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INDUCTION AND IDENTIFICATION OF BIOACTIVE COMPOUNDS FROM CALLUS EXTRACT OF *Piper betle* L. var. Nigra

(Induksi Dan Pengenalpastian Sebatian Bioaktif dari Ekstrak Kalus *Piper betle* L. var. Nigra)

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Abstract

Black betel (*Piper betle* L. var. Nigra) has been shown to have the potential for medicinal use. The black betel leaves contain 4.2% essential oil, alkaloids, flavonoids, saponins, tannins, eugenol, and chavicol. The application of plant tissue culture is needed to increase the production of secondary metabolites. This study aimed to investigate the impact of the combination of growth regulators (IBA, BAP, and kinetin) on the bioactive compounds of black betel callus extract. The experimental parts consisted of four treatments: I_2B_2, $I_{1.5}B_{1.5}$, $I_2K_{1.5}$, $I_1K_{1.5}$, and a control group. Callus induction time, fresh weight, dry weight, morphology, and bioactive compounds of black betel callus extract were observed. The findings showed that the combination of IBA and BAP, and IBA and kinetin influenced the growth of black betel leaves explants. The best concentration of growth regulators was the combination of 2 mg/L IBA and 2 mg/L BAP, which give the results of 9.33 days callus induction time, 650.68 mg fresh weight, and 54.22 mg dry weight. The grown callus had white to light brown colour with a compact texture. Furthermore, different treatments resulted in different bioactive compounds contained in the black betel leaves callus extract. Supplementation of I_2B_2 resulted in an extract containing 15 compounds, $I_{1.5}B_{1.5}$ supplementation yielded 8 compounds, $I_2K_{1.5}$ 12 compounds, and $I_1K_{1.5}$ 13 compounds. The bioactive compounds were predominated by methyl-β-D-Glucopyranoside (28.69%), 9,12-octadecadienoic acid (Z, Z; 15.07%), and hexadecanoic acid (11.03%).

Keywords: callus induction, bioactive compounds, *Piper betle* L. var. Nigra, IBA, BAP, kinetin

Abstrak

Sirih hitam (*Piper betle* L. var Nigra) telah terbukti berpotensi dalam kegunaan perubatan. Daun sirih hitam mengandungi 4.2% minyak pati, alkaloid, flavonoid, saponin, tanin, eugenol, dan chavikol yang penting. Penggunaan kultur tisu tumbuhan diperlukan untuk meningkatkan pengeluaran metabolit sekunder. Tujuan kajian ini adalah untuk mengkaji kesan gabungan pengawal atur tumbesaran (IBA, BAP, dan kinetin) pada sebatian bioaktif ekstrak kalus sirih hitam. Bahagian eksperimen terdiri daripada empat rawatan: I_2B_2, $I_{1.5}B_{1.5}$, $I_2K_{1.5}$, $I_1K_{1.5}$ dan kumpulan kawalan. Masa induksi kalus, berat segar, berat kering, morfologi, dan sebatian bioaktif ekstrak kalus sirih hitam telah diperhatikan. Hasil kajian menunjukkan bahawa gabungan IBA dan BAP, dan IBA dan kinetin mempengaruhi pertumbuhan eksplan daun sirih hitam. Kepekatan pengawal atur tumbesaran terbaik ialah gabungan 2 mg/L

IBA dan 2 mg/L BAP yang memberikan hasil masa induksi kalus selama 9.33 hari, berat segar 650.68 mg, dan 54.22 mg berat kering. Kalus yang tumbuh mempunyai warna putih hingga coklat muda dengan tekstur yang padat. Selanjutnya, rawatan yang berbeza menghasilkan sebatian bioaktif berbeza yang terkandung dalam ekstrak kalus daun sirih hitam. Tambahan I_2B_2 menghasilkan ekstrak yang mengandungi 15 sebatian, tambahan $I_{1.5}B_{1.5}$ menghasilkan 8 sebatian, $I_2K_{1.5}$ 12 sebatian, dan $I_1K_{1.5}$ 13 sebatian. Sebatian bioaktif didominasi oleh metil-β-D-glukopiranosida (28.69%), asid 9,12-oktadekanoic (Z, Z; 15.07%), dan asid heksadekanoik (11.03%).

