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Effects of Salicylic Acid Concentration and Elicitation Duration on Total Flavonoid Content in Binahong (*Anredera cordifolia* (Ten.) Steenis) Callus Cultures

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Abstract: This study aimed to determine the optimal elicitor concentration and elicitation duration for increasing the total flavonoid content of binahong (*Anredera cordifolia*) callus cultures. A two-factor completely randomized design (CRD) was employed, with salicylic acid concentration and elicitation duration as the experimental factors. Nine treatment combinations were tested with six replicates each, consisting of salicylic acid concentrations of 0, 20, and 40 ppm applied for 7, 14, and 21 days. The observed parameters included callus morphology (color and texture), callus biomass, and total flavonoid content determined using UV–Vis spectrophotometry. The results showed that the callus was predominantly compact in texture and green in color. Callus fresh weight differed significantly among treatments ($p = 0.008$), with the highest value recorded in treatment E2W3 (14 days, 40 ppm), at 0.9367 g. In contrast, callus dry weight did not differ significantly among treatments ($p = 0.170$). Two-way ANOVA further showed that elicitation duration ($p = 0.374$), salicylic acid concentration ($p = 0.691$), and their interaction ($p = 0.330$) had no significant effect ($p > 0.05$) on the total flavonoid content of binahong (*Anredera cordifolia*) callus cultures. Overall, the combination of salicylic acid concentration and elicitation duration significantly affected callus fresh weight but did not significantly influence callus dry weight or total flavonoid content.
Keywords: Elicitor; flavonoid; salicylic acid; callus culture; binahong (*Anredera cordifolia*)

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INTRODUCTION

Indonesia possesses exceptionally rich plant diversity, including numerous species traditionally used for medicinal purposes. More than 1,000 plant species have been reported to have medicinal properties in Indonesia, approximately 300 of which have been utilized for therapeutic applications. Among these medicinal plants, *Anredera cordifolia* (Ten.) Steenis, commonly known as binahong, is a member of the family Basellaceae and occurs widely in tropical and subtropical regions (Alba et al., 2020). Binahong has attracted considerable attention because it contains various bioactive secondary metabolites associated with antioxidant, antidiabetic, antibacterial, and wound-healing activities that have been investigated both *in vivo* and *in vitro* (Fitria et al., 2025). The major secondary metabolites reported in *A. cordifolia* include flavonoids, steroids, phenolic compounds, and saponins.

Flavonoids are particularly important because of their strong antioxidant properties, including their ability to scavenge reactive free radicals and modulate cellular enzyme activity. These biological properties have supported their extensive application in nutraceutical, pharmaceutical, medical, and cosmetic products (Karak, 2019). More broadly, plant secondary metabolites represent an important source of high-value bioactive compounds for pharmaceutical and other industrial applications (Pramanik et al., 2024). However, their natural abundance in plants is often low, and

conventional production through direct extraction from field-grown plants presents several limitations. These include low metabolite concentrations, compound losses during extraction, long cultivation cycles, seasonal and environmental variation, limited cultivation areas, and differences among plant species or genotypes (Tariq et al., 2023). In addition, extensive utilization of flavonoid-rich plants that are also used as food sources may create competition between medicinal and food-related demands.

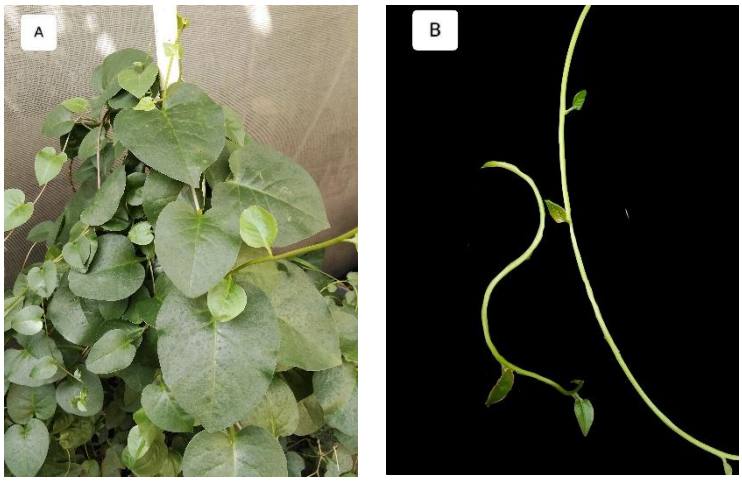

Figure 1. A. Binahong plant (*Anredera cordifolia*); B. Young stem of *A. cordifolia*

Secondary metabolites are specialized organic compounds that are not directly involved in primary plant growth and development but play essential roles in ecological adaptation and defense (Angin et al., 2019). They are synthesized in various plant organs, including roots, leaves, flowers, fruits, and seeds, and contribute to defense against herbivores and pathogens, attraction of pollinators and seed-dispersing organisms, and protection against environmental stresses such as ultraviolet radiation. Nevertheless, the accumulation of these compounds is strongly influenced by plant developmental stage, physiological status, genotype, and environmental conditions (Selwal et al., 2025). Consequently, the yield and composition of metabolites obtained from conventionally cultivated medicinal plants may vary substantially.

