

Qualitative and quantitative analysis of Precocene II, estimation of enzymatic, nonenzymatic antioxidant, and cytotoxic potentials of methyl jasmonate-elicited shoot culture of *Ageratum conyzoides* Linn.

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ABSTRACT

Ageratum conyzoides Linn. is well known for its insecticidal properties due to the rich content of chromenes. The work mainly concentrated on the increased production of Precocene II from methyl jasmonate (MeJa)-elicited shoot culture. The mass spectroscopy (MS) medium supplemented with 4.6 μ M kinetin-induced multiple shoots was subjected to MeJa (100 μ M) treatment for 10 days. Gas chromatography-MS report showed almost twofold increased production of Precocene II (6,7-dimethoxy-2,2-dimethyl-chromene) (29.92%) in elicited culture over control (13.44%). Likewise, high-performance liquid chromatography analysis of the elicited shoot culture also reported to have a high amount of Precocene II (13.5 ± 0.007 μ g/mg) when compared with the control (4 ± 0.002 μ g/mg). The increased level of antioxidant enzymes was observed in the elicited shoot culture. Followed by pure compound, Precocene II and methanol extract of elicited shoot culture exhibited better *in vitro* antioxidant activity and considerable cytotoxic activity on HeLa (cervical cancer) cells when compared with control. Overall, MeJa-elicited shoot culture had more of Precocene II and better biological activity.

1. INTRODUCTION

Ageratum conyzoides Linn. (Asteraceae) is commonly called as goat weed and it is a soft hairy annual weed with strong scented smell and distributed usually in damp places. The plant is native to tropical America and widely distributed in tropics and subtropics. Several researchers across the world have studied the pest and disease-resistant potential of *A. conyzoides* [1–5]. The plant is used in traditional medicine to treat many diseases [6]. *Ageratum conyzoides* also reported to have various secondary metabolites like phenols, glycosides, and resins [7]. Liu and Liu [8] reported the insecticidal, feeding deterrent, and repellent activities of *A. conyzoides* against important agricultural insects and pests. The literature has reported on high-performance thin-layer chromatography (HPTLC) studies of Precocene I and II

variations in *A. conyzoides* taken from the Western Himalaya range [9] and the notable effect of Precocenes on disturbing the production of juvenile hormones of many insects has been studied. The acaricidal property of the plant against *Rhipicephalus (boophilus)* was reported by Ajith *et al.* [10]; antitick activity and variation in biochemicals of *A. conyzoides* from Eastern and Western Ghats of India was reported by Bhanu Kumar *et al.* [11]. Ndacnoua *et al.* [12] reported the antifungal activity of ethanol extract against *Phytophthora megakarya*.

The precocious metamorphosis of *Rhodnius prolixus* and *Triatoma dimidiata* was induced by Precocene II [13]. Antifungal activity of Precocene II against *Rhizoctonia solani* and *Sclerotium rolfsii* has been reported by Iqbal *et al.* [14]. Hypoglycemic activity in rats treated with Precocene II was studied by Adebayo *et al.* [15]. Antifungal activity by mitochondrial degradation in *Aspergillus flavus* was induced by Precocene II-rich essential oil of *A. conyzoides* [16].

Based on the medicinal values and presence of bioactive compound Precocene II in *A. conyzoides*, the purpose of the study was to increase the production of Precocene II in methyl jasmonate (MeJa)-elicited

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shoot cultures, followed by qualitative and quantitative analysis using Gas chromatography-mass spectroscopy (GC-MS) and high-performance liquid chromatography (HPLC), respectively. In addition to that, the enzymatic and nonenzymatic antioxidant and cytotoxicity studies were also carried out.

2. MATERIALS AND METHODS

2.1. Plant Material

Ageratum conyzoides Linn. (Asteraceae) (Fig. 1a) was collected from the local areas of Coimbatore (latitude and longitude are 11.004556 and 76.961632, respectively), Tamil Nadu, India. The plant specimen was identified and a voucher specimen (KASC/Bot/43-2019) has been prepared and deposited in the herbarium of Department of Botany, Kongunadu Arts and Science College (Autonomous), Coimbatore, Tamil Nadu, India. Plants were grown in a green house to avoid causalities.