Kata kunci: induksi kalus, sebatian bioaktif, *Piper betle* L. var. Nigra, IBA, BAP, kinetin

Introduction

Betel leaves are mainly used as mouth refresher and medicine for both infectious and non-infectious diseases, such as influenza, cough, asthma, bronchitis, rheumatic, conjunctivitis, constipation, wound, and others [1]. *Piper betle* L. can be used to treat various health conditions as its leaves contain 4.2% essential oil, alkaloids, flavonoids, saponins, tannins, eugenol, and chavicol [2]. The essential oil component in betel leaves is composed mostly of phenolic and terpenoid compounds [3]. Moreover, the key components of betel leaves consist of starch, sugars, diastases and essential oil composed of terpinen-4-ol, safrole, allyl pyrocatechol monoacetate, eugenol, eugenyl acetate, hydroxyl chavicol and the betle oil contains cadinene carvacrol, allyl catechol, chavicol, p-cymene, caryophyllene, chavibetol, cineole, estragol, and others [4].

The phenolic content of *P. betle* L. can protect the human body from pathogenic bacteria, either Gram-positive or -negative [1]. Betel leaves are known to contain various nutrients and enzyme substances, such as diastase and catalase, besides several types of amino acids, including lysine, histidine, and arginine [1]. In addition, the extract of betel leaves also contained fatty acids, e.g., stearate and palmitate, which exhibited antimicrobial activity against *Streptococcus mutans*. Betel leaves extract may also inhibit the growth of *Staphylococcus aureus* and *Escherichia coli* bacteria in disk-diffusion test [5].

Conventionally, secondary metabolites are obtained via direct extraction from plant organs. However, this method requires massive raw plant material, in addition to costly extraction, isolation, and purification processes [6]. Therefore, plant tissue culture can be applied as an alternative to the production of secondary metabolites. Growth regulators from the auxin group play a role in callus induction, chlorophyll formation, and morphogenesis. Low concentration of auxin will trigger adventive roots formation, whereas a high concentration of it will stimulate callus formation and suppress or inhibit morphogenesis [7]. Meanwhile, the use of cytokinin in plant tissue culture will affect cell division and differentiation of adventive shoots from callus and organ [8]. The optimum ratio of auxin and cytokinin played a significant role in plant morphogenesis. The application of exogenous plant growth substances (auxin and cytokinin) of an intermediate ratio can promote callus induction. In addition, a high ratio of auxin:cytokinin or cytokinin:auxin ratios could induce root and shoot regeneration [9].

Callus induction of *P. betle* L. can be performed by a combination of IBA and kinetin growth regulators. A study reported that the addition of 2 mg/L IBA and 1 mg/L kinetin resulted in the shortest callus induction period, i.e., 7.5 days [10]. On the other hand, a combination of 2 mg/L IBA and 1.5 mg/L kinetin resulted in the highest callus fresh weight at 177 mg and dry weight at 46.6 mg, where the grown callus had a compact texture [10]. The combination of growth regulators of 2 mg/L IBA and 2 mg/L BAP could induce *P. betle* L. callus at the shortest period of 10 days, and the response of callus formation was 100%. The same combination also resulted in the highest callus fresh weight (0.8507 grams) and dry weight (0.0769 grams). Callus morphology from this treatment was friable textured and white [11].

The combination of 2 mg/L IBA and 2 mg/L BAP growth regulators given to *Piper crocatum* Ruiz and Pav explants resulted in the shortest induction time of 28

days. Meanwhile, the combination of 1.5 mg/L IBA and 1.5 mg/L BAP produced the highest fresh weight (226 mg) and dry weight (43.2 mg). GC-MS analysis showed that the latter combination of growth regulators yielded the callus extract with 19 types of compound (19 peaks). From the 19 peaks, 2 of the highest peaks were n-hexadecanoic acid and cyloeicosane [12].

The supplementation of growth regulators 6-benzylaminopurine (BAP), 6-furfurylaminopurine (kinetin), and indole-3-butyric acid (IBA) can induce callus from several callus explants in the *Piper* genus [10,11,12]. Thus, this study was designed using the combination of growth regulators BAP, kinetin, and IBA to determine the effect of growth regulator combinations in inducing callus and to identify secondary metabolites contained in black betel (*P. betle* L. var. Nigra) leaves callus. This is a novel approach as no research has been done on black betel leaves callus culture using these growth regulators.

Materials and Methods

Materials

Young black betel leaves (*P. betle* L. var. Nigra) taken from the second or third position from the top of the plant were chose and cut into small pieces. The plants were obtained from the Bratang Flower Market, Surabaya. Chemicals used were Murashige-Skoog (MS) medium, growth regulators (BAP, IBA, and kinetin), distilled water, 20% clorox, 70% alcohol, 1 N KOH (potassium hydroxide), 5 N HCl (hydrochloric acid), and 1 N methanol.

