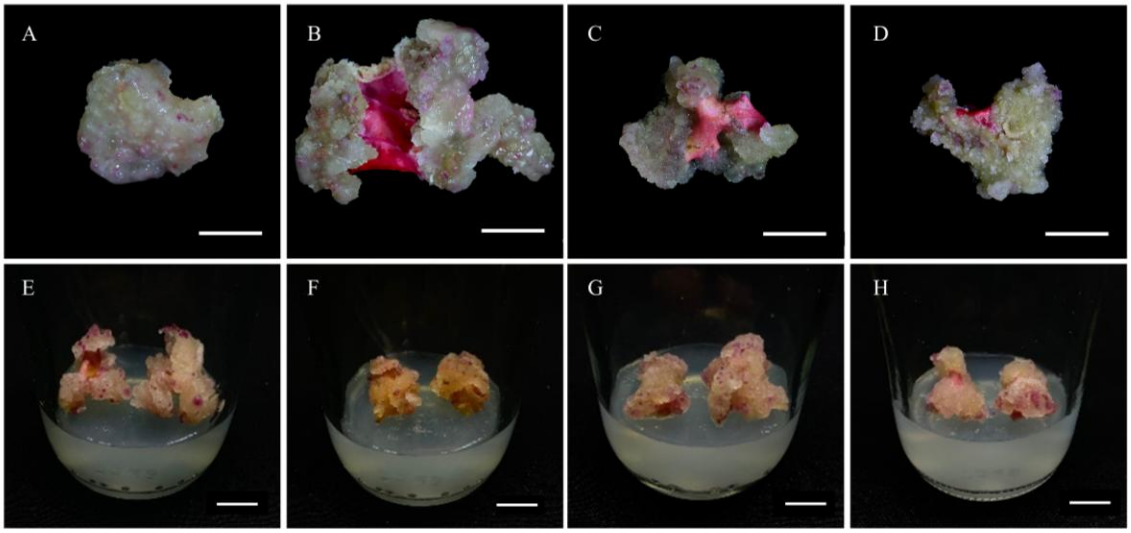

Figure-1. Effects of different elicitors on pokeweed callus development. The calli were treated with three elicitors as (A) 50 mM NaCl for 7 days, (B) 50 mM NaCl for 21 days, (C) 200 mM NaCl for 7 days, (D) 200 mM NaCl for 21 days, (E) 0 mM MeJA for 9 days, (F) 400 mM MeJA for 9 days, (G) 0 mM SA for 9 days, and (H) 200 mM SA for 9 days.

HPLC method validation for the determination of EsA

The retention time (R_t) of EsA ranged from 24.887 to 24.931 min, with %RSD below 2%. The average peak area was 3154.64, with %RSD 1.71%. The asymmetric factor of the EsA peaks ranged from 1.14 to 1.18, and the theoretical plates varied between 5064.37 and 5312.30 (Table 2). The limits of detection (LOD) and quantification (LOQ) were determined based on the slope and standard deviation of the linearity curve at 30.76 μg/mL and 93.21 μg/mL, respectively.

Five concentrations of EsA (200, 400, 600, 800, and 1000 μg/mL) were used for the linear regression analysis. A linear relationship was established between EsA concentration and the corresponding peak area providing R^2 = 0.9994. The same concentrations were used to evaluate repeatability (intra-day precision) and intermediate precision. The results showed that %RSD values for repeatability ranged from 0.70% to 1.57% and for intermediate precision from 0.80% to 1.82%. Both precision metrics exhibited %RSD values below 2% (Table 3). Accuracy was assessed at concentrations of 500, 700, and 900 μg/mL, with recovery percentages ranging from 99.52% to 103.49% (Table 4), demonstrating minimal variation. These findings confirmed the high accuracy and reliability of the method for the quantification of EsA.

Table-1. Effects of elicitation using NaCl, MeJA and SA on fresh and dry weight of pokeweed callus at different durations.

NaCl (mM)	Duration (days)	FW (g)	DW (mg)
Control		0.87 ± 0.03 d	48.8 ± 4.77 f
0	7	1.48 ± 0.11 bc	78.28 ± 8.30 ef
50	7	1.56 ± 0.34 bc	91.86 ± 15.98 c-e
100	7	1.57 ± 0.10 bc	102.26 ± 7.03 c-e
150	7	1.22 ± 0.13 cd	91.60 ± 4.93 c-e
200	7	1.21 ± 0.12 cd	89.88 ± 7.99 c-e
0	14	1.91 ± 0.35 b	104.50 ± 23.01 c-e
50	14	1.76 ± 0.29 b	103.90 ± 16.89 c-e

100	14	1.75 ± 0.22 b	105.44 ± 16.22 c-e
150	14	1.09 ± 0.02 cd	84.82 ± 3.17 c-e
200	14	1.13 ± 0.07 cd	95.34 ± 8.16 c-e
0	21	2.61 ± 0.38 a	138.38 ± 16.05 ab
50	21	2.64 ± 0.49 a	145.14 ±16.05 a
100	21	1.52 ± 0.10 bc	110.28 ± 12.49 b-d
150	21	1.48 ± 0.18 bc	112.54 ± 16.22 b-d
200	21	1.45 ± 0.19 bc	117.68 ± 14.13 a-c
MeJA (µM)	**Duration (days)**	**FW (mg)**	**DW (mg)**
Control		0.83 ± 0.06 ef	46.68 ± 3.65 ef
0	3	1.35 ± 0.18 b	72.64 ± 8.11 b
100	3	0.83 ± 0.03 ef	50.08 ± 4.23 d-f
200	3	0.83 ± 0.06 ef	51.24 ± 3.05 d-f
300	3	0.86 ± 0.05 def	51.28 ± 2.70 d-f
400	3	0.76 ± 0.08 f	44.88 ± 1.96 f
0	6	1.39 ± 0.11 b	79.48 ± 8.81 b
100	6	1.03 ± 0.05 cd	58.44 ± 3.74 d
200	6	0.98 ± 0.10 c-e	60.12 ± 8.78 cd
300	6	0.84 ± 0.05 ef	52.42 ± 1.66 d-f
400	6	0.87 ± 0.04 d-f	53.76 ± 2.77 d-f
0	9	1.81 ± 0.06 a	107.94 ± 7.94 a
100	9	1.09 ± 0.07 c	71.78 ± 4.32 bc
200	9	0.87 ± 0.07 d-f	56.92 ± 4.07 de
300	9	0.91 ± 0.04 d-f	57.36 ± 2.86 de
400	9	0.87 ± 0.06 d-f	54.50 ± 4.98 d-f
SA (µM)	**Duration (days)**	**FW (g)**	**DW (mg)**
Control		0.85 ± 0.05 de	50.26 ± 4.90 f
0	3	1.35 ± 0.18 b	72.64 ± 8.11 b-d
50	3	0.95 ± 0.04 de	57.64 ± 5.70 ef
100	3	0.95 ± 0.10 de	56.60 ± 3.86 ef
150	3	0.78 ± 0.05 e	47.50 ± 4.42 f
200	3	0.85 ± 0.06 de	50.74 ± 2.74 f
0	6	1.39 ± 0.11 b	79.48 ± 8.81 b
50	6	1.22 ± 0.08 bc	79.30 ± 3.91 b
100	6	1.30 ± 0.09 b	78.58 ± 5.81 bc
150	6	0.99 ± 0.09 d	65.00 ± 6.29 de
200	6	0.90 ± 0.05 de	56.88 ± 6.86 ef
0	9	1.81 ± 0.06 a	107.94 ± 7.94 a
50	9	1.29 ± 0.06 b	80.70 ± 3.74 b
100	9	1.33 ± 0.07 b	85.54 ± 3.48 b
150	9	0.94 ± 0.05 de	60.38 ± 3.63 d-f
200	9	1.04 ± 0.11 cd	66.16 ± 6.64 c-e
Concentration		< 0.001	< 0.001
Duration		< 0.001	< 0.001
Interaction		< 0.05	< 0.01

