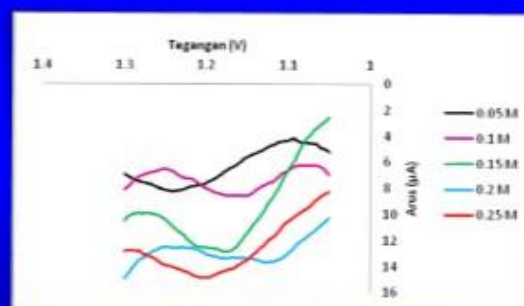
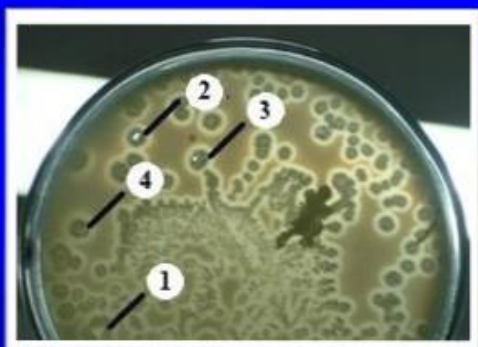
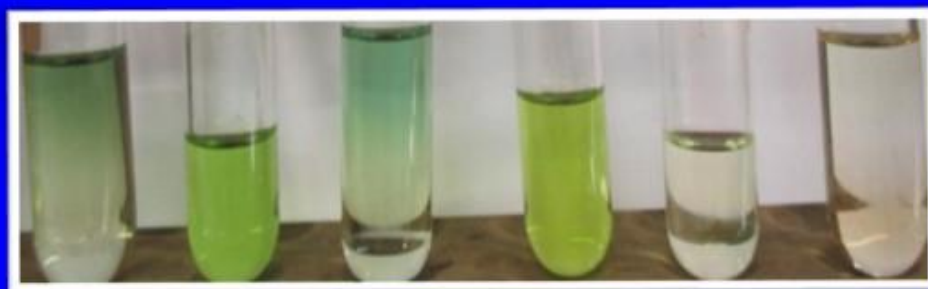
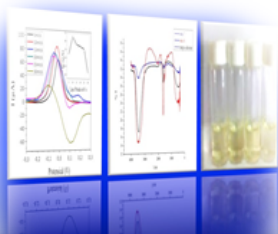


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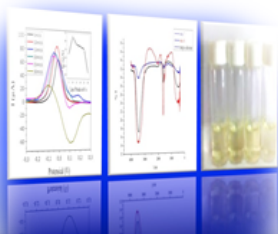
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ISOLATION AND IDENTIFICATION OF SECONDARY METABOLITES OF BLACK BETEL (*Piper betle* L. var Nigra)

¹Junairiah*, ¹Ni'matuzahroh, ¹Nabilah Istighfari Zuraidassanaaz, ²Lilis Sulistyorini
¹Department of Biology, Faculty of Science and Technology, Airlangga University, Mulyorejo 60115
Surabaya, Indonesia
²Faculty of Public Health, Airlangga University, Mulyorejo 60115 Surabaya, Indonesia
*E-mail: alip.jun1@gmail.com

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Abstract

Piper betle L. var Nigra commonly known as black betel have a potential as drug raw materials. The leave of black betel can be used to resist bleeding so as to accelerate the healing of wounds on the skin, sputum, other uses are for epistaxis, as well as for dialysis, asthma, bronchitis, cough, and high blood pressure. Many uses of black betel leaves make it interesting to learn its metabolites compounds by phytochemical screening. So, this research aimed to isolate and identificate secondary metabolites of *Piper betle* L. var Nigra. This research used three kind of organic solvents, there were methanol, ethyl acetate, and n-hexane. Several screening tests were used to isolate and identificate the phytochemical compound, including screening of alkaloids, terpenoids/steroids, flavonoids, polyphenols, tannins, and saponins. The results showed that methanol extract of black betel leaves contained of alkaloids, terpenoids/steroid, flavonoids, polyphenols and tannins compounds. The ethyl acetate extract of black betel leaves contained of terpenoids/steroid, flavonoids and tannins compounds. The n-hexane extract of black betel leaves contained of terpenoids/steroid. The methanol extract of *Piper betle* L. var Nigra contained more secondary metabolites than n-hexane and ethyl acetate extracts.

