

# Effect of salicylic acid, jasmonic acid, and a combination of both on andrographolide production in cell suspension cultures of *Andrographis paniculata* (Burm.f.) Nees

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## ARTICLE INFO

### Article history:

Received on: April 19, 2022

Accepted on: September 15, 2022

Available online: January 22, 2023

### Key words:

*Andrographis paniculata*,  
Andrographolide,  
Abiotic elicitors,  
Salicylic acid,  
Jasmonic acid,  
Cell suspension cultures.

## ABSTRACT

Elicitors act as signaling compounds that can induce and enhance the production of metabolites by activating biochemical pathways in response to external stress. Salicylic acid (SA) acts as a signaling molecule in plants in response to an attack by biotrophic pathogens whereas jasmonic acid (JA) is released in plants in response to wounds and herbivory. Both the signaling molecules aid in plant chemical defenses by increasing the production of secondary metabolites. The present study investigates the effect of SA, JA, and the combination of both (SA + JA) on the andrographolide content in cell suspension cultures of *Andrographis paniculata*. Four different concentrations (25, 50, 75, and 100  $\mu\text{M}$ ) of SA and JA individually, and an equimolar combination of both SA and JA together, were administered to cell suspension cultures taken in triplicates at shake flask scale. Andrographolide content was estimated using high performance liquid chromatography. Both SA and JA showed a positive effect on andrographolide content with the increase in their concentrations. SA at its highest concentration resulted in just 0.18-fold increase ( $83.33 \pm 6.7 \mu\text{g/g DCW}$ ) in andrographolide content compared to control, whereas JA resulted in a 3-fold increase ( $211 \pm 5.8 \mu\text{g/g DW}$ ); the combination of both SA and JA had an intermediate effect at all concentrations except one concentration (75 + 75  $\mu\text{M}$ ) which resulted in a 3.8-fold increase ( $280 \pm 2.7 \mu\text{g/g DW}$ ), in andrographolide content.

## 1. INTRODUCTION

*Andrographis paniculata* has a wide array of phytochemicals of pharmacological effects. In the past decade, many techniques have been proposed to extract the phytochemicals of medicinal importance [1,2]. The important phytochemicals of this plant have found to be labdane diterpenes, flavonoids, derivatives of quinic acid, xanthenes (only in roots) [3,4], and some rare noriridoids [5]. The herb reportedly shows anti-inflammatory, antioxidant, antidiabetic, antileishmanial, anti-diarrheal, antifertility, antivenom, anti-HIV, antimalarial, antibacterial, antifungal, anti-filaricidal, antiparasitic, vasorelaxant, hepatoprotective, antihyperglycemic, immunomodulatory, and anticancer activities [6]. It is effective against common cold and fever and possesses mosquito larvicidal activities [7]. Andrographolide is the key secondary metabolite of the plant that is of pharmacological significance. It is reported to have anti-inflammatory effects against lipopolysaccharide-induced neurotoxicity [8]. It caused apoptosis of lymphoma cell lines [9] and is anti-proliferative to cancer cells [10]. Apart from these, andrographolide inhibited carcinoma [11],

hepatoma [12], and leukemia [13]. It also inhibited Epstein Barr virus which causes glandular fever [14].

*In vitro* tissue culture studies generally aim to enhance the production of a given secondary metabolite that is of therapeutic significance. One of the common methods employed to increase the bioproduction of secondary metabolites in cell suspension cultures is to simulate stress conditions. Elicitors are signaling compounds that activate certain pathways to counter external stress, both abiotic and biotic, leading to secondary metabolite biosynthesis [15], for instance, to counter abiotic stresses caused by salinity, heavy metals, drought, temperature, micronutrient toxicity, wounds, etc., jasmonic acid (JA) and its derivatives act as signaling molecules in triggering plant chemical defenses [16,17]. Along with other plant hormones, they also regulate various metabolic pathways [18]. Similarly, salicylic acid (SA) is involved in plant defense against hemi-biotrophic and biotrophic pathogens, establishment of Systemic Acquired Resistance [19], and growth responses [20]. A wide variety of other elicitors has also been used in plant cell suspension cultures to enhance the production of secondary metabolites. Elicitation with various abiotic elicitors such as  $\text{HgCl}_2$ ,  $\text{CdCl}_2$ ,  $\text{CuCl}_2$ , and  $\text{AgNO}_3$  and biotic elicitors such as yeast and bacteria has been attempted to increase andrographolide content in suspension culture grown in liquid MS media supplemented with 2,4-D (2 ppm) and BAP (0.4 ppm), respectively [21]; Yeast in particular resulted in 8-fold increase in andrographolide content compared to the untreated grown cultures [21]. Vakil and Medhulkar

