

Research Article

Callus induction and secondary metabolite profile from *Elephantopus scaber* L.

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ABSTRACT

Elephantopus scaber L. is a plant that has potential as traditional medicine. Callus induction and production of secondary metabolite content can be increased by culture callus using plant growth regulators. This study was purposed to investigate the effect of IBA and kinetin concentration on the induction and secondary metabolite profile of callus from *E. scaber* L. leaves. Leaves explant of *E. scaber* L. were cultured on MS medium with various combination concentrations of IBA and kinetin for 6 weeks and then callus was extracted using methanol. Secondary metabolite content from the resulting extract was analyzed using the phytochemical screening method. The result showed that the treatment of IBA 2.0 mg/L and kinetin 1.0 mg/L and treatment of IBA 2.0 mg/L and kinetin 2.5 mg/L are the fastest combination concentration to induce callus at 5.33 ± 0.577 days. Treatment of IBA 2.0 mg/L and kinetin 2.5 mg/L produced callus with the highest fresh weight and dry weight at 0.7016 ± 0.0588 grams and 0.0766 ± 0.0062 grams, respectively. The morphology of calluses grown during this study was compact with various colors appearance, such as light green, brownish green, and brown. Secondary metabolite content of methanol extract of callus *E. scaber* L. are flavonoids, alkaloids, terpenoids, and saponins.

Keywords: Callus, *Elephantopus scaber* L., IBA, kinetin, secondary metabolites

INTRODUCTION

Elephantopus scaber L. is a type of plant that can be easily found in Indonesia (Nurtamin et al. 2018). This plant is also potentially used as a traditional medicine to treat influenza, headaches, cough, fever, diarrhea, jaundice, hepatitis, laryngitis, anemia, and fluor albus (Arisandi & Andriani 2006; Wang et al. 2014). In previous studies, showed that *E. scaber* L. can reduce LDL (Low Density Lipoprotein) level and also promising hepatoprotection activity in mice (Daisy et al. 2007; Ho et al. 2012; Nasri 2012; Puspita 2004; Sankar et al. 2001; Sulastru 2008). The leaves of *E. scaber* L. containing terpenoids, flavonoids, saponins, and tannins which function as antibacterial and antiinflammatory (Bigam et al. 2003; Grayson 2000; Lim et al. 2006; Nurtamin et al. 2018).

There are an increasing use and unrestricted exploitation of natural population for medicinal purposes due to the high medicinal value of this plant, there has been causing an increase in the use (Abraham & Thomas

2015). Poor seed viability and early death of young seedlings in these natural environmental conditions can be inhibiting factors for the propagation of this species (Parashurama et al. 2013). Therefore, alternative propagation methods are necessary for the rapid multiplication and conservation of this plant. One of them is the technique of plant tissue culture. This method has been recognized as an alternative for plant propagation, production, characterization, elite clones, and secondary metabolites in a limited period (Abraham et al. 2010; Cheruvathur & Thomas 2014; Kumar & Thomas 2012; Murthy et al. 2014).

Plant propagation and an efficient callus induction have been standardized for an ethnomedicinal plant (Abraham & Thomas 2015). In previous studies was reported that callus of *E. scaber* L. can be induced by adding 1.5 mg L⁻¹ 2,4-Dichlorophenoxy acetic acid and 1.5 mg L⁻¹ kinetin, or also can be induced by adding 5.0 µM 2,4- Dichlorophenoxy acetic acid and 0.5 µM kinetin (Abraham & Thomas 2015; Rout & Sahoo 2013). In this study, callus induction of *E. scaber* L. is using the addition of indole butyric acid/IBA (auxin) and kinetin (cytokinins) as plant growth regulators. The purpose of this study was to investigate the effect of IBA and kinetin concentration on the induction and secondary metabolite profile of callus from *E. scaber* L. leaves.

MATERIALS AND METHODS

Materials

In this study, the plant materials used were the young leaves of *Elephantopus scaber* L. obtained from the Bratang flower market, Surabaya, Indonesia. Which had been identified in the plant physiology laboratory, Universitas Airlangga. The chemical materials for Murashige and Skoog (MS) medium, chlorox, alcohol 70%, IBA (PhytoTechnology Laboratories®, US), kinetin (PhytoTechnology Laboratories®, US), methanol, filter paper, distilled water, pH indicator strips, liquid detergent, aluminium foil, KOH 1 N, and HCl 1 N.

