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*by Ni'matuzahroh Ni'matuzahroh*

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## Biomass and terpenoids profile of callus extract of *Piper betle* L. var. Nigra with abiotic elicitor cobalt (II) chloride

Junairiah<sup>a\*</sup>, Nabilah Istighfari Zuraidassanaaz<sup>a</sup>, Y. Sri Wulan Manuhara<sup>a</sup>, Ni'matuzahroh<sup>a</sup>, Lilis Sulistyorini<sup>b</sup>

<sup>a</sup>Departement of Biology, Faculty of Science and Technology, Universitas Airlangga, Surabaya, Indonesia, 60115

<sup>b</sup>Faculty of Public Health, Universitas Airlangga, Surabaya, Indonesia, 60115

### Abstract

Black betel (*Piper betle* L. var. Nigra) is traditional medicinal plant potentially produce secondary metabolites. Its secondary metabolite content can be promoted using callus culture with abiotic elicitor. This study was aimed to determine the effect of cobalt (II) chloride (CoCl<sub>2</sub>) concentration towards biomass and terpenoids profile of leaf callus of *Piper betle* L. var. Nigra. Leaf explants of *Piper betle* L. var. Nigra were cultured in solid Murashige and Skoog (MS) medium supplemented with growth regulators 2,4-D (0.5 mg/L) and BAP (2.0 mg/L) to induce callus formation. Elicitation was performed after callus reached five weeks of age by performing subculture on MS medium added with CoCl<sub>2</sub> (0.5 mg/L; 1.0 mg/L; 2.5 mg/L). Calli of *Piper betle* L. var. Nigra were harvested after one, two, or three weeks before they were extracted using methanol and analyzed of its terpenoids content using Gas Chromatography-Mass Spectrometry (GC-MS) method. Results showed that calli had compact and crumb texture with green, white, brown, and white color. Callus added 1.0 mg/L CoCl<sub>2</sub> at two weeks age produce terpenoids at 5.95%. Callus with 1.0 mg/L CoCl<sub>2</sub> at three weeks of age produced higher fresh and dry weight at 916.4±162.3 mg and 93.3±11.7 mg respectively. Callus supplemented with 1.0 mg/L CoCl<sub>2</sub> at two weeks of age had higher terpenoids percentage compared to control. Two of the highest terpenoid types found in the callus of black betel leaf explant were 1,2-epoxy-1-vinylcyclododecene and hexadecanoic acid.

\* Corresponding author:

alip.jun1@gmail.com

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## 1. Introduction

One of the plants which have potential as secondary metabolites-producing medicinal plant is black betel (*Piper betle* L. var. Nigra). Secondary metabolites content of black betel including alkaloids, flavonoids, saponins, tannins,

steroids, triterpenoids, and polyphenolates [1]. Terpenoids are group of secondary metabolites with the highest number of compound types ( $\pm 25,000$  types) compared to alkaloids ( $\pm 12,000$  types) and phenols ( $\pm 8,000$  types). Due to its high number of types, terpenoids have high potential for utilization in pharmacology and industry [2–5]. Terpenoids are useful for fragrance and coloring additives, insecticides in farming, and medicine industry [6]. Several studies stated that terpenoids were able to combat various diseases, such as cancer, malaria, and HIV [7–9]. Essential oil in betel leaves had antibacterial activity against *Streptococcus mutans*, *Streptococcus sanguis*, and *Actinomyces viscosus* which caused periodontal diseases, and *Streptococcus viridans* and *Staphylococcus aureus* which caused respiratory tract disease in human [10]. In addition, ethanol extract of black betel leaves could also inhibit the growth of *Candida albicans* [11]. Based on these, more intensive effort is needed using the right alternative technique to fulfill demand of plant raw materials to produce secondary metabolites, one of them via callus culture [12–15]

Callus culture is one of the initial effective biotechnology methods that can suppress plant exploitation in its original habitat, produce biomass in large scale, and accumulate secondary metabolites component [16,17]. Resulting secondary metabolites from optimum callus culture can be increased using elicitation strategy, which is based on natural phenomenon of host cells defense mechanism against pathogen by producing secondary metabolites [18–21]. The quality and type of secondary metabolite compounds is affected by environmental, physical, pathogenic, and insect irritant factors entering plant cells which affecting biosynthesis of plant secondary metabolites [22]. Secondary metabolites commonly produce in plant cells as a response from various biotic and abiotic environmental stresses, known as elicitor [23–25]. Elicitor is a signal inducing stress response in defense mechanism of plant cells [26]. Plants will respond by inducing certain changes that cause stress and trigger a series of morphological, physiological, biochemical, and molecular alteration in plants [27].