Culture medium preparation

Plants require nutrition for their growth and development. In the laboratory setting, in vitro culture medium supplies the nutrition required by the plant cells to imitate the whole plant grown in nature [8]. The MS medium (1000 mL) was prepared to incorporate macronutrients as follows; 1650 mg NH_4NO_3, 1900 mg KNO_3, 440 mg $CaCl_2.2H_2O$, 370 mg $MgSO_4.7H_2O$, and 170 mg KH_2PO_4 dissolved one by one in 500 mL distilled water using a magnetic stirrer until the solution was homogenous. Then, 5 mL of iron stock solution, 1 mL of micronutrient stock, and 4 mL of vitamin stock solution were added, followed by 100 mg myo-inositol and 30 grams sucrose. The growth regulators were added at various levels according to treatment. The pH of the solution was recorded using pH-indicator strips (Merck) and adjusted into the optimum pH of 5.6-5.8 using 1 N KOH and/or 1 N HCl. Lastly, distilled water was topped up until 1000 mL. Approximately 8 grams of agar was added into the solution while heated to solidify the medium. The agar was mixed well until it dissolved. The liquid medium was poured into culture bottles (about ± 10 mL/bottle). The bottles were covered with aluminium foil and labelled accordingly. The bottles were then sterilised in the autoclave at 121 °C, 1.2 atm for 15 minutes. The sterile medium was left at room temperature to solidify.

Explants planting

This stage was conducted according to previous research with modifications [13]. The young black betel leaf explants were planted aseptically in the laminar airflow (LAF) cabinet. Before planted, the leaves were sterilised by soaking it in detergent water for 3 minutes while its surface was rubbed gently using hands before rinsing using tap water. The leaves were soaked again in detergent water for 2 minutes while shaken, then rinsed again. Next, they were soaked in 70% alcohol for 5 minutes and washed three times using sterile distilled water. The leaves were soaked again in a 20% clorox solution and shaken for 7-10 minutes. Lastly, the leaves were rinsed using distilled water for three times. They were then transferred to a petri dish lined with filter paper. The leaves were cut into small pieces of ± 1cm^2, before putting into medium-filled culture bottle. Each treatment used four culture bottles filled with three leave explants. The explants were incubated at room temperature (20-25 °C) with lighting from a 40-watt neon lamp positioned at 1.5-2 m above the bottle shelf.

Extraction of black betel callus

Callus grown from the leaf explants was harvested after 8 weeks of incubation and dried, according to previous research with modifications [14]. The callus was crushed into powder using mortar and pestle. The powdered callus was extracted using maceration for 24 hours using methanol. After maceration, the callus extract was filtered using a filter paper. The pulp was re-macerated using the same solvent and then re-filtered. If

the filter result was still soupy, maceration was repeated. The product was air-dried to remove the remaining solvent. The extract was then identified for secondary metabolites content using Gas Chromatography-Mass Spectrophotometry (GC-MS) method [15].

Identification of secondary metabolites of *P. betle* L. var. Nigra callus using Gas Chromatography-Mass Spectrophotometry

The result of *P. betle* L. var. Nigra callus maceration from each combination treatment was identified for secondary metabolites using GC-MS, according to previous research with modifications [15]. The methanol extract of *P. betle* L. var. Nigra callus was analysed using Agilent 190915-105 with HP-5MS column silica capillary (60 m × 200 µm × 3 µm). A volume of 1 µL sample was injected into the column. Helium was used as the carrier gas at 1 mL/minute velocity. The injector temperature was set at 280 °C, while the detector temperature was 350 °C. The oven temperature was programmed at 40 °C for 5 minutes before raised to 250 °C at the rate of 2 °C/min and then hold for 20 minutes. Compounds were identified based on mass spectrum comparison to Wiley ver. 10 mass spectrum library.

Results and Discussion

Callus induction time and percentage of callus

Comparison of mean callus induction time and percentage of callus grown from black betel leaf explants on MS medium is presented in Table 1. Black betel leaf explants responded towards the combination of IBA and BAP, also IBA and kinetin in the MS medium. The initial reactions observed were explant curving, thickening at the margin of the explant, and broadening of the leaf vein and midrib. Small white spots emerged near scars and midrib. These spots are the first feature of callus formation. Initial callus growth was marked with explant swelling, followed by white callus emergence at the margin of the explant. Table 1 shows that a 100% callus formation is found in the explants from each treatment except for the control [16]. IBA and BAP combination (0, 0.5, 1.0, 2.0, and 3.0) could induce explants to form 100% callus [17]. Similarly, the addition of 1 mg/L IBA and 1.5 mg/L kinetin induced callus in *Justicia gendarussa* leaf explants with 100% callus formation [18].