2.2. Shoot Culture

Healthy green nodal explants were collected and washed with tap water before being surface sterilized in Tween 20 for 15 minutes and subsequently washed thrice in sterile distilled water and given treatment with 0.01% mercuric chloride for 1 minute. MS medium [17] was added with sucrose (3%) and agar (0.8%) for shoot culture. Medium pH was set to 5.8, followed by autoclaving (121°C for 15

minutes). The nodes were then inoculated on MS medium fortified with various concentrations cytokinins, such as benzyl aminopurine (BA; 2.22–8.88 μM), Kinetin (KIN; 2.32–9.29 μM), Thiadiazuran (TDZ; 2.27–9.08 μM), and Zeatin (2.28–9.12 μM) for shoot induction and multiplication. Inoculated cultures were kept under 16/8 hours light/dark photoperiod with $25^\circ \pm 25^\circ$. Experiments were conducted in triplicate with 10 replicates per treatment.

2.3. Elicitation

Sixty-day-old *A. conyzoides* shoot cultures were elicited with 100 μM MeJa [18] in liquid MS medium for 10 days and maintained at $25^\circ \pm 2^\circ\text{C}$ under 16/8 hours light/dark photoperiod. Each experiment consisted of three replicates and repeated thrice.

2.4. Crude Extract Preparation

5 g of elicited and nonelicited shoots were extracted individually with 25 ml of methanol to yield crude extract which has been dried and stored at 4°C for further study.

2.5. Chemical Composition Analysis

2.5.1. Gas chromatography

GC analysis was carried out with Varian 3800 GC with mass selective detector coupled to front injector type 1079. GC was equipped with

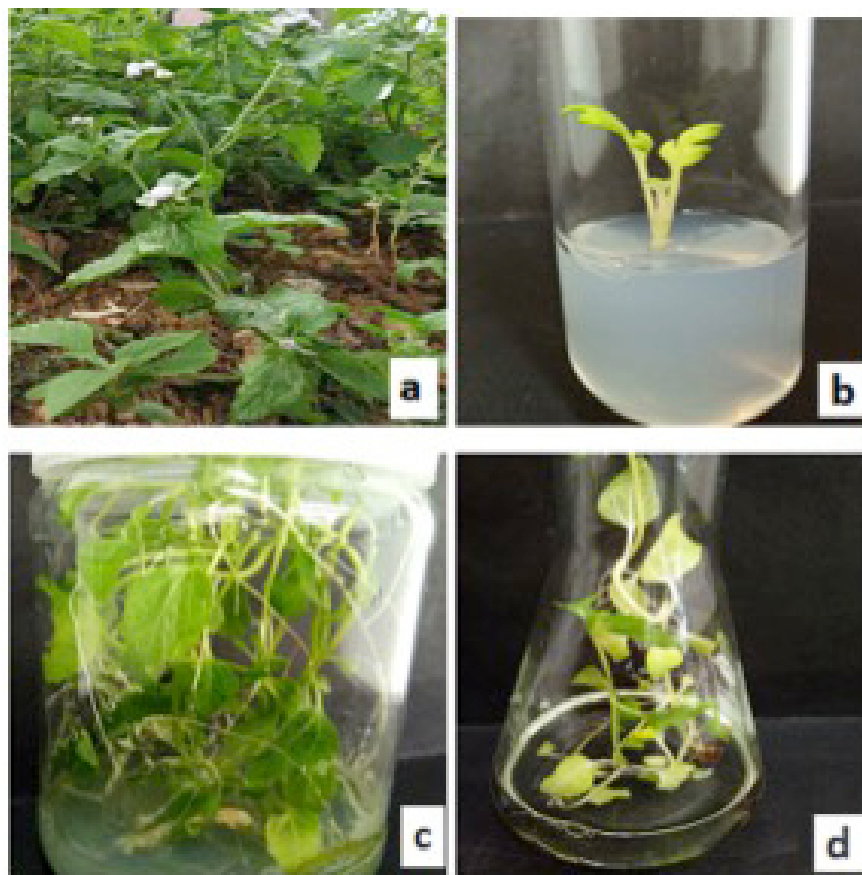


Figure 1: a) *Ageratum conyzoides* Linn. Habit. b) Shoot induction on MS + 4.6 μM KIN after 10 days of culture. c) Multiple shoot induction on the same medium after 25 days of culture. d) *In vitro* developed shoot subjected to MeJa (100 μM) elicitation in the liquid MS medium