Field-based production of medicinal plants is also constrained by susceptibility to pathogens, dependence on agricultural inputs, heavy-metal contamination, and environmental instability. These factors may affect both the quantity and quality of secondary metabolites intended for medicinal applications (Wang et al., 2022). Advances in plant biotechnology have therefore encouraged the development of alternative strategies for producing valuable phytochemicals, including the selection of superior genotypes, plant cell and organ culture, metabolic engineering, and genetic approaches (Anggraito et al., 2018). Recent studies have further emphasized plant tissue culture as a promising platform for the controlled and reproducible production of high-value secondary metabolites under defined environmental conditions (Selwal et al., 2025; Paek & Murthy, 2024).

Among these approaches, *in vitro* culture provides a useful system for enhancing secondary metabolite production because environmental and nutritional conditions can be precisely controlled (Kadapi et al., 2024). Callus culture is one of the most widely used tissue culture techniques for this purpose and can provide a continuous source of undifferentiated plant cells capable of synthesizing bioactive compounds. Under optimized conditions, callus cultures may accumulate secondary metabolites at levels comparable to or greater than those found in intact plants (Kousalya et al., 2025). Furthermore, plant cell cultures can reduce dependence on seasonal cultivation and

enable manipulation of culture conditions to regulate metabolic pathways (Raza et al., 2022; Pramanik et al., 2024).

Secondary metabolite accumulation in *in vitro* cultures can be further enhanced through elicitation. Elicitors are biotic or abiotic signals that activate plant defense-related signaling pathways and stimulate the biosynthesis of specialized metabolites (Rosniawaty et al., 2024). Elicitation has become an important strategy in plant biotechnology because it can modulate metabolic pathways without requiring permanent genetic modification. Recent evidence indicates that elicitor responses are highly dependent on elicitor type, concentration, exposure duration, plant species, tissue type, and developmental stage; therefore, optimization is essential to obtain the desired metabolic response (Selwal et al., 2025; Rafiq et al., 2023). Elicitors may also affect plant growth and stress responses, indicating that enhancement of secondary metabolism may occur together with physiological trade-offs.

Salicylic acid (SA) is one of the most commonly investigated chemical elicitors because it plays an important role in plant defense signaling and stress responses. Exogenous SA can activate defense-associated metabolic pathways and promote the biosynthesis and accumulation of phenolics, flavonoids, and other specialized metabolites in both intact plants and *in vitro* cultures (Ali, 2021). Because of its relatively low cost, ease of application, and effectiveness at appropriate concentrations, SA has been widely explored as an elicitor for improving secondary metabolite production. However, its effects are not universally positive; the response depends strongly on concentration and exposure time, and excessive elicitation may impose physiological stress that limits metabolite accumulation. Thus, determining an appropriate combination of SA concentration and elicitation duration is critical for optimizing its application (Ali, 2021; Selwal et al., 2025).

The potential of SA to enhance secondary metabolite production has been demonstrated in several *in vitro* systems. Linardi et al. (2022) reported that SA increased flavonoid accumulation in Javanese ginseng (*Talinum paniculatum*) callus cultures, with the highest response obtained at 20 ppm SA after 2 days of elicitation. Similarly, Gorni et al. (2017) showed that exogenous application of 0.25 mM SA improved biomass-related responses and enhanced the accumulation of secondary metabolites, including flavonoids and phenolic compounds, in *Foeniculum vulgare*. Collectively, these findings indicate that SA can stimulate secondary metabolism, although the magnitude and direction of the response vary according to the elicitation conditions.

Despite the medicinal importance of *A. cordifolia* and the established role of SA in stimulating secondary metabolite biosynthesis, information on the use of SA elicitation in *A. cordifolia* callus cultures remains limited. In particular, the combined effects of SA concentration and elicitation duration on total flavonoid accumulation have not been adequately characterized. Therefore, this study aimed to evaluate the effects of different combinations of SA concentrations and elicitation durations on the total flavonoid content of *A. cordifolia* callus cultures. The results are expected to provide a scientific basis for optimizing elicitation strategies for the production of flavonoid-related secondary metabolites in binahong callus cultures.

METHOD

The study was conducted at the Biology Laboratory, Faculty of Mathematics and Natural Sciences, Universitas Negeri Semarang, from October 2025 to January 2026. This experimental study employed a two-factor completely randomized design (CRD), with salicylic acid concentration and elicitation duration as the experimental factors.