Callus induction

Preparation of MS medium with addition of IBA and kinetin

MS medium was prepared by adding macronutrient stock, 1 mL micronutrient stock, 5 mL iron stock, 4 mL vitamin in 500 mL distilled water was homogenized. Then, added 100 mg myoinositol and 30 grams of sucrose, after all the materials have dissolved then added distilled water to 1 L and the concentration of IBA and kinetin according to the specified treatment was added (Table 1). Furthermore, the acidity of the medium solution was adjusted to the range 5.6-5.8 using pH indicator strips, then 8 grams of agar powder was added to condense the solution, then it was covered with aluminium foil and labelled. The medium was sterilized using an autoclave at a pressure of 1.2 atm and a temperature of 121°C for 15 minutes (Manuhara 2014).

Table 1. Combination concentration of IBA and kinetin applied to each treatment group.

Treatment (mg/L)	Code
IBA 0.0 + Kinetin 0.0	I _{0,0} K _{0,0}
IBA 0.5 + Kinetin 0.5	I _{0,5} K _{0,5}
IBA 0.5 + Kinetin 1.0	I _{0,5} K _{1,0}
IBA 0.5 + Kinetin 1.5	I _{0,5} K _{1,5}
IBA 1.5 + Kinetin 0.5	I _{1,5} K _{0,5}

Table 1. Contd.

Treatment (mg/L)	Code
IBA 1.5 + Kinetin 1.0	I _{1.5} K _{1.0}
IBA 1.5 + Kinetin 1.5	I _{1.5} K _{1.5}
IBA 2.0 + Kinetin 0.5	I _{2.0} K _{0.5}
IBA 2.0 + Kinetin 1.0	I _{2.0} K _{1.0}
IBA 2.0 + Kinetin 2.5	I _{2.0} K _{2.5}

Planting leaves explant of *E. scaber* L.

E. scaber L. leaves were washed with liquid detergent for five minutes then rinsed three times using tap water, then it was soaked in 70% alcohol for 5 minutes and rinsed using sterile distilled water three times, then soaked in 20% chlorox for 10 minutes and rinsed using sterile distilled water again three times. Then the leaves explant was cut ($\pm 1 \text{ cm}^2$) and planted on MS medium for 6 weeks of culture periods, at a regulated temperature of $25 \pm 2^\circ\text{C}$ and lighting 3000-3500 lux for 24 hours ([Manuhara 2014](#)).

Analysis of secondary metabolites content

Callus extraction

The dried callus was weighed and recorded, then mashed into powder. Then 5 mL of methanol was added to the vial containing the callus powder to be macerated for 24 hours. Then the extract was filtered using filter paper. The extract was concentrated until the remaining volume of 2 mL. Extraction was also carried out on *E. scaber* L. leaves that had dried with the same extraction method. This is necessary to compare the compounds contained in the mother plant and callus of *E. scaber* L.

Identification of secondary metabolites content using phytochemical screening

Flavonoids compounds test

The methanol extract from callus *E. Scaber* L. has put in as much as 1 mL into a test tube, then 0.5 mL concentrated HCl and 4 pieces of Mg bands were added. The appearance of red, orange, or green indicates the presence of flavonoids in the sample extract ([Kristanti et al. 2008](#)).

Alkaloids compounds test

1 mL of the methanol extract from callus *E. scaber* L., 3 drops of Mayer's reagent, and 3 drops of chloroform were added to 3 test tubes and then homogenized until blended. The positive alkaloid content was indicated by the presence of white sediment in a test tube ([Darwis 2000](#)).

Terpenoids and steroids compounds test

3 drops of sample methanol extract of *E. scaber* L. callus were put on a spot test board. Then added 3 drops of anhydrous acetate (Ac₂O) and a drop of concentrated sulphuric acid (H₂SO₄) into it. After well homogenized the terpenoids content was marked by red or brown colors, while steroids were indicated by blue color ([Kristanti et al. 2008](#)).

Saponins compounds test

The Forth method was used to analyze the saponins content by putting 1 mL methanol extract into the test tube then added 5 ml hot water and the test tube was shaken until 60 seconds. The extracted sample was positive for saponins if it showed foam that had formed in the test tube for 30 seconds ([Darwis 2000](#)).

