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Research Report

## BETLE LEAF ESSENTIAL OIL FOR HEMOPHILIAC PATIENTS AND ITS ANTIBACTERIAL EFFECTS ON *MYCOBACTERIUM TUBERCULOSIS*

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### ABSTRACT

*Betle leaf (Piper betle L.) is a medicinal plant. It contains essential oil and shows various biological activities, such as antibacterial, anticoagulant, etc. It is further reported to have low anticoagulant activities; thus, it is highly potential as a candidate for coagulant drug. Coagulant is used to prevent bleeding for patients with blood clotting disorders like hemophilia. In Indonesia, 1,236 people were reported with hemophilia. The standard parameters of anticoagulant activity are the freezing period and the compound concentrations. The purpose of this study was to determine the effect of betle leaf's essential oil on blood coagulation in patients with factor VIII and IX of blood plasma disorders. The isolation of essential oil is conducted through steam distillation method with two kinds of solvents, namely distilled water and n-hexane. The obtained n-hexane extract is then separated from the liquid-liquid extraction and rotary evaporator. Essential oil is diluted with citrate plasma solution. The test results of blood clots increase as the concentration of essential oils increase. The results are recorded as such: essential oils 1/2 times dilution of 99.67 seconds; 1/4 times dilution of 127 seconds; 1/8 times dilution of 179 seconds; and 1/16 times dilution of 242.67 seconds. The test above proves that the piper betle extract possesses a coagulant activity. The ethanol extract contained in the piper betle could stimulate clotting in the blood cells. It is caused by the increase of blood plasma concentration which further escalate the plasma fluid into the blood cells. Based on this study, the activity of Mycobacterium tuberculosis can be obstructed by betle leaf in 1/2 times dilution. The extract significantly reduces acid which accelerates bacteria development.*

**Keywords:** Betle Leaf, Liquid-Liquid Extraction, Blood Clotting, Coagulant, Anti-Mycobacterium tuberculosis

### ABSTRAK

*Daun Sirih (Piper betle L.) merupakan tanaman obat. Daun sirih terdapat kandungan minyak atsiri dan menunjukkan berbagai aktivitas biologi, diantaranya adalah antibakteri, antikoagulan, dan lain sebagainya. Di Indonesia, jumlah penderita hemofilia dilaporkan 1.236 orang. Koagulan digunakan untuk mencegah pendarahan pada penderit kelainan pembekuan darah seperti hemofilia. Daun sirih dilaporkan memiliki aktivitas antikoagulan rendah, sehingga sangat potensial untuk kandidat obat koagulan. Parameter standar untuk aktifitas antikoagulan adalah waktu pembekuan dan konsentrasi senyawa. Tujuan dari penelitian ini adalah mengetahui pengaruh minyak atsiri daun sirih terhadap pembekuan darah pada penderita kelainan faktor VIII dan IX plasma darah. Isolasi minyak atsiri dilakukan dengan metode destilasi uap menggunakan dua macam pelarut yaitu aquades dan n-heksana. Ekstrak n-heksana yang diperoleh dipisahkan dengan ekstraksi cair-cair dan rotary evaporator. Minyak atsiri didilusi dengan larutan plasma sitrat. Hasil uji pembekuan darah minyak atsiri meningkat seiring konsentrasi minyak atsiri yaitu pengenceran 1/2 kali 99.67 detik; pengenceran 1/4 kali 127 detik; pengenceran 1/8 kali 179 detik; dan pengenceran 1/16 kali 242.67 detik. Pengujian di atas menunjukkan bahwa ekstrak piper betle memiliki aktivitas koagulan. Ekstrak etanol yang terkandung dalam piper betle dapat menyebabkan pembekuan dalam sel-sel darah. Hal ini disebabkan konsentrasi plasma darah naik, yang meningkatkan cairan plasma ke dalam sel darah. Berdasarkan penelitian ini, aktivitas Mycobacterium tuberculosis dapat dihambat oleh ekstrak daun sirih pada pengenceran 1/2 kali. Ekstrak secara signifikan mengurangi sifat asam yang dapat mempercepat perkembangan bakteri.*