Key words : methanol ; Phytochemical screening ; *Piper betle* L. var Nigra.

Introduction

Piper is one of the genera of the familia Piperaceae. Piperaceae is one of the largest angiosperm families, with 1050 species divided into three genera in Asia (Suwanphakdee *et al.*, 2016). In Indonesia there is a black betel (*Piper betle* L. var Nigra), The leaves were reported to resist bleeding so as to accelerate the healing of wounds on the skin, sputum, other uses are for epistaxis, as well as for dialysis, asthma, bronchitis, cough, and high blood pressure (Moeljanto dan Mulyono, 2004). Black betel is a semi-woody dioecious creeper, the colour of stems are blackish red and the colour of leaves are blackish green. Falling

leaf will leave a ring-shaped marks on the stem, the leaves have a distinctive aromatic odor and it is rapidly growing 5 up to 15 meters (Abdullah, 2011). It is distributed in the eastern part of the coast of Africa, Zanzibar island, Bonin island, Fuji islands, and the Indonesian islands (Moeljanto dan Mulyono, 2004). It is one of the important plants in the Asiatic region after coffee and tea respectively in terms of daily consumption (Kumar *et al.*, 2009). Lots of black betel varieties in various countries, *Piper betle* L. var Nigra is one of endemic species of Indonesia.

Piper betle L. var Nigra has been known as ornamental plants and herbal remedies.

Plants are recognized as aromatic as well as source of medicine. The extracts obtained from various plant parts possess medicinal properties and are used as colouring agent, preservative, sweetening agent and as an additive in many medicinal formulations. Plants restrain abundant amount of secondary metabolites, they are considered to be principal source of therapeutically active compounds (Craker, 2007). However, secondary metabolites of n-hexane, ethyl acetate and methanol extract of black betel leaf still remain to be investigated. So this research aimed to determine the secondary metabolites of n-hexane ethyl acetate and methanol extract of black betel leaves.

Material and Methods

Plant Materials

The whole plants of *Piper betle* L. var Nigra were collected from the Kayon flowers market, Surabaya, East Java, Indonesia.

Extraction

All of the black betel leaves were washed with running water to separate it from the rest of the land attached, then all of them were dried, after which the black betel leaves in small pieces like powder and inserted into each bottle containing of n-hexane, ethyl acetate, and methanol solvent. After two days, the extract solution of each black betel was separated from the pulp using filter paper.

Screening of Alkaloids and Terpenoids / Steroids

To screen alkaloids, 0.2 gram sample was dissolved in 4 mL of 96% ethanol using an ultrasonic vibrator. Then, 4 µl extract was spotted on TLC plates. Kiesel gel GF 254 was used in stationary phase, chloroform-methanol (9:1) was used in mobile phase and Dragendorff reagent was used in color stain.

Orange color indicated the alkaloids in the extract.

To screen terpenoids/steroids, 0.3 gram sample was dissolved in 15 mL of ethanol, and then divided into three parts, each part was 5 mL, referred to as a solution of II-A, II-B, and II-C. Then, it was identified by TLC. 0.2 g sample was dissolved in 4 mL of n-hexane. 2 µl extract was spotted on the stationary phase. Kiesel gel GF 254 was used as stationary phase, n-hexane - ethyl acetate (4:1) was used as mobile phase and anisaldehyde sulfuric acid was used as color stain. The existence of terpenoids/steroid was indicated by the occurrence of red-purple or purple. For Salkowski test, solution II-A is used as a blanco, 5 mL solution II-C was added 1-2 mL concentrated H₂SO₄ through the wall of the tube. The presence of unsaturated steroids was characterized by the emergence of the red color ring.

Screening of Flavonoids and Polyphenols

To screen flavonoids, 0.2 g sample was dissolved in 10 mL of 96% ethanol using an ultrasonic vibrator. Then, TLC testing method was used. 25 µl extract was spotted on a TLC plate. This TLC test used a thin layer of Kiesel Gel GF 254 in stationary phase, chloroform - methanol (9:1) in mobile phase, and FeCl₃ 2% as color stains. Color spot was observed by UV 366 nm and 254 nm. The presence of flavonoids was showed by spotting brown to black with FeCl₃ 2% as a color stains.