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reported 6.94 and 6.23-fold increase in andrographolide content in cell suspension cultures of *A. paniculata* when two fungal species were administered in the MS media supplemented with 2,4-D and BAP [22]; while these elicitors increase secondary metabolite production, most often they incur huge growth costs. The growth in terms of biomass of the cell cultures remains compromised [23]. Nevertheless, a consensus as such does not exist. JA and SA and their derivatives have enhanced the secondary metabolite content in many plant-cell cultures [24-26]. Limited work exists with regard to the direct effects of SA and JA on the biomass and andrographolide content in *A. paniculata* cell suspension cultures.

The combined action of both SA and JA on the biomass and andrographolide has not been reported. It is widely established that SA and JA act antagonistically [27,28]. Many studies have reported JA dependent defenses are largely compromised because of already accumulated endogenous SA leading to believe that SA-dependent-resistance is prioritized over JA-dependent defense [29] and vice versa [30-34]; some studies have also reported synergistic effects of SA and JA [35]. The antagonistic effect of SA and JA plays an important role in modulation of immune signaling networks in plants [36]. The present study deals with the effect of SA and JA administered individually, and in combination on andrographolide production in *A. paniculata* cell suspension cultures.

## 2. MATERIALS AND METHODS

### 2.1. *In Vitro* Establishment of Callus Cultures from *A. paniculata*

The leaves of *A. paniculata* were collected from the plants grown in the polyhouse of CHRIST (Deemed to be University), Bangalore. They were surface sterilized with sodium hypochlorite (2%) for 7 min, 0.1% HgCl<sub>2</sub> for 30 s and washed with sterile distilled water 5–6 times. Explants of 0.5cm<sup>2</sup> were cultured on MS media supplemented with various concentrations of PGRs such as picloram (Pic), 2,4-dichlorophenoxyacetic acid (2,4-D), kinetin (Kn), benzylaminopurine (BAP), isopentenyl adenine (2iP), and thidiazuron (TDZ). The cultures were maintained at 25 ± 2°C under 16/8 h (light/dark) photoperiod provided by white fluorescent tubes (3000 lux).

### 2.2. Establishment of Cell Suspension Cultures

Cell suspension cultures were established from actively dividing callus obtained from leaf explants cultured on MS media supplemented with PIC: TDZ (1.0:1.0 ppm). 0.5 g of callus was taken in 50 ml liquid MS media without agar, supplemented with the same hormonal combination and concentration in 250 ml Erlenmeyer flasks. The cell suspension cultures were kept under a 16 h photoperiod provided by 40 Watts white fluorescent lamps in continuous agitation at 110 rpm in an orbital shaker and incubated at 25 ± 2°C for 28 days. The cell suspension cultures were sub-cultured at every 15 days interval. After this period, the cells were harvested for biomass and extracted and analyzed for andrographolide content.

### 2.3. Elicitation by Signal Compounds

SA, JA, and JA+SA, respectively, were evaluated for their effect on andrographolide production in cell suspension cultures of *A. paniculate*. 1000 µM/ml stocks of SA, JA, and SA+JA were prepared. Filter sterilized SA (25 µM, 50 µM, 75 µM, 100 µM), JA (25 µM, 50 µM, 75 µM, 100 µM), and SA + JA (25 + 25 µM, 50 + 50 µM, 75 + 75 µM, 100 + 100 µM) were added aseptically in the culture vessel on day 0. The cell suspension cultures were then incubated at 25 ± 2°C in an orbital shaker under continued agitation at 110 rpm. Sixteen h

photoperiod was provided by 40 watts white fluorescent lamps for 28 days. The experiments were done in triplicates. After this period, the cells were harvested for the biomass, extracted, and analyzed for andrographolide production.

