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GCMS ANALYSIS AND ANTIMICROBIAL ACTIVITY OF FRACTIONS OF *PIPER BETLE* L. VAR NIGRA

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ABSTRACT

Black betel (*Piper betle* L. var Nigra) belong to Piperaceae family and one of endemic species of Indonesia. Previous research showed that the antimicrobial activity of methanol extract was better compared to ethyl acetate and n-hexane extracts. This study was aimed to determine antimicrobial activity of fractions of methanol extracts of *Piper betle* L. var Nigra toward pathogenic microbes *Candida albicans* ATCC 10231, *Staphylococcus aureus* ATCC 25923, and *Escherichia coli* ATCC 25922. Leaf powder of black betel was macerated using methanol solvents and the methanol extracts was fractionated by vacuum liquid chromatography (VLC). The fractionation process produces 11 fractions. Each fraction was analyzed by GCMS to identify the number and types of bioactive compounds. Antifungal and antibacterial activity were determined using diffusion and dilution tests. Data collected from both tests including diameter of inhibition zone (mm) and Minimal Inhibitory Concentration (MIC), Minimal Bactericidal Concentration (MBC), Minimal Fungicidal Concentration. Data was then analyzed statistically. Result showed that each fraction produces varying amounts and types of compounds. The highest inhibition of *E. coli* was found in fractions of 5, 6 and 11. The highest inhibition of *S. aureus* was found in fraction 7. The highest inhibition of *C. albicans* was found in fractions 7 and 11. The MIC and MBC values of the three test microbes were <0.1 mg/mL and 0.1 mg/mL, respectively.

KEY WORDS : Antibacteria, Fractions, Methanol Extracts, *Piper Betle* L. Var Nigra.

INTRODUCTION

Piperaceae consisted of 10 genera and 2000 species. *Piper* genus is known to contain secondary metabolites of polyphenol, alkaloid, steroid, saponin, and tannin (Navickiene *et al.*, 2006; Regasini *et al.*, 2008).

Betel variety currently recorded including green, red, and black betel. Black betel (*Piper betle* L. var Nigra) is one of Piperaceae family contained various secondary metabolites, such as alkaloid, flavonoid, saponin, terpenoid, and steroid. Thus, this plant has potential as antibacterial, antifungal, antidiabetic, antiulcer, antiplatelet, antifertility, antitumor, antimutagenic, and antihelminthic agents (Lei *et al.*, 2003; Majumdar *et al.*, 2002).

Water, methanol, ethyl acetate, and ether extracts of *Piper betle* L. were found to be able to inhibit growth of *Streptococcus pyrogenes, Staphylococcus aureus, Proteus vulgaris*, and *Escherichia coli* (Chakraborty and Shah, 2011), while ethanol and chloroform extracts of *Piper nigrum* were also able to inhibit *Escherichia coli, Salmonella typhii, Pseudomonas sp.*, and *Staphylococcus aureus* (Ganesh *et al.*, 2014). Ethanol, hexane, chloroform, ethyl acetate, methanol, and water extracts of *Piper hayneanum* could suppress growth of *Staphylococcus aureus* and *Candida albicans* (Bastos *et al.*, 2011), while ethanol extract of black pepper (*Piper nigrum* L.) was able to suppress *Staphylococcus aureus, Bacillus subtilis, Pseudomonas aeruginosa, Escherichia coli, Alternaria alternata, Aspergillus niger, Aspergillus flavus*, and *Fusarium oxysporum* (Rani *et al.*, 2013). In previous study has shown that methanol extract of black betel

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were known to have antimicrobial activity against *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, *Candida albicans* ATCC 10231 which is better when compared with n-hexane and ethyl acetate extract (Junairiah *et al.*, 2017).

Based on previous study, few study has been conducted to identify the number and types of bioactive compounds of each fraction and determine antimicrobial activity of fractions of methanol extracts of *Piper betle* L. var Nigra toward pathogenic microbes *Candida albicans* ATCC 10231, *Staphylococcus aureus* ATCC 25923, and *Escherichia coli* ATCC 25922.

MATERIALS AND METHODS

Plant Collection

Black betel (*Piper betle* L. var Nigra) was obtained from Kayon Flower Mart, Surabaya, East Java, Indonesia. This plant was identified in Plant Physiology Laboratory, Faculty of Science and Technology, Universitas Airlangga.

Pathogenic Microbes

Pathogenic microbes used were *Candida albicans* ATCC 10231, *Staphylococcus aureus* ATCC 25923, and *Escherichia coli* ATCC 25922. The three microbes were collection of Microbiology Laboratory, Department of Biology, Faculty of Science and Technology, University of Airlangga.