Salicylic acid was applied at three concentrations (0, 20, and 40 ppm), while elicitation was conducted for three durations (7, 14, and 21 days). Each treatment combination was replicated six times, resulting in 54 experimental units. Each experimental unit consisted of one culture bottle containing approximately 0.5 g of binahong (*Anredera cordifolia*) callus.

Equipment and Materials

The equipment used in this study included an autoclave, oven, culture bottles, laminar airflow cabinet (LAF), analytical balance, scalpel, pH indicator, Erlenmeyer flasks, beakers, Petri dishes, stirring rods, micropipettes, micropipette tips, Bunsen burner, hot plate, aluminum foil, filter paper, forceps, mortar and pestle, and a UV–Vis spectrophotometer.

The plant material consisted of stem explants from two-month-old binahong (*Anredera cordifolia*) plants (Figure 1B). Fresh explants were collected from the youngest portion of the stem, extending from the shoot tip to the third stem segment. The plant material was obtained from the greenhouse of the Biology Laboratory, Faculty of Mathematics and Natural Sciences, Universitas Negeri Semarang.

The culture medium consisted of Murashige and Skoog (MS) basal medium supplemented with 1.5 ppm picloram and 0.5 ppm kinetin as plant growth regulators, together with glucose, agar, and myo-inositol. Additional materials used for explant sterilization included powdered detergent, fungicide, bactericide, 10% Bayclin solution, and 70% and 96% ethanol. Materials used for callus extraction and total flavonoid analysis included methanol, HCl, 10% aluminum chloride ($AlCl_3$), 5% $NaNO_2$, 4% NaOH, and quercetin as the reference standard.

Preparation of Callus-Induction and Elicitation Media

Media were prepared for both callus induction and salicylic acid elicitation treatments. To prepare 1 L of callus-induction medium, 4.43 g of MS medium, 30 g of sucrose, and 0.1 g of myo-inositol were placed in an Erlenmeyer flask, dissolved initially in 500 mL of sterile distilled water, and mixed until homogeneous. The pH of the medium was adjusted to 5–6 using a pH indicator. A pH above the desired range was adjusted with 0.1 N HCl, whereas a pH below the desired range was adjusted with 0.1 N NaOH.

The medium was subsequently supplemented with 1.5 ppm picloram and 0.5 ppm kinetin, followed by the addition of 7 g of agar. The mixture was heated for 20 min while continuously stirred until all components were dissolved and the medium was homogeneous. The medium was then dispensed into sterile culture bottles, tightly sealed, and autoclaved at 121°C for 20 min. After sterilization, the medium was incubated for at least 3 days before explant inoculation.

The elicitation medium was prepared using the same formulation and procedure as the callus-induction medium, with the addition of salicylic acid at the predetermined concentrations of 0, 20, or 40 ppm.

Explant Sterilization

Explant sterilization was performed to eliminate microorganisms present on the explant surfaces (Solin & Siregar, 2025). Binahong stem explants were first washed under running water for 30 min. The explants were then immersed for 10 min in a solution containing 0.2 g of powdered detergent dissolved in 100 mL of distilled water. Subsequently, the explants were rinsed three times with sterile distilled water for 3 min per rinse.

The explants were then immersed for 30 min in 200 mL of a bactericide–fungicide solution containing 0.5 g of each agent, followed by rinsing with sterile distilled water. Surface sterilization was continued inside the laminar airflow cabinet by immersing the explants in 10% Bayclin solution for 10 min. The sodium hypochlorite contained in Bayclin acts as a disinfectant that helps prevent microbial contamination by eliminating bacteria and other contaminating microorganisms (Widiastiti et al., 2023). After treatment, the explants were rinsed three times with sterile distilled water for 3 min per rinse.

Explant Inoculation

All instruments, materials, and the laminar airflow cabinet were sterilized before inoculation. Explant inoculation was carried out aseptically inside the LAF cabinet. Sterilized stem explants were transferred using sterile forceps onto sterile Petri dishes. The internodal portions of the stems were excised with a sterile scalpel into approximately 1-cm-long segments.

Three stem explants were inoculated into each culture bottle containing callus-induction medium. The cultures were then incubated for 58 days at approximately 20–25°C, with relative humidity maintained at 52–58% and a 16-h light/8-h dark photoperiod under LED illumination at an intensity of 2,000 lux.

Elicitation and Determination of Callus Biomass

Fifty-eight-day-old calli were used for the elicitation treatments. The calli were subcultured onto treatment media containing salicylic acid at concentrations of 0, 20, or 40 ppm. Elicitation periods were 7, 14, or 21 days after subculture. Approximately 0.5 g of callus was transferred to each salicylic acid-containing treatment medium and incubated according to the designated elicitation duration.