RESULTS AND DISCUSSION

Based on the result in this study, shown that callus can be induced in all treatments except control (Table 2). The table shows that the fastest callus induction time period was 5.33 ± 0.577 days in combination with IBA 2.0 mg/L + kinetin 1.0 mg/L and IBA 2.0 mg/L + kinetin 2.5 mg/L. Whereas the slowest callus induction period was 8.0 ± 0 days in combination with IBA 1.5 mg/L + kinetin 1.5 mg/L. Explants planted on MS medium without the addition of growth regulators (as a control) did not form a callus until the end of the observation time at the sixth weeks after planting. The percentage of explant forming callus in each treatment was 100%.

Table 2. Mean callus induction time and percentage of explant forming callus of *E. scaber* L. leaves from various combination concentration of IBA and kinetin treatments.

Treatment (mg/L)	Mean callus induction time (days)	Percentage of explant forming callus (%)
I _{0.0} K _{0.0}	0 ± 0^a	0
I _{0.5} K _{0.5}	6.66 ± 0.577^{bc}	100
I _{0.5} K _{1.0}	7.33 ± 0.577^{cd}	100
I _{0.5} K _{1.5}	7.66 ± 0.577^{cd}	100
I _{1.5} K _{0.5}	6.66 ± 0.577^{bc}	100
I _{1.5} K _{1.0}	5.66 ± 0.577^b	100
I _{1.5} K _{1.5}	8.0 ± 0^{cd}	100
I _{2.0} K _{0.5}	6.66 ± 0.577^{bc}	100
I _{2.0} K _{1.0}	5.33 ± 0.577^b	100
I _{2.0} K _{2.5}	5.33 ± 0.577^b	100

*) Number followed by different letters indicating significant difference based on Mann-Whitney test at 5% significant level.

Callus culture is used to obtain callus from explants grown in a controlled environment. The formation of callus is to induce certain plant parts by providing growth regulators. Callus induction is caused by cuts or explant slices in response to hormones both exogenously and endogenously. The selection of growth regulators is one of the important factors in determining the formation of plant callus. IBA has chemical properties and more stable mobility in plants, besides the longer working power. This is causes that IBA more successful to be used. In addition, the cytokinins play a role in stimulating cell division and callus proliferation. Kinetin added to culture medium increases the rate of protein synthesis so that it encourages cell enlargement and division (mitosis). Interaction between the role of auxin and cytokinin which are both added to the medium with the right combination will cause the explants to experience callus induction ([Rosyidah et al. 2014](#)).

Table 3. Mean fresh weight and dry weight of *E. scaber* L. callus.

Treatment (mg/L)	Mean	
	Fresh weight (grams)	Dry weight (grams)
I _{0.0} K _{0.0}	0 ± 0^a	0 ± 0^a
I _{0.5} K _{0.5}	0.1850 ± 0.0103^b	0.0288 ± 0.0046^b
I _{0.5} K _{1.0}	0.2627 ± 0.0117^b	0.0525 ± 0.0027^d
I _{0.5} K _{1.5}	0.2473 ± 0.0069^b	0.0400 ± 0.0088^{bcd}
I _{1.5} K _{0.5}	0.2652 ± 0.0481^b	0.0416 ± 0.0141^{bcd}
I _{1.5} K _{1.0}	0.2696 ± 0.0222^b	0.0431 ± 0.0055^{cd}
I _{1.5} K _{1.5}	0.2332 ± 0.0217^b	0.0362 ± 0.0028^{bc}
I _{2.0} K _{0.5}	0.2215 ± 0.0219^b	0.0352 ± 0.0045^{bc}

Table 3. Contd.

Treatment (mg/L)	Mean	
	Fresh weight (grams)	Dry weight (grams)
I _{2.0} K _{1.0}	0.3215 ± 0.0108 ^b	0.0440 ± 0.0092 ^{cd}
I _{2.0} K _{2.5}	0.7016 ± 0.0588 ^{bc}	0.0776 ± 0.0062 ^c

*) Number followed by different letters indicating significant difference based on Mann-Whitney test at 5% significant level.

The addition of a combination concentration of IBA and kinetin growth regulators on fresh weight and dry weight of callus in each treatment was different. The highest mean fresh weight of callus was found in the treatment of IBA 2.0 mg/L + kinetin 2.5 mg/L with 0.7016 ± 0.0588 grams, this treatment also had the highest average dry weight with a weight of 0.0776 ± 0.0062 grams. While the treatment of IBA 0.5 mg/L + kinetin 0.5 mg/L had the lowest fresh weight and dry weight of callus with 0.1850 ± 0.0103 grams and 0.0288 ± 0.0046 grams, respectively. Table 3 shows that there is a tendency to increase the average fresh weight and dry weight of callus along with an increase in the concentration of growth regulators IBA and kinetin.