Studies using abiotic elicitors had been conducted extensively, such as in leaf callus of *Cordia myxa* L. which showed increase of robinin content when 1 mg/L  $\text{CoCl}_2$  was added into solid medium [28]. In-vitro propagation of *Phoenix dactylifera* L. cv. Ashgar with  $\text{CoCl}_2$  addition at 0.5  $\mu\text{M}$  could increase phenols contained in the callus [29].

Abiotic elicitor used in the current study was  $\text{CoCl}_2$  (Cobalt (II) Chloride), one of essential micronutrients that can activate ATPase enzyme using  $\text{Co}^{2+}$  ions during secondary metabolites production. The addition of  $\text{CoCl}_2$  in high level also caused plant cells to produce secondary metabolites as response of defense mechanism against its toxicity [28,30]. Abiotic elicitor was added to MS medium separately to compare the effect of elicitor concentration in producing biomass and terpenoids profile of black betel leaf callus. Related study on the effect of different  $\text{CoCl}_2$  elicitor concentrations on biomass production and terpenoids profile of black betel leaf callus has never been conducted previously. This study was aimed to determine the effect of  $\text{CoCl}_2$  elicitor on biomass and terpenoids profile of black betel leaf callus.

## 2. Methods

### 2.1. Materials

Materials used in this study were black betel leaves (*Piper betle* L. var. Nigra) obtained from Kayon Flower Market, Surabaya, Murashige and Skoog medium, chlorox, 70% alcohol, 2,4-Dichlorophenoxyacetic acid (2,4-D), Benzylaminopurine (BAP),  $\text{CoCl}_2$ , distilled water, methanol, filter paper, aluminum foil, liquid detergent, spiritus, pH indicator strips, 1 N KOH, 1 N HCl.

### 2.2. Black betel callus induction

#### 1. Explants preparation

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Explants used were meristematic black betel (*Piper betle* L. var. Nigra) leaves, taken from third and fourth leaves from tip of plants.

## 2. Preparation of MS medium added with growth regulators

About 1 L MS medium was prepared with macronutrient stock (10 mL of macro I, macro II, and macro III respectively), 1 mL micronutrient stock, 5 mL iron, 4 mL vitamin, 0.5 mg/L 2,4-D and 2 mg/L BAP growth regulators which were homogenized in 500 mL distilled water one by one. Then, myoinositol and sucrose were added into mixture before distilled water was added until total volume of 1 L and mixture was properly homogenized. Acidity of solution was recorded using pH indicator and adjusted accordingly until pH was 5.5-5.6. Agar powder was added to solidify solution, then medium was placed into 1000 mL Erlenmeyer glass, covered with aluminum foil, and labelled accordingly. Medium was sterilized using autoclave at pressure of 1.2 atm and temperature of 121°C for 15 minutes (Manuhara, 2014).

## 3. Black betel explants planting

Black betel leaves were washed with liquid detergent for five minutes and rinsed three times using tap water, before being submerged in 70% alcohol for 6 minutes. Explants were rewashed three times using sterile distilled water, then soaked in 20% chlorox for 10 minutes. Leaves were then cut into  $\pm 1$  cm<sup>2</sup> pieces and planted on MS medium, then cultured for five weeks in temperature of 25 $\pm$ 2°C, lighting of 3000-3500 lux for 24 hours.

### 2.3. Elicitation using CoCl<sub>2</sub>

#### 1. Preparation of CoCl<sub>2</sub> stock solution

Stock solution of CoCl<sub>2</sub> was prepared by dissolving 0.1 g CoCl<sub>2</sub> into 50 mL distilled water, then it was homogenized and added distilled water up to final volume of 100 mL. Stock was stored in refrigerator. Preparation of MS medium with addition of CoCl<sub>2</sub> at various concentration

6  
CoCl<sub>2</sub> was added into 1 L MS medium at various concentration of 0.5 mg/L, 1.0 mg/L, and 2.5 mg/L respectively ( $\pm 20$  mL/bottle). Bottle mouth was covered with aluminum foil and labelled accordingly. Medium filled into bottle was sterilized using autoclave for 15 minutes at 121°C and 1.2 atm, then stored in sterile room. Abiotic and biotic elicitors used in the current study were applied into medium separately.