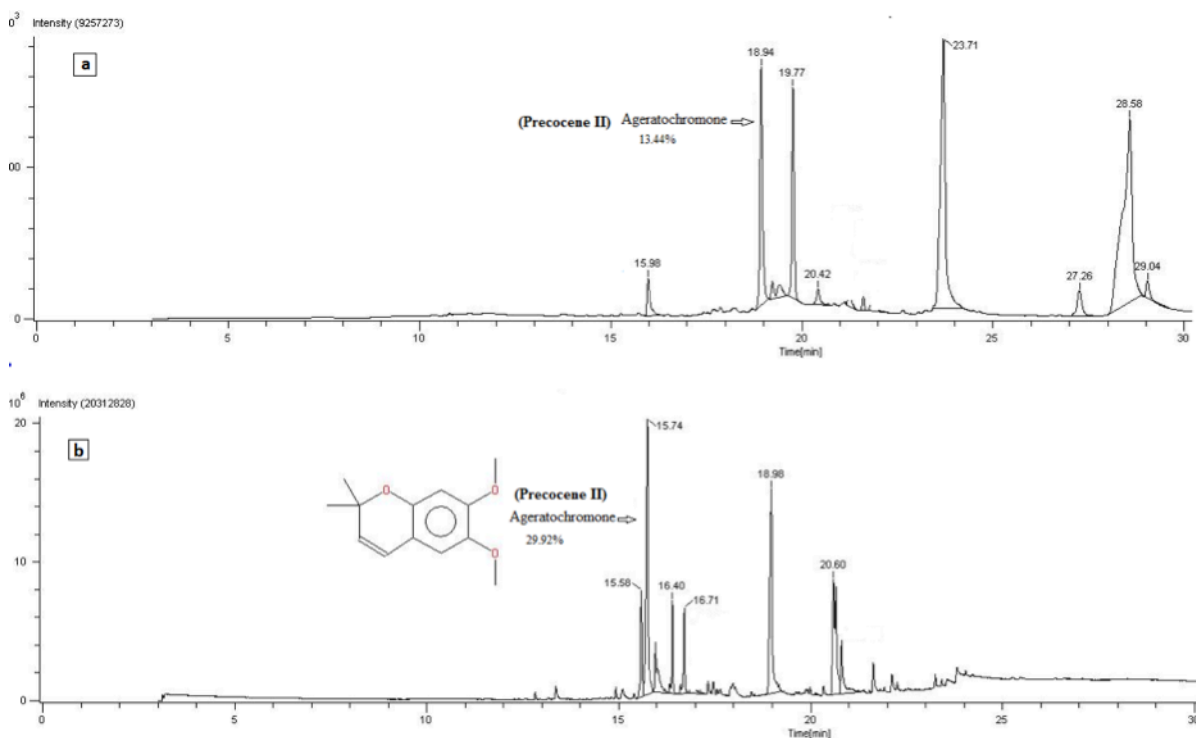


Figure 2: a GC-MS chromatogram of *in vitro* cultured control shoot showing Precocene II along with other compounds. b Increased amount of Precocene II in MeJa elicited *in vitro* cultured shoots

DB-5 column (30 m × 0.25 mm). The injector port temperature and column oven temperature were initially maintained at 280°C and 45°C, respectively, and programmed to 300°C at the rate of 10°C/minute and finally held at 200°C for 5 minutes. Helium was used as the mobile phase and the flow rate was 1.0 ml/minute. The obtained GC peak areas were used to compute the percentage of compounds in the extracts.

2.5.2. GC-MS analysis

GC-MS was carried out using Varian 3800 GC equipped with Varian 1200 I single quadrupole mass spectrometer. GC and column conditions were like GC analysis. Mass spectrophotometer operated at 70 eV mode. The temperature of the ion source and transfer line was kept at 250°C. The identification of compounds was carried out by mass spectra with those of computer library and retention time [19].