Extraction and Fractionation Procedure and GCMS Analysis

Leaves of *Piper betle* L. var Nigra were firstly washed and air-dried, then crushed into powder. Betel powder weighed to 29.5 grams then extracted using methanol solvents. Extraction was performed using maceration method for three days and repeated for three times. The methanol extracts was fractionated by vacuum liquid chromatography (VLC), Each fraction was analyzed by GCMS. Each fraction was made into a series of concentration; 2 µg/disc; 5 µg/disc, 10 µg/disc, 15 µg/disc and 20 5 µg/disc.

Microbe Culture

Examined microbe was made into stock solution. Microbe suspension was produced by mixing several ose of bacteria from slanted agar into 20 mL saline water. Suspension was homogenized, then taken several mL for measuring its optical density using spectrophotometer. Stock used had OD 0.1 at 600 nm wavelength for fungi and 625 nm for bacteria.

Diffusion Test

Medium used for diffusion test was Mueller Hinton agar (MHA). 1 mL microbe suspension was placed in petri dish, then 15 mL MHA medium was added, mixture was homogenized and left to be solidified. On the surface of medium, 3 paper discs (6 mm diameter) saturated with 20 µl extract of respective solvent at each concentration of 2 µg/disc; 5 µg/disc; 10 µg/disc; 15 µg/disc; 20 µg/disc. Diameter of inhibition zone was measured using caliper.

Dilution test

1 mL microbe suspension in Mueller Hinton Broth (MHB) medium was placed into reaction tube filled with 1 mL betel extract of each solvent type and concentration. Each culture was incubated for 24 hours. Next, 0.1 mL of each culture was placed into petri dish and added 15 mL medium, then incubated for 24 hours for bacteria and 48 hours for fungi. If microbe growth was observed in medium, then concentration used was MIC value. If microbe growth was not observed, then concentration used was MBC/MFC value.

RESULTS AND DISCUSSION

The results of GCMS analysis of fractions produced from black betel methanol extract can be seen in Tables 1 to 10. Each fraction contains different amounts and types of compounds. The major compounds in the fraction are crotanaldehyde, acetylene, 1-heyine, 1, 5 hexadiyne, hexyne and

Table 1. Phytocomponents identified in the fraction 1

Peak	RT	Area (%)	Phytocomponent
1	1.08	0.50	1-Propanamine
2	1.34	60.25	1-Hexyne
3	1.45	32.14	1,3-Butadiene
4	1.58	0.34	2-Butenedinitrile
5	3.04	0.14	Arsenous acid
6	7.88	0.06	Cyclotetrasiloxane
7	13.68	0.21	Cyclohexasiloxane
8	15.88	0.18	Cycloheptasiloxane
9	17.87	0.09	Cyclooctasiloxane
10	19.09	0.03	Tenamfetamine
11	19.59	0.07	Morphinan
12	20.82	0.03	2-Ethylacridine
13	21.11	0.06	Silicone grease

methoxycyclohexane. Crotanaldehyde is a chemical compound with the molecular formula CH_3CH = CHCHO. This compound has biological activity as an antimicrobial (Svyatoslav *et al.*, 2016).

Acetylene is an alkyne hydrocarbon, molecular formula C2H2. Alkyne compounds isolated from *Permalia peltata* have biological activities as antibacterial on *Staphylococcus aureus*, *Bacillus subtilis*, *Streptomyces grieveces*, *Escherichia coli* and *Aspergillus niger*, *Trichoderma reseei*, *Fusarium oxysporum*, *Penicillium funiculosam* (Pratibha and Mahesh, 2016). Hexadiyne is found in *Artemisia capillaris*. This plant contains 1-oxo-1-phenyl-2,4-hexadiyne (capilin) and 1-phenyl-2,4-hexadiyne (capillen) (Ueda and kato, 1984).

Table 2. Phytocomponents identified in the fraction 2

Peak	RT	Area (%)	Phytocomponent
1	0.62	0.05	Cyclotrisiloxane
2	1.07	0.54	Benzenemethanol
3	1.14	17.72	Ethyl ester
4	1.34	47.21	3-Methoxycyclohexene
5	1.45	30.07	Methylenecyclopropane
6	1.58	0.57	2-Butenedinitrile
7	2.36	0.13	Tetramethylguanidine
8	2.47	0.10	Butoxy-N
9	3.04	0.29	Cyclotrisiloxane
10	3.40	0.09	N-Cyano-N′
11	7.88	0.04	Cyclotetrasiloxane
12	13.70	0.12	Cyclohexasiloxane
13	15.90	0.13	cycloheptasiloxane
14	17.89	0.06	2-Ethylacridine
15	19.63	0.03	Anisole