To screen polyphenols, TLC testing method was used. 5 µl extract was spotted on a TLC plate. This TLC test used a thin layer of Kiesel Gel GF 254 in stationary phase, ethyl acetate - methanol - formic acid (16:4:1) as mobile phase, and FeCl₃ 2% as color stain. Color spot was observed by UV 366 nm and 254 nm. The presence of polyphenols was showed by brown to black spot with FeCl₃ 2% as a color stains.

Screening of Tannins and Saponins

To Screen Tannins, three tubes were provided. They were tube A (for blanco), tube B (for comparison tannins) and tube C (for samples of guava leaf extract). A tube contained 5 mL water, the tube B contained tannin reference solution in 5 mL water, and tube C contained guava leaf extract plus 5 mL water. Then each tube was added with 1 mL Folin solution, vortex 1 minute, let stand for 5 minutes. Then 2 mL sodium carbonate was added, vortex 1 minute, let stand for 10 minutes. Blue color indicated content of tannin.

To screen saponins, Forth test was used, 0.25 g sample was dissolved in 10 mL of boiling water and then centrifuged at 5000 rpm. The supernatant was taken as much as 10 mL and strongly shaken for 30 seconds. Positive result of forth test was showed by stable forth height at 1-10 cm above the surface for more than 30 minutes and when it was added by 1 drop of 2N hydrochloric acid, the forth did not disappear. Then Liebermann-Burchard test was used, using a solution of II-A and II-B test group of terpenoids and steroids solution II-A is used as a blanco. 3 drops of acetic acid anhydride and 1 drop of concentrated H₂SO₄ was added to 5 ml II-B solution and shaken slowly. The blue green color occurred showed the presence of steroid saponins, red-purple color showed the presence of saponinriterpenoids and light yellow color indicates saturated saponin/sapogenin.

Results and Discussion

Screening of Alkaloids and Terpenoids/Steroids

The emergence of an orange stain on TLC test showed the presence of alkaloids compounds. In the Fig. 3(a) showed that methanol extract of black betel was stained orange, whereas the samples of ethyl acetate (Fig. 2(a)) and n-hexane (Fig. 1(a)) extracts were not stained orange stain. The emergence of red-purple/purple on the TLC test showed a group of terpenoids/steroid compounds. In the image shown that methanol, ethyl acetate, and n-hexane extract of black betel leaves were stained purplish red (Fig. 1(b), 2(b), and 3(b)). The emergence of the red ring on Salkowski test showed the presence of unsaturated steroids compounds. In Fig. 4 red ring was not showed on methanol, ethyl acetate, and n-hexane extract of black betel leaves.

Screening of Flavonoids and Polyphenols

The emergence of brown to blackish stain on TLC test showed a flavonoid compound. In Fig. 3(c) and 2(c) showed that methanol and ethyl acetate extracts of black betel leaves were stained brown, meanwhile the n-hexane extract of black betel leaves were not stained brown (Fig. 1(c)). The emergence of brown to blackish stain on TLC test showed the presence of polyphenols compounds group. In Fig. 3(d) showed that methanol extract of black betel leaves was stained black, while the stain of ethyl acetate (Fig. 2(d)) and n-hexane (Fig. 1(d)) extract were not stained black.

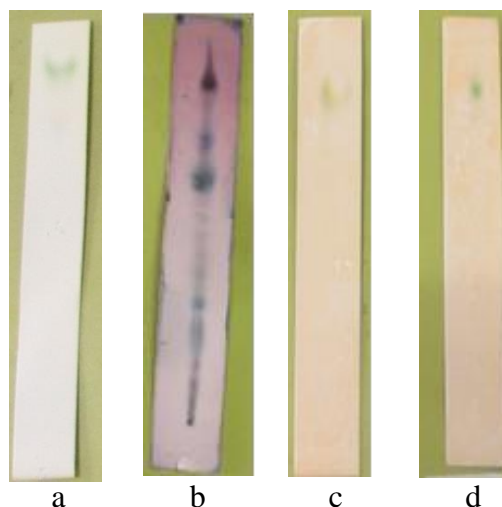


Figure 1. Phytochemical screening of n-hexane extract of black betel leaves (*Piper betle* L. var Nigra) with TLC method test. (a) Alkaloids, (b) Terpenoids/steroids, (c) Flavonoids, and (d) Polyphenols.