2.6. Quantitative Analysis

2.6.1. High-performance liquid chromatography (HPLC) analysis

The analytical column such as Waters Nova-Pack C18 (4 µm, 3.9 × 150 mm) and Waters Nova-Pack C18 60Å (3.9 × 20 mm) guard columns were employed. Elicited and nonelicited shoot culture extracts and pure compound Precocene II were dissolved in methanol, filtered and examined at 280 nm. HPLC conditions were as follows: acetonitrile and methanolic acid (0.1%) were solvent A; and water and methanolic acid (0.01%) were solvent B. The gradient program is 0–10 minutes, 50% → 0% solvent B; 10–15 minutes, 100% solvent A; and 15–20 minutes, 0% → 50% solvent B. To keep the system stable, the latter solvent was kept in place for another 30 minutes. The sample run was carried out at 25°C and 1 ml/minute flow rate. Each sample (20 µl) was injected thrice individually. The compounds were confirmed by

matching the retention times with Precocene II standard. To prepare the calibration curve, Precocene II was dissolved in methanol between the concentration range of 5.0–1,000.0 µg/ml [20].

2.7. Antioxidant Enzyme Estimation

2.7.1. Enzyme extraction

Elicited and nonelicited shoots (200 mg each) were homogenized individually with potassium phosphate buffer (100 mM; pH 7.5) in ethylenediaminetetraacetic acid (1 mM) and polyvinylpyrrolidone (5%, w/v). The homogenate was centrifuged at 13,000 rpm for 10 minutes at 4°C. Clear supernatant was taken as enzyme extracts and used for subsequent assays. The standard used to quantify total protein was bovine serum albumin using Lowry's method [21].

2.7.2. Catalase activity

Catalase activity [22] was assayed with minor alterations. 10 µl of 30% hydrogen peroxide and 100 µl of enzyme extract were added to potassium phosphate buffer to make a 2 ml reaction mixture (100 mM; pH 7). Absorbance was read at 240 nm for 5 minutes.

2.7.3. Peroxidase activity

Peroxidase activity was studied according to Kar and Mishra [23]. In a reaction mixture containing 1 µl of phosphate buffer, 1 ml of enzyme extract (100 mM; pH 6.8), 1 µl of pyrogallol (50 mM), and 1 µl of hydrogen peroxide (50 mM). Further incubation of the reaction mixture was carried out for 5 minutes at 25°C and reaction was terminated by adding 0.5 ml of sulfuric acid (5% v/v). Absorbance value was read at 420 nm for 5 minutes.

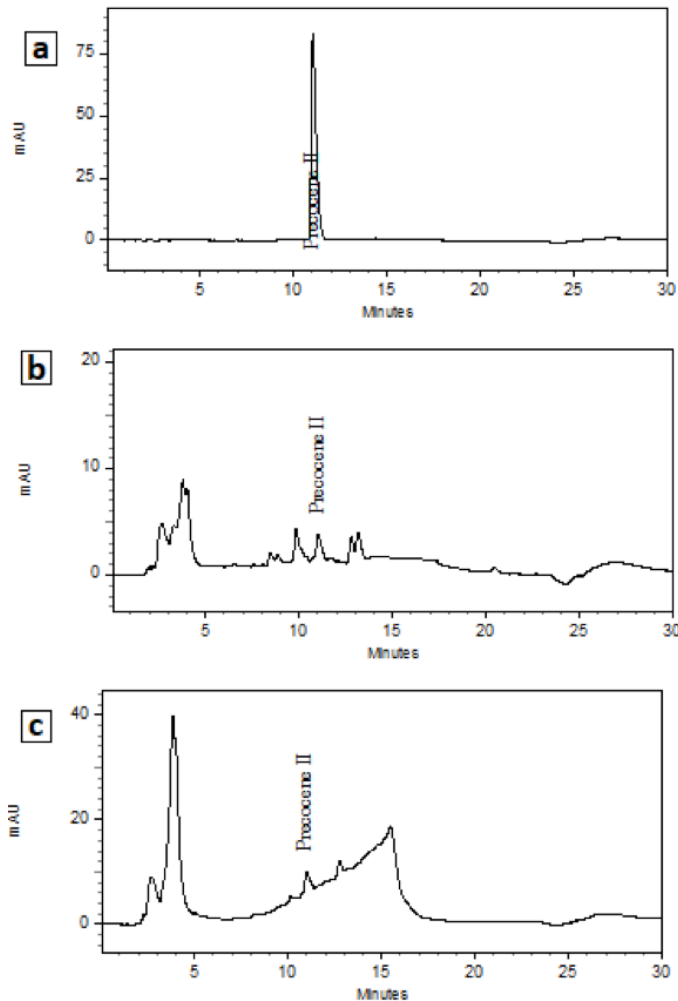


Figure 3: HPLC quantification of Precocene II in the *in vitro* cultured control and MeJa elicited shoot cultures. **a** Chromatogram of Precocene II standard. **b** Chromatogram of Precocene II in the MeJa elicited *in vitro* shoot culture. **c** Chromatogram of Precocene II in the *in vitro* cultured control shoots (values expressed as mean±SD)

