

Research article

Effect of Growth Regulators on *Calotropis gigantea* (Linn.) Aiton f. Callus Formation and Its Phytochemical Content

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Abstract

Crown flower (*Calotropis gigantea* (Linn.) Aiton f.) is a weed that has potential as a source of medical materials. *In vitro* culture through callus induction can be an alternative method for rapid multiplication and producing phytochemical compounds in a shorter time. The great potential of crown flower plants is still unutilized optimally in Indonesia. The research aimed to determine the effect of various concentrations of plant growth regulators on callus formation and the phytochemical contents of crown flower. Young stem explants of crown flower were cultured on Murashige and Skoog (1962) medium supplemented with 6-benzyl amino purine and 2,4-dichlorophenoxyacetic acid. The research used a completely randomized design and callus formation time, fresh callus weight, callus morphology, flavonoids, and phytochemical contents were observed. The data were analyzed using ANOVA and DMRT with a 5% level test. Treatments with 0.5 mg/L BAP and 0.5 mg/L 2,4-D could induce faster and bigger calli than other treatments with 5.82 g callus weight, yellowish-green colour, and compact texture. The addition of BAP and 2,4-D into the culture medium was able to produce different compounds compared to the field grown plants. Based on the results, PGR's combination are required for callus formation and the production of different phytochemical compounds. Therefore, crown flower can be more effectively utilized as a medicinal or industrial plant.

Keywords: *Calotropis gigantea*; flavonoid; GC-MS; plant tissue culture; secondary metabolites

1. Introduction

Crown flower (*Calotropis gigantea* (Linn.) Aiton f.), known as biduri in Indonesia, is a shrub whose all parts produce a milky white sap. Crown flower leaves are classified as a single leaf and the stem is thick (Kundu, 2021). This plant can reach 2.4-3.0 m in height and is found on low mountain slopes, deserts, grasslands, and on the coast. Crown flower is a large shrub from Asia that is found in Indonesia, India, and Sri Lanka (Adejoh et al., 2021;

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Wijeweera et al., 2021; Ramesh et al., 2022). Crown flower has been categorized as a wild plant or weed but it has diverse benefits for utilization in each part. Crown flower extract can be used as an antidote to snake bites and to treat skin infections (Ali-Seyed & Ayesha., 2020). The flowers and leaves could be used to treat mellitus diabetes (Hii et al., 2018). Based on its economic value, crown flower has also been used as a textile material (Vinod et al., 2018). However, the potential of crown flower plants has not been optimally utilized in Indonesia.

Secondary metabolites are phytochemicals produced by plant cells through metabolic pathways (Hussein & El-Anssary, 2019). They are a common source of natural chemicals. Crown flower contains flavonoids, alkaloids, terpenoids, and cardiac glycosides (Ayemele et al., 2020). Flavonoids are secondary metabolites, especially phenolic compounds, that are widely distributed in plants (Proença et al., 2022). Previous studies reported that flavonoids were used as herbal medicines for asthma, and for anti-inflammatory and antioxidant purposes (Liskova et al., 2021; Tian et al., 2021). However, the production of total compounds was limited.

Environmental factors such as temperature, light, and culture medium have influenced the secondary metabolite production (Toscano & Romano., 2021). These factors contribute to the efficiency of secondary metabolite production (Afrin et al., 2015). *In vitro* culture has been an alternative technology for large-scale rapid propagation and secondary metabolite production. Phytochemical compounds can be extracted from callus, eliminating the need to utilize entire plant organs (Efferth, 2019). Thus, *in vitro* culture is an alternative option for improving the production of phytochemical compounds (Nielsen et al., 2019). Callus can be achieved by incorporating auxin and cytokinin into a growth medium, leading to the development of callus with optimal results.

A combination of auxin and cytokinin at a specific ratio has an essential role in tissue culture (Hesami et al., 2018). Using an appropriate ratio of these growth regulators was shown to be beneficial for large-scale propagation of crown flower (Priya et al., 2019). 6-benzyl amino purine (BAP) and 2,4-dichlorophenoxyacetic acid (2,4-D) are plant growth regulators that are expected to enhance optimal callus growth and increase phytochemical content. BAP and 2,4-D are a combination that can produce complex effects on plant hormonal systems. This combination of growth regulators can induce callus formation in plant tissues, both embryogenic callus that can develop into new plants and callus with high and diverse secondary metabolite content. The appropriate concentration of this growth regulator combination needs to be considered to achieve optimal callus formation. Determining the concentration of growth regulators was done with a 0.5 mg/L interval to ensure precise and controlled results regarding how growth regulators affect the callus formation process. Using a narrow interval was expected to help achieve consistency in results among different treatments. In order to avoid toxic effects on tissue-cultured plants due to a wide gap interval and excessively high concentrations, the concentrations used in the treatment were limited at 0, 0.5, 1, 1.5, and 2 mg/L. This study aimed to determine the effect of various concentrations of BAP and 2,4-D on callus formation, callus morphology, and the phytochemical content of *Calotropis gigantea* (Linn.) Aiton f.