#### 2. Elicitation of black betel leaf callus

Callus from black betel leaf was moved onto MS medium contained 0.5 g CoCl<sub>2</sub> at five weeks of age. Callus was harvested after one, two, or three weeks culture period (Ravi *et al.*, 2011).

#### 3. Black betel callus harvest

Harvested callus was weighed its fresh weight, dried in oven at 60°C for 72 hours, then weighted its constant dry weight using analytical balance. Dried callus was stored and labelled accordingly for then to be extracted.

### 2.4. Analysis of terpenoids content profile

#### 1. Terpenoids extraction

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Dried callus was weighed at about 0.5 g, then crushed into powder using mortar. Powder was macerated by adding 5 mL methanol. Extraction was performed on water heater at 60°C for 5 minutes, then solution was filtered using filter paper. Extract was concentrated until volume left was 2 mL. Extraction was also performed on dried black betel leaves with the same method as callus extraction to compare compounds contained in mother plant and callus.

#### 2. Analysis of terpenoids content profile

About 1  $\mu\text{L}$  sample from resulting callus extraction of each treatment was taken and injected into column type HP-5 MS (60 m x 200  $\mu\text{m}$  x 0.33  $\mu\text{m}$ ) to be analyzed its content compounds and areal percentage (%) of terpenoids content using GC-MS Agilent Technologies 7890A and Willy Mass Spectral Data Base. Oven temperature was set at 40<sup>o</sup>C for 1 minute, then raised to 160<sup>o</sup>C for 1 minute, and increased for second time to 270<sup>o</sup>C for 5 minutes. Detector temperature was set at 300<sup>o</sup>C and injector at 230<sup>o</sup>C. Running time of each test extract was  $\pm$ 41 minutes [31].

Result of GC-MS chromatogram showed peaks indicating generally identified compounds. Compounds then categorized into terpenoids group by comparing compound name and molecular formula to secondary data from *Terpenoid Library List* based on its chemical structure and *U.S National library of medicine*.

### 3. Results and Discussion

Black betel callus given abiotic elicitor  $\text{CoCl}_2$  at 0.5 mg/L, 1.0 mg/L and 2.5 mg/L concentration showed varying fresh and dry weight. Analysis indicated significant difference of mean fresh weight between control callus (1066.4 $\pm$ 45.3 mg) and the three  $\text{CoCl}_2$  treatment with recorded fresh weight of 916.4 $\pm$ 162.3 mg (1.0 mg/L  $\text{CoCl}_2$ ), 772.7 $\pm$ 104.6 mg (0.5 mg/L  $\text{CoCl}_2$ ), and 804.4 $\pm$ 150.8 mg (2.5 mg/L  $\text{CoCl}_2$ ) respectively, at the same age of three weeks. In the other hand, mean dry weight of callus at three weeks of age showed no difference between control (112.0 $\pm$ 20.7 mg) and 1.0 mg/L  $\text{CoCl}_2$  (93.3 $\pm$ 11.7 mg), but significant difference was found in 0.5 mg/L  $\text{CoCl}_2$  (77.4 $\pm$ 7.7 mg) and 2.5 mg/L  $\text{CoCl}_2$  (84.6 $\pm$ 17.3 mg) (Table 1).

All callus given elicitor  $\text{CoCl}_2$  showed lower fresh and dry weight compared to control at three weeks of age. Control callus after being subcultured had the highest fresh and dry weight with significant difference compared to  $\text{CoCl}_2$  callus, at mean of 1066.4 $\pm$ 45.3 mg and 112.0 $\pm$ 20.7 mg, respectively. Compared to biotic elicitor (*S. cerevisiae* extract), callus given 0.5, 1.0 and 2.5 mg/L  $\text{CoCl}_2$  also showed lower fresh and dry weight. Callus grown on MS medium added with 1.0 mg/L  $\text{CoCl}_2$  at three weeks of age had higher fresh (916.4 $\pm$ 162.3 mg) and dry weight (93.3 $\pm$ 11.7 mg) compared to other  $\text{CoCl}_2$  concentration.