2.7.4. Superoxide dismutase activity

0.5 ml of enzyme extract was mixed with 1 ml of sodium carbonate (125 mM), 0.4 ml of nitro blue tetrazolium chloride (25 μ M), and 0.2 ml of ethylenediaminetetraacetic acid (0.1mM) to set as reaction mixture. The reaction was carried out by adding 0.4 ml of hydroxylamine hydrochloride (1 mM) and was read at 560 nm with a 5-minute interval. SOD activity has been represented as the amount of enzyme needed to inhibit 50% reduction of nitro blue tetrazolium chloride [24].

2.7.5. Glutathione S-transferase activity

Glutathione-S-transferase assay was carried out according to Mannervik [25]. The reaction solution contained 980 μ l of phosphate buffered saline (pH 6.5), 10 μ l of 1-chloro, 2,4-dinitrobenzene (100 mM), and 10 μ l of glutathione (100 mM). Then, 900 μ l of the reaction solution was taken and subjected to incubation at 30°C for 5 minutes. The absorbance was read at 340 nm for 5 minutes after addition of enzyme extract of 100 μ l.

Table 1: a) Chemical composition analysis of *in vitro* cultured control shoots by GC-MS. b) Chemical composition analysis of *in vitro* cultured MeJa-elicited shoots by GC-MS.

S.No	Retention time	Compound	Percentage
1.	15.98	Precocene-I	2.25
2.	18.94	Precocene II	13.44
3.	19.77	3,5-di-tert-Butyl-4-hydroxyacetophenone	10.77
4.	20.42	Oleic acid	1.11
5.	23.71	Hexadecanoic acid	27.02
6.	27.26	Phytol	2.50
7.	28.58	Linolenic acid	32.63
8.	29.04	Dihomo-gamma linolenic acid	1.23
Total identified			90.95

S.No	Retention time	Compound	Percentage
1.	15.58	Cyclopentylacetic acid	6.76
2.	15.74	Precocene II	29.92
3.	16.40	3,5-ditert-Butyl-4-hydroxyacetophenone	4.8
4.	16.71	Bicyclo (4.4.0) dec-5ene-1-acetic acid	4.5
5.	18.98	Hexadecanoic acid	22.48
6.	20.60	Linoleic acid	23.90
Total identified			92.36

2.8. In Vitro Antioxidant and Cytotoxicity Studies

Both elicited and nonelicited shoots (5 g each) were extracted with 25 ml of methanol to yield crude extract. The preparation of stock solution was carried out by dissolving 1 mg of methanol extract in 1 ml of dimethyl sulfoxide (10%), and 10 mg stock solution of Precocene II was also prepared by using 10% dimethyl sulfoxide and then five test concentrations (25–125 μ g/ml) of both extracts and pure compound; Precocene II were prepared separately. The percentage of antioxidant activity was calculated as follows: % Inhibition = $[(A_B - A_A)/A_B] \times 100$, where A_B = absorption of blank sample and A_A = absorption of test sample.

Antioxidant activities was carried out for study samples as per the following methods: scavenging of 1,1-Diphenyl-2-picrylhydrazyl (DPPH) free radicals [26], metal chelation activity [27], scavenging of hydroxyl radicals [28], inhibition of linoleic acid peroxidation [29], and prevention of deoxyribose degradation [30].