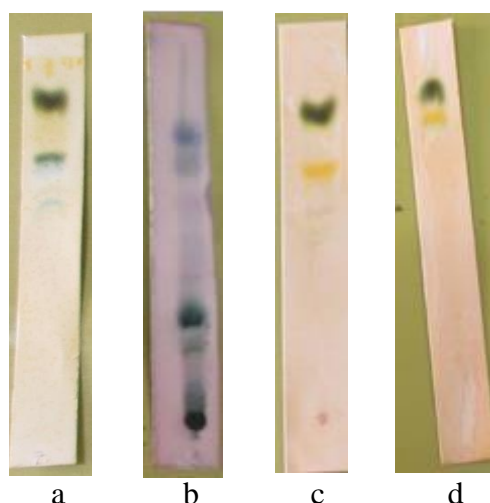


Figure 2. Phytochemical screening of ethyl acetate extract of black betel leaves (*Piper betle* L. var Nigra) with TLC method test. (a) Alkaloids, (b) Terpenoids/steroids, (c) Flavonoids, and (d) Polyphenols.

Screening of Tannins and Saponins

In Fig. 5 showed that the solutions were blue at methanol and ethyl acetate extract of black betel leaf. It indicated the presence of tannin compounds. In screening of saponins, if the solution of extract showed forth in the forth test, it indicated the presence of saponins compounds. In Fig. 6 (a-c) showed that all of extract solutions (methanol, ethyl acetate, and n-hexane

extract of black betel leaf) did not produce a froth. Positive result in Liebermann-Burchard test showed blue-green color, red-purple color and pale yellow color that indicated the presence of steroidal saponins, saponinriterpenoids and saturated saponin respectively. Meanwhile in this research, the methanol and ethyl acetate extract of black betel leaves showed green

color and n-hexane extract showed clear color (Fig. 6 (d), (f), (h)).

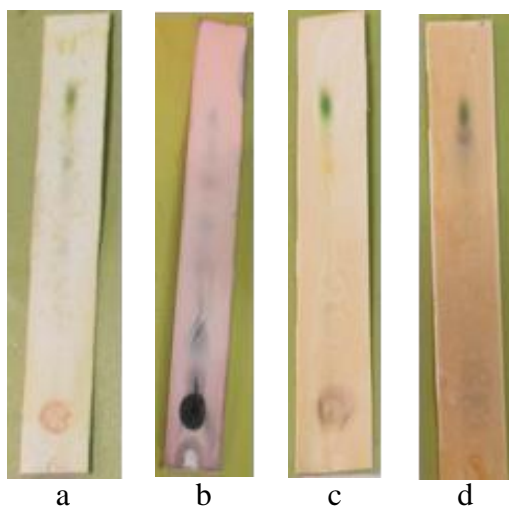


Figure 3. Phytochemical screening of methanol extract of black betel leaves (*Piper betle* L. var Nigra) with TLC method test. (a) Alkaloids, (b) Terpenoids/steroids, (c) Flavonoids, and (d) Polyphenols.