### 2.4. Extraction and Quantification of Andrographolide and High Performance Liquid Chromatography (HPLC) Analysis

Andrographolide was extracted using the method employed by Zaheer and Giri [37] and analyzed using HPLC. Dried callus obtained from each flask, including control was taken and a proportional amount of methanol was added to give a 1% solution. It was incubated at room temperature for 1 h, sonicated for 30 min, and filtered through Whatman filter paper number 41. The extract was then filtered using a 0.45 µm membrane before injecting it into the HPLC system for analyzing andrographolide content. HPLC analysis of a total of 39 samples was performed on Shimadzu chromatographic system. Chromatographic separation was achieved on Phenomenex column 18 (5 µm, 4.6 × 250 mm). Mobile phase consisting of acetonitrile: water (40:60) with an injection volume of 10 µl. A chromatographic condition of 1 ml/min flow rate at 230 nm. 1 mg/ml stock solution of standard andrographolide prepared in HPLC grade methanol was used for analysis. HPLC grade reagents were procured from HiMedia India.

### 2.5. Statistical Analyses of Data

The results for quantification of andrographolide content using HPLC were performed in triplicates. The data of the experiments were statistically analyzed using one-way ANOVA. Duncan's multiple range test (DMRT) using IBM's SPSS software version 21 was used for significance. The results were expressed as mean ± SE.

## 3. RESULTS AND DISCUSSION

### 3.1. Callus Initiation and Proliferation

Cultured leaf explants were able to induce callus on the MS media. Among the various concentrations and combinations of auxins and cytokinins used PIC (1.0 ppm) in combination with 1.0 ppm TDZ was highly efficient for induction of callus [Table 1]. Callus obtained from this combination was used for the establishment of cell suspension cultures, which were maintained by frequent subculturing. The same hormonal combination was used for elicitation studies.

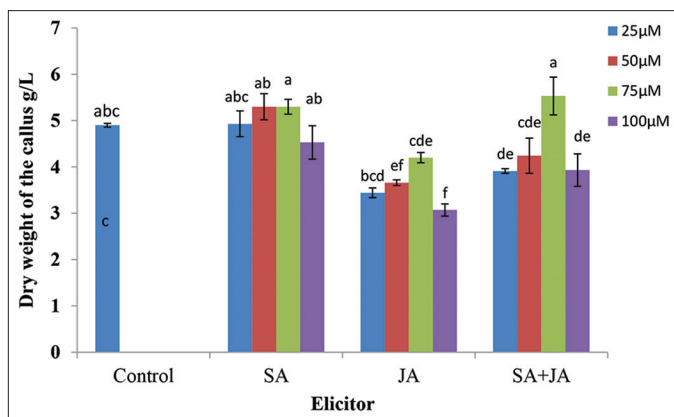
### 3.2. Effect of Elicitors on Biomass

All the elicited cell suspension cultures were unable to yield high biomass. Most of the elicited cell suspension cultures had a negative impact on the biomass production but cultures elicited with 50 and 75 µM of SA had a minimal positive influence on biomass accumulation where 0.08-fold increment (5.3 ± 0.28 g/L DW) was observed. A similar effect with negligible increase in dry cell weight at a much higher concentration was also reported by Al-Khayri and Naik in cell suspension cultures of *Phoenix dactylifera* [38]. JA imparted a net negative effect on the dry cell weight; 100 µM of it yielded just over 3.07 g/L DCW, a decrease by 0.37-fold compared to the control. A similar negative effect of JA on biomass has also been reported in the cell suspension cultures of *Taxus* [39] and *Thevetia peruviana* [40]. 75 µM of SA and 75 µM of JA, respectively, resulted in an increase in dry weight by 0.1-fold (5.53 ± 0.406 g/L DW) compared to control [Figure 1]. All other concentrations showed a relative decrease in biomass compared to the control. Equimolar amounts of both SA and JA administered together at all four concentrations imparted an intermediate effect on the biomass accumulation; it was lower than