Different letters in columns represent significant differences between treatments according to the analysis of variance and Duncan's multiple range test ($p < 0.05$). Data are expressed as mean ± standard deviation (SD).

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Table-2. System suitability of EsA.

Injection	Retention time (R_t; min)	Peak area	Asymmetric factor	Theoretical plates (N)
1	24.906	3112.23	1.17	5312.30
2	24.887	3067.02	1.14	5150.78
3	24.906	3208.00	1.18	5061.67
4	24.891	3167.53	1.14	5194.20
5	24.891	3189.82	1.16	5064.37
6	24.931	3183.25	1.15	5108.16
Mean	24.901	3154.64	1.16	5148.58
SD	0.017	53.94	0.02	95.04
%RSD	0.069	1.71	1.43	1.85

Table-3. Repeatability and intermediate precision of EsA.

Theoretical concentration (µg/mL)	Repeatability		Intermediate precision	
	Peak area	%RSD	Peak area	%RSD
200	587.85 ± 9.05	1.54	579.99 ± 5.97	1.03
400	1218.56 ± 14.31	1.17	1219.96 ± 22.17	1.82
600	1839.14 ± 15.48	0.84	1861.55 ± 17.88	0.96
800	2426.42 ± 38.11	1.57	2452.35 ± 19.66	0.80
1000	3119.74 ± 21.86	0.70	3172.17 ± 36.51	1.15

Data are expressed as mean ± standard deviation (SD).

Table-4. Recovery percentages of EsA.

Theoretical concentration (µg/mL)	Calculated concentrations (µg/mL)	%RSD	Recovery percentage range
500	509.32 ± 6.00	1.18	100.28-102.82
700	713.05 ± 10.17	1.42	99.52-103.35
900	917.79 ± 11.95	1.30	100.22-103.49

Data are expressed as mean ± standard deviation (SD).

Total phenolic content (TPC), antioxidant activities and EsA content of pokeweed callus extracts elicited with different elicitors for different durations

Total phenolic content and antioxidant activity

Significant interaction effects between NaCl concentration and elicitation duration were observed for total phenolic content (TPC) and antioxidant activities (DPPH, ABTS, and FRAP assays) ($p < 0.001$; Table 5). TPC peaked at 13.13 µg GAE/mg DW in calli treated with 50 mM NaCl for 7 days but declined with higher NaCl concentrations (Table 5). Similar trends were observed during elicitation periods of 14 and 21 days, with 50 mM NaCl yielding optimal phenolic contents of 12.34 and 11.33 µg GAE/mg DW, respectively before declining at NaCl concentrations exceeding 100 mM (Table 5). The highest phenolic content (13.13 µg GAE/mg DW) was achieved with 50 mM NaCl treatment for 7 days, significantly higher than the other treatments and natural roots (*p*-value < 0.001).

Antioxidant activities, as determined by the ABTS and FRAP assays, closely corresponded to the patterns observed for TPC. During the 14-day elicitation, ABTS and FRAP activities increased when calli were elicited with 50 and 100 mM NaCl, ranging from 898.44 to 953.20 and 613.82 to 618.54 ng TE/mg DW, respectively. These activities were statistically comparable or significantly higher than in calli elicited for 7 days at the same concentration. When the NaCl concentration increased to 150 and 200 mM, the calli

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subjected to 14-day treatment exhibited significantly lower activities compared to those elicited for 7 days (p-value < 0.001; Table 5), with significantly lowest antioxidant activities noticed in elicitation treatments for 21 days across all NaCl concentrations (p-value < 0.001; Table 5). The DPPH assay activity displayed a distinct trend compared to the other assays. The DPPH activity of calli treated with NaCl for 7 days demonstrated a progressive increase, reaching a peak of 701.96 ng TE/mg DW at 150 mM NaCl treatment (p-value < 0.001; Table 5). For treatments of 14 and 21 days, the activity was significantly lower compared to the 7-day treatments but increased when the calli were treated with 200 mM NaCl. The optimal DPPH activity was observed in the 150 mM NaCl treatment at 7 days (701.96 ng TE/mg DW) while the highest ABTS and FRAP activities were found in the 50 mM NaCl treatment at 14 days (953.20 and 618.54 ng TE/mg DW, respectively). These values were significantly higher than the control and natural pokeweed roots (p-value < 0.001).