**Kata kunci :** Daun Sirih, Ekstraksi Cair-Cair, Pembekuan Darah, Koagulan, Anti-Mycobacterium tuberculos

## INTRODUCTION

Indonesia has a tropical climate suitable to grow various medicinal plants, one of them is Betle leaf (*Piper betle* L.).<sup>1</sup> Indonesian people who live in rural areas particularly use betle leaf to cure various diseases. A part of the leaf is mainly used for some health treatments, such as nosebleed (epistaxis). The leaf is rolled up and put into one's nostrils.<sup>2</sup> Moreover, betle leaves can also be used as a mouthwash. A dried betle leaf can also be used as a traditional medicine, such as cough medicine, drugs, or eye wounds. Betle is a chemical plant which consists of saponins, flavonoids, polyphenols, and essential oil.<sup>3</sup> There is an increase usage of natural materials through a large scale of fabrication. The use of traditional medicine is considered having fewer side effects compared with the chemical drugs and more affordable.<sup>4</sup> Modern drug is widely believed to cause spasm of the bile duct sphincter and impede bile flow; whereas the effect on renal development receives less attention.<sup>5</sup>

Meanwhile, hemophilia is a hereditary disorder which is heavily associated with a deficiency or an abnormality of biological factor VIII and factor IX in blood plasma.<sup>6</sup> This genetic disorder affects many people. In Indonesia, the number of hemophiliac was reported at 1,236 people.

The contents of the essential oil in a betle plant are chavicol, eugenol, cineol, and carvacrol. Essential oils functions as antibacterial, antioxidant, antifungal, anti-ulcerogenic, anti-amoebic, anti-inflammatory, anti-filarial, anti-microbial, anti-fertility, anti-hyperglycemic, anti-dermatophytid, anti-naceptive, and radioprotective properties.<sup>1</sup> Tuberculosis (TB), which is caused by *Mycobacterium tuberculosis*, is a highly infectious disease. Its morbidity and mortality continue being a cause of concern. There has been a substantial increase in these last decades in the investigation of medicinal plants to find out their biological efficacies for the treatment of various disorder. In the field of anti-TB agents, several studies on potential medicinal plants have been reported from various parts of the world. *Piper nigrum* extract, a combination of acetone and ethanol extracts of 50 µg/ml each, was effectively tested against anti-*Mycobacterium tuberculosis*.<sup>7</sup> The antibacterial activity from the plant is caused by secondary metabolic compounds with phenolic compounds. This study chooses betle plant leaves as the research object, as it is often used as nosebleed cure.<sup>8</sup> This study uses *Piper betle* L. species from Jajar village, Kediri District, East Java, Indonesia.

On the description above, the researchers look for a new solution by leveraging the existing knowledge which increases the potential betle leaf's essential oil extracts (*Piper betle* L.) on hemophiliac patients. The purpose of this study is to determine the effects of betle leaf's essential oil on hemophiliac patients in vitro using clotting time method and study of anti-*Mycobacterium tuberculosis* activity.

## MATERIAL AND METHOD

### Betle Leaf's Essential Oil Extraction

Betle leaf's essential oil is isolated using steam distillation technique. Prior to the first steam distillation, the betle leaves are cut into small pieces to facilitate the distillation and insulate the essential oil inside the betle leaf. Solvents are used for the distillation. Time required to isolate the essential oil is about 2 hours until the solution in the distillation equipment condenser becomes colorless. The color indicates that the essential oil has been all isolated.

Isolation is separated between water phase and organic phase using liquid-liquid extraction with an organic n-hexane solvent. The extraction is performed 5 times to perfectly separate the essential oil in the water. The essential oil will be mixed with the n-hexane solvent.