2. Materials and Methods

2.1 Plant material and surface sterilization

Calotropis gigantea (Linn.) Aiton f. seeds were sown using compost as the seedling medium in the greenhouse at Faculty of Agriculture, Universitas Sebelas Maret, Indonesia.

Young stems at 1.5 months old were used as explants. The explants were sterilized outside a Laminar Air Flow Cabinet for preliminary cleaning and disinfection, starting with cleaning them from residual planting medium with running water, followed by immersion in a detergent solution for 1 min, then soaking in bactericide and fungicide (1 g fungicide and 1 g bactericide for 1000 mL distilled water) for 45 min, and rinsed with aquadest. While inside the Laminar Air Flow Cabinet, the explants were surface sterilized with 100% Clorox for 1 min followed by 70% alcohol for 1 min and then rinsed twice with sterilized distilled water.

2.2 Culture medium

The explants were cultured on Murashige and Skoog (MS) medium (1962) supplemented with BAP and 2,4-D as plant growth regulators (Tandon et al., 2021). The process of making medium culture was done by mixing 30 g/L of sugars, 50 mL/L of macronutrients, 10 mL/L of micronutrients, 50 mL/L of vitamins, 50 mL/L of Fe-EDTA, growth regulators according to each treatment concentration, and distilled water until the volume reached 1000 mL (Raji & Siril, 2021). The stock solutions, consisting of macronutrients, micronutrients, vitamins, FeEDTA, and growth regulators, were prepared with a dilution of 100 times. The combination of BAP and 2,4-D was applied to the medium at 5 different concentrations (0; 0.5; 1; 1.5; 2 mg/L). The pH of the medium was adjusted to between 5.5 and 6.2 using NaOH and HCl solution, and then 4 g of agarose was added. MS medium was autoclaved at 121°C for 30 min to sterilize it.

2.3 Explant initiation

The initial explants were accomplished under sterilized conditions. Each stem was isolated using a sterilized scalpel knife. The 2 cm lengths of stems were placed onto culture medium. Thereafter, all explants were incubated in the incubation room at 25-28°C for six weeks.

2.4 Total flavonoid identification

Identification materials were field grown plants and callus. The field grown plants used were the same seedlings as the explants with plant parts of leaves, stems, and roots. Calli were harvested six weeks after culturing. Following this, they were dried in an oven at 60°C for 2 days or until they reached the constant weight and then underwent extraction using a maceration method (Jha & Sit, 2022). The extract was added with 5% sodium nitrite, 10% aluminum chloride, 1M sodium hydroxide, and aquadest. Eventually, the UV-Vis Spectrophotometric method was used to identify the total flavonoids. First, the sample was placed into the cuvette, and then the wavelength was set at 510 nm (Dhianawaty et al., 2021). Finally, the results that appeared on the display were recorded.

2.5 GC-MS analysis

The phytochemical compounds of extract were determined using gas chromatography-mass spectrometry (GCMS-QP2010 Ultra, Shimadzu with an Agilent DB-5MS UI column of 30 m length) (Jaradat, 2021). Each sample was analyzed with a GC system. Dried callus (1.61 g) was extracted, then 50 mg of extract was dissolved in 4.5 mL isopropyl alcohol and sterilized by ultrasonic bath for 30 min. GC-MS sample analysis was performed using a GC system with an autosampler and detector. The identified compounds were verified

by comparing their mass spectra with the existing library. Lastly, the relative percentage of the total peak area in the chromatogram was calculated (Ibrahim et al., 2019).

2.6 Data analysis

The research was carried out with a completely randomized design (CRD) with 3 replicates. The variables observed were callus formation time, fresh callus weight, callus color, callus texture, total flavonoid content, and phytochemical compounds. Analyses of the qualitative data included descriptive analysis, while the quantitative data obtained were analyzed with the F test at the 5% level. DMRT 5% level was used if there was a significant difference. The application used was SPSS Statistics 25.