ANOVA results revealed significant interaction effects between MeJA concentration and elicitation duration on TPC and antioxidant activities (p-value < 0.001; Table 6). Pokeweed callus cultured on MeJA-free medium for 9 days exhibited the highest TPC (13.99 µg GAE/mg DW), significantly higher than calli elicited for 3 and 6 days at the same MeJA concentration (p-value < 0.001; Table 6). The TPC in the 9-day treatments decreased significantly with increasing MeJA concentration. Interestingly, TPC increased in calli treated with 100 µM MeJA for 3 and 6 days (14.26 and 12.69 µg GAE/mg DW, respectively), despite the overall trend of decreasing TPC with higher MeJA concentrations. The 100 µM MeJA treatment for 3 days yielded the highest TPC but was not significantly different from the 0 µM MeJA treatment for 9 days (p-value > 0.05).

Consistent trends were observed in the DPPH and FRAP antioxidant assays. After 9 days of MeJA elicitation, calli treated with 0 µM MeJA exhibited the highest antioxidant activity, with values of 639.10 and 539.61 ng TE/mg DW for DPPH and FRAP, respectively. Antioxidant activities decreased with 100 to 400 µM MeJA treatments and were significantly lower compared to the same concentrations at 3- and 6-day elicitation (p-value < 0.001; Table 6). DPPH activity in calli treated for 3 and 6 days remained stable across MeJA concentrations. The highest DPPH activity, 639.10 ng TE/mg DW, was observed in calli treated with 0 µM MeJA for 9 days (p-value < 0.001; Table 6). FRAP activity increased with MeJA concentration, with the highest activity (675.83 ng TE/mg DW) observed in calli treated with 100 µM MeJA for 3 days, significantly higher than the other treatments (p-value < 0.001; Table 6). The ABTS assay results did not show clear trends, with the highest antioxidant activity (886.16 ng TE/mg DW) recorded in the 0 µM MeJA treatment with 9 days of elicitation, similar to the trends observed in the DPPH and FRAP assays (p-value < 0.001). The TPC and antioxidant activities of the elicited calli were significantly higher than the control and natural pokeweed roots (p-value < 0.001).

Interaction effects on TPC and antioxidant activities were observed in the combined treatments of SA concentrations and elicitation durations (p-value < 0.001). The TPC increased with SA concentration during the 3 and 6 day treatments but decreased when calli were treated with 200 µM SA for 3 and 6 days. Elicitation of pokeweed calli with SA for 9 days resulted in higher TPC compared to the 3 and 6 day treatments across various SA concentrations, with the highest (15.82 µg GAE/mg DW) observed at 150 µM SA. The treatment of 150 µM SA for 9 days of elicitation gave higher TPC compared to the control and natural pokeweed roots (p-value < 0.001; Table 7).

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Table-5. Effects of pokeweed callus elicitation with NaCl at 0-200 mM for 7-21 days on phytochemical contents and antioxidant activity using three different assays.

NaCl (mM)	Duration (days)	TPC (µg GAE/mg DW)	DPPH (ng TE/mg DW)	ABTS (ng TE/mg DW)	FRAP (ng TE/mg DW)	EsA content (µg/mg DW)
Natural roots		1.95 ± 0.03 j	393.01 ± 14.00 i	432.47 ± 13.29 i	49.79 ± 1.23 i	1.98 ± 0.02 k
Control		12.91 ± 0.18 ab	545.00 ± 6.28 g	799.22 ± 1.67 e	339.4 ± 20.74 h	5.55 ± 0.01 f-h
0	7	11.81 ± 0.08 b-d	615.03 ± 8.26 cd	817.73 ± 8.31 e	507.53 ± 23.53 c-f	5.99 ± 0.27 fg
50	7	13.13 ± 0.28 a	617.89 ± 6.65 cd	893.88 ± 9.34 bc	631.62 ± 5.28 a	4.99 ± 0.16 hi
100	7	10.84 ± 0.24 d-f	672.55 ± 7.21 b	861.14 ± 22.95 d	561.52 ± 43.82 a-d	5.71 ± 0.09 f-h
150	7	10.30 ± 0.79 e-g	701.96 ± 12.07 a	819.02 ± 9.13 e	506.95 ± 15.03 c-f	8.06 ± 0.38 de
200	7	11.44 ± 0.24 cd	685.69 ± 2.23 ab	880.76 ± 8.72 b-d	625.38 ± 19.85 ab	7.37 ± 0.37 e
0	14	11.82 ± 0.43 b-d	583.40 ± 4.86 ef	817.91 ± 4.65 e	536.73 ± 42.69 b-e	3.61 ± 0.20 j
50	14	12.34 ± 0.45 a-c	618.11 ± 3.37 cd	953.20 ± 9.55 a	618.54 ± 22.94 ab	5.23 ± 0.14 g-i
100	14	11.39 ± 0.18 c-e	628.17 ± 3.29 c	898.44 ± 8.12 b	613.82 ± 20.02 ab	6.16 ± 0.35 f
150	14	9.61 ± 0.41 gh	573.38 ± 5.71 f	733.69 ± 13.21 f	512.91 ± 30.64 c-e	7.98 ± 0.21 de
200	14	9.28 ± 0.31 gh	615.83 ± 7.48 cd	745.13 ± 5.25 f	472.09 ± 70.81 d-g	10.13 ± 0.28 c
0	21	10.10 ± 0.57 fg	492.00 ± 2.82 h	693.03 ± 8.91 g	449.66 ± 26.65 e-g	4.64 ± 0.33 i
50	21	11.33 ± 0.47 c-e	563.49 ± 8.12 fg	865.39 ± 2.15 cd	567.11 ± 40.99 a-c	5.30 ± 0.37 f-i
100	21	10.75 ± 0.36 d-f	565.42 ± 4.88 fg	818.84 ± 14.93 e	579.75 ± 20.17 a-c	8.54 ± 0.18 d
150	21	8.04 ± 0.05 i	509.47 ± 8.20 h	645.49 ± 11.51 h	390.15 ± 11.31 gh	14.06 ± 0.68 b
200	21	8.91 ± 0.10 hi	602.80 ± 14.34 de	672.58 ± 7.77 gh	418.88 ± 12.53 f-h	16.73 ± 0.27 a
Concentration		< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
Duration		< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
Interaction		< 0.001	< 0.001	< 0.001	< 0.001	< 0.001

Different letters in columns represent significant differences between treatments according to the analysis of variance and Duncan's multiple range test ($p < 0.05$). Data are expressed as mean ± standard deviation (SD).

Table-6. Effects of pokeweed callus elicitation with MeJA at 0-400 mM for 3-9 days on phytochemical contents and antioxidant activity using three different assays.