Callus fresh and dry weight

Comparison of the mean fresh and dry weight of *P. betle* L. var. Nigra callus supplemented with various combinations of IBA, BAP, and kinetin growth regulators on MS medium is presented in Table 2. The highest mean of fresh and dry weight was found from explants given 2 mg/L IBA and 2 mg/L BAP, at 650.68 mg and 54.22 mg (Table 2). Plant production is usually more accurately referred to in dry weight than fresh weight. Callus fresh weight is also affected by the environment, as well as metabolic activity and humidity [19]; thus, the dry weight is more stable compared to fresh weight. The increased dry weight of the callus is caused by rising cell activity. Inside the cell, auxin plays a role in enhancing protein synthesis, improving plasticity, and developing cell walls, while cytokinin functions in cell division and protein synthesis [6].

Morphology of black betel (*P. betle* L. var. Nigra) leaf explant callus on MS medium

Table 3 shows that *P. betle* L. var. Nigra leaf explants planted for 8 weeks on MS medium with the combination of 2 mg/L IBA and 2 mg/L IBA can form callus (Figure 1). Callus grown from this combination had a brownish white compact texture. Callus can form in response to other hormones or wounding. Transcription factors regulate wound-induced calli. Wounding upregulates the biosynthesis and signalling of cytokinin, leading to cell proliferation activation and callus formation [20]. Callus induced by injury originated from various type of cells, including vessel and cortex cells [21]. Grown callus undergoes increased cell mass and callus colour change. Indicators of growth in the explants during in vitro culture can also be seen from the colour and texture of the callus. The while explant in the control group could not form callus (Figure 2).

Table 1. Mean callus induction time and percentage of callus grown from black betel leaf explants on MS medium added with growth regulators combination of IBA and BAP, and IBA and kinetin

No.	Concentration of Growth Regulator (mg/L)			Group	Mean Induction Time (days)*	Percentage of Explants Formed Callus (%)
	IBA	BAP	Kinetin			
1.	2	2	-	I_2B_2	9.33 ± 0.47^b	100
2.	1.5	1.5	-	$I_{1.5}B_{1.5}$	13.00 ± 1.00^c	100
3.	2	-	1.5	$I_2K_{1.5}$	14.08 ± 0.83^d	100
4.	1	-	1.5	$I_1K_{1.5}$	14.75 ± 1.09^d	100
5.	-	-	-	Control	0.00 ± 0.00^a	0**

*) Mean is followed by letters, different letter indicates significant difference based on Mann-Whitney test at 5% significance level. (-) = concentration 0 mg/L. **) not formed callus

Table 2. Mean callus fresh and dry weight from black betel leaf explants on MS medium supplemented with concentration combination of IBA and BAP, and IBA and kinetin growth regulators

No.	Concentration of Growth Regulator (mg/L)			Group	Mean Fresh Weight (mg)*	Mean Dry Weight (mg)*
	IBA	BAP	Kinetin			
1.	2	2	-	I_2B_2	650.68 ± 312.07^b	54.22 ± 9.11^b
2.	1.5	1.5	-	$I_{1.5}B_{1.5}$	429.78 ± 1.00^b	48.30 ± 11.53^b
3.	2	-	1.5	$I_2K_{1.5}$	164.53 ± 49.15^c	26.14 ± 4.18^c
4.	1	-	1.5	$I_1K_{1.5}$	60.94 ± 31.56^d	10.90 ± 4.62^d
5.	-	-	-	Control	0.00 ± 0.00^a	0.00 ± 0.00^a

*) Mean is followed by letters, different letter indicates significant difference based on Games-Howell test at 5% significance level. (-) = concentration 0 mg/L.

Table 3. Description of black betel leaves callus of I_2B_2 group from 1st to 8th week

Week	Description		
	Morphology	Texture	Colour
1st	Explants margin thickened slightly, surface smooth, explants slightly levitated, explants colours remain unchanged.	-	-
2nd	Explants margin thickened, margin coloured green, midrib and vein enlarged, explants slightly curved, transparent pustules emerged in the margin.	-	Transparent
3rd	Callus emerged at explants margin, margin curved, explants surface slightly smooth, explants coloured dark green.	Friable	White

Table 3 (cont'd). Description of black betel leaves callus of I_2B_2 group from 1st to 8th week

Week	Description		
	Morphology	**Texture**	**Colour**
4th	Callus in explants margin thickened more but still not prevalent, very few calli in the underside of explants, margin became wavy, and explants curved.	Compact	Greenish white
5th	Callus thickened more in the margin of explants; callus growth was uneven.	Compact	White
6th	More callus formed in the margin and underside of explants; callus growth was uneven.	Compact	White
7th	More callus grew, explants rolled.	Compact	White
8th	More callus grew, callus colours started to be brownish.	Compact	Brownish white