3. Results and Discussion

3.1 Callus formation time

Callus were formed from the young stem explants in the culture medium by the application of BAP and 2,4-D. Previous research reported that auxins and cytokinins were able to induce callus by encouraging cell division and elongation through synergistic interactions, antagonists, and additives to promote cell growth (Castro et al., 2016). Explants used were from the young stems of 1.5 months old seedling. Young plants have high regeneration power and thereby can produce *Styrax benzoin* callus with a percentage of 76.25 (Ferial et al., 2020; Nurwahyuni et al., 2020).

The interaction effect of BAP and 2,4-D on crown flower callus formation were studied using stem explants. The results showed that the combination of BAP and 2,4-D did not significantly affect callus induction. However, the application of individual treatment with BAP or 2,4-D significantly affected on the induction of callus formation time (Tables 1 and 2).

Table 1 shows that application of 0.5 mg/L BAP could induce the callus formation faster than other treatments by 6.13 days after culturing. However, statistically, there were no significant differences. The increment of BAP concentration in medium culture took longer periods for callus formation (Table 1). However, BAP still had an important role in callus induction because, without adding BAP, callus was formed more slowly and took 10.6 days after culturing.

Moreover, the 0.5 mg/L 2,4-D concentration exhibited the fastest crown flower callus formation, occurring within 8.53 days after culturing. However, the result did not show a significant difference compared to 2,4-D concentrations at 1 and 1.5 mg/L. Without the addition of 2,4-D, callus formation in crown flower did not occur until the end of the observation period (Table 2). The medium without the addition of 2,4-D was unable to induce callus formation. Auxins play an important role in inducing callus formation. The absence of auxin resulted in unformed callus in the explant (Table 2). According to Ma et al. (2018), the presence of auxin especially 2,4-D is essential in callus formation and callus proliferation at specific concentrations. The addition of cytokinin combined with auxins also has an important role because without cytokinins, the crown flower callus formation took longer time and has a low fresh weight. In addition, the presence of cytokinins in a culture medium positively induces callus when combined with auxin because they can encourage cell growth by promoting cell division and elongation (Hemmati et al., 2020).

Table 1. Effect of BAP in Murashige and Skoog medium on callus formation time of crown flower

BAP Concentration (mg/L)	Callus Formation Time (days)
0	10.60±6.32 ^b
0.5	6.13±3.52 ^a
1	6.80±4.06 ^a
1.5	7.06±4.23 ^a
2	7.20±4.83 ^a

Note: Numbers followed by the same letter in each treatment show no significant effect on DMRT at the 5% level ($P < 0.05$).

Table 2. Effect of 2,4-D in Murashige and Skoog medium on callus formation time of crown flower

2,4-D Concentration (mg/L)	Callus Formation Time (days)
0	No formation
0.5	8.53±3.70 ^a
1	9.00±2.62 ^a
1.5	9.13±2.70 ^a
2	11.13±3.81 ^b

Note: Numbers followed by the same letter in each treatment show no significant effect on DMRT at the 5% level ($P < 0.05$).

3.2 Callus fresh weight

The application of different growth regulators (BAP and 2,4-D) during callus induction resulted in variation of callus formation and morphologies. For instance, plant growth regulator concentration is particularly important in callus formation, proliferation, and regeneration (Ningtiyas et al., 2022). Table 3 shows that the application of BAP and 2,4-D combination on MS medium significantly affected the crown flower again fresh callus weight, generally. In contrast, the combination of 0.5 mg/L BAP and 0.5 mg/L 2,4-D (5.82 g) were not significantly different compared to 1 mg/L BAP and 1.5 mg/L 2,4-D (5.51 g) but significantly higher than other treatments.

Based on the results, the application of various treatments of BAP and 2,4-D resulted in various fresh callus weights. The highest fresh callus weight (5.82 g) was from the combination of 0.5 mg/L BAP and 0.5 mg/L 2,4-D. The interaction of auxin and cytokinin in the medium had a significant impact on callus formation. Unlike 2,4-D, the application of auxin without cytokinin was able to induce callus formation but in a small size and low fresh weight.