Table 3. Phytocomponents identified in the fraction 3

Peak	RT	Area (%)	Phytocomponent
1	1.08	0.52	Hydrastininic acid
2	1.35	61.19	1-Hexyne
3	1.45	31.60	1,3-Butadiene
4	3.04	0.13	Cyclotrisiloxane
5	7.89	0.05	Cyclotetrasiloxane
6	13.74	0.06	Cyclohexasiloxane
7	15.91	0.07	Cycloheptasiloxane

Diffusion test was a method commonly used for determining microbe activity, due to its inexpensive cost. Result of diffusion test was presented in Table 12. It was showed that n-hexane, ethyl acetate, and methanol extracts was able to inhibit the three species of pathogenic microbes. However, each extract was found to have different sensitivity towards specific microbe. N-hexane extract of black betel was found to inhibit *E. coli* ATCC 25922 better at 5 µg/disc, with inhibition zone diameter of 8.92 mm. Extract of ethyl acetate inhibited *S. aureus* ATCC 25923 at 10 µg/disc, with zone diameter of 10.63 mm, while methanol extract was able to inhibit *S. aureus* ATCC 25923 at 10 µg/disc concentration, with zone diameter of 12.69 mm.

Table 4. Phytocomponents identified in the fraction 4

Peak	RT	Area (%)	Phytocomponent
1	1.08	0.53	2-Butanamine
2	1.34	60.65	1,5-Hexadiyne
3	1.45	33.65	2-Pentyne
4	1.58	0.48	Phenyl hydride
5	3.04	0.15	Cyclotrisiloxane
6	7.88	0.04	Cyclotetrasiloxane
7	13.49	0.07	Folic Acid
8	13.72	0.11	2,6-diphenylpyridine
9	15.07	0.11	Tenamfetamine
10	15.93	0.07	Cycloheptasiloxane

Table 5. Phytocomponents identified in the fraction 5

Peak	RT	Area (%)	Phytocomponent
1	1.08	0.73	Pentylamine
2	1.35	63.47	N-methyl-N
3	1.45	32.35	1,3-Butadiene
4	2.11	0.08	Satratoxin
5	3.04	0.13	s-Triazine
6	7.89	0.06	Silane
7	13.69	0.11	Cyclohexasiloxane
8	15.90	0.18	Cycloheptasiloxane
9	17.90	0.02	Benzoic acid

Table 6. Phytocomponents identified in the fraction 6

Peak	RT	Area (%)	Phytocomponent
1	1.08	0.46	Phenethylamine
2	1.34	63.42	1-Hexyne
3	1.45	33.31	1,3-Butadiene
4	1.58	0.52	Pyrobenzol
5	3.04	0.12	5-Methyl-2-phenylindolizine
6	7.88	0.03	Cyclotetrasiloxane
7	13.69	0.10	Cyclohexasiloxane
8	15.90	0.09	Silane
9	17.89	0.06	Cholest-2-en-19-oic acid

Based on result of current study, methanol extract was found as the best extract solvent to be used compared to other solvent n-hexane and ethyl acetate. This was due to methanol as universal solvent, thus was able to bind various compound or secondary metabolites. Methanol extract was found

Table 7. Phytocomponents identified in the fraction 7

Peak	RT	Area (%)	Phytocomponent
1	1.08	0.43	2-Ethoxyamphetamine
2	1.34	60.32	1-Hexyne
3	1.45	31.40	1,3-Butadiene
4	1.58	0.43	But-2-enedinitrile
5	3.04	0.12	2,3-dimethyl-4-azaphenanthrene
6	7.89	0.03	Cyclotetrasiloxane
7	13.70	0.08	Cyclohexasiloxane
8	15.91	0.11	Megastrol acetate
9	17.89	0.05	Benzenamine

Table 8. Phytocomponents identified in the fraction 8

Peak	RT	Area (%)	Phytocomponent
1	1.08	0.46	12-Methylaminododecanoic acid
2	1.34	60.93	Acetylene
3	1.45	32.92	Cyclopentene
4	1.58	0.33	Benzene
5	3.04	0.10	Cyclotrisiloxane
6	7.89	0.03	Silane
7	13.71	0.07	Acetic acid
8	15.91	0.09	Cycloheptasiloxane
9	17.90	0.05	2-phenylimino-1,2-dihydroanthra (1,2-d) thiazole-6,11-dione