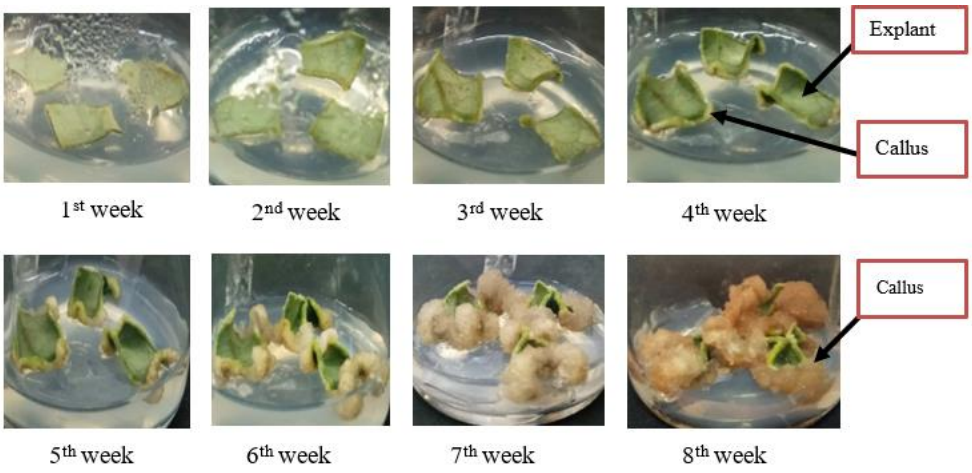

Figure 1. Callus morphology grown from black betel leaf explants from 1st to 8th week given 2 mg/L IBA and 2 mg/L BAP combination

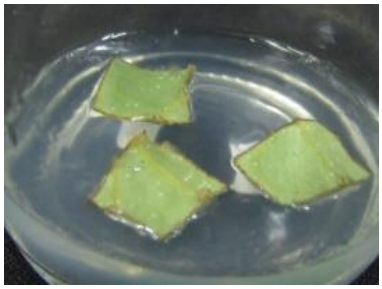

Figure 2. Explant morphology grown from black betel leaf explants without additional plant growth regulators (control group)

Secondary metabolites analysis

Compounds contained in the methanol extract of black betel callus is presented in Table 4. The positive control is the chemical content identified from the methanol extract of black betel leaves [22]. Three major secondary metabolite compounds in the 2 mg/L IBA and 2 mg/L BAP combination were 14-beta-H-pregna, n-hexadecanoic acid, and 1-docosane. Hexadecanoic acid belongs to the saturated fatty acid group. Fatty acids are the primary metabolite compounds known to possess antibacterial and antifungal properties. Fatty acids can modulate the immune response by acting directly on T cells [23]. The GC-MS analysis of the black betel callus methanol extract in combination with 1.5 mg/L IBA and 1.5 mg/L BAP showed that there were three compounds with a high percentage; methyl-β-D-glucopyranoside at 28.69%, 9,12-Octadecadienoic acid (Z, Z) at 15.07%, and hexadecanoic acid at 11.03%. Methyl-β-D-glucopyranoside belongs to the monosaccharide group. A monosaccharide is the monomer of polysaccharide and a type of primary metabolite. On the contrary, hexadecanoic acid and 5-octadecene belonged to the fatty acid group. Methyl-β-D-glucopyranoside was found in *Agrocybe aegerita* edible mushroom [24], and it was also the main compound found in *Geum montanum* L. leaves and will be accumulated throughout the lifetime of the plant. Synthesis of methyl-β-D-glucopyranoside and its derivates could reduce methanol in the cytoplasm of plant cells [25].

The main secondary metabolite present in the black betel callus methanol extract administered with 2 mg/L IBA and 1.5 mg/L kinetin combination was found to be chloroquine at 19.40%. Chloroquine is one of several malaria drug types [26]. Chloroquine is a common drug used for the treatment of *Plasmodium vivax*, which may interrupt the cycle of haemoglobin digestion process by parasite through interfering with β-hematein or inhibiting hemozoin formation [27]. Chloroquine could also inhibit plasmepsin and falcipain enzymes, which function in globin breakdown into amino acids. Hemozoin and amino acids are necessary for parasite growth; thus, inhibition of their formation will cause the parasite to die [28].

Analysis of callus extract given the combination of 1 mg/L IBA and 1.5 mg/L kinetin resulted in the production of primary metabolites, such as 9,12-Octadecadienoic acid, methyl ester, methyl-beta-D-thiopyranoside, and others. Similarly, 9,12-Octadecadienoic acid and methyl ester, which have a chemical formula of $C_{19}H_{34}O_2$, were identified from the methanol extract of *Cenchrus ciliaris* L. stem. Primary metabolite compounds identified from the GC-MS method are predominantly fatty acids, in addition to amino acids and carbohydrate polymers [29].