The application of BAP and 2,4-D could form callus in a shorter time (6 days after planting) and high fresh weight (5.82 g) at a concentration of 0.5 mg/L. In contrast, the application of BAP and 2,4-D at higher concentrations reduced the callus formation time and fresh weight. This was likely because the application of high auxin concentration to the medium inhibited the growth and development of callus (Kona et al., 2019). In the same way, fresh callus weight was affected by the time required for cell division, proliferation, and enlargement. In turn, a growth regulators have a role in encouraging growth and development, ranging from cell division to the development of specific tissues and organs (Mroue et al., 2018).

3.3 Callus morphology

Callus first appeared with the swelling of the explant and followed by the formation of a white callus. Then the callus further developed and turned green. Further results showed that the callus color change indicated a difference in the growth phase in the cells. The callus color changes with increasing of callus lifespan. The final changes were color change to brown and eventually the cessation of growth in the culture medium (Liu et al., 2022). The different colors in the crown flower callus during growth are shown in (Figure 1). Furthermore, the colors of the callus were compared with the standard color scale bar shown in Figure 2.

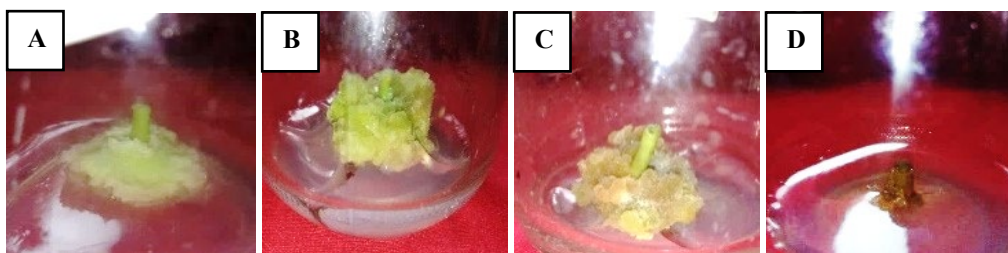


Figure 1. The color of crown flower callus, (A) greenish-white; (B) yellowish-green; (C) brownish-yellow; (D) brown

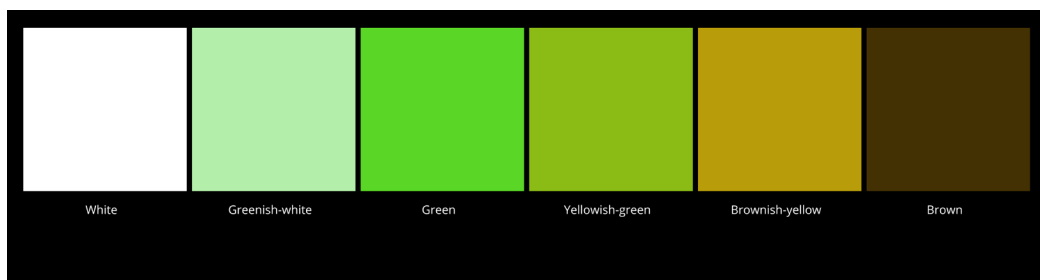


Figure 2. Color scale bar

Brown callus was found only in the medium supplemented with 2 mg/L 2,4-D concentration. In general, the application of a single and higher concentrations of 2,4-D caused the development of brown callus (Yaacob et al., 2018; Ashokhan et al., 2020).

The combination of BAP and 2,4-D significantly affected the fresh weight, color, and texture of the crown flower callus. As shown in Table 3, different concentrations of BAP and 2,4-D caused variations in callus formation, where higher concentrations generally produced dense callus with greenish-yellow to brownish color, while lower concentrations tended to form fragile callus. Morphological differences in callus texture are shown in Figure 3.

Table 3. The effect of the combination of BAP and 2,4-D on the average fresh weight and callus morphology of crown flower