MeJA (µM)	Duration (days)	TPC (µg GAE/mg DW)	DPPH (µg TE/mg DW)	ABTS (µg TE/mg DW)	FRAP (µg TE/mg DW)	EsA content (µg/mg DW)
Natural roots		1.95 ± 0.03 j	393.01 ± 14.00 j	432.47 ± 13.29 h	49.79 ± 1.23 i	1.98 ± 0.02 i
Control		12.91 ± 0.06 c	545.00 ± 8.01 g	753.10 ± 0.89 g	339.40 ± 24.43 g	5.55 ± 0.01 d
0	3	11.67 ± 0.06 ef	605.71 ± 10.35 c-e	822.05 ± 4.46 d	322.53 ± 2.45 g	7.19 ± 0.01 b
100	3	14.26 ± 0.14 a	603.26 ± 2.88 c-e	829.24 ± 0.98 cd	675.83 ± 24.22 a	3.71 ± 0.07 f
200	3	13.25 ± 0.16 b	580.38 ± 4.40 ef	833.10 ± 1.70 cd	435.58 ± 15.47 de	1.53 ± 0.11 j
300	3	12.39 ± 0.07 d	606.59 ± 12.69 b-d	880.33 ± 2.75 ab	452.55 ± 16.81 d	1.25 ± 0.04 k
400	3	11.61 ± 0.09 ef	575.11 ± 4.45 f	842.84 ± 0.99 c	410.53 ± 11.83 de	1.04 ± 0.09 l
0	6	11.28 ± 0.04 g	587.27 ± 6.43 def	841.90 ± 3.47 c	360.09 ± 3.96 fg	8.51 ± 0.06 a
100	6	12.69 ± 0.14 c	631.70 ± 2.99 ab	868.36 ± 3.07 b	539.63 ± 20.13 c	2.55 ± 0.06 g
200	6	11.83 ± 0.02 e	624.99 ± 4.58 a-c	793.67 ± 5.42 ef	363.92 ± 16.93 fg	2.14 ± 0.09 hi
300	6	11.74 ± 0.11 e	599.20 ± 3.05 d-f	870.93 ± 2.08 b	392.30 ± 24.61 ef	0.77 ± 0.05 mn
400	6	11.42 ± 0.10 fg	590.84 ± 3.08 d-f	870.85 ± 4.82 b	326.61 ± 8.96 g	0.65 ± 0.01 n
0	9	13.99 ± 0.08 a	639.10 ± 11.96 a	886.16 ± 2.79a	594.91 ± 10.28 b	5.89 ± 0.08 c
100	9	8.47 ± 0.03 i	514.81 ± 11.96 h	802.65 ± 1.87 e	250.34 ± 0.85 h	4.09 ± 0.13 e
200	9	9.05 ± 0.04 h	506.20 ± 6.53 hi	783.16 ± 5.96 f	213.65 ± 3.04 h	2.24 ± 0.08 h
300	9	8.55 ± 0.08 i	482.18 ± 3.20 i	796.94 ± 3.94 ef	215.93 ± 6.72 h	0.94 ± 0.03 lm
400	9	9.29 ± 0.11 h	549.27 ± 5.01 g	839.37 ± 6.81 c	235.73 ± 11.47 h	0.73 ± 0.02 n
Concentration		< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
Duration		< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
Interaction		< 0.001	< 0.001	< 0.001	< 0.001	< 0.001

Different letters in columns represent significant differences between treatments according to the analysis of variance and Duncan's multiple range test ($p < 0.05$). Data are expressed as mean ± standard deviation (SD).

Table-7. Effects of pokeweed callus elicitation with SA at 0-200 mM for 3-9 days on phytochemical contents and antioxidant activity using three different assays.

SA (µM)	Duration (days)	TPC (µg GAE/mg DW)	DPPH (µg TE/mg DW)	ABTS (µg TE/mg DW)	FRAP (µg TE/mg DW)	EsA content (µg/mg DW)
Natural roots		1.95 ± 0.03 k	393.01 ± 14.00 h	432.47 ± 13.29 k	49.79 ± 1.23 i	1.98 ± 0.02 k
Control		12.91 ± 0.07 f	545.00 ± 3.00 f	753.10 ± 1.54 j	339.40 ± 20.74 h	5.55 ± 0.01 d
0	3	10.36 ± 0.05 i	603.38 ± 10.31 e	819.24 ± 4.45 i	344.07 ± 2.61 h	7.19 ± 0.01 b
50	3	11.93 ± 0.05 h	669.92 ± 7.54 d	874.25 ± 5.99 f	382.60 ± 22.28 gh	4.60 ± 0.04 f
100	3	12.61 ± 0.04 g	677.84 ± 5.48 cd	891.70 ± 3.84 de	408.72 ± 7.79 g	4.87 ± 0.02 e
150	3	13.01 ± 0.07 f	665.39 ± 3.93 d	855.12 ± 1.75 g	431.23 ± 38.21 g	4.98 ± 0.03 e
200	3	11.73 ± 0.05 h	615.70 ± 9.07 e	851.45 ± 8.08 gh	490.20 ± 41.86 f	4.40 ± 0.01 g
0	6	10.51 ± 0.04 i	614.30 ± 6.73 e	839.02 ± 3.46 h	403.38 ± 4.43 g	8.51 ± 0.06 a
50	6	13.82 ± 0.04 e	723.06 ± 18.08 ab	961.68 ± 1.14 a	605.09 ± 12.70 e	5.44 ± 0.15 d
100	6	12.79 ± 0.12 fg	746.98 ± 3.88 a	970.60 ± 4.15 a	808.43 ± 15.74 d	3.59 ± 0.08 i
150	6	12.89 ± 0.02 f	680.67 ± 23.82 cd	897.73 ± 1.84 d	850.88 ± 16.56 cd	4.68 ± 0.03 f
200	6	9.92 ± 0.02 j	476.42 ± 35.72 g	914.40 ± 3.81 bc	651.59 ± 9.11 e	5.02 ± 0.06 e
0	9	14.19 ± 0.03 d	682.48 ± 12.77 b-d	889.21 ± 1.82 de	838.09 ± 8.83 cd	6.04 ± 0.11 c
50	9	14.49 ± 0.20 c	716.58 ± 3.59 a-c	902.75 ± 1.85 cd	952.89 ± 10.88 a	4.20 ± 0.03 h
100	9	14.42 ± 0.11 cd	680.52 ± 12.67 cd	882.08 ± 1.81 ef	890.93 ± 3.50 bc	3.27 ± 0.04 j
150	9	15.82 ± 0.09 a	753.89 ± 7.63 a	958.71 ± 1.13 a	906.17 ± 5.70 ab	3.38 ± 0.03 j
200	9	15.28 ± 0.07 b	713.22 ± 9.30 a-c	921.96 ± 3.29 b	887.85 ± 4.25 bc	4.61 ± 0.07 f
Concentration		< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
Duration		< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
Interaction		< 0.001	< 0.001	< 0.001	< 0.001	< 0.001

Different letters in columns represent significant differences between treatments according to the analysis of variance and Duncan's multiple range test ($p < 0.05$). Data are expressed as mean ± standard deviation (SD).