In the current study, the addition of IBA, BAP, and kinetin plant growth regulators (PGRs) could affect chemical formation in black betel leaf explant callus. Table 4 shows that several compounds are only formed in specific treatment. This is greatly influenced by the addition of PGRs in the MS medium. The formation of bioactive compounds and their numbers are closely related to the addition of variations in the combination of concentrations of IBA and BAP, and IBA and kinetin, which will affect endogenous hormones in explant cells that are cultured in MS medium. This current phenomenon is often studied as an essential factor in green biotechnology. PGRs play an important role in research that focuses on micropropagation of the plant [30]. One example was 14-beta-H-pregna, which can only be found in callus given the combination of 2 mg/L IBA and 2 mg/L BAP. The area percentage of 14-beta-H-pregna was found to be the highest. This indicated that 1 µL methanol extract sample of black betel callus given 2 mg/L IBA and 2 mg/L BAP contained 14-beta-H-pregna at the concentration of 15.34%. Beside 14-beta-H-pregna, another compound only found in one treatment was chloroquine. Based on GC-MS analysis, chloroquine was only produced by callus given 2 mg/L IBA and 1.5 mg/L kinetin combination. In this treatment, chloroquine was found to have the highest area percentage. This indicated that in 1 µL methanol extract sample of callus from 2 mg/L IBA and 1.5 mg/L kinetin treatment contained chloroquine at 19.4% level.

Table 4. Secondary metabolites content of black betel callus analysed based on GC-MS method

Compounds	Area (%) / Retention Time (minutes)				
	I_2B_2	$I_{1.5}B_{1.5}$	$I_2K_{1.5}$	$I_1K_{1.5}$	Positive Control
Pyridine	0.08/8.669	0.33/8.629	0.38/8.806	0.64/8.766	0.26/7.29
Methyl-pyrazine	0.03/11.404	0.18/11.455	0.38/11.478	0.75/11.633	-
Imidazole	0.04/65.282	-	-	-	0.02/21.64
Chloroquine	-	-	19.4/85.498	-	-
Piperidine	-	-	-	0.77/49.787	-
2-Picoline	-	-	-	-	0.16/6.13
Piperazine	-	-	-	-	0.01/20.23
Caryophyllene oxide	-	-	0.17/61.471	-	-
Hexacosane	1.11/110.377	-	-	-	-
Squalene	-	0.17/117.458	0.60/117.771	0.40/117.466	-
Neophytadine	0.39/72.284	-	1.69/77.418	-	-
Linuron	-	-	-	0.13/5.356	-
Dihydro-2-(3H)-thiphenone	-	-	-	0.32/10.403	-
Dihydro-2(3H)-furanone	-	-	-	0.13/16.411	-
Phytol	0.75/88.319	1.73/88.301	1.02/88.319	0.89/75.278	-
Gamma -muurolene	-	0.07/57.574	0.37/57.580	-	-
n- Hexadecanoic acid	3.26/81.990	2.47/80.033	5.83/81.887	15.62/81.801	-
Hexadecanoic acid	-	11.03/80.113	-	5.98/79.621	-
Nonadecane	0.06/78.305	-	-	-	-
Tetradecanal	0.04/86.757	-	-	-	-
Octadecanoic acid	-	-	1.06/90.894	-	-
Myrcene	-	-	-	-	0.09/7.76
Undecane	-	-	-	-	0.01/7.94
2-Dodecanone	-	-	-	-	0.01/21.02
Tetradecane	-	-	-	-	0.01/21.02
1,2-Epoxy-1 vinylcyclododecane	-	-	-	-	0.02/22.39
1-Docosane	3.58/97.548	-	-	-	-
Oxirane, 2-ethyl-2-methyl	0.03/16.262	-	-	-	-
2-Propenoic acid	-	-	0.29/97.169	0.55/97.056	-

Table 4 (cont'd). Secondary metabolites content of black betel callus analysed based on GC-MS method

Compounds	Area (%) / Retention Time (minutes)				
	I_2B_2	$I_{1.5}B_{1.5}$	$I_2K_{1.5}$	$I_1K_{1.5}$	Positive Control
Piperlonguminine	-	-	0.35/103.745	-	-
Myristcin	-	-	-	2.91/57.557	-
Ethanol	2.87/100.724	-	-	-	-
Hexadecane	1.51/109.318	-	-	-	-
Dodecanoic acid	-	0.43/60.297	-	-	-
Ethyl Alcohol	-	-	-	-	0.19/1.25
Tricosane	2.64/119.057	-	-	-	-
4-Hexenoic acid	0.23/98.641	-	-	-	-
12-Metyltricosane	0.24/102.664	-	-	-	-
14-beta-H-Pregna	15.34/129.854	-	-	-	-