**Table 1:** Effect of combinations of PGRs on callus induction from leaf explants of *Andrographis paniculata*.

Hormonal combination (ppm)	Pic (1)		2,4-D (1.5)		2,4-D (2)	
	Weight (g)	% Callus	Weight (g)	% Callus	Weight (g)	% Callus
Kn (0.5)	0.16±0.03 <sup>c</sup>	34	0.68±0.4 <sup>b</sup>	66	0.12±0.05 <sup>c</sup>	100
Kn (1.0)	0.13±0.03 <sup>c</sup>	67	0.2±0.02 <sup>c</sup>	34	0.09±0.02 <sup>c</sup>	56
Kn (1.5)	0.21±0.14 <sup>b</sup>	67	--	--	0.26±0.09 <sup>c</sup>	34
Kn (2)	0.19±0.08 <sup>c</sup>	55	--	--	--	--
BAP (0.5)	--	--	--	--	--	--
BAP (1.0)	--	--	--	--	--	--
BAP (2.0)	--	--	--	--	--	--
2-iP (0.5)	--	--	--	--	--	--
2-iP (1.0)	--	--	--	--	--	--
2-iP (2.0)	--	--	--	--	--	--
TDZ (0.5)	--	--	--	--	--	--
TDZ (1.0)	1.38±0.7 <sup>a</sup>	100	--	--	--	--
TDZ (2.0)	--	--	--	--	--	--

Data in the table represent the mean values SE of 9 replicates. Mean values with common letters are not significantly different at  $P \leq 0.05$  according to Duncan's multiple range test (DMRT).



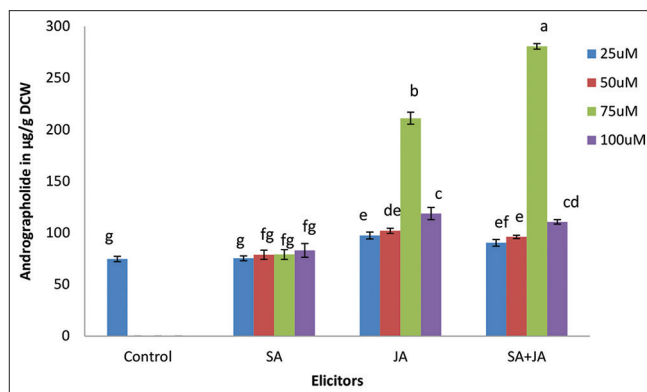
**Figure 1:** Effect of elicitors (SA, JA, and SA+JA) on the accumulation of biomass from cell suspension cultures of *Andrographis paniculata*. Data in the figure represent the mean of triplicates. Bar represents the standard error and mean values with common letters are not significantly different at  $P \leq 0.05$  according to Duncan's multiple range test (DMRT).

the biomass obtained when SA alone was used and higher than the biomass obtained when JA alone was used. JA seems to inhibit the proliferative action of SA, pointing to some antagonism.

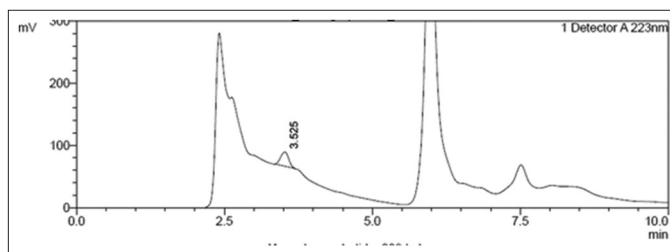
### 3.3. Effect of Elicitors on Andrographolide Content

Elicitors act as signaling compounds that can induce and enhance the production of metabolites by activating biochemical pathways in response to external stress [15]. JA acts as a signaling molecule to defend plants against necrotrophic pathogens and herbivory [29], while SA acts as a signaling molecule to defend the plants against biotrophic pathogens [19]. Both the signaling molecules aid in plant chemical defenses by enhancing the biosynthesis of secondary metabolites [16,17].