FRAP assay results demonstrated a strong influence of elicitation duration on antioxidant activity. After 9 days of elicitation, the FRAP activity was significantly higher across all SA concentrations compared to the 3 and 6 day treatments. The optimal FRAP activity was obtained from the treatment of 50 and 150 µM SA for 9 days as 952.89 and 906.17 ng TE/mg DW, respectively. The 3-day elicitation exhibited significantly lower FRAP activity than longer durations at all SA concentrations (p-value < 0.001; Table 7). The DPPH and ABTS activities did not exhibit distinct trends, with the highest activities for both assays observed in the same treatment. The FRAP activity at 150 µM SA treatment for 9 days yielded 753.89 and 958.71 ng TE/mg DW for DPPH and ABTS, respectively. These values were significantly higher than the control and natural pokeweed roots (p-value < 0.001; Table 7).

EsA contents detected with HPLC

Pokeweed callus extracts were prepared in methanol (MeOH), and the peak area under the curve for EsA in the extracts was determined using the validated HPLC method. EsA content was calculated from the peak area using the linear equation outlined in the previous section. ANOVA results demonstrated the significant effects of interaction between elicitor concentrations and elicitation durations on EsA contents (p-value < 0.001; Table 5). During NaCl-induced callus elicitation for 7 and 14 days, EsA content increased with higher NaCl concentrations. After 21 days of elicitation, a substantial increase in EsA content was observed with increasing NaCl concentration, and the significantly highest content, 16.73 µg/mg DW, was observed at 200 mM NaCl (p-value < 0.001; Table 5). By contrast, callus elicitation in elicitor-free medium (without SA and MeJA) yielded the highest EsA content at each elicitation duration, with the optimal content of 8.51 µg/mg DW observed after 6 days (p-value < 0.001; Tables 6 and 7). Increasing SA concentrations led to a progressive decline in EsA content across all elicitation durations, ranging from 3.27 to 4.98 µg/mg DW (Table 7). Similar to SA, callus elicitation with MeJA also exhibited consistent results while a drastic decrease in EsA content was observed in the treatment of 300 and 400 µM MeJA, providing 0.65 to 0.94 µg/mg DW (Table 6). Compared to the EsA content from the control treatment and natural roots, the content from the 200 mM NaCl treatment elicited for 21 days was significantly higher, whereas the elicitation with SA and MeJA resulted in significantly lower EsA levels. (p-value < 0.001; Tables 6, 7).

Pearson's correlation among the phytochemical parameters

Correlations among the phytochemical parameters of the elicited calli were expressed in the correlation matrix (Figure 2). A strong positive correlation was shown between TPC and antioxidant activities, providing 0.705 to 0.718 correlation coefficients (p-value < 0.001). Positive moderate correlation coefficients (0.545-0.642) were observed among the antioxidant activity assays (p-value < 0.001). The EsA content demonstrated an insignificant correlation with the DPPH and FRAP assays, and a significantly negatively weak to moderate correlation coefficient with the TPC (-0.341) and the ABTS assay (-0.482) (p-value < 0.001).

A significantly positive correlation was noticed between TPC and the antioxidant activities for all elicitors, with correlation coefficients varying between 0.341 and 0.919 (p-value < 0.05). EsA content and the other parameters from treatments of MeJA showed insignificant correlation, while moderately and strongly negative correlations were observed in treatments of SA and NaCl, respectively (p-value < 0.001).