Conclusion

The studied bioactive compounds of *P. betle* L. var. Nigra leaves methanol extract using GC-MS method resulted in the detection of several compounds. The compound with the highest area percentage was methylamine, at 85.08%. Methylamine is an organic compound from the amine group that was previously found to have antimicrobial bioactivity. Based on GC-MS analysis, methanol extract of black betel callus given IBA, BAP, and kinetin growth regulators combination did not contain methylamine. However, two similar compounds were identified from the methanol extract of black betel leaf, i.e., pyridine and methyl pyrazine. Both compounds belong to the alkaloid secondary metabolite group. Pyridine is essential in the manufacture of medicines and has a wide range of bioactivities, such as anti-inflammatory, analgesic, anticancer, and antimicrobe [22]. Meanwhile, methyl pyrazine can act as an anticancer medicine [31].

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References

1. Das, S., Parida, R., Sandeep, I. S., Nayak, S. and Mohanty, S. (2016). Biotechnological intervention in betelvine (*Piper betle* L.): A review on recent advances and future prospects. *Asian Pacific Journal of Tropical Medicine*, 9(10): 938-946.
2. Khomsatun, Wahyudi, Hari R. I. W., and Gustomo Y. (2019). Effectiveness of betel leaves extract (*Piper betle*, Linn) as mosquito *Anopheles spp* repellent. *International Journal of Innovative Research in Science, Engineering and Technology*, 8(5): 5144-5152.
3. Alighiri, D., Cahyono, E., Eden, W. T., Kusuma, E. and Supardi, K. I. (2018). Study on the improvement of essential oil quality and its repellent activity of betel leaves oil (*Piper betle* L.) from Indonesia. *Oriental Journal of Chemistry,* 34 (6): 2913 - 2926.
4. Sugumaran, M., Poornima, M., Venkatraman, S., Lakshmi, M., and Srinivasansethuvani (2011). Chemical composition and antimicrobial activity of sirugamani variety of *Piper betle* Linn leaf oil. *Journal of Pharmacy Research*, 4(10): 3424-3426.

5. Hermawan, A. (2007). Pengaruh ekstrak daun sirih (*Piper betle* L.) terhadap pertumbuhan *Staphylococcus aureus* dan *Escherichia coli* dengan metode difusi disk. Thesis of Undergraduate Degree. Universitas Airlangga.
6. Utomo, B. (2015). Pengaruh kombinasi zat pengatur tumbuh *Napthalene Acetic Acid* (NAA) dan kinetin terhadap pembentukan dan kandungan metabolit sekunder kalus sirih merah (*Piper crocatum* Ruiz dan Pav.). Thesis of Undergraduate Degree. Universitas Airlangga.
7. Overvoorde, P., Fukaki, H. and Beeckman, T. (2010). Auxin control of root development. *Cold Spring Harbor Perspectives in Biology*, 2(6): a001537.
8. Saad, A. I. M. and Elshahed, A. M. (2012). Plant tissue culture media. In *Recent Advances in Plant in vitro Culture*. IntechOpen: pp. 29-40.
9. Ikeuchi, M., Sugimoto, K. and Iwase, A. (2013). Plant callus: Mechanisms of induction and repression. *The Plant Cell,* 25 (9): 3159-3173.
10. Fatin, U. (2016). Induksi kalus eksplan daun sirih hitam (*Piper betle* L.) dengan kombinasi konsentrasi zat pengatur tumbuh indol butyric acid (IBA) dan kinetin. Thesis of Undergraduate Degree. Universitas Airlangga.
11. Rachmah, A. (2016). Induksi kalus eksplan daun sirih hitam (*Piper betle* L.) dengan kombinasi konsentrsi zat pengatur tumbuh indol butyric acid (IBA) dan *6-Benzylaminopurine* (BAP). Thesis of Undergraduate Degree. Universitas Airlangga.
12. Tantri, N. A. (2015). Pengaruh variasi konsentrasi zat pengatur tumbuh *Indol Butyric Acid* (IBA) dan *Benzylaminopurine* (BAP) terhadap induksi kalus dan kandungan senyawa sirih merah (*Piper crocatum* Ruiz dan Pav.). Thesis of Undergraduate Degree. Universitas Airlangga.
13. Junairiah, J., Zuraidassanaaz, N. I., Izdihar, F. N. and Manuhara, Y. S. W. (2017). Callus induction of leaf explant *Piper betle* L. var Nigra with Combination of Plant Growth Regulators Indole-3-acetic Acid (IAA), Benzyl Amino Purine (BAP) and Kinetin. *AIP Conference Proceedings*, 1888: 020028.
14. Junairiah, J., Ni'matuzahroh, N., Zuraidassanaaz, N. I., and Sulistyorini, L. (2018). Isolation and identification of secondary metabolites of black betel (*Piper Betle* L. var. Nigra). *Jurnal Kimia Riset*, 3(2): 131-138.
15. Wahyuni, D. K., Ansori, A. N. M. and Vidiyanti, F. (2017). GC-MS Analysis of phytocomponents in methanolic extracts of leaf-derived callus of *Justicia gendarussa* Burm. f. *Bioscience Research.* 14(3): 668-677.
16. Wahyuningtyas, L., Resmiari, R. S. and Nashichuddin, A. (2014). Induksi kalus akasia (*Acacia mangium*) dengan penambahan kombinasi 2,4-D dan BAP pada media MS. https://etheses.uinmalang.ac.id/ 376/12/10620033%20Rangkuman.pdf. [Access online 26 December 2018].
17. Widyarso, M. (2010). Kajian penggunaan IBA dan BAP untuk merangsang pembentukan tunas lengkeng (*Dimocarpus longan* Lour.) varietas pingpong secara *In Vitro*. Thesis of Undergraduate Degree. Universitas Sebelas Maret.
18. Rohmatin, N. (2014). *Induksi kalus dari eksplan daun gandarusa (Justicia Gendarussa Burm. F.) dengan pemberian kombinasi zat pengatur tumbuh 2,4-D, IBA, dan kinetin***.** Thesis of Undergraduate Degree**.** Universitas Airlangga.
19. Muryanti, S., and Anggarwulan, E. (2005). Pertumbuhan dan produksi reserpin kalus pule pandak (*Rauvolia serpentine* (L.) Bentham ex. Kurz) pada pemberian metil jasmonat secara *In Vitro*. *Bioteknologi*, 2(2): 58-64.
20. Fehér, A. (2019). Callus, dedifferentiation, totipotency, somatic embryogenesis: What these terms mean in the era of molecular plant biology?. *Frontiers in Plant Science*, 10: 536.
21. Ikeuchi, M., Sugimoto, K., and Iwase, A. (2013). Plant callus: Mechanisms of induction and repression. *The Plant Cell*, 25(9): 3159-3173.
22. Junairiah, Nurhariyati, T., Ni'matuzahroh and Sulistyorini, L. (2018). GC-MS analysis of bioactive compounds in n-hexane, ethyl acetate and methanol extract of *Piper betle* L. Var. Nigra. *Bioscience Research*, 15(3): 1472-1479.