The last phase of separation is conducted using a rotary evaporator. The essential oil in n-hexane is separated using the principle of boiling point. The heating process is carried out at approximately 60 to 70°C. N-hexane's boiling point is recorded at 63°C in which it is still in the form of gas; while the essential oil remains in liquid form due to the extremely high boiling point of the volatile oil. The heating process produces two products, namely n-hexane and pure essential oil of betle leaf. The essential oil obtained in this isolation is 4.5 mL with a percentage of 0.9%. This is because the properties of essential oil is volatile, thus, it reduces their products. Volatile chemical compounds have a high vapor pressure at ordinary room temperature.<sup>6</sup>

### Extract Dilutions

Extract dilutions is conducted using PZ solution (saline), because this solution is deemed to have the same osmotic pressure with the fluid contained in human body. Dilution is done by extracting the essential oil of betle leaf as much as 1 cc, added with 1 cc solution of PZ, then ½ times dilution of the extract concentrated essential oil is obtained. A quarter times, 1/8 times, and 1/16 times dilutions are also conducted to determine the most effective dilution to speed up blood clotting.<sup>6</sup>

### Separation of Plasma from Red Blood

Twenty-five cc blood from normal individual is mixed with 3.8% sodium citrate in 9:1 ratio; then, the mixture is put in a tube of blood plasma and made sure it is perfectly blended. The mixture is centrifuged for about 30 minutes at 1500 rpm. Tubes are excluded from clinical centrifuges. At the top of the tube, there is clear yellowish liquid; while at the bottom, red sediment can be seen. The clear liquid, which is called citrate plasma, is then extracted and stored in a refrigerator.

### Control Solution

Citrate plasma of 0.8 ml for each blood group is mixed with 0.2 cc PZ solution, then they are shaken and left for some time to mix. Next, 0.2 cc and 0.2 cc plus  $\text{CaCl}_2$  are taken for control solution until the first fiber is formed. The fiber is in the form of white threads called fibrin.

### Blood Coagulation Experiment using the Essential Oil of Betle Leaf

The 0.8 cc citrate plasma solution is added to 0.2 cc betle leaf's essential oil extract. After making sure it is blended well, 0.2 cc from the mixed solution is added to 0.2 cc  $\text{CaCl}_2$ . The researchers then observe and record the freezing time. The same procedures are performed to  $\frac{1}{2}$  times,  $\frac{1}{4}$  times,  $\frac{1}{8}$  times, and  $\frac{1}{16}$  times dilutions. Citrate plasma uses all blood types. The experiments are performed at  $37^\circ\text{C}$ .

### Anti-*Mycobacterium tuberculosis* test

*Mycobacterium tuberculosis* refers to the strain H37Rv. The preparation for medium 7H10 was dissolved with aquadest, then autoclaved in  $121^\circ\text{C}$  for 10 minutes. Medium 7H10 is added with essential oil and incubated for 4-3 weeks at  $37^\circ\text{C}$  in a  $\text{CO}_2$  incubator. The tested essential oil concentrations consist of  $\frac{1}{2}$  times dilution,  $\frac{1}{4}$  times dilution,  $\frac{1}{8}$  times dilution, and  $\frac{1}{16}$  times dilution.

## RESULT AND DISCUSSION

Blood plasma is the most important [object] in this research, because it consists of plasma proteins which have a big impact on blood clotting. The blood plasma was separated by a mixture of blood centrifuged between normal and 3.8% sodium citrate. This study uses the normal blood group B. Therefore, sodium citrate anticoagulant of 3.8%, which slows down clotting process, is added to make the normal blood to have the same nature with the blood with clotting factor disorders. Although both citrate and heparin are used as anticoagulants during apheresis, citrate is preferred for most exchange procedures because of its safety and effectiveness.<sup>9</sup> The 9:1 (blood to sodium citrate 3.8%) ratio is used for the anticoagulants as an ideal comparison, because the anticoagulation in a greater portion of blood clotting takes longer processing time.<sup>10</sup> The plasma from the reaction above is called citrate plasma (Figure

The control solution is used as a comparison to the blood clotting process using betle leaf's essential oil. A control solution is considered as successfully made if the color is brownish yellow (Figure 2).