During elicitation, cultures were maintained at approximately 20–25°C and 52–58% relative humidity under a 16-h light/8-h dark photoperiod with LED illumination at an intensity of 2,000 lux. Culture bottles were randomly positioned during incubation to minimize the effects of spatial variation in environmental conditions. Contaminated cultures were replaced with new cultures.

At the end of each elicitation period, calli were harvested and weighed to determine fresh weight. For dry-weight determination, harvested calli were dried in an oven at 60°C until a constant weight was achieved. Fresh and dry weights were measured using an analytical balance and used as indicators of *A. cordifolia* callus biomass.

Callus Extraction

Dried calli were extracted using methanol as the solvent. The dried material was ground using a mortar and pestle and subsequently macerated with 5 mL of methanolic HCl and 5 mL of 2 N HCl. The extracts were incubated in an oven at 37°C for 24 h.

After incubation, the extracts were filtered through filter paper and transferred into vials. The filtrates were evaporated to dryness in an oven at 60°C with the vial caps left open. The dried extracts were subsequently reconstituted in 2–5 mL of methanol.

Determination of Total Flavonoid Content

Total flavonoid content was determined using a UV–Vis spectrophotometer at a wavelength of 510 nm. The analysis was performed using the $AlCl_3$ colorimetric method, with quercetin used as the flavonoid reference standard. Measurements were conducted in triplicate.

A quercetin calibration curve was prepared using standard solutions at concentrations of 2, 4, 6, 8, and 10 ppm. The absorbance obtained from each sample was substituted as the *y* value in the calibration equation, and the corresponding *x* value was used to determine the flavonoid concentration in the working sample solution. Total flavonoid content was calculated using the linear regression equation derived from the quercetin calibration curve:

$$y = bx + a$$

Data Analysis

Callus morphology, including texture and color, was observed directly from day 0 until the end of each treatment period (7, 14, or 21 days). Callus color was further characterized using the Munsell Color Chart to determine the corresponding color code.

Fresh- and dry-weight data were statistically analyzed using IBM SPSS Statistics version 26, with statistical significance set at $p < 0.05$. Before comparative analyses, data normality was assessed using the Kolmogorov–Smirnov test, while homogeneity of variance was evaluated using Levene's test. Data that met the assumptions of normality and homogeneity were analyzed using two-way analysis of variance (ANOVA). When the data did not meet either assumption, the Kruskal–Wallis test was applied, followed by Dunn's post hoc test when significant differences were detected.

According to Dalming et al. (2022), the total flavonoid content of *A. cordifolia* callus was calculated using the following equation:

$$\boldsymbol{F = \frac{c\,.V.f}{m} \times 100\%}$$

Note:

F = total flavonoid content
c = sample concentration
V = sample volume
f = dilution factor
m = sample weight

RESULTS AND DISCUSSION

Callus Morphology

Callus texture and color are important morphological indicators of callus quality. The morphological characteristics of *Anredera cordifolia* calli subjected to different salicylic acid concentrations and elicitation durations are presented in Table 1 and Figures 2 and 3. Most elicited *A. cordifolia* calli exhibited a compact texture, except for treatment E1W2, which produced friable callus. Friable callus is characterized by loosely associated cells that can readily separate into individual cells, large intercellular spaces, and a relatively high water content (Setiawati et al., 2021). Consistent with Silvina et al. (2021), these characteristics, together with good oxygen aeration, make friable callus particularly suitable for cell suspension culture. In contrast, compact callus is characterized by nodular protrusions, small and densely arranged cells that cannot easily be separated into individual cells, and a firm, dense texture (Julianti et al., 2021). Compact callus may accumulate higher levels of secondary metabolites than friable callus because the latter generally contains more water, which may result in a lower concentration of secondary metabolites (Arieswari et al., 2018). Thus, compact callus may be more suitable for secondary metabolite production.

Callus color can also provide information on the physiological condition of callus cells, particularly whether the cells remain actively dividing or have entered a less active developmental stage (Afiyah et al., 2022). In the present study, elicited calli exhibited colors ranging from green to brownish white. Callus color was evaluated visually (Figures 2 and 3), and the corresponding color codes were determined using the Munsell Color Chart (Table 1). Changes in callus color may reflect cellular growth and developmental changes and can therefore serve as an indicator of successful regeneration (Rismayanti & Nafi'ah, 2021). Yellowish-green callus is generally associated with active cell division and the presence of chlorophyll, whereas white callus may indicate that the plastids have not yet differentiated and that the tissue remains in a meristematic or embryonic state (Prashariska et al., 2021; Setiawati et al., 2021).