23. Aparna, V., Dileep, K. V., Mandal, P. K., Karthe, P., Sadasivan, C. and Haridas, M. (2012). Anti-inflammatory property of n-hexadecanoic acid: Structural evidence and kinetic assessment. *Chemical Biology & Drug Design*, 80 (3): 434-439.
24. Diyabalanage, T., Mulabagal, V., Mills, G., DeWitt, D. L. and Nair, M. G. (2008). Health-beneficial qualities of the edible mushroom, *Agrocybe aegerita*. *Food Chemistry*, 108(1): 97-102.
25. Aubert, S., Choler, P., Pratt, J., Douzet, R., Gout, E. and Bligniy, R. (2004). Methyl-b-D-glucopyranoside in higher plants: Accumulation and intracellular localization in *Geum montanum* L. leaves and in model systems studied by 13C nuclear magnetic resonance. *Journal of Experimantal Botany*, 55(406): 2179- 2189.
26. Isnawati, A. and Rooslamiati, I. (2013). Penetapan kadar artemisinin dalam ekstrak heksan tanaman *Artemisia annua L.* menggunakan metode densitometri. *Media Litbangkes*, 23(1): 15-22.
27. Hasugian, A. R. and Tjitra, E. (2014). Artesunat-amodiakuin dan klorokuin untuk pengobatan malaria vivaks di Puskesmas Kopeta, Maumere, Nusa Tenggara Timur, 2007. *Media Penelitian dan Pengembangan Kesehatan*, 24(4): 161 - 168.
28. Muti'ah, R. (2012). Penyakit malaria dan mekanisme obat-obat antimalaria. *Alchemy*, 2(1): 80-91.
29. Kumar, S. and Pandey, A. K. (2013). Chemistry and biological activities of flavonoids: An overview. *The Scientific World Journal*, 2013: 1-16.
30. Szopa, A., Kubica, P., Snoch, A. and Ekiert, H. (2018). High production of bioactive depsides in shoot and callus cultures of *Aronia arbutifolia* and *Aronia × prunifolia*. *Acta Physiologiae Plantarum*. 40(48): 1-11.
31. Urban, S., Hickford, S. J. H., Blunt, J. W. and Munro, M. H. G. (2000). Bioactive marine alkaloids. *Current Organic Chemistry*, 4(7): 765-807.