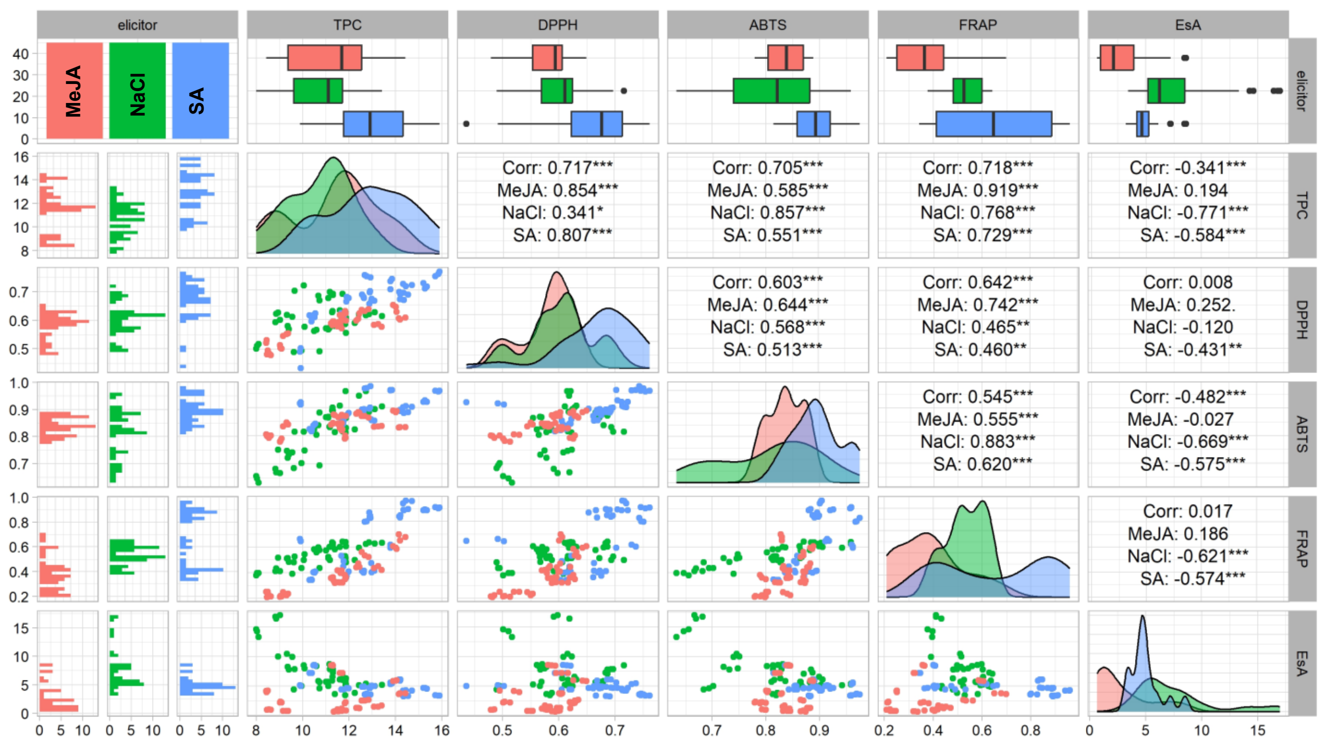

Figure-2. Pearson's correlation exhibiting a pairwise correlation matrix, density, and scatter plots of the phytochemical parameters of pokeweed callus elicitation using NaCl, MeJA, and SA. *, **, and *** represented significance levels at 0.5, 0.01, and 0.001, respectively.

Discussion

Fresh and dry weight of pokeweed callus elicited with NaCl, MeJA and SA

Pokeweed callus growth was evaluated based on fresh and dry biomass after elicitation. At low salinity levels (0–50 mM NaCl), fresh and dry weights increased over time, indicating no adverse effect on callus growth. In contrast, higher NaCl concentrations (100–200 mM) significantly reduced biomass. The application of NaCl-induced salinity stress in the callus, resulting in the accumulation of Na^+ in the elicitation medium. Excessive accumulation of Na^+ increases negative water potential which drives water

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from the plant cells (higher water potential) to the medium (lower water potential). Exposure of plant cells to salinity stress results in water deficit, impairment of the photosynthetic system and plant growth (Balasubramaniam et al., 2023). Salinity stress induces reactive oxygen species (ROS) accumulation and oxidative stress that damages the cell membrane and other biomolecules, leading to severe membrane degradation, chloroplast dysfunction, and alterations in the thylakoid structure, ultimately inhibiting plant cell growth (Cao et al., 2023; Zhou et al., 2024). The biomass of *Stevia rebaudiana* and *Azadirachta indica* calli decreased with increasing NaCl concentration (Gupta et al., 2014; Omar et al., 2024). Salinity stress also resulted in decreases in *Capsicum annuum* callus fresh weight. Proline accumulation was applied to maintain water potential in *C. annuum* callus because proline content in the callus increased with higher salt levels in the media (Al Hattab et al., 2015). In this study, pokeweed callus biomass decreased in the treatment of 100-200 mM NaCl for 14 and 21 days reaching levels comparable to those observed at lower NaCl concentrations. The highest biomass accumulation was found in pokeweed calli treated with 50-100 mM NaCl for 21 days. These findings suggested that pokeweed calli use particular adaptive mechanisms to lessen the effects of salinity stress while eliciting responses.

Because MeJA-treated pokeweed callus exhibited a similar response, the negative effects of high elicitor concentrations were not restricted to NaCl. MeJA is a signaling molecule and plant growth regulator (PGR) that maximizes photosynthetic efficiency, increases endogenous abscisic acid levels in response to stress, and promotes shoot growth (Kamińska, 2021; Sirhindi et al., 2020). However, excessive MeJA concentrations inhibit cell division and suppress callus proliferation, as previously observed in *Taxus cuspidata* cells and *Allium jesdianum* callus cultures (Patil et al., 2014; Yazdanian et al., 2022). Excessive MeJA intake also causes oxidative stress, which results in chlorophyll precursor degradation and lipid peroxidation (Kim et al., 2023). For example, high MeJA levels decreased both fresh and dry weights of *Phyllanthus pulcher* (Danaee et al., 2015) and increased malondialdehyde (MDA) accumulation along with reduced callus growth in *Carum carvi* (Rahmati et al., 2023). Similarly, MeJA treated callus of Artemisia maritima exhibited decreased biomass but raised proline and sugars contents as well as enhanced antioxidant enzyme activities (SOD, CAT, and APX), which indicating oxidative stress responses (Nabi et al., 2025).

Meanwhile, salicylic acid (SA) also acts as a stress-related signaling molecule, accumulating in plants during pathogen infection and contributing to defense pathways that regulate growth and development (Radojičić et al., 2018; Jeyasri et al., 2023). However, high SA concentrations negatively affected pokeweed callus biomass in our study, which is consistent with the growth inhibition reported in *Fagonia indica* and *Salvia miltiorrhiza* calli (Khan et al., 2019; Szymczyk et al., 2021). Interestingly, SA stimulated cell and callus growth in *Hypericum perforatum* at an early stage. However, prolonged exposure (21 days) resulted in a decrease in cell viability and fresh weight, particularly in suspension cultures (Gadzovska et al., 2013).

Method validation of EsA with HPLC

To confirm the accuracy and reproducibility of EsA quantification by HPLC, the necessary monitoring parameters were checked. A standard solution of EsA (1 mg/mL in MeOH) was injected to examine the retention time (Rt), peak area, and peak asymmetry. The Rt showed only minor fluctuations (24.887-24.931 min) with a relative standard deviation (RSD) of 0.069%. In addition, the symmetry of the consistent peak and the theoretical number of more than 5,000 plates demonstrate the reliability and efficiency of the system. The method's robustness and suitability for precise EsA analysis were further demonstrated by negligible deviations in peak areas.

The lowest concentration of EsA to establish a linearity curve and equation should be greater than the LOQ, which was 93.21 mg/mL. The concentrations range of concentrations was 200 - 1000 μg/mL. With an R^2 of 0.9994, the linear regression model demonstrated strong predictive power and outstanding goodness of fit. Under the same circumstances, repeatability is equivalent to precision. In this study, the repeatability of EsA determination was performed on the same day, while intermediate precision was performed under different conditions or on different days. Accuracy of determination means the closeness of the observed and exact concentration as expressed by the percentage of recovery (Naz et al., 2014). Both parameters varied between 0.70 and 1.82 %RSD, lower than 2%RSD, while recovery percentages ranged from 99.52 to 103.49%. The results indicated the reliability and robustness of the analytical method to evaluate EsA content in pokeweed calli.