Blood clotting test is conducted by mixing the essential oil dilution with plasma citrate in four different reaction tubes. A solution of  $\text{CaCl}_2$  is then added into the mixture. The fourth solution is formed in yellow-brown color with different intensities of concentration. The color intensity for the solution concentration of essential oil with  $\frac{1}{2}$  times dilution is higher or more concentrated than the essential oil

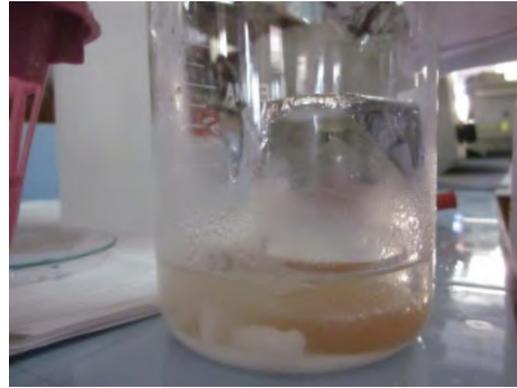


Figure 1. Citrate plasma



Figure 2. Control solution



Figure 3. Dilution of essential oil with citrate plasma

with  $\frac{1}{16}$  times dilution. This solution results in the same color with the earlier control solution (Figure 3-4).

Blood clotting mechanism cannot be shown directly in this study, as this study is only conducted in vitro; however, there are some visual data obtained.  $\text{CaCl}_2$  solution acts as the activation for prothrombin. The study is conducted at  $37^\circ\text{C}$ , matching the temperature of human body. The result of blood clotting test is showed in Table 1.

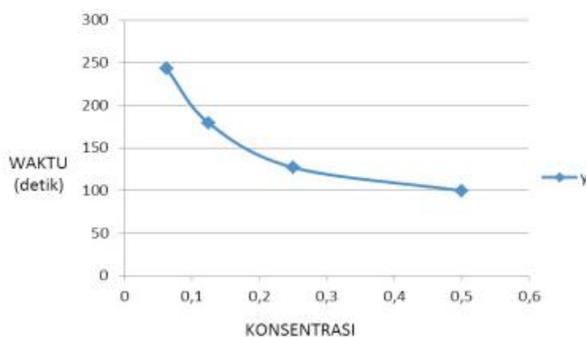
ANOVA test is then performed to analyze the results. A significant difference between four conditions of essential oil is obtained (F-count is recorded at 232.69, greater than the F-table at 4:07).



**Figure 4.** Freezing blood test with essential oil

**Table 1.** The results of blood clotting in seconds

Concentration of Essential Oil	Time (second)
1/2 times	99.67
1/4 times	127
1/8 times	179
1/16 times	242.67



**Figure 5.** Curve blood clotting in betle leaf's essential oil

According to Table 1, the frozen blood from each donor was shown in time difference. Normal blood clotting occurs from 3 to 18 minutes.<sup>11</sup> Blood clots occurred because plasma protein prothrombin is changed into thrombin. Thrombin is an enzyme which catalyzes the forming of fibrinogen. It is a soluble protein which changes into fibrin. In a few second, fibrin polymerized a mesh which is composed by some fibrin threads. The thread runs to every direction and forms a net which catches blood element and forms a clot.<sup>12</sup>

Based on blood clotting curve of the betle leaf's essential oil, it can be concluded that the higher the concentration is, the faster the blood clotting process takes place.<sup>13</sup> The test results of blood clots increase as the concentration of essential oil increases. The results are as follows: essential oils 1/2 times dilution for 99.67 seconds; 1/4 times dilution for 127 seconds; 1/8 times dilution for 179 seconds; and 1/16 times dilution for 242.67 seconds. This research successfully demonstrates that the essential oil of betle leaf can be used as blood clotting. The betle leaf is known to have antibacterial activity. *Staphylococcus aureus*' activity was inhibited by betle leaf in 200 mg/ml concentration. The extract is found to significantly reduce acid production of the bacteria.<sup>14</sup> The test above indicates that the piper betle

extract possesses the coagulant activity. Blood clotting is a complex procedure which involved numerous factors in the plasma and tissues. Both intrinsic and extrinsic pathways play vital roles. Inhibitors of the blood coagulation affect some factors in blood (Figure 5).<sup>15</sup>