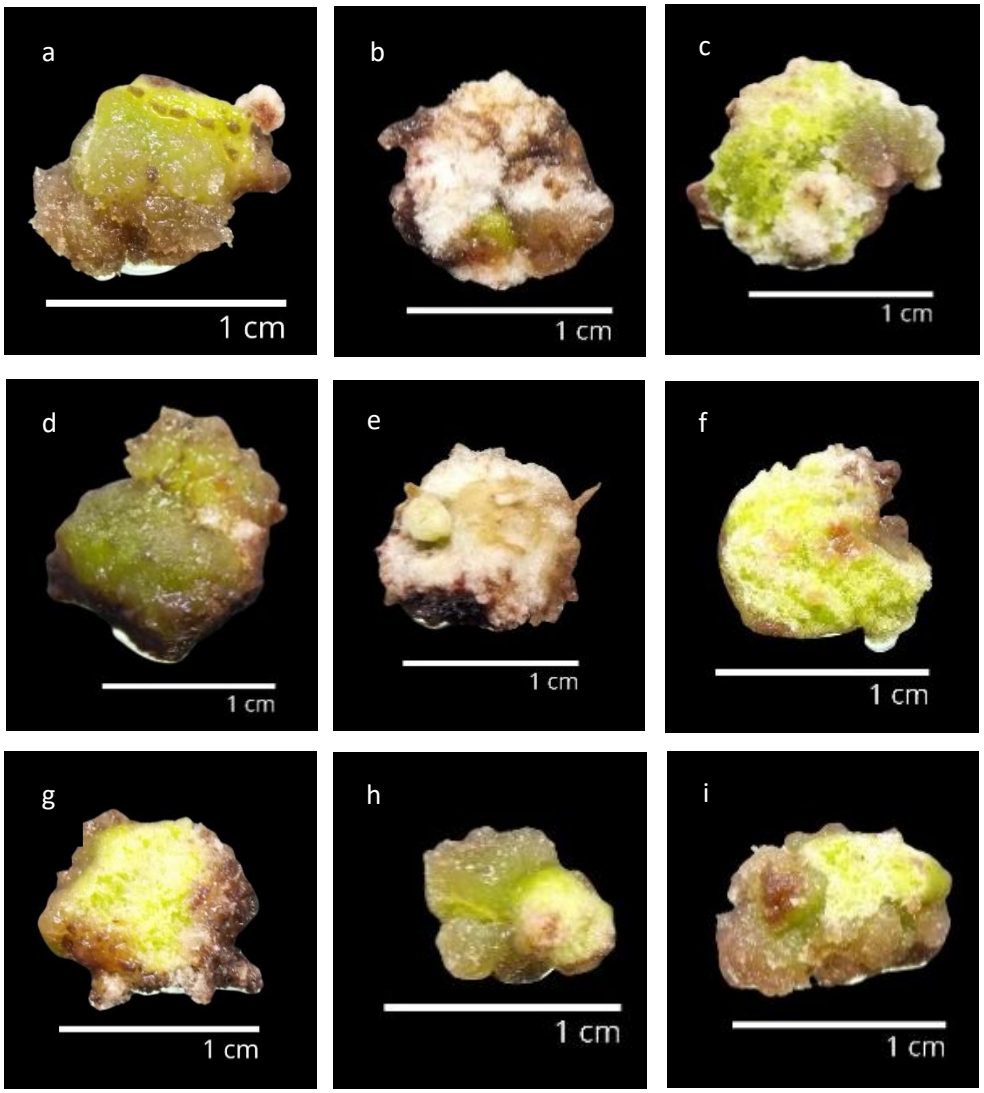

Figure 2. Morphology of *A. cordifolia* callus after elicitation: (a) E1W1, (b) E1W2, (c) E1W3, (d) E2W1, (e) E2W2, (f) E2W3, (g) E3W1, (h) E3W2, and (i) E3W3

Figure 3. Morphology of control *A. cordifolia* callus

According to Oktafiana et al. (2022), white callus may indicate embryogenic potential and the capacity to proliferate and develop into somatic embryos through somatic embryogenesis. Yellowish-white callus may indicate that the tissue is approaching the end of the active cell-division phase, whereas yellow coloration may be associated with flavonoid pigments in callus parenchyma cells (Ekawati et al., 2022). In the present study, brown coloration was observed in several peripheral regions of the callus (Figures 2 and 3), suggesting the onset of tissue senescence. Browning at the callus margins may occur because peripheral cells respond more rapidly to environmental conditions owing to their direct contact with the culture medium (Dewi et al., 2023). Such color changes may reflect a transition from young, actively dividing cells to more mature cells (Girsang et al., 2023).