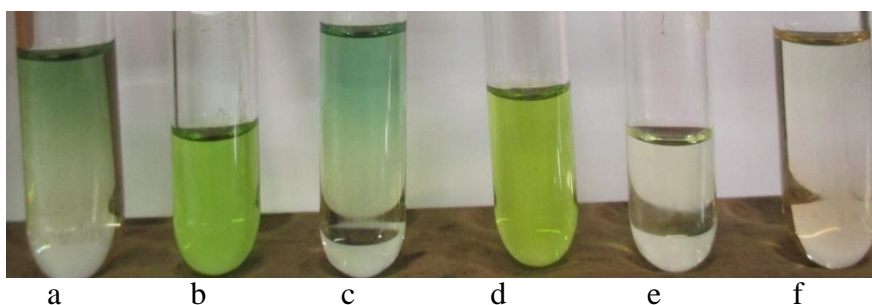


Figure 4. Screening of steroids using Salkowski test on various organic solvents extract of black betel leaves (*Piper betle* L. var Nigra). (a) Methanol extract of black betel (*Piper betle* L. var Nigra) + 1-2 mL of H₂SO₄ concentrated, (c) Ethyl acetate extract of black betel (*Piper betle* L. var Nigra) + 1-2 mL of H₂SO₄ concentrated, (e) n-hexane extract of black betel (*Piper betle* L. var Nigra) + 1-2 mL of H₂SO₄ concentrated, and (b), (d), (f) blanco.

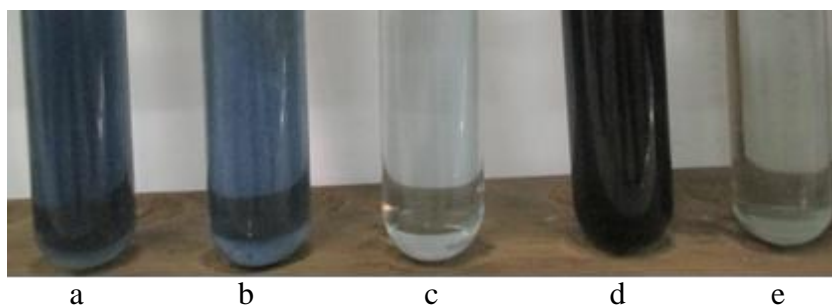


Figure 5. Screening of tannins on various organic solvents extract of black betel leaves (*Piper betle* L. var Nigra). (a) Methanol extract of black betel (*Piper betle* L. var Nigra), (b) Ethyl

acetate extract of black betel (*Piper betle* L. var Nigra), (c) n-hexane extract of black betel (*Piper betle* L. var Nigra), (d) standard solution of tannins, and (e) blanco.