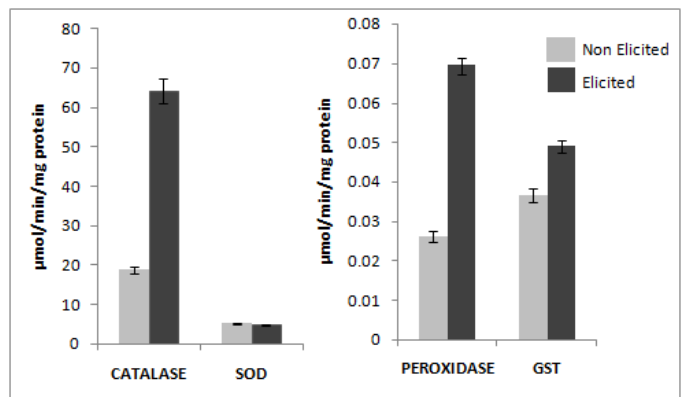


Figure 4: Antioxidant enzymes level in the *in vitro* cultured control and MeJa elicited shoots

Table 2: Antioxidant activity of *in vitro* cultured control shoot, MeJa-elicited shoot, and Precocene II. ^aIC₅₀ values are expressed as the mean ± SD of three replicates.

Antioxidant assays	Control shoot IC ₅₀ (µg/ml) ^a	Elicited shoot IC ₅₀ (µg/ml) ^a	Precocene II IC ₅₀ (µg/ml) ^a
DPPH free radical scavenging activity	29.73 ± 0.01	14.26 ± 0.01	10.26 ± 0.01
Metal ion chelation activity	37.97 ± 0.02	14.83 ± 0.01	14.16 ± 0.01
Hydroxyl radical scavenging activity	23.19 ± 0.01	16.07 ± 0.01	10.07 ± 0.01
Inhibition of linolenic peroxidation	31.81 ± 0.01	14.31 ± 0.01	12.43 ± 0.01
Prevention of deoxyribose degradation	39.77 ± 0.02	14.78 ± 0.01	13.59 ± 0.01

2.8.1. Cell culture

Cervical cancer (HeLa) cell line was purchased from the National Centre for Cell Science, Pune, India, and grown in culture flask (25 cm²) using Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 µg/ml). Furthermore, the cell culture was incubated at 37°C with 5% CO₂.

2.8.2. MTT assay

Each well of the 96-well plate was added with 1 × 10⁴ cells/100 µl medium and incubated for 24 hours. Furthermore, the cells were subjected to treatment with different concentrations (5–25 µg/ml) of the compound Precocene II; the methanol extract of control and elicited shoots individually were further incubated for 48 hours. The addition of about 20 µl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (5 mg/ml) in phosphate buffered saline to each well and the culture plate was kept at 37°C for 4 hours. The medium was discarded followed by adding dimethyl sulfoxide (100 µl) to all wells. The culture plate was read at 570 nm after 37°C incubation for 10 minutes [31]. Percentage (%) of cell viability = [(A_B - A_A)/A_B] × 100, where A_B = absorption of blank sample and A_A = absorption of test sample.

2.9. Statistical Analysis

The results of antioxidant and cytotoxicity experiments are expressed as mean ± SE in triplicate. Inhibitory concentration 50% (IC₅₀) values were calculated by SPSS (16.00).

3. RESULTS AND DISCUSSION

3.1. Multiple Shoot Formations and MeJa Treatment

Plant tissue culture is a valuable and alternate source for production of commercially, pharmaceutically, industrially, and biologically important secondary metabolites. The nodal explants did not respond to the MS medium devoid of hormones. Out of four different cytokinins tested, KIN showed better response on shoot induction, elongation and number of shoots. All the tested concentrations of KIN (2.3–9.2 µM/l) induced the shoot after 10 days of culture, followed by multiple shoot inductions in the fourth week of culture. Exactly 4.6 µM KIN was observed as optimal and induced more number shoots with the highest shoot length (Fig. 1b and c). When increasing the concentration of KIN, shoot number, and length got reduced. BA showed the best response, followed by KIN and subsequently Zeatin and TDZ. KIN was observed as the best for multiple shoot inductions in several Asteraceae members [32–35]. To produce increased amounts of secondary metabolites using *in vitro* culture, application of elicitors

is a good choice. Among several elicitors, MeJa is a promising one for the induction of secondary metabolites in plants. Sixty-day-old *A. conyzoides* shoot cultures were elicited with 100 µM MeJa in liquid MS medium for 10 days (Fig. 1d). The shoot culture without MeJa treatment was served as control. After 10 days, both elicited and control shoots were taken for chemical composition analysis.