SA's effect on andrographolide content does not correspond to its effect on biomass. Andrographolide content increased gradually with the increase in concentration, peaking at 100 μM concentration of SA ( $83.33 \pm 6.7$  μg/g DCW) [Figure 2]. The increase in andrographolide

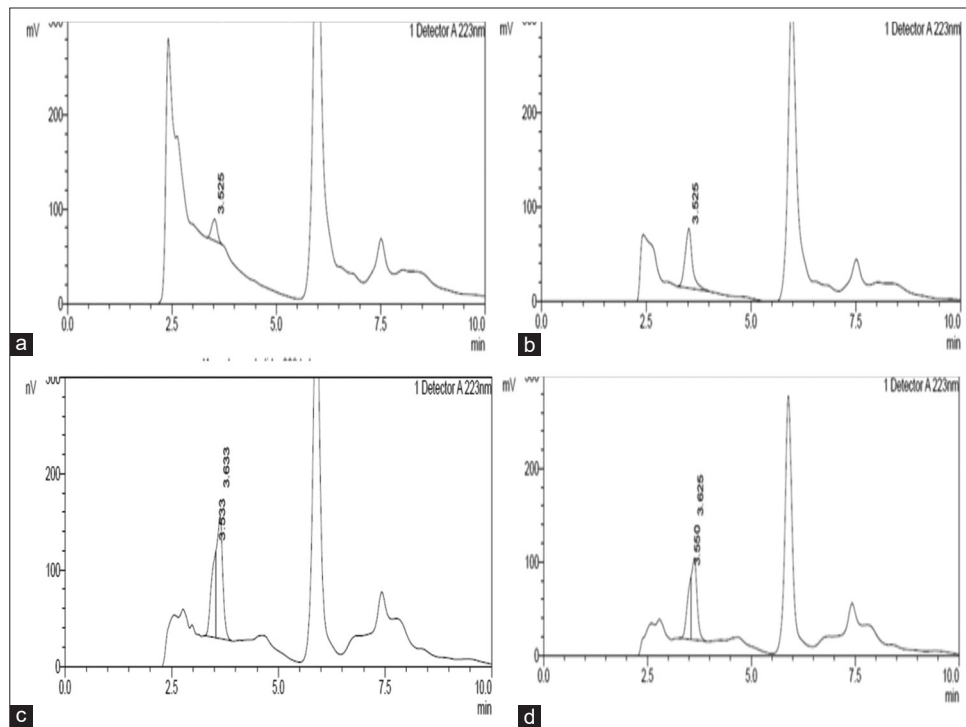


**Figure 2:** Effect of elicitors (SA, JA, and SA+JA) on the production of andrographolide from cell suspension cultures of *Andrographis paniculata*. Data in the figure represent the mean values of triplicates. Bar represents the standard error and mean values with common letters are not significantly different at  $P \leq 0.05$  according to Duncan's multiple range test (DMRT).



**Figure 3:** Control chromatogram

content was not very significant as only 18% increment was observed when compared with control [Figure 3]. In a previous study, 8-fold increase in andrographolide content was seen in hairy root cultures of *A. paniculata*; however, the amount of SA administered was much higher (100 mM) than the present study [40]. Our results are not in line with Vakil and Medhulkar [25] who reported an increase in andrographolide content by 18.5-folds when *A. paniculata* cell



**Figure 4:** HPLC Chromatograms at 100µM a: Control b: SA treated c: JA treated d: SA+JA treated

suspension cultures were grown in MS media supplemented with 2, 4-D (1ppm); BAP (0.5ppm) and 50 µM of SA. In our study, JA was reportedly superior in enhancing the andrographolide content. JA and its derivatives have been highly effective in enhancing secondary metabolite production in cell cultures of different plants [24]. JA shows the exact opposite effect on the metabolite content compared to its effect on biomass in our study. Andrographolide content increases with increase in JA concentration. It peaks at 75 µM of JA ( $211 \pm 5.8$  µg/g DW), resulting in a 3-fold higher andrographolide content than the control. The metabolite content dips at 100 µM to yield only a 1.68-fold increment over control [Figures 2 and 4]. Our results for the effect of JA on andrographolide content are in line with the previous studies where *Salvia officinalis* shoots treated with 20 µM JA produced higher diterpenoid content (8 mg/g DW) compared to the control (5 mg/g) [41], and *A. paniculata* hairy root cultures treated with 100 mM JA resulted in a 5-fold increment in andrographolide [42]. In yet another study on *A. paniculata*, genes involved in andrographolide (diterpene) synthesis were reported to have been influenced by JA treatment [43]. Equimolar concentrations of SA plus JA show a trend similar to that of JA alone with the andrographolide content peaking at 75 µM + 75 µM of SA and JA ( $280 \pm 2.7$  µg/g DW), a 3.8-fold increase compared to the control [Figure 2].