Table 9. Phytocomponents identified in the fraction 9

Peak	RT	Area (%)	Phytocomponent
1	1.08	0.61	2-Pyridinepropanoic acid
2	1.35	62.65	N-methyl-N′
3	1.45	30.65	1,3-Butadiene
4	3.05	0.14	Cyclotrisiloxane
5	7.89	0.04	Cyclotetrasiloxane
6	13.70	0.05	Cyclohexasiloxane
7	15.91	0.13	Phenol

Table 10. Phytocomponents identified in the fraction 10

Peak	RT	Area (%)	Phytocomponent
1	1.08	0.43	2-Butanamine
2	1.35	60.91	Crotonaldehyde
3	1.45	31.10	1,3-Butadiene
4	3.04	0.12	Cyclotrisiloxane
5	7.89	0.05	Cyclotetrasiloxane
6	13.71	0.06	Cyclohexasiloxane
7	15.91	0.08	6-ethyl-5-(4′-trifluoromethylphenyl) pyrimidine-2,4-diamine

Table 11. Phytocomponents identified in the fraction 11

Peak	RT	Area (%)	Phytocomponent
1	1.08	0.53	Methanimidamide
2	1.34	60.65	Crotonaldehyde
3	1.45	32.34	1,3-Butadiene
4	1.58	0.41	Divinylacetylene
5	3.04	0.11	Cyclotrisiloxane
6	7.88	0.03	Cyclotetrasiloxane
7	13.71	0.09	Acetic acid
8	15.91	0.09	Pentasiloxane

to contain terpenoid, steroid, flavonoid, polyphenol, tannin, and alkaloid. Steroid was found to have function as antibacterial agent (Silvia *et al.*, 2003). Steroid was able to act as antibacterial by preventing spore germination process (Subisha and Subramoniam, 2005). Other than them, alkaloid also had cytotoxic property against bacteria (Ezekiel *et al.*, 2009).

For determining MIC and MBC, dilution test was performed for each solvent and concentration used. MIC and MBC was presented in Table 13.

Indoquinolone alkaloid specifically previously found to induce cell lysis and morphological alteration on *S. aureus* (Sawer *et al.*, 2005). Flavonoid inhibited function of cytoplasm membrane and energy metabolism (Cushnie and Lamb, 2009). Flavonoid possessed ability as anti-microbe (Ogundipe *et al.*, 2001; *Silvia et al.*, 2003) and was able to suppress growth of *S. aureus* and *E. coli* (Chattopadhay *et al.*, 2001). Flavonoid was currently applied as new compound in antimicrobial therapy (Ozcelik *et al.*, 2008). Polyphenol had antibacterial activity (Taguri *et al.*, 2004), while tannin had previously hypothesized as antibacterial agent (Akiyama, 2001; Futanogawa, 2004; Guittat *et al.*, 2003; Lisgarten *et al.*, 2002).

Previous study had shown that water, methanol, petroleum ether, diethyl acetate, chloroform, and methanol extract of *Tamarindus indica* leaves at 100 mg/mL concentration were found to be able to inhibit growth of various Gram positive (*Bacillus subtilis* ATCC 11774, MRSA ATCC 977, *Staphylococcus aureus* ATCC 29213, and *Micrococcus luteus* ATCC 4698) and Gram negative bacteria species (*Escherichia coli* ATCC 8739, *Klebsiella pneumoniae* ATCC 700603, and *Pseudomonas aeruginosa* ATCC 27853). Lowest inhibition was found from petroleum ether extract towards *Bacillus subtilis* with 11.67 mm zone diameter, while highest inhibition was recorded from methanol extract

Table 12. Antifungal and antibacterial activities of three extracts from *Piper betle* L. var Nigra

Microbe strain	Diameter of inhibition zone (mm)														
	n-hexane (µg/disc)					ethyl acetate (µg/disc)					methanol (µg/disc)				
	2	5	10	15	20	2	5	10	15	20	2	5	10	15	20
E. coli ATCC 25922	8.88	8.92	7.20	7.53	6.00	9.57	9.56	6.00	6.00	6.00	7.02	10.20	9.80	7.47	8.22
S. aureus ATCC 25923	6.00	6.00	6.00	7.00	6.00	6.00	6.00	10.63	7.53	10.03	6.00	6.00	12.69	12.00	8.52
C. albicans ATCC 10231	6.00	6.00	6.00	6.00	6.00	6.00	7.42	6.00	6.00	6.00	6.82	7.87	7.68	6.00	6.00

against *Micrococcus luteus*, with zone diameter of 43.33 mm. In addition, ethanol, petroleum ether, diethyl acetate, ethyl acetate, and chloroform extracts were also found to be able to inhibit fungi species *Aspergillus flavus* ATCC 2000226, *Aspergillus fumigatus* ATCC 204305, *Aspergillus niger* ATCC 1015, and *Candida albicans* ATCC 10231. Highest inhibition was reported from ethanol extract of *Tamarindus indica* towards *Aspergillus fumigatus*, while lowest inhibition was reported from diethyl acetate extract towards *Aspergillus niger* (Gumgumjee *et al.*, 2012).