This is in line with previous study, which found that fresh and dry weight of *Cordia myxa* leaf callus were relatively lower than of control [28]. This was due to different response of each plant even though they were given the same concentration. Taha (2017) also explained that the addition of heavy metals to culture medium caused reduce of callus weight, due to excessive toxicity towards plant cells, thus heavy metals was only applied at low concentration [28].

**Table 1:** Fresh and dry weight of black betel leaf callus given various  $\text{CoCl}_2$  concentration

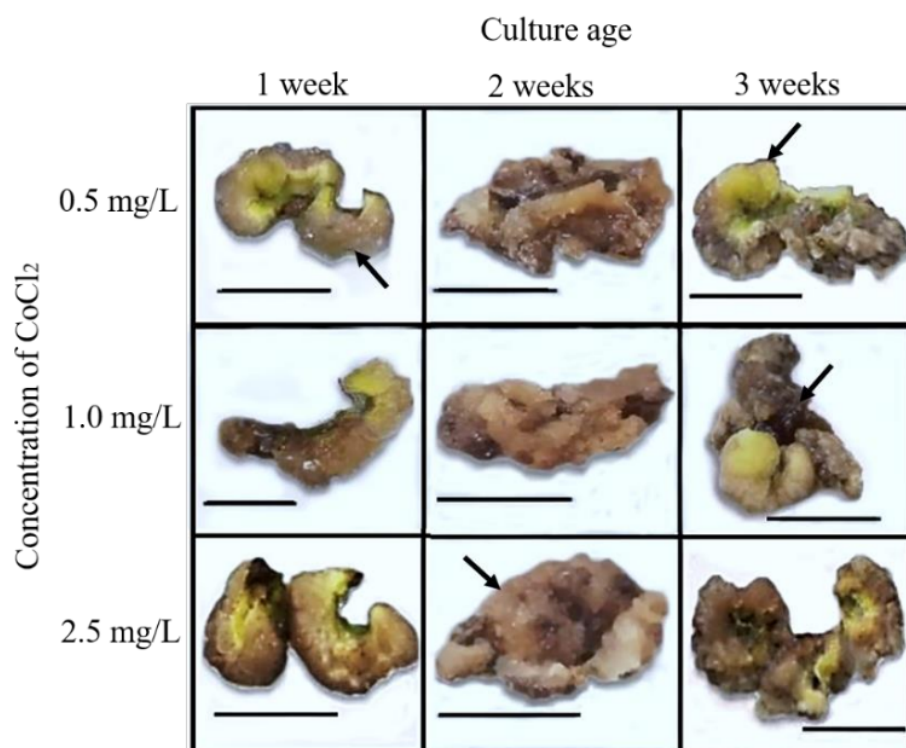
Treatment	Concentration	Callus age (weeks)	Fresh weight (mg)	Dry weight (mg)
Control		1	470.9 $\pm$ 19.9 <sup>f</sup>	51.7 $\pm$ 2.5 <sup>efg</sup>
		2	674.6 $\pm$ 94.9 <sup>d</sup>	69.5 $\pm$ 21.5 <sup>cde</sup>
		3	1066.4 $\pm$ 45.3 <sup>a</sup>	112.0 $\pm$ 20.7 <sup>a</sup>
$\text{CoCl}_2$	0.5 mg/L	1	488.3 $\pm$ 86.9 <sup>ef</sup>	60.3 $\pm$ 16.9 <sup>def</sup>
		2	459.7 $\pm$ 69.5 <sup>f</sup>	49.4 $\pm$ 4.2 <sup>fg</sup>
		3	772.7 $\pm$ 104.6 <sup>c</sup>	77.4 $\pm$ 7.7 <sup>c</sup>
$\text{CoCl}_2$	1.0 mg/L	1	480.2 $\pm$ 53.6 <sup>f</sup>	45.1 $\pm$ 4.0 <sup>h</sup>
		2	548.8 $\pm$ 49.7 <sup>e</sup>	60.0 $\pm$ 9.6 <sup>e</sup>
		3	916.4 $\pm$ 162.3 <sup>b</sup>	93.3 $\pm$ 11.7 <sup>ab</sup>

	1	460.4±13.6 <sup>f</sup>	45.8±11.3 <sup>gh</sup>
2.5 mg/L	2	734.2±82.5 <sup>bcd</sup>	73.7±6.8 <sup>cd</sup>
	3	804.4±150.8 <sup>c</sup>	84.6±17.3 <sup>bc</sup>

\*) Different letters indicate significant difference based on result of Mann-Whitney test ( $\alpha = 0,05$ ).