The chemical components of betle leaf's essential oil are monoterpenes, sesquiterpenes, alcohols, esters, aldehydes, and phenols.<sup>16</sup> According to Tangkery in 2013, ethanol causes clotting in the blood cells to stick to each other; however, the red blood cells, or erythrocytes, no longer have any forms, because the cell's wall has been destroyed. It is caused by the increase of blood plasma concentration which further escalates plasma fluid into the blood cells.<sup>11</sup>

#### Anti-*Mycobacterium Tuberculosis* Test

Amidst the emerging drug resistance in infectious diseases field, the use of medicinal plants provides an alternative therapy. Unfortunately, there are limited report on the anti-*Mycobacterium tuberculosis* in Indonesian medicinal plants. Essential oil from *Piper betle L.* was shown to have anti-*Mycobacterium tuberculosis* activity (Figure 6).

The essential oil with 1/2 times dilution concentration demonstrates an inhibitory activity against *Mycobacterium tuberculosis*. It proves its effectiveness for the inhibited activity of *Mycobacterium tuberculosis*. Some drops of fungal are shown in the 1/2 times dilution bottle, however, there are no bacteria showed.

*Mycobacterium tuberculosis* and fungal are shown in 1/4 times dilution, 1/8 times dilution and 1/16 times dilution. Non-active essential oil and activity of *Mycobacterium tuberculosis* are demonstrated in the lower concentration of the essential oil.

Meanwhile, piperine is an active compound in *Piper betle L.* extract. In the literature, piperine of 1.0 and 10 µg/ml showed an up-regulation of IFN-γ and IL-2 production in *Mycobacterium tuberculosis*. An effective immunostimulant can complement the host cellular immune response by specifically inducing the type 1 (Th-1) response.<sup>17</sup> In



1/16 times 1/8 times 1/4 times 1/2 times

**Figure 6.** Culture of *Mycobacterium tuberculosis* containing essential oil from *Piper betle L.*

this regard, the key cytokine in mice and humans seems to be gamma interferon (INF- $\gamma$ ) which activates bactericidal effector mechanisms in the mycobacterial host cell, the macrophage.<sup>18</sup> Piperine (1 mg/kg) in mice which are infected with *Mycobacterium tuberculosis* activates the differentiation of the T cells into Th-1 sub-population (CD4<sup>+</sup>/CD8<sup>+</sup> subsets).<sup>17</sup> Protective immunity against *Mycobacterium tuberculosis* requires the generation of cell-mediated immunity. The secretion of Th-1 cytokines by antigen-specific T cells plays an important role in protective granuloma formation and stimulates the antimicrobial activity of the infected macrophages.<sup>19</sup>

## CONCLUSION

The dilution of the betle leaf's essential oil extraction at 1/2 dilution have the most rapid blood clotting effectiveness for hemophilia treatment in vitro. It is caused by the capability of ethanol compound in the betle leaf's extract to yield clotting in blood cells. The blood clot further increases blood plasma concentration and plasma fluid in blood cells. Plasma fluid is an important component for blood clot factor, because it contains prothrombin and fibrinogen. Betle leaf extract provides anti-infection activity, mainly against *Mycobacterium tuberculosis*.