SA shows a significantly lesser effect on andrographolide content while JA shows a considerable increase in the metabolite content. This could mean that andrographolide may not be playing a significant role in defense against biotrophic pathogens, given that SA concentration in plants increases during a pathogen attack as mentioned earlier [19]. JA's positive effect on andrographolide content could mean that it could be implicated in plant defense against wounds, given that JA is released in plants in response to wounds. More molecular work in this regard is needed to validate the claims.

SA and JA usually show antagonistic response [29-31]; higher SA signaling in response to biotrophic pathogens is correlated

with decreased JA signaling and lower resistance to necrotrophic pathogens [44]. *Arabidopsis thaliana* treated with SA was unable to show JA-induced resistance to an insect *Spodoptera exigua* [32]. Conversely, *A. thaliana* already treated with JA showed reduced resistance to *Pseudomonas syringae*, a biotrophic pathogen which triggers the release of SA [33,34]. Mur *et al.* [35], however, reported synergistic effect between SA and JA pathways. Interestingly, in our study, equimolar concentration of SA plus JA used in combination yields higher metabolite content than the amount yielded when SA alone was used, and lower metabolite content when JA alone was used; this, however, changes at the concentration of 75 + 75 µM of SA and JA. More molecular level research would, therefore, be needed to understand if SA and JA have antagonistic responses in terms of andrographolide production.

#### 4. CONCLUSION

The results in the study have indicated that none of the elicitors at the given concentrations acted as good promoters for growth in terms of biomass (dry weight) compared to untreated control. However, SA at all concentrations showed an increase in andrographolide content. Similarly, JA was found to be a very effective elicitor for andrographolide production and generated higher metabolite compared to the control. The concentrations of 25 µM, 50 µM, and 75 µM showed an increase in andrographolide content while having an opposite effect on biomass. Equimolar concentrations of SA and JA administered together, at all four concentrations, showed an intermediate effect on the metabolite content higher than what was yielded when SA alone was treated and lower than what was yielded when JA alone was used; except at 75 + 75 µM which yielded the highest andrographolide content. Further molecular studies on *A. paniculata* would help us understand the exact mechanism through which these elicitors bring about higher andrographolide content.

## 5. ACKNOWLEDGMENT

We thank the HOD, Dept of Life Science, CHRIST (Deemed to be University), Bangalore, India, for providing us with facilities and resources. Nihal is extremely grateful to the Department of Minority, Karnataka (CR-11/2019-20) for his doctoral fellowship.

## 6. AUTHORS' CONTRIBUTIONS

Both the authors have contributed to the conception and design of the study. Nihal Ahmed prepared the material, collected the data, analyzed it, and wrote the first draft of the manuscript. Both the authors gave comments on the previous versions of manuscript and approved the final version of the manuscript.

## 7. FUNDING

This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

## 8. CONFLICTS OF INTEREST

The authors Nihal A and Praveen N declare that there is no conflict of interest.

## 9. ETHICAL APPROVALS

The present study does not involve any experiments on animals or human subjects.

## 10. DATA AVAILABILITY

All data generated and analyzed are included in the form of tables and figures within this research article.

## 11. PUBLISHER'S NOTE

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**How to cite this article:**

Ahmed N, Praveen N. Effect of salicylic acid, jasmonic acid, and a combination of both on andrographolide production in cell suspension cultures of *Andrographis paniculata* (Burm.f.) Nees. *J App Biol Biotech*. 2023;11(2):198-203. DOI: 10.7324/JABB.2023.110220