Total phenolic content, antioxidant activities and EsA contents of the elicited pokeweed callus

As discussed above, salinity stress can induce the overaccumulation of ROS that damages cell compartments and biomolecules, thereby inducing oxidative stress (Zhou et al., 2024). Plant cells employ various strategies to cope with stress. The production of phenolic compounds is a non-enzymatic antioxidant strategy to scavenge ROS through hydrogen atom donation to reactive species and neutralize their activities (Kumar and Goel, 2019). In this study, the TPC in elicited pokeweed callus was measured together with antioxidant assays to investigate the effects of elicitor concentration and elicitation duration. A low concentration of NaCl (50 mM) induced improvement in TPC and antioxidant assay activities. Elevated levels of Na^+ can initiate stress-induced signaling pathways. Transcription factors (TFs) such as MYB-TFs are activated to regulate the expression of genes involved in the biosynthesis of osmolytes including phenolic compounds (Zhou et al., 2024). Shoots of *Inula crithmoides* elicited with NaCl at 50-200 mM provided higher phenolic acids including shikimic acid, gentisic acid, caffeic acid, and coumaric acid along with improved flavonoid contents. The phenolic and flavonoid contents increasing in *I. crithmoides* was found to be more active with of phenylalanine ammonia lyase (PAL) activity increased, which is a key enzyme in the phenylpropanoid pathway (Rodrigues et al., 2024). Similarly, a progressive increase in *PAL* gene expression was found in *Moringa oleifera* seedlings under increased salinity stress (Osman and El-Naggar, 2022). Additionally, when exposed to increasing concentrations of NaCl, the callus of *Salsola baryosma*, *Trianthena triquetra* and *Zygophyllum simplex* showed high total phenolic content (TPC), including antioxidant activities by DPPH and FRAP measurement (Sharma and Ramawat, 2013). However, several phenolic compounds of *Rumex thyrsiflorus* calli increased to 43-129 mM NaCl but decreased to 172 mM (Gozdur et al., 2024). Our study also showed that longer elicitation times and greater NaCl concentrations both led to a reduction in TPC and antioxidant activity. The cause of this is probably excessive salinity stress, which harms cells and interferes with metabolic functions that are required to combat oxidative stress.

In vitro grown plants under stress are commonly elicited by the PGRs, MeJA and SA (Jeyasri et al., 2023; Kamińska, 2021). When exogenous MeJA is applied, the expression of the *MYC2* and *JARI* genes is upregulated, which causes the JAZ protein to degrade and permits the upregulation of JA-responsive genes. These genes include important phenylpropanoid pathway enzymes like PAL, cinnamate4-hydroxylase (C4H), and 4-coumarate coenzyme A ligase (4CL). Enhanced activities of these enzymes result in an increase in TPC and antioxidant activities (Li et al., 2022). Our results demonstrated that pokeweed calli elicited with MeJA at 100 µM provided higher TPC and antioxidant activities, especially in the FRAP assay. Supplementation of MeJA at 10 µM improved phenolic and flavonoid contents along with the DPPH antioxidant activity of *Phyllanthus plucher* calli (Danaee et al., 2015). Similar to the elicitation of *Allium jesdianum* and *Carum caivi* calli, MeJA at 50 µM provided the highest contents of phenolics, flavonoids, flavonols, and antioxidant activity. These parameters declined when MeJA concentrations reached 100 µM (Rahmati et al., 2023; Yazdanian et al., 2022). Adverse effects of excessively high concentrations of MeJA were found in callus elicitation of pokeweed. A prolonged elicitation with MeJA for 6-9 days reduced the phenolic compounds in pokeweed calli. The negative effects observed in phenolic content resulted from excessive oxidative stress (Nabi et al., 2025), also found in pokeweed callus biomass.

Contrary to MeJA, pokeweed callus elicitation with SA for 9 days gave higher TPC and FRAP activity assays compared to 3 and 6 days across all concentrations. SA is a phenolic compound synthesized from shikimic acid via phenylpropanoid or isochorismate pathways in plastids and acts as a signaling molecule to respond to biotic and abiotic stress. Application of SA as an elicitor contributes to the accumulation of phenolic compounds and antioxidant activity (Saleem et al., 2021). Activities of PAL and chalcone isomerase (CHI), key enzymes in the phenylpropanoid pathway, were enhanced in treatments of prolonged elicitation (7-21 days) with high concentrations of SA ranging from 100-250 µM in callus of *Hypericum perforatum*. The improved activities resulted in higher phenolic and flavonol contents (Gadzovska et al., 2013). A progressive increase in SA concentration caused positive effects on PAL activity, phenolics, and aervine contents in *Aerva sanguinolenta* callus (Maqbool et al., 2023).

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LC-MS/MS profiling of *Pelargonium graveolens* callus revealed that exogenous SA at 25 µM resulted in an improvement of phenolic compounds such as gallic acid, vanillic acid, and rutin (Elbouzidi et al., 2025). These results proved that the positive effects of SA as an elicitor for phenolic compound production in plant callus cultures. However, the optimal duration and elicitor concentration dependent on plant species and explant variation.

The EsA contents in pokeweed calli elicited with NaCl were measured following validation of the EsA determination process. During 7-14 days after elicitation, the EsA content did not increase with increasing NaCl concentrations. The EsA content increased and reached the optimal value when elicited with 100-200 mM NaCl for 21 days. To respond to salinity stress, plants accumulate certain osmolytes to adjust intracellular osmotic potential (Balasubramaniam et al., 2023). The accumulation of EsA, a triterpene glycoside, was associated with osmotic adjustment in pokeweed calli. The content of azadirachtin, a tetranortriterpenoid, from *Azadirachta indica* calli improved when treated with 1.5% (w/v) NaCl. Proteomic analysis revealed that the number of proteins involved in photosynthesis, redox homeostasis, and plant stress defense increased after NaCl treatment. (Omar et al., 2024). Additionally, it was discovered that salinity stress altered several enzymes involved in terpenoid biosynthesis. Hydroxymethylglutaryl-CoA reductase (HMGR) and 2C-methyl-D-erythritol 2,4-cyclodiphosphate synthase (MES), key enzymes in mevalonate (MVA) and methylerythritol phosphate (MEP) pathways, were upregulated when *Dendrobium huoshanense* was treated with NaCl (Song et al., 2022). Several genes encoding enzymes in the artemisinin biosynthetic pathway, a monoterpene, as well as key triterpene-synthesizing enzymes, namely squalene synthase (SQS) and beta-amyrin synthase (BAS), were upregulated in response to NaCl treatment in *Artemisia annua* (Yadav et al., 2017). These results indicate that NaCl elicitation activates terpenoid biosynthetic pathways, linking terpenoid accumulation to the plant salinity stress responses.