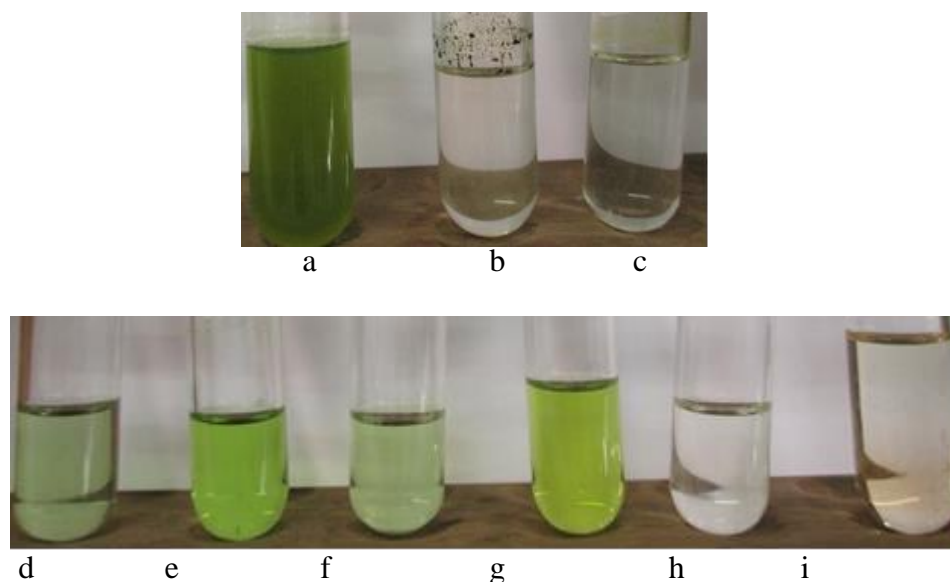


Figure 6. Screening of saponins on various organic solvents extract of black betel leaves (*Piper betle* L. var Nigra). (a) Forth test of methanol extract of black betel (*Piper betle* L. var Nigra), (b) Forth test of ethyl acetate extract of black betel (*Piper betle* L. var Nigra), (c) Forth test of n-hexane extract of black betel (*Piper betle* L. var Nigra), (d) Liebermann-Burchard test methanol extract of black betel (*Piper betle* L. var Nigra) + 3 drops of acetic acid anhydride + 1 drop of concentrated H_2SO_4 , (f) Liebermann-Burchard test ethyl acetate extract of black betel (*Piper betle* L. var Nigra) + 3 drops of acetic acid anhydride + 1 drop of concentrated H_2SO_4 , (h) Liebermann-Burchard test n-hexane extract of black betel (*Piper betle* L. var Nigra) + 3 drops of acetic acid anhydride + 1 drop of concentrated H_2SO_4 , (e), (g), and (i) blanco.

Table 1. Identification of screening phytochemical in various organic solvents of extract black betel leaves.

Phytochemical	Organic solvents of extract black betel leaves		
	n-hexane	Ethyl acetate	Methanol
Alkaloids	-	-	+
Terpenoids/ Steroids	+	+	+
Flavonoids	-	+	+
Polyphenols	-	-	+
Tannins	-	+	+
Saponins	-	-	-

Plant can be used as traditional medicine because it contain chemical compounds that have biological activity. The biologically active compound is a secondary metabolite which includes alkaloids, flavonoids, terpenoids, tannins and saponins. The content of secondary metabolite compounds in plant can be known by approaching method that provide information on the presence of secondary metabolite compounds. One of the method that can be used is the phytochemical screening method (Harbone, 1973). Before isolation and identification, the extraction was done first in this research. There are many extraction method, one of them is maseration.

After the black betel leaves was dried and mashed, then it divided into three equal parts to add three organic solvents to each part. Pulverization was useful to increase area surface of the particles contacted with solvent. After entering the powder, solvent exported the chemical compounds to be mixed with the substance of the attractants so that the process of escape can be more effective. The advantages of the maceration method are easy to perform, high enough rendamen product and low possibility of damaged chemical compounds contained in the material because did not accompanied by giving heat. In table 1. showed that methanol extract of black betel leaves was contained of alkaloids, terpenoids/steroid, flavonoids, polyphenols and tannins compounds. The ethyl acetate extract of black betel leaves was contained of terpenoids/steroid, flavonoids and tannins compounds. The n-hexane extract of black betel leaves was contained of terpenoids/steroid. In general, methanol solvents were used in the process of isolating organic compounds of natural materials because they could dissolve secondary metabolites (Prashant *et al.*,

2011). Methanol was a universal solvent which had a polar group (-OH) and a nonpolar group (-CH) So that it could attract those analytes polar and nonpolar. There are several study about secondary metabolites, like *Piper longum* L. (Lee *et al.*, 2001), *Peperomia pellucida* L., *Marsilea quadrifolia* L. (Jothi *et al.*, 2013), *Mentha longifolia* L. (Al-bayati, 2009), and *Euphorbia hirta* L. (Basma *et al.*, 2011).

Flavonoids and tannins are part of the phenolic compounds. Flavonoids that have an ortho-shaped hydroxy group will give intensive yellow fluorescence on UV366 when reacts with boric acid. Flavonoids have a variety of types and present in free form (aglikon) or bound as glycosides. The polymethoxyaglicone is nonpolar, the polyhydroxyaglycone is semi-polar, while the flavonoid glycoside is polar, because it contains a number of hydroxyl groups and sugar (Harbone, 1973). Therefore, the flavonoid group may be extracted in a universal methanol solvent. The tannins were shown after addition of FeCl_3 which reacted with one of the hydroxyl groups on the tannin compound. FeCl_3 addition produced a blackish-green color indicated the presence of a tannin condensed. Saponin is polar because it is a glycoside form of saponin. Saponin is active compound on the surface and cause foam in shaken water. The presence of forth on the saponin test indicated the presence of glycosides. Glycosides had the ability to form foam in water that was hydrolyzed to glucose and other compounds. The compound of saponins will tend to be extracted by semi-polar solvents such as methanol. While, alkaloid screening had same principle with precipitation reaction that occurs due to the replacement of the ligand (Agostini-costa *et al.*, 2012).

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