BAP (mg/L)	2,4-D (mg/L)	Callus Fresh Weight (g)	Callus Color	Callus Texture
0	0	No formation	No formation	No formation
	0.5	0.50±0.08abc	Brownish-yellow	Friable
	1	0.30±0.20abc	Brownish-yellow	Friable
	1.5	0.20±0.11ab	Brownish-yellow	Friable
	2	0.40±0.38abc	Brown	Friable
0.5	0	No formation	No formation	No formation
	0.5	5.82±4.12h	Yellowish-green	Compact
	1	1.33±0.18a-d	Yellowish-green	Compact
	1.5	4.00±1.00e-h	Brownish-yellow	Friable
	2	2.00±0.98a-e	Brownish-yellow	Compact
1	0	No formation	No formation	No formation
	0.5	3.80±1.43d-h	Yellowish-green	Compact
	1	1.95±0.60a-e	Yellowish-green	Friable
	1.5	5.51±2.32h	Yellowish-green	Compact
	2	2.63±0.16b-g	Brownish-yellow	Compact
1.5	0	No formation	No formation	No formation
	0.5	4.76±1.47fgh	Brownish-yellow	Compact
	1	1.30±0.62abc	Brownish-yellow	Compact
	1.5	2.60±1.08b-g	Brownish-yellow	Compact
	2	2.74±1.27c-g	Brownish-yellow	Compact
2	0	No formation	No formation	No formation
	0.5	2.38±1.41a-f	Greenish-white	Compact
	1	2.32±0.30a-f	Greenish-white	Compact
	1.5	5.00±1.16gh	Yellowish-green	Compact
	2	2.70±2.41c-g	Greenish-white	Compact

Note: Numbers followed by the same letter in each treatment show no significant effect on DMRT at the 5% level ($P < 0.05$).

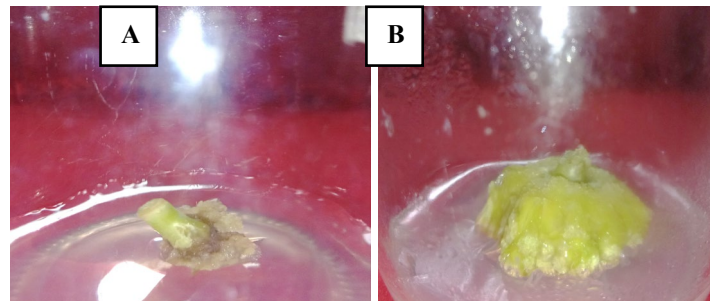


Figure 3. The texture of crown flower callus: (A) friable callus on treatment 0 mg/L BAP + 1 mg/L 2,4-D, (B) compact callus on treatment 2 mg/L BAP + 1.5 mg/L 2,4-D

The results showed that most of the calli had compact texture. Junairiah et al. (2021) revealed that the application of cytokinin as an exporter nutrient to the medium caused the compact texture. Moreover, the addition of sucrose to the medium caused turgor pressure in which variations of solution concentration allows nutrients and water entering the medium to the cell by osmosis. As a result, the cell wall becomes stiffer, causing the callus cells to compact. Eventually, metabolites were present in compact callus (Reyes-Martínez et al., 2019).

3.4 Total flavonoid content

One of the secondary metabolite compounds that are found in the crown flower plant is the flavonoids. Flavonoids are positively found in various plant components like flowers and leaves (Patil, 2020; Kemala et al., 2022). Flavonoids in plants have multiple benefits including antioxidants. Not all treatments were tested for their flavonoid content. Only 4 samples out of 25 treatments were tested. The selection of these 4 samples was based on the magnitude of the BAP and 2,4-D concentration combinations, namely low BAP with low 2,4-D, low BAP with high 2,4-D, high BAP with low 2,4-D, and high BAP with high 2,4-D. The combination of 0,5 mg/L BAP + 2 mg/L 2,4-D produced the highest total flavonoid content at 1.21% (Table 4) compared to the other 3 tested treatments with flavonoids from calli with yellowish-brown color (Table 3). Additionally, the application of growth regulators to the medium resulted in a higher flavonoid level than in field plants. Devi et al. (2022) stated that *in vitro* plants have a higher production of secondary metabolites due to the stimulating role of plant growth regulators, especially cytokinins. Talitha et al. (2022) added that the secondary metabolites in plants can change after tissue culture propagation with the addition of auxin and cytokinin.

Table 4 shows that 0.5 BAP in combination of 2 mg/L 2,4-D produced the highest flavonoid content by 1.21% but did not significantly differ from other treatments. The application of different concentrations of BAP and 2,4-D into the medium produced different levels of flavonoids. This research found that the flavonoid content of crown flower cultivated in the field (*in vivo*) was different to that of callus (*in vitro*). This study confirmed that the callus phase could produce flavonoids higher than field plant. A brownish callus indicated that there were phenol compounds in the callus. According to Al-Ramamneh et al. (2017), browning in callus arises due to oxidized phenol compounds. An adaptive response of callus cells under unfavorable conditions is to form an oxidative enzyme that can react with phenols to form quinones, which make the callus brown.