In this study, the addition of a combination concentration of IBA 2.0 mg/L + kinetin 2.5 mg/L growth regulators produced the highest mean fresh weight and dry weight, 0.7016 ± 0.0588 grams and 0.0776 ± 0.0062 grams, respectively. This is presumably because at these concentrations the endogenous auxin and cytokinin conditions become balanced so that it can produce a good amount of callus mass formation ([George & Sherrington 2007](#)). The results of fresh weight and dry weight of callus were obtained from a combination of the lower concentration of IBA compared to kinetin. This is in line with previous studies that produce fresh weight and dry weight at a ratio of IBA combination of 1 mg/L + kinetin 1.5 mg/L with fresh weight 0.4688 grams and IBA concentration 1 mg/L + kinetin 2 mg/L with dry weight 0.0895 grams ([Rohmatin 2014](#)).

Table 4. Callus morphology of *E. scaber* L. at 6th weeks of the culture period (bar = 1 cm).

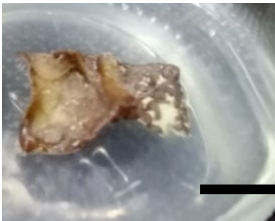









No.	Treatment (mg/L)	Figure	Description of callus morphology
1.	I _{0.0} K _{0.0}		The leaves explant planted on MS medium was colored brown and failed to induce callus
2.	I _{0.5} K _{0.5}		The brown callus has compact texture
3.	I _{0.5} K _{1.0}		The brown callus has compact texture

Table 4. Contd.

No.	Treatment (mg/L)	Figure	Description of callus morphology
4.	I _{0.5} K _{1.5}		The brown callus has compact texture
5.	I _{1.5} K _{0.5}		The brownish green callus has compact texture
6.	I _{1.5} K _{1.0}		The brownish green callus has compact texture
7.	I _{1.5} K _{1.5}		The light green callus has compact texture
8.	I _{2.0} K _{0.5}		The brownish green callus has compact texture
9.	I _{2.0} K _{1.0}		The brownish green callus has compact texture
10.	I _{2.0} K _{2.5}		The light green callus has compact texture

All treatments produced callus with a compact callus texture while the control in this study did not experience growth so that no callus was formed. The callus color of the *E. scaber* L. explants observed in each treatment was brownish, brownish green, and light green. Observation of callus morphology at the sixth weeks after culture was presented in Table 4. For

efficient callus induction and plant regeneration, callus would be developed in light conditions rather than in the dark. In previous studies, callus induction on *Nicotina tabacum* L., *Stelechocarpus burabol*, and *Brassica napus* L. which showed the best callus induced in the light conditions, while explants grown in the dark did not show optimum results and some of the treatment was not even callus induced (Siddique & Islam 2015; Habibah et al. 2018; Afshari et al. 2011). The abilities of photosynthesis increased plant morphological features gradually in *Ginkgo biloba* L. (Yang & Chen 2014). According to their reports and claimed that the callus developed in light condition might carry photosynthetic pigments that influenced the growth and development of callus.

The compact callus texture is an effect of cytokinins which play a role in nutrient transport. The cytokinin transport system from the basal to the apex will carry water and nutrients through the transport vessels and influence the osmotic potential in the cells. The addition of sucrose will flow through the phloem vessels and causes turgor pressure. This pressure arises due to differences in solution concentration so that water and nutrients such as sucrose from the medium will enter the cell through osmosis. This will make the cell walls more rigid so that the callus cells will become compact. Callus *E. scaber* L. was induced at different lengths of time, but each callus in each treatment had the same characteristic, the initial color of the light green callus was shown in each early induction. As the culture period increases until the sixth weeks the callus appears to have a variety of colors. These color changes indicate changes in the growth phase of cells and cell regeneration power that is influenced by low nutrient intake during culture and the presence of explant tissue injury that triggers the callus to experience stress so that the callus is brown, while the green color of the callus indicates the formation of chlorophyll in the tissue (Payghamzadeh & Kazemitabar 2011).