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## REFERENCES

- Sripradha S. Betel Leaf – The Green Gold. *J Pharm Sci Res*. 2014;6(1):36–7.
- A TP, Farida Y. Total Phenolic, Flavonoids Content and Antioxidant Activity of The Ethanolic Extract of Betel Leaf (Piper betle L.). In: Total Phenolic, Flavonoids Content and Antioxidant Activity of The Ethanolic Extract of Betel Leaf (Piper betle L). Jakarta: The International Conference in Nanotechnology; 2013. p. 1–4.
- Ovedoff D. Kapita Selekt Kedokteran. In: Kapita Selekt Kedokteran. Binarupa Aksara; 2002.
- Oktora L, Kumala R. Pemanfaatan Obat Tradisional Dan Keamanannya. *Maj Ilmu Kefarmasian*. 2006;III(1):1–7.
- Schreuder MF, Bueters RR, Huigen MC, Russel FGM, Masereeuw R, van den Heuvel LP. Effect of Drugs on Renal Development. *Clin J Am Soc Nephrol*. 2011 Jan 1;6(1):212–7.
- Ramström S. Clotting time analysis of citrated blood samples is strongly affected by the tube used for blood sampling. *Blood Coagul Fibrinolysis*. 2005 Sep;16(6):447–52.
- Birdi T, D'souza D, Tolani M, Daswani P, Nair V, Tetali P, et al. Assessment of the Activity of Selected Indian Medicinal Plants against *Mycobacterium tuberculosis*: A Preliminary Screening Using the Microplate Alamar Blue Assay. *European J Med Plants*. 2012 Jan 10;2(4):308–23.
- Tedjasulaksana R. Ekstrak Etil Asetat dan Etanol Daun Sirih (Piper betle L.) dapat Memperpendek Waktu Pendarahan Mencit (*Mus Musculus*). *J Kesehat Gigi*. 2013;1(1):32–9.
- Lee G, Arepally GM. Anticoagulation techniques in apheresis: From heparin to citrate and beyond. *J Clin Apher*. 2012;27(3):117–25.
- Félix-Silva J, Souza T, Camara RBBG, Cabral B, Silva-Júnior AA, Rebecchi IMM, et al. In vitro anticoagulant and antioxidant activities of *Jatropha gossypifolia* L. (Euphorbiaceae) leaves aiming therapeutical applications. *BMC Complement Altern Med*. 2014 Dec 20;14(1):405.
- Rossi C, Holbrook M, James SH, Mabel and D. Medical and Forensic Aspects of Blood Clot Formation in the Presence of Saliva – A Preliminary Study. *J Bloodstain Pattern Anal*. 2012;28(3):3–12.
- Tangkery RAB, Paransa DS, Rumengan A. Uji Aktivitas Antikoagulan Ekstrak Mangrove *Aegiceras corniculatum*. *J Pesisir dan Laut Trop*. 2013;1:7–14.
- Miranda CAS, Cardoso MG, Mansanares ME, Gomes MS, Marcussi S. Preliminary assessment of *Hedychium coronarium* essential oil on fibrinolytic and coagulant activity induced by Bothrops and Lachesis snake venoms. *J Venom Anim Toxins Incl Trop Dis*. 2014;20(1):39.
- Nalina T, Rahim ZHA. The Crude Aqueous Extract of Piper betle L. and its Antibacterial Effect Towards *Streptococcus mutans*. *Am J Biochem Biotechnol*. 2007 Jan 1;3(1):10–5.
- Jesonbabu J, Spandana N, Reddy MS. A Bioactive Compound from Piper Betel with Anticoagulant Activity. *Int J Pharm Pharm Sci*. 2012;4(3):2012.
- Satyap P, Setzer WN. Chemical Composition and Biological Activities of Nepalese Piper betle L. *IJPHA*. 2012;1(2):23–6.
- Sharma S, Kalia NP, Suden P, Chauhan PS, Kumar M, Ram AB, et al. Protective efficacy of piperine against *Mycobacterium tuberculosis*. *Tuberculosis (Edinb)*. 2014 Jul;94(4):389–96.
- Holten-Andersen L, Doherty TM, Korsholm KS, Andersen P. Combination of the cationic surfactant dimethyl dioctadecyl ammonium bromide and synthetic mycobacterial cord factor as an efficient adjuvant for tuberculosis subunit vaccines. *Infect Immun*. 2004 Mar;72(3):1608–17.
- Quiroga MF, Jurado JO, Martínez GJ, Pasquinelli V, Musella RM, Abbate E, et al. Cross-talk between CD31 and the signaling lymphocytic activation molecule-associated protein during interferon-gamma production against *Mycobacterium tuberculosis*. *J Infect Dis*. 2007 Nov 1;196(9):1369–78.