Table 1. Morphology, texture, and color of *Anredera cordifolia* callus

Treatment	Texture	Callus Color	Munsell Color Chart Code
E0W0	Compact		Green-2.5GY 7/10
E1W1	Compact		Green-2.5GY 8/10
E1W2	Friable		Brownish white-7.5YR 9/2
E1W3	Compact		Green-10Y 8/8
E2W1	Compact		Brownish green-5Y 6/4
E2W2	Compact		Brownish white-10YR 9/2
E2W3	Compact		Green-2.5GY 9/8
E3W1	Compact		Yellowish green-10Y 9/6
E3W2	Compact		Green-10Y 7/8
E3W3	Compact		Green-2.5GY 9/10

Note: E0W0 = control; E1W1 = 7-day elicitation, 0 ppm; E1W2 = 7-day elicitation, 20 ppm; E1W3 = 7-day elicitation, 40 ppm; E2W1 = 14-day elicitation, 0 ppm; E2W2 = 14-day elicitation, 20 ppm; E2W3 = 14-day elicitation, 40 ppm; E3W1 = 21-day elicitation, 0 ppm; E3W2 = 21-day elicitation, 20 ppm; and E3W3 = 21-day elicitation, 40 ppm.

Callus Biomass

Callus biomass was evaluated based on the fresh and dry weights of elicited calli. Fresh and dried calli were weighed using an analytical balance, while dry weight was determined after oven-drying at 60°C. The fresh- and dry-weight data are presented in Table 2 and were statistically analyzed using IBM SPSS Statistics 26.

The fresh-weight data were normally distributed and homogeneous. Statistical analysis showed a significant difference among treatments (p = 0.008 < 0.05). Post hoc analysis indicated that calli elicited for 14 days with 40 ppm salicylic acid (E2W3)

had the highest fresh weight (0.9367 g) and differed significantly from some of the other treatments. In contrast, the lowest fresh weight (0.5817 g) was recorded for calli maintained for 7 days at 0 ppm salicylic acid (E1W1).

Because the dry-weight data did not meet the homogeneity assumption, they were analyzed using the Kruskal-Wallis test. No significant difference in callus dry weight was detected among treatments ($p = 0.170 > 0.05$). Nevertheless, the highest mean dry weight was observed in calli elicited for 14 days with 20 ppm salicylic acid (E2W2; 0.1133 g), whereas the lowest value was observed after 7 days of elicitation with 20 ppm salicylic acid (E1W2; 0.0833 g). Although the differences were not statistically significant, mean dry weights after 14 days of elicitation tended to be higher than that of the control, whereas those after 7 and 21 days of elicitation generally tended to be lower.

Overall, elicitation significantly affected callus fresh weight but did not significantly influence dry weight. A reduction in callus biomass under elicitation may be associated with the allocation of cellular resources toward stress-defense responses. Under stressful conditions, plants may suppress growth while increasing the synthesis of secondary metabolites as part of their defense strategy (Linardi et al., 2022). Elicitor application can redirect cellular metabolism from primary growth-related processes toward secondary metabolism, thereby promoting defense-related metabolite production at the expense of biomass accumulation (Restiani et al., 2022).

Table 2. Mean biomass of *A. cordifolia* callus

Elicitation Duration (days)	Salicylic Acid Concentration (ppm)	Fresh Weight (g)	Dry Weight (g)
0 (E0)	0 (W0)	0.7583 ± 0.16^{ab}	0.0983 ± 0.02
7 (E1)	0 (W1)	0.5817 ± 0.04^{b}	0.0867 ± 0.03
	20 (W2)	0.7400 ± 0.12^{ab}	0.0833 ± 0.03
	40 (W3)	0.8050 ± 0.19^{ab}	0.0900 ± 0.02
14 (E2)	0 (W1)	0.6683 ± 0.09^{ab}	0.0950 ± 0.03
	20 (W2)	0.6950 ± 0.08^{ab}	0.1133 ± 0.08
	40 (W3)	0.9367 ± 0.28^{a}	0.1117 ± 0.03
21 (E3)	0 (W1)	0.6300 ± 0.04^{b}	0.0983 ± 0.19
	20 (W2)	0.7483 ± 0.09^{ab}	0.0917 ± 0.15
	40 (W3)	0.7347 ± 0.16^{ab}	0.0867 ± 0.02
Mean		**0.7347 ± 0.16^{ab}**	**0.0955 ± 0.03**

Note: Values sharing the same superscript letter are not significantly different among treatments.

Determination of Total Flavonoid Content

Total flavonoid content in *A. cordifolia* callus was determined after extracting the dried callus by maceration. Extraction is performed to transfer target chemical constituents from the sample matrix into a suitable solvent. The process is based on mass transfer of dissolved compounds across the sample-solvent interface. Methanol was selected as the extraction solvent because of its ability to dissolve a broad range of polar and relatively non-polar compounds, making it suitable for extracting secondary metabolites from plant material (Dalming, 2022).

A quercetin calibration curve was prepared using standard concentrations of 2, 4, 6, 8, and 10 ppm, with absorbance measured in triplicate for each concentration. The calibration curve was used to establish the relationship between quercetin concentration and absorbance, thereby enabling the concentration of flavonoids in the samples to be estimated. Table 3 presents the absorbance values obtained by UV-Vis

spectrophotometry at 510 nm. Absorbance increased with increasing quercetin concentration, indicating a positive relationship between concentration and analytical response.