Table 4. Total flavonoid content of crown flower

Sample	Flavonoid Content (% w/w)
Field plant	1.08
0.5 mg/L BAP + 0.5 mg/L 2,4-D	0.90
0.5 mg/L BAP + 2 mg/L 2,4-D	1.21
2 mg/L BAP + 0.5 mg/L 2,4-D	1.00
2 mg/L BAP + 2 mg/L 2,4-D	0.96

3.5 Phytochemical compounds

Crown flower (*Calotropis gigantea* (Linn.) Aiton f.) is a plant that has various benefits but has not been widely cultivated and utilized. Crown flower contains flavonoids, alkaloids, phenols, tannins, and saponins (Chandrawat & Sharma., 2018; Khasanah et al., 2021). However, there has been no previous study conducted on the total levels of each of these compounds. Therefore, *in vitro* cultures can be an alternative for improving the extraction of phytochemical compounds (Nielsen et al., 2019). This study was conducted to identify phytochemical compounds from callus of crown flower through *in vitro* cultures.

Crown flower has the potential to be a productive medicinal plant characterized by the presence of various phytochemical compounds in the plant. According to Kumari et al. (2017) and Verma et al. (2021), the flower extract of crown flower plant analyzed with GC-MS contained tetrazolo (1,5-b) pyridazine, pyranone, 5-hydroxymethylfurfural, 2-methoxy-4-vinylphenol, folic acid, 6-acetyl- β -d-mannose, 1-dodecanol, 3,7,11-trimethyl-, and tetraacetyl-d-xylonic nitrile compounds. In the present study, the phytochemical compounds in crown flower callus were observed by gas chromatography-mass spectrometry. Consequently, the phytochemical compounds extracted from a fresh weight of 4.76 g of brownish-yellow color and compact texture callus were induced by treatment of 1.5 mg/L BAP and 0.5 mg/L 2,4-D (Table 3). GC-MS analysis was also carried out to determine the components of the phytochemical compounds of field grown plants (*in vivo*) as a comparison. The field grown plant parts identified were the roots, stems, and leaves of crown flower. Identification of the phytochemical compound of callus and field grown plant in *Calotropis gigantea* (Linn.) Aiton f. using GC-MS was carried out in this research. The callus of crown flower contained 3 major and 11 minor compounds, while plants grown in the field with plant parts of leaves, stems, and roots had 4 major compounds and 9 minor compounds based on percentage area (Figure 4).

Major compounds identified by the GC-MS method were found positive in field plants and calluses (Table 5). The major compounds of callus were tetracosamethylcyclododecasiloxane (23.95%), ethylbenzene (15.14%), and 1,3-dimethylbenzene (99.0%). Minor compounds were methyl isobutyl carbinol; methylbenzene; tert-butyl isopropyl ether; 1,2-dimethylbenzene; tetradecamethylcyclohepta-siloxane; propanoic acid, 2-methyl-,1-(1,1-dimethyl ethyl)-2-methyl-1,3-propanediyl ester; hexadecamethylcyclooctasiloxane; octadecamethyl-cyclononasiloxane; pentadecanoic acid; and icosamethylcyclodecasiloxane. Major compounds of crown flower field grown

plant were octadecamethylcyclonona-siloxane (19.28%), tetracosamethylcyclododecasiloxane (13.95%), pentadecanoic acid (9.93%), and ethylbenzene (9.43%). Ethylbenzene and 1,3-dimethylbenzene are groups of hydrocarbon compounds. Tetracosamethylcyclododecasiloxane and octadecamethylcyclo-nonasiloxane are siloxane compounds. Siloxane is a compound that composed of silicon atoms connected to oxygen atoms with a straight or cyclic chain. It is commonly used for coating and textile materials (Elzaabalawy & Meguid, 2020). Pentadecanoic acid is a class of fatty acids that plays a part as antioxidant, anticancer, anti-inflammatory, and antifungal properties (Chetoui et al., 2019). By contrast, the minor compounds were methyl isobutyl carbinol; methylbenzene; 1,3-dimethylbenzene; 1,2-dimethylbenzene; 1,2-benzenedicarboxylic acid, diethyl ester; octadecamethylcyclononasiloxane; icosamethylcyclo-decasiloxane; and 2-hydroxyclopentadecanone.