The same case was also found in *Catharanthus roseus* callus with heavy metals HgCl<sub>2</sub> added into its medium; callus weight was lowered [32]. However, *T. paniculatum* callus given 5 mg/L CuSO<sub>4</sub> had increase of weight compared to control instead, because the callus was still tolerant towards heavy metals due to specific mechanism developed by plant cells [33].

Callus grown during current study showed compact (1.0 mg/L CoCl<sub>2</sub> at three weeks of age) and crumb (0.5 mg/L CoCl<sub>2</sub> at two weeks of age) texture with various color, such as green, white, brown, and black. Generally, callus given CoCl<sub>2</sub> had green color. Callus colored green indicated that callus cells contained high amount of chlorophyll, while white color indicated starch content in the cells with chlorophyll had yet to be developed [34].



**Figure 1:** Morphological difference of texture and color found in black betel callus at various CoCl<sub>2</sub> level. Arrow indicates difference of callus color. Scale bar = 1 cm

<sup>10</sup> Methanol extract of black betel leaves contained higher level of terpenoids (20.59%) compared to callus given CoCl<sub>2</sub> at various level. This was due to callus harvested at three weeks of age; thus, it was still in exponential phase (growth stage), causing terpenoids level of callus given CoCl<sub>2</sub> far lower compared to leaf of mother plant. Several calli from control at one week, 1.0 mg/L CoCl<sub>2</sub> at one week, and 2.5 mg/L CoCl<sub>2</sub> at one and two weeks of age were not

identified of their terpenoids content, but contained other compounds instead such as alkaloids, flavonoids, saponins, and various fatty acids.

Extract of callus given 1.0 mg/L CoCl<sub>2</sub> at two weeks of age showed the highest areal percentage compared to other treatment, at 5.95% (Table 2). Selection of abiotic elicitor was thought to be one of the effective strategies to raise secondary metabolites production in in-vivo culture (Al-Khayri and Naik, 2016). Increase of abiotic elicitor concentration in MS medium was able to promote secondary metabolites biosynthesis. However, no further information present on elicitation mechanism of abiotic factor in enhancing secondary metabolites content.

The improvement of secondary metabolites biosynthesis is affected by several factors, for example determination of explant type and age, culture photoperiod, callus subculture time, elicitor type and concentration, and precursor (Bosila *et al.*, 2003; Karuppusamy, 2009). Based on that, it was hypothesized that unidentified terpenoids content of calli at several CoCl<sub>2</sub> concentration was caused by CoCl<sub>2</sub> level added into MS medium at level too low to induce terpenoids formation within black betel callus, in addition of early harvest after subculture. These affected terpenoids content, as secondary metabolites generally started to be synthesized at the end of growth phase. In the other hand, study previously performed showed that 1.0 mg/L CoCl<sub>2</sub> was able to induce higher robinin content in *Cordia myxa* leaf callus (Taha, 2017). Other study of callus culture at *Phoenix dactylifera* L. cv. Ashgar found that addition of 0.5 μM CoCl<sub>2</sub> could also promote higher phenols content (Al-Mayahi, 2014).

**Table 2:** Terpenoids profile of methanol extract of black betel leaf callus with various CoCl<sub>2</sub> level in solid MS medium

Group	Concentration (mg/L)	Culture age (weeks)	Number of Terpenoids	Terpenoids type	Area of terpenoids (%)	Total (%)
Leaf of black betel mother plant	0	0	12	Caryophyllene	0.19	20.59
				β-bisabolene	0.28	
				1,3-Cyclohexadiene, 5-(1,5-dimethyl-4-hexenyl)-2-methyl-	0.28	
				Cyclohexanol, 3-ethenyl-3-methyl-2-(1-methylethenyl)-6-(1-methylethyl)-, [1r-(1α,2α,3β,6α)]-	0.23	
				4-[1(E)-1,5-Dimethyl-1,4-hexadienyl]-1-methyl-1-cyclohexene	0.25	
				1,6,10-Dodecatrien-3-ol, 3,7,11-trimethyl-, [S-(Z)]-	2.32	
				1,2-Epoxy-1-vinylcyclohexene	0.10	
				Naphthalene, decahydro-4a-methyl-1-methylene-7-(1-methylethenylidene)	0.32	
				α-Cadinol	0.29	
				2-Hexadecen-1-ol, 3,7,11,15-tetramethyl-, [R-[R,R-(E)]]-phytol	0.54	
				Neophytadiene	15.34	
				Cyclohexanol, 5-methyl-2-(1-methylethenyl)-	0.45	
Control	0	0	β-bisabolene	2.26	4.99	
			(E)-1-Methyl-4-(6-methylhept-5-en-2-ylidene) cyclohex-1-ene	2.73		
			Bicyclo[7.2.0]undec-4-ene, 4, 11, 11-trimethyl-8-methylene-, (E)-(1R, 9S)-	0.65		
			(-)-			
			β-bisabolene	0.71		
			Cyclopentadecanone, 2-hydroxy-	0.14		
			D-Campholic acid	0.33		
			(E)-1-Methyl-4-(6-methylhept-5-en-2-ylidene) cyclohex-1-ene	1.02		
			trans-Geranylgeraniol	0.26		