Piperin isolated from *Piper nigrum* at 25 µL volume was found to be able to inhibit *S. aureus* with zone diameter of 4 mm, however was not able to inhibit *Escherichia coli* (Rani *et al.*, 2013). Water, methanol, ethyl acetate, and ether extracts of *Piper betle* leaves at 5 mg/mL concentration was able to inhibit growth of *S. aureus* and *E. coli* (Chakraborty and Shah, 2011). N-hexane, ethyl acetate, and methanol extract was found unable to suppress *S. aureus*, but able to inhibit *Candida albicans* with inhibition zone diameter of 1.0 up to concentration of 350 µg/disc. MIC of n-hexane, ethyl acetate, and methanol extract were 350 µg/disc, 350 µg/disc, and 1.0 µg/disc respectively (Bastos *et al.*, 2011). Black pepper extract at 1 mg/mL up to 4 mg/mL concentration could suppress *E. coli*, *S. typhi*, *Pseudomonas sp.*, and *Proteus sp.* (Ganesh *et al.*, 2014).

MIC of hexane extract from fruits and leaves of *Piper arboretum* was 250 µg/mL, while MIC of hexane and ethyl acetate extracts of *Piper tuberculatum* was >1000 µg/mL (Regasini *et al.*, 2009). Ethanol extract of *Tamarindus indica* fruits was able to suppress *S. aureus* and *E. coli* have inhibition zone of 18.0 mm and 13.0 mm. MIC of ethanol extract against both bacteria were 500 mg/mL (Gupta *et al.*, 2014). Methanol extract of *Origanum vulgare* could suppress *E. coli* and *S. aureus* at 250 µg/mL concentration (Ashraf *et al.*, 2011). Ethanol extract of leaf, stem, and fruit of *Tamarindus indica* were able to inhibit *E. coli* and *S. aureus* (Nwodo *et al.*, 2011). Water, acetone, and ethanol extract of stem and leaf of *Tamarindus indica* could inhibit *E. coli* and *S. aureus*, but unable to suppress *C. albicans*. MIC of ethanol extract against *E. coli* and *S. aureus* were 15.5 mg/mL and 8 mg/mL respectively. MBC of both extract against *E. coli* and *S. aureus* were 18 mg/mL and 20 mg/mL (Doughari, 2006).

MIC and MBC of ethanol leaf extract from *Tamarindus indica* against *E. coli* were 15.5 mg/mL and 18 mg/mL respectively, while against *S. aureus* was 8 mg/mL and 20 mg/mL (Doughari, 2006). MIC and MBC of hexane and ethyl acetate leaf extracts of *Piper arboretum* against *C. albicans* were respectively 250 µg/mL and >1000 µg/mL. MIC and MBC of leaf extract from *Piper tuberculatum* against *C. albicans* was > 1000 µg/mL (Regasini *et al.*, 2009). MIC and MBC ethanol and water extract of *Tamarindus*

Table 13. MIC and MBC of each extract from *Piper betle* var Nigra towards *E. coli*. *S. aureus* and *C. albicans*.

Microbe strain	Extract solvent					
	n-hexane		ethyl acetate		methanol	
	MIC (mg/mL)	MBC (mg/mL)	MIC (mg/mL)	MBC (mg/mL)	MIC (mg/mL)	MBC (mg/mL)
E. coli ATCC 25922	0.05	0.1	0.015	0.02	0.0275	0.03
S. aureus ATCC 25923	0.05	0.1	0.02	0.04	0.035	0.02
C. albicans ATCC 10231	-	-	0.02	0.015	0.0275	0.03

indica towards *E. coli* ATCC 11775 were 31.25 and 125 mg/mL respectively (Nwodo *et al.*, 2011). Based on data in Table 2, MIC and MBC of ethyl acetate and methanol extracts of black betel was higher compared to extracts from *Tamarindus indica*, *Piper arboretum*, and *Piper tuberculatum*. Based on result of current study, three types of extract using n-hexane, ethyl acetate, and methanol extracts had antibacterial and antifungal activities. From those varieties of extracts examined, methanol extract had highest antibacterial and antifungal activities.

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