3.2. Qualitative and Quantitative Estimation of Precocene II

GC-MS analysis of *in vitro* cultured control and MeJa-elicited *in vitro* shoots showed various compounds. The major compounds were Precocene II and Precocene I and their percentage of variations of concentration was calculated (Table 1a and b). When compared with control shoot, MeJa-elicited shoot showed a twofold increase in Precocene II (from 13.44% to 29.92%) (Fig. 2a and b). On the contrary, compounds like Precocene I and the linoleic acid level had a reduced percentage in elicited shoot. Followed by HPLC, the quantification of Precocene II (Fig. 3a) from elicited shoot culture was calculated as 13.5 ± 0.007 µg/mg (Fig. 3b) which is threefold higher than the control (4 ± 0.002 µg/mg) (Fig. 3c). Both qualitative and quantitative analyses showed increased production of Precocene II in MeJa-elicited culture.

3.3. Antioxidant Enzymes

MeJa is proved to be a promising plant signaling molecule which can induce oxidative stress and involve plant antioxidant metabolism [36]. In this study, the increased levels of CAT, POX, and GST were observed in MeJa-elicited *in vitro* shoot cultures and compared with *in vitro* cultured control shoots (Fig. 4). Higher CAT activity of 64.032 µmol minute⁻¹ mg⁻¹ protein was found in elicited shoots, whereas 18.705 µmol minute⁻¹ mg⁻¹ protein was observed in the control. Meanwhile, SOD level was high in control (5.163 µmol minute⁻¹ mg⁻¹ protein), whereas the elicited shoot showed 4.823 µmol minute⁻¹ mg⁻¹ protein. Significant elevation of peroxidase was seen in shoots from 0.026 µmol minute⁻¹ mg⁻¹ protein to 0.069 µmol minute⁻¹ mg⁻¹ protein after elicitation and the GST level also increased in elicited shoots (0.048 µmol minute⁻¹ mg⁻¹ protein) than control (0.036 µmol minute⁻¹

Table 3: Cytotoxic activity of methanol extract of *in vitro* cultured control shoot, MeJa-elicited shoot, and Precocene II.

Samples	IC ₅₀ value (µg/ml) ^a
Control shoot	14.80 ± 0.02
Elicited shoot	14.36 ± 0.02
Precocene II	11.29 ± 0.01

^aIC₅₀ values are expressed as the mean ± SD of the three replicated.

mg⁻¹ protein). Similar to the findings of this study, previous reports stated that exogenous stress induction by elicitors such as MeJa led to the activation of superoxide dismutase, catalase, ascorbate peroxidase and glutathione peroxidase enzymes in plants [37,38,36].

3.4. *In Vitro* Antioxidant Capacity and Cytotoxicity

The antioxidant activities of Precocene II and methanol extract of both control and MeJa-elicited *in vitro* cultured shoots were studied using five different *in vitro* antioxidant assays. Significant effect on scavenging free radicals, lipid peroxidation inhibition, metal ion chelation, and prevention of deoxyribose degradation activities were shown by elicited shoot than control. Meanwhile the overall antioxidant activity of Precocene II was higher than that of both control and elicited shoots (Table 2). The antioxidant activity of Precocene II might be due to methyl groups. Similarly, Zhang *et al.* [39] proved the antioxidant potential of betaine with the presence of methyl groups. Like antioxidant activity, Precocene II showed higher cytotoxic effect on HeLa cells over control and elicited shoots (Table 3).

4. CONCLUSION

In this study, the amount of Precocene II has been significantly increased in the MeJa-elicited *in vitro* cultured shoots. The secondary metabolite accumulation and biological activities were also increased after elicitation. Hence, *A. conyzoides* could be cultured in large scale *in vitro*, followed by MeJa elicitation, which may result in the increased production of bioactive compounds to meet future needs.

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7. CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

8. ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

9. DATA AVAILABILITY

All data generated and analyzed are included within this research article.

10. AUTHOR CONTRIBUTIONS

Selvaraj Vasantharani collected the plant material and carried out entire experimental work. Ramaraj Thirugnanasampandan carried out work design, data analysis, and manuscript preparation. Gunasekaran Bhuvanewari assisted with the antioxidant and cytotoxic studies and drafted the manuscript.

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