Table 3. Absorbance of quercetin standard solutions

No.	Concentration (ppm)	Absorbance
1	2	0.020
2	4	0.021
3	6	0.044
4	8	0.064
5	10	0.079

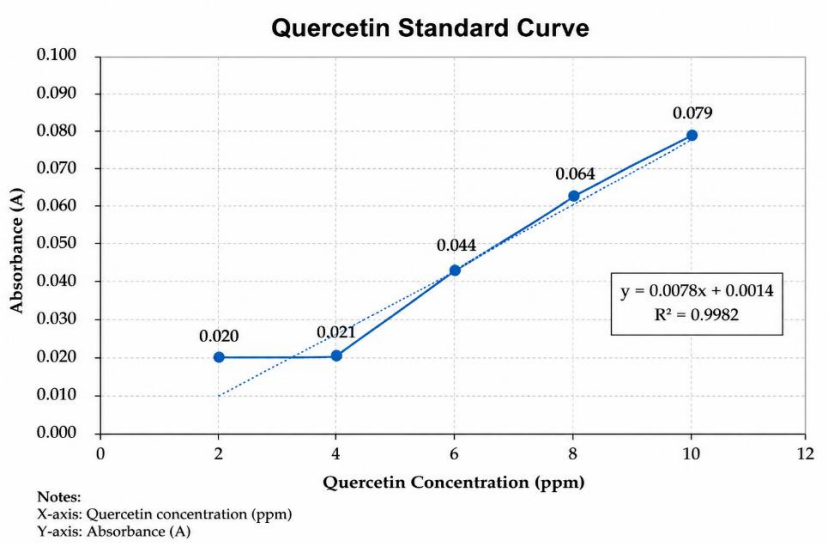

Figure 4. Quercetin standard calibration curve

The calibration curve (Figure 4) generated the linear regression equation $y = 0.0078x + 0.0014$, with an R^2 value of 0.9982. This high coefficient of determination indicates strong linearity between quercetin concentration and absorbance over the tested concentration range. According to Nita (2018), as cited in Muna et al. (2023), compliance with the Beer-Lambert relationship is indicated by a correlation coefficient (r) approaching 1, demonstrating a strong linear relationship between concentration (x) and analytical response (y).

Table 4. Total flavonoid content of *A. cordifolia* callus

Treatment	Mean Absorbance	Sample Concentration	Total Flavonoid Content (%) (Mean ± SD)
Blank	0.000	–	0.000
Control	0.061	7.705	3.89 ± 0.021
E1W1	0.098	12.363	0.71 ± 0.093
E1W2	0.143	18.126	10.89 ± 0.101
E1W3	0.172	21.416	12.15 ± 0.069
E2W1	0.135	17.100	9.02 ± 0.089
E2W2	0.121	15.277	6.77 ± 0.076
E2W3	0.119	15.020	6.75 ± 0.088
E3W1	0.090	11.359	5.78 ± 0.050

Treatment	Mean Absorbance	Sample Concentration	Total Flavonoid Content (%) (Mean ± SD)
E3W2	0.153	19.500	10.60 ± 0.099
E3W3	0.104	13.083	7.58 ± 0.073

Note: E0W0 = control; E1W1 = 7-day elicitation, 0 ppm; E1W2 = 7-day elicitation, 20 ppm; E1W3 = 7-day elicitation, 40 ppm; E2W1 = 14-day elicitation, 0 ppm; E2W2 = 14-day elicitation, 20 ppm; E2W3 = 14-day elicitation, 40 ppm; E3W1 = 21-day elicitation, 0 ppm; E3W2 = 21-day elicitation, 20 ppm; and E3W3 = 21-day elicitation, 40 ppm.

The total flavonoid content of the callus samples is presented in Table 4. Statistical analysis showed that the data were normally distributed ($p = 0.140 > 0.05$) and homogeneous ($p = 0.343 > 0.05$); therefore, a two-way ANOVA was performed. Elicitation duration had no significant effect on total flavonoid content ($p = 0.374$), and salicylic acid concentration likewise showed no significant effect ($p = 0.691$). Moreover, no significant interaction between elicitation duration and salicylic acid concentration was detected ($p = 0.330$). Thus, within the concentration and duration ranges evaluated in this study, neither factor nor their interaction significantly affected total flavonoid accumulation in *A. cordifolia* callus.

The absence of a statistically significant elicitation effect may be associated with the concentration of the elicitor and the duration of exposure, which could have induced excessive oxidative stress in the callus. Moreno-Escamilla et al. (2020) reported that elicitation does not invariably produce beneficial effects because high elicitor concentrations may promote excessive accumulation of reactive oxygen species (ROS), potentially resulting in cellular damage and reduced phytochemical accumulation. Salicylic acid application can induce ROS production as part of the plant stress-response signaling network. ROS comprise reactive molecules, free radicals, and ions derived from molecular oxygen and are generated in several subcellular compartments, including mitochondria, chloroplasts, and peroxisomes.