Group	Concentration (mg/L)	Culture age (weeks)	Number of Terpenoids type	Terpenoids type	Area of terpenoids (%)	Total (%)
CoCl <sub>2</sub>	0.5	1	7	Octadecanoic acid	0.06	5.94
				Myristic acid	0.15	
				Ethyl (9Z,12Z)-9, 12-octadecadienoa	2.49	
				Longifolene	0.31	
				Cyclohexanol, 3-ethenyl-3-methyl-2-(1-methylethenyl)-6-(1-methylethyl)-, [1r-(1 $\alpha$ ,2 $\alpha$ ,3 $\beta$ ,6 $\alpha$ )]-	0.26	
				trans- $\gamma$ -bisabolene	0.36	
				(1R,2S,4R,6S)-4-Isopropenyl-1-methylbicyclo[4.1.0]heptan-2-ol	0.32	
				Cyclopentadecanone, 2-hydroxy-	0.83	
				Octadecanoic acid	1.66	
				Squalene	2.20	
				Butanal, 3-methyl-	0.16	
				[1,1'-Bicyclopentyl]-2-one	0.10	
				1,2-Epoxy-1-vinylcyclododecene	4.34	
CoCl <sub>2</sub>	1.0	2	7	Butanal, 3-methyl-	0.20	5.95
				2-Pinen-4-one	0.14	
				Cyclopentadecanone, 2-hydroxy-	3.05	
				Copaene	0.23	
				Bicyclo[7.2.0]undec-4-ene, 4, 11,11-trimethyl-8-methylene-, (E)-(1R, 9S)-	1.17	
CoCl <sub>2</sub>	1.0	2	7	(-)-	0.79	5.95
				Longifolene	0.79	
				trans- $\gamma$ -bisabolene	0.59	
				Spathulenol	0.19	

Group	Concentration (mg/L)	Culture age (weeks)	Number of Terpenoids type	Terpenoids type	Area of terpenoids (%)	Total (%)
				Cyclopentadecanone, 2-hydroxy-	0.31	
				Hexadecanoic acid	2.67	
				Butanal, 3-methyl-	0.44	
				$\beta$ -bisabolene	0.29	
				trans- $\gamma$ -bisabolene	0.34	
	3	3	6	2,6,10-Dodecatrinal,3,7,11-trimethyl-	0.09	2.51
				1,2-Epoxy-1-vinylcyclohexene	1.34	
				(-)-5-Oxatricyclo [8.2.0.0(4,6)]dodecane,12-trimethyl-9-methylene-,	0.01	
				Butanal, 3-methyl-	0.20	
	2.5	3	4	1,2-Epoxy-1-vinylcyclohexene	2.12	2.91
				Cyclopentadecanone, 2-hydroxy-	0.15	
				Octadecanoic acid	0.44	

## 4. Conclusion

The difference in  $\text{CoCl}_2$  concentration affects the biomass and callus terpenoid profile of *Piper betle* L. var leaves. Nigra. Treatment of 1.0 mg/L  $\text{CoCl}_2$  concentration in two weeks old culture was able to produce higher types of terpenoid compounds but callus biomass of *Piper betle* leaf L. var. Nigra produced less than control. In the callus culture of *Piper betle* L. var leaves. Nigra produces the two largest types of compounds namely Hexadecanoic acid and 1,2-Epoxy-1-vinylcyclododecene. Higher percentages of total types of terpenoid compounds were found at concentrations of 1.0 mg/L  $\text{CoCl}_2$ .

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