Based on Table 5 it can be seen that the *E. scaber* L. leaves extract which was used as a positive control, produces secondary metabolites of flavonoids, alkaloids, terpenoids, and saponins. In the flavonoid test, all treatments are positive contained flavonoids except for the treatment of IBA 0.5 mg/L + kinetin 0.5 mg/L. This is due to the provision of kinetin with low concentrations that cause flavonoid compounds cannot be identified by using phytochemical screening methods. In the alkaloids, saponins,

Table 5. Phytochemical screening of *E. scaber* L. callus extract supplemented with combination concentration of IBA and kinetin plant growth regulator.

Treatment (mg/L)	The presence of secondary metabolites from the callus extract				
	Flavonoids	Alkaloids	Steroids	Terpenoids	Saponins
Leaves extract of <i>E. scaber</i> L.	+	+	-	+	+
I _{0,0} K _{0,0}	-	-	-	-	-
I _{0,5} K _{0,5}	-	+	-	+	+
I _{0,5} K _{1,0}	+	+	-	+	+
I _{0,5} K _{1,5}	+	+	-	+	+
I _{1,5} K _{0,5}	+	+	-	+	+
I _{1,5} K _{1,0}	+	+	-	+	+
I _{1,5} K _{1,5}	+	+	-	+	+
I _{2,0} K _{0,5}	+	+	-	+	+
I _{2,0} K _{1,0}	+	+	-	+	+
I _{2,0} K _{2,5}	+	+	-	+	+

terpenoids, and steroids tests all treatments showed positive results containing these compounds. Kinetin is one of the growth regulator types from cytokinin. In previous studies, authors stated that cytokinin and low auxin concentration have a possible synergistic interaction on accumulation and distribution of recorded secondary metabolites, but at low kinetin concentration not shown an optimum accumulation and distribution of recorded secondary metabolites ([Radić et al. 2016](#)). The concentration of IBA and kinetin in this study also affected to secondary metabolites profile in the methanol extract of *E. scaber* L. callus. The accumulation of secondary metabolites contained in *E. scaber* L. callus was influenced by the increase in the biosynthetic pathway process in each type of secondary metabolites therein. IBA and kinetin added from outside the cell (exogenous) will have a more effective impact if they can be balanced with the levels of auxin and cytokinin hormones in the explant cells (endogenous). In previous studies also stated that the production of secondary metabolites in vitro will significantly increase if added exogenous of various types and concentrations of auxin and cytokinins ([Radić et al. 2016](#); [Jamwal et al. 2018](#); [Jain et al. 2012](#)).

According to Harborne ([2006](#)), terpenes are generally fat-soluble compounds. Then based on the level of solubility, in testing compounds, terpenes are withdrawn with ether. But in this study, the withdrawal of terpenes was carried out using methanol as a solvent. This is because methanol is a universal solvent so that it can dissolve polar and nonpolar compounds. Terpenoids have antibacterial activity, inhibit cancer cells, inhibition of cholesterol synthesis, anti-inflammatory, menstrual disorders, skin disorders, liver damage, and malaria ([Rumondang 2013](#)). In previous studies, *E. scaber* L. leaves contained saponins, terpenoids, polyphenols, flavonoids luteolin-7-glucoside, epipriedelinol, lupeol, stigmaserin, lupeol acetate, deoxyelephantopin, isodeoxyelephantopin compounds ([Asmaliyah et al. 2010](#); [Yuniarti 2008](#)). These leaves also contain secondary metabolites of terpenoids and flavonoids which act as antibacterial. In previous study, showed that terpenoid compounds have antibacterial activity, there are monoterpenoid linalool, phytol, hardwicklic acid diterpenoid, triterpenoid glycoside, and triterpenoid saponin ([Grayson 2000](#); [Lim et al. 2006](#); [Bigham et al. 2003](#)).

CONCLUSION

The combination of IBA and kinetin plant growth regulators has a significant effect on the induction time, the percentage of explants forming callus, fresh weight, dry weight, morphology, texture, and secondary metabolite profile of *E. scaber* L. leaves callus. The optimum combination treatment is the concentration IBA 2.0 mg/L and kinetin 2.5 mg/L with a mean of fresh weight callus at 0.7016 ± 0.0588 grams and dry weight callus at 0.0776 ± 0.0062 grams. *E. scaber* L. callus extract contains secondary metabolites such as flavonoids, alkaloids, terpenoids, and saponins.

AUTHORS CONTRIBUTION

J.J. designed the research and supervised all the process, D.A.W. collected and analyzed the data, E.S.W.U. designed the research and supervised the data, and N.I.Z. analyzed the data and wrote the manuscript.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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