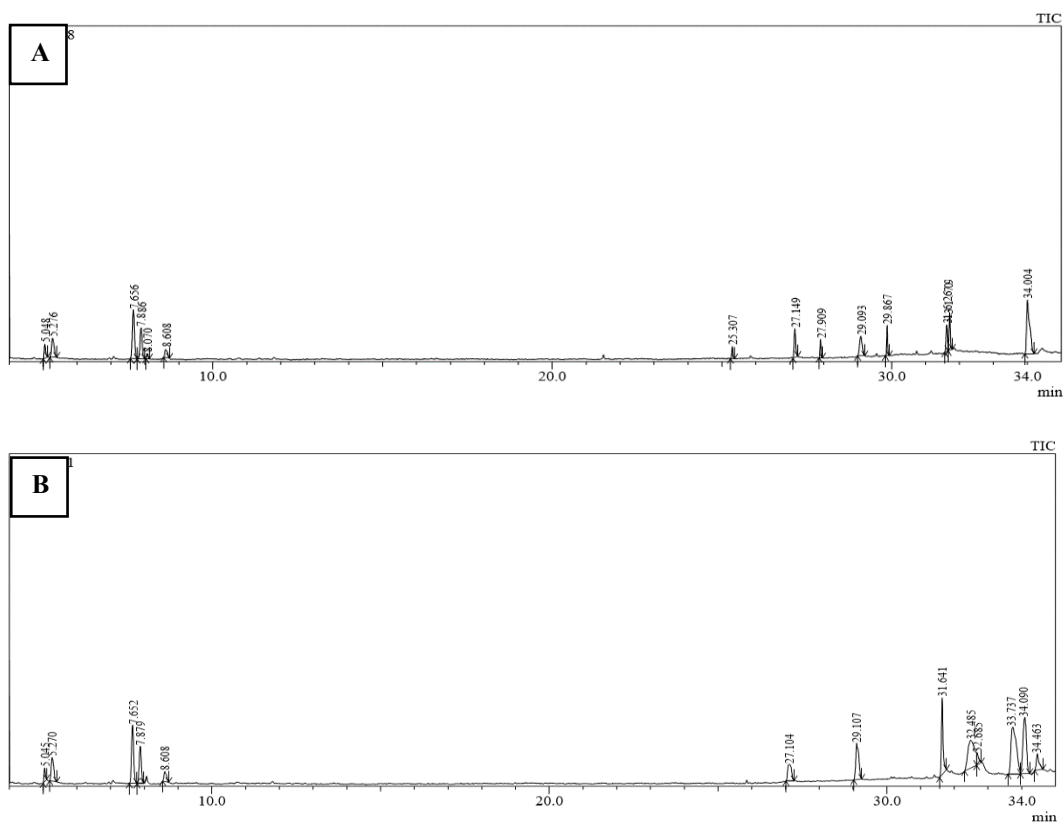
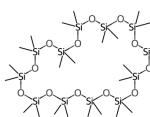
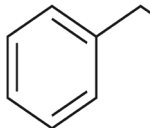
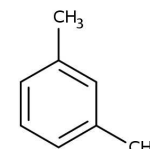
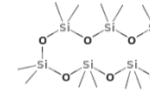


Figure 4. Representative chromatograms of crown flower, (A) callus (*in vitro*) and (B) field plants (*in vivo*)

Table 5. Major compounds in field grown plants and calli of crown flower identified by GC-MS

No.	Name	Structure	Formula	Group	Field Grown Plant	Callus
1	Tetracosamethylcyclododecasiloxane		$C_{24}H_{72}O_{12}Si_{12}$	Siloxane	+	+
2	Ethylbenzene		C_8H_{10}	Hydrocarbons	+	+
3	1,3-Dimethylbenzene		C_8H_{10}	Hydrocarbons	-	+
4	Octadecamethylcyclonona-siloxane		$C_{18}H_{54}O_9Si_9$	Siloxane	+	-
5	Pentadecanoic acid		$C_{15}H_{30}O_2$	Fatty acid	+	-

4. Conclusions

In the present study, callus was induced from stem explant of *Calotropis gigantea* (Linn.) Aiton f. species. The application of 0.5 mg/L BAP and 0.5 mg/L 2,4-D produced the callus with the fastest callus formation time (6 days after planting), the highest fresh weight (5.82 g), and compact callus texture. At the same time, identification of phytochemical compounds using callus induction with the application of PGR was able to produce different compounds that were not found in field plants (*in vivo*).

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6. Authors' Contributions

The authors declare that we have no conflict of interest.

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7. Conflicts of Interest

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