Several previous studies showed the positive effects of MeJA on triterpenes production. The contents of asiaticoside and total triterpenoids in *Centella asiatica* hairy roots increased after treating 50 µM MeJA (Nguyen et al., 2019). However, total triterpenoids in *Cyclocarya paliurus* leaves were not significantly changed but individual triterpenoids such as oleanolic acid exhibited higher content after extended elicitation with MeJA from 20 to 30 days. Elevated relative expression of HMGR genes in response to MeJA treatment may contribute to the enhanced biosynthesis of triterpenoids compared to control conditions (Xia et al., 2023). SA has also been widely applied to elicit triterpenoid production. SA treatment at 100 µM caused improvement of deoxyactein and actein contents in *Actaea racemosa* rhizomes (De Capite et al., 2016). Treatments of SA for 8-24 h resulted in the upregulation of several genes in MVA and MEP pathways such as acetyl-CoA acyltransferase (AACT), HGMS, and 1-deoxy-D-xylulose 5-phosphate synthase (DXS), leading to an increase in total saponin content in *Psammosilene tunicoides* hairy roots (Su et al., 2021). Application of exogenous MeJA and SA caused the upregulation of transcription factors including *WRKY*, *MYB*, and *NAC* with downstream triterpene biosynthetic genes (Su et al., 2021; Yamada et al., 2021). Previous studies exhibited the positive effects of MeJA and SA in triterpene production in various plant species but our investigation revealed distinctly different response patterns. The EsA content in pokeweed callus cultures progressively decreased with increasing concentrations of SA and MeJA and when elicitation periods were extended to 9 days. Detrimental effects were also observed in several studies when MeJA and SA were applied at high concentrations. Adverse effects of these elicitors at excessively high concentrations or prolonged elicitation were noticed in *Centella asiatica* hairy roots and *Actaea racemosa* rhizomes (De Capite et al., 2016; Nguyen et al., 2019). Moderate concentrations of both elicitors activate transcription factors and induce oxidative burst that regulate triterpenoid biosynthetic pathways. High concentrations and prolonged exposure may result in excessive ROS production, disrupting biological activities and consequently reducing secondary metabolite accumulation (Nabi et al., 2025).

Correlation analysis between TPC, EsA and antioxidant activity

NaCl induced salinity stress promotes the generation of reactive oxygen species (ROS) and disturbs the redox balance in plant cells (Patel et al., 2020). To cope with this oxidative stress, plants produce and accumulate antioxidant compounds that help eliminate excess ROS (Balasubramaniam et al., 2023). Antioxidant capacity was assessed using multiple assays, including DPPH, ABTS, and FRAP, each using different mechanisms to evaluate radical-neutralizing potential.

In this study, Pearson's correlation analysis revealed moderate to high and significant correlations between total phenolic content (TPC) and all three antioxidant assays. Previous reports proved that numerous plant species have shown a positive correlation between TPC and antioxidant activity. For example, in *Hibiscus cannabinus* shoots (Birhanie et al., 2022) and *Carthamus tinctorius* callus, the TPC showed a strong correlation with DPPH activity after treated with salt stress (Golkar and Taghizadeh, 2018). However, this study found a weak or negative correlation between EsA content, antioxidant activity and TPC. These results are in line with earlier findings in *Juglans regia* leaves, which revealed a significant inverse relationship between total saponins and DPPH activity (Elouafy et al., 2023). Overall, TPC was largely responsible for the elevated antioxidant activity in elicited pokeweed cultures treated with different elicitors. While the accumulation of EsA only marginally influenced the observed antioxidant effects.

Conclusions

This study applied NaCl, methyl jasmonate (MeJA), and salicylic acid (SA) in pokeweed callus elicitation for various durations. These elicitors did not cause positive effects on callus biomass but significant effects on phytochemical parameters were noted. TPC significantly improved in the treatments of NaCl (50 mM for 7 days), MeJA (100 µM for 3 days), and SA (150 µM for 9 days) along with enhanced activities of antioxidation assays compared to non-elicited calli and natural roots. Higher concentrations and prolonged elicitation with NaCl and MeJA caused negative effects on TPC and antioxidant activities. The analytical procedure for EsA quantification using HPLC was validated before the evaluation of EsA content in elicited callus cultures. Elicitation with NaCl, especially for 21 days, significantly improved EsA contents compared to the other treatments. MeJA and SA caused a progressive decrease in EsA contents in calli. The application of these elicitors on TPC was associated with the upregulation of genes in phenylpropanoid pathways. The biosynthetic pathway of EsA remains unclear but evidence suggests that NaCl induces ROS bursts that act as secondary messengers to upregulate the genes related to MVA and MEP pathways. Therefore, strategic elicitation with NaCl, MeJA, and SA could be an effective approach to enhance phenolic and EsA production in pokeweed callus cultures, providing a promising platform for large-scale, sustainable production of this valuable metabolite without depending on natural plant resources. Although this study successfully optimized the callus culture conditions for enhanced secondary metabolite production, it is limited by the lack of advanced metabolite profiling due to resource and time constraints. Future research should consider applying comprehensive analytical techniques, such as LC–MS/MS-based metabolomic profiling, to further elucidate the qualitative and quantitative diversity of metabolites under optimized conditions. Such an approach would deepen our understanding of the biochemical mechanisms underlying stress and hormones induced metabolic responses in pokeweed callus cultures.

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Trunjaruen A: Conceptualization, methodology, investigation, data curation, formal analysis, writing – original draft, writing – review and editing.
Kongnok T: Methodology, investigation, data curation, writing – original draft.
Yaowachai W, Panomai P, Maneerattanarungroj P, Tungpairojwong N & Monthatong M: Investigation, data curation and validation.
Taratima W: Conceptualization, funding acquisition, supervision, validation, writing – original draft, writing – review and editing.

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