The physiological effects of ROS depend strongly on their intracellular concentration. At low to moderate levels, ROS function as secondary messengers in intracellular signaling cascades and contribute to stress-response regulation. At excessive concentrations, however, ROS can damage cellular biomolecules and disrupt normal physiological processes. Therefore, although ROS levels were not directly measured in the present study, excessive ROS accumulation may be one possible explanation for the lack of a significant salicylic acid-induced increase in total flavonoid content. When ROS production exceeds the capacity of cellular antioxidant systems to maintain ROS below a tolerable threshold, oxidative damage may occur (Singh et al., 2023). Excessive ROS formation can disturb cellular redox homeostasis and impair normal cellular functions, indicating that ROS must be maintained within an appropriate physiological range to support normal plant metabolism (Hajam et al., 2023).

Despite the absence of statistically significant effects, descriptive differences in mean total flavonoid content were observed among treatments. Several salicylic acid treatments produced higher mean flavonoid levels than the control. The highest mean total flavonoid content was recorded in treatment E1W3, corresponding to 7 days of elicitation with 40 ppm salicylic acid, at 12.15%. However, because the two-way ANOVA detected no significant effects of elicitation duration, salicylic acid concentration, or their interaction, this treatment should be interpreted as having the highest numerical mean rather than as a statistically superior or optimal treatment.

Comparable responses to salicylic acid elicitation have been reported in other plant species. Linardi et al. (2022) found that variation in salicylic acid concentration

and elicitation duration influenced flavonoid accumulation in *Talinum paniculatum* callus, with the highest response observed at 20 ppm salicylic acid after 2 days of elicitation. Sharifi and Nematzadeh (2019) also reported increased flavonoid content in *Ruta graveolens* callus following elicitation with 1 mg/mL salicylic acid compared with the control. Similarly, Khyahrii and Shetty (2025) reported a 1.34-fold increase in flavonoid content following treatment with 100 µM salicylic acid. More generally, elicitor concentration and exposure duration are important factors determining secondary metabolite production in plant cell and callus cultures (Ramirez-Estrada & Vidal-Limon, 2016). These findings suggest that elicitation responses are highly dependent on plant species, tissue type, elicitor concentration, and treatment duration.

Elicitors function as stress signals that activate plant defense mechanisms, including the synthesis of antimicrobial compounds and other secondary metabolites. Recognition of elicitor molecules can initiate signal-transduction pathways and regulate the expression of genes involved in secondary metabolite biosynthesis (Husain et al., 2023). When an elicitor interacts with cellular receptors, downstream signaling pathways are activated, leading to defense-related physiological and biochemical responses (Gowthami, 2018). Accordingly, salicylic acid may stimulate defense-related metabolic pathways in callus tissue; however, the magnitude and direction of the response depend on elicitor concentration, duration of exposure, and the physiological condition of the cultured cells.

CONCLUSION

Salicylic acid elicitation affected the growth characteristics of *Anredera cordifolia* callus cultures, as reflected in callus morphology and fresh biomass. Most elicited calli exhibited a compact texture and predominantly green coloration. Callus fresh weight differed significantly among treatments ($p = 0.008$), with the highest mean fresh weight (0.9367 g) observed in the E2W3 treatment (14 days of elicitation with 40 ppm salicylic acid). In contrast, callus dry weight did not differ significantly among treatments ($p = 0.170$).

Two-way ANOVA demonstrated that elicitation duration ($p = 0.374$), salicylic acid concentration ($p = 0.691$), and their interaction ($p = 0.330$) had no significant effects on the total flavonoid content of *A. cordifolia* callus cultures ($p > 0.05$). Although the E1W3 treatment (7 days of elicitation with 40 ppm salicylic acid) produced the highest mean total flavonoid content (12.15%), this increase was not statistically significant. Therefore, under the experimental conditions used in this study, salicylic acid elicitation significantly influenced callus fresh biomass but did not significantly affect callus dry biomass or total flavonoid accumulation.

RECOMMENDATION

Future studies should investigate a broader range of salicylic acid concentrations and shorter elicitation periods to better characterize the response of *A. cordifolia* callus cultures to salicylic acid elicitation. Increasing the number of replicates is also recommended to improve statistical power and the reliability of treatment comparisons. Furthermore, flavonoid profiles should be analyzed using high-performance liquid chromatography (HPLC), which provides greater analytical specificity and resolution than UV–Vis spectrophotometry. Further research should also evaluate other explant types, such as leaves, and investigate secondary metabolites other than flavonoids to provide a more comprehensive understanding of elicitor-induced secondary metabolite production in *A. cordifolia*.

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