Antidiabetic and cytotoxic activities of ethyl acetate extract of Piper betle Leaves

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Abstract. Antidiabetic and cytotoxic activity examination of ethyl acetate extract of betel leaf (Piper betle L.) has been done. The antidiabetic activity test was performed in vitro by investigating the inhibition of the extract against the activity of α-glucosidase enzyme. The IC50 of the ethyl acetate extract of betel leaves against the α-glucosidase were 48.42 ppm. The cytotoxic activity of ethyl acetate extract was carried out by using Brine Shrimp Lethality Test (BSLT) method. Data analysis was done with Probit Analysis, it was found that ethyl acetate extract of betel leaf had high cytotoxic activity with LC50 value of 3.92 ppm and its standard error of 0.42 ppm.

1. Introduction

Betel plants have been very popular among Asian people such as Indonesia, Malaysia, India, China, and Nepal. Since several centuries ago betel has been used as a remedy by the Chinese and Indians [1]. Research with the subject of betel plant has been done many of them, the antioxidant properties of betel leaf extract, cytotoxic ethanol extract of red betel leaf, and antidiabetic activity of ethanol extract and water extract of betel leaf [2, 3, 4].

Green betel leaf (Piper betle L.) is commonly consumed daily by some Asian societies [5]. Traditionally, green betel leaf is believed to be used to overcome bad breath and body odor, constipation, headache, nervous disorders, itching, rash, boils, rheumatism, blisters, and others [6]. Betel leaf is widely known to have antifungal, antioxidant, antiplatelet, antipyretic, anti-inflammatory, antithrombotic, and depressent properties [7]. Piper betle ethanol extract was report to showed strong antibacterial activity Gram-positive methicillin-resistant Staphylococcus aureus and vancomycin-resistant Enterococcus bacteria [8]. It is known that the potential and relevance of alternative drugs are in correspondence with disease progression. Such as diabetes can be enhanced by the determination of the chemical structure of bio-actives potentially as antidiabetic from plants that are easily obtained in everyday life such as betel leaf [9]. However, so far there has been no report on compounds with antidiabetic activity from betel leaves. Therefore, at this early stage the authors are interested to examine the antidiabetic and cytotoxic activity of ethyl acetate extract of betel leaf, especially green betel commonly consumed by Indonesian.
2. Experiments

2.1. Material

The sample material of betel leaf was taken from the Betel field of Matangdrien, Tanah Jambo Aye, North Aceh, Aceh. Chemicals used were various organic solvents such as methanol, n-hexane, and ethyl acetate. FeCl$_3$, aquadest, α-glucosidase enzyme, phosphate buffer, p-nitrophenyl-α-D-glucosidase, Na$_2$CO$_3$, and DMSO. All chemicals used were of analytical grade.

2.2. Equipment

The tools used in this research include reflux, rotary evaporator (Buchi R-114), water bath, separating funnel, chemical glass, erlenmeyer (Pyrex), micropipette, test tube, measuring cup, analytical scales, incubator (Jouan), and Ultraviolet-Light Visible Spectrophotometer (Hitachi U-3900H).

2.3. Sample preparation

A total of 6 kg of betel leaves were air dried for 3 to 5 days in the shade. Samples were then mashed up into a powder using a leaf grinder. The resulting powder were then subjected to extraction by maceration.

2.4. Extraction of betel leaf powder

Betel leaf powder (1 kg) were soaked in 3000 ml of 96% methanol at room temperature for 2 x 24 hours. The sample were then filtered. The extract was extracted again with the same volume of 96% methanol. The extraction was repeated until the solvent was clear.

2.5. Phytochemical test of betel leaves

Prior to the drying process, maceration filtrates were screened for its phytochemical contents using several reagents as follows: FeCl$_3$, Meyer, Bouchardat, Dragendorf / Wagner reagents, and aquadest, in the following manner:

1. Methanol extract of betel leaves (10 ml) was added with 3 drops of 5% FeCl$_3$ solution. The changes that occur was then noted.
2. The same procedure were repeated using the Meyer, Bouchardat, and dragendorf / Wagner reagents.

Methanol extract (10 ml) was added with 2 ml of distilled water, the solution was then vigorously shaken for 5 second around. Formation of foam was then observed.

2.6. Fractination of betel leaves extract

The extraction of betel leaves was done in stages with steps as previously performed [10, 11], with slight modification. The concentrated methanol extract of the betel leaves was diluted with aquadest, filtered and the partitioned with ethyl acetate (1:1) gradually until the ethyl acetate fraction showed a negative result of the phenolic test, in which the test solution color did not turn blackish when depleted with FeCl$_3$ reagent.

The ethyl acetate fraction was concentrated and then reconstituted with methanol and partitioned with n-hexane to separate the non-polar compound. The result was concentrated to obtain the desired betel leaf extract.

2.7. The antidiabetic activity test of ethyl acetate extract of betel leaf

The antidiabetic activity test was performed in vitro on the basis of the absorbance of the UV-Vis light spectrum from the test solution made from the reaction mixture as in Table 1. Furthermore, the reaction results are read absorbance at λ 400 nm by using a spectrophotometer. The percentage of inhibitory power is calculated by the equation (1).
\[
\text{% inhibition} = \frac{C - S}{C} \times 100 \ldots \ldots (1)
\]

where, \( C \) = absorbance control – blank
\( S \) = absorbance \( S_1 \) - \( S_0 \)

Table 1. Preparation of solution for determination of UV-Vis spectrum absorbance.

| Control (µL) | Blank (µL) | \( S_1 \) (µL) | \( S_0 \) (µL) |
|-------------|------------|----------------|----------------|
| Sample *)   | -          | 25             | 25             |
| DMSO 1%     | 25         | 25             | -              |
| Phosphate buffer 0.1 M | 475 | 475 | 475 | 475 |
| Substrate of pNPG 0.5 mM | 250 | 250 | 250 | 250 |
| Incubate temperature 37oC for 5 minutes | Enzyme \( \alpha \)-glucosidase 0.04 units/ml | 250 | - | 250 | - |
| Phosphate buffer is 0.01 M | - | 250 | - | 250 |
| Incubate temperature 37oC for 25 minutes | \( \text{Na}_2\text{CO}_3 0.2 \text{ M} \) | 1000 | 1000 | 1000 | 1000 |

*) Samples are made in concentrations: 50, 100, 250, 500 and 1000 ppm.

2.8. Cytotoxic activity test of ethyl acetate extract of betel leaf
Toxicity test was performed using Brine Shrimp Lethality Technique (BSLT) method. Ethyl acetate extract of betel leaf (100 mg) was added with 3 drops of DMSO, and was then diluted with 100 ml of sea water to get final concentration of 1000 ppm. The solution was then serially diluted with sea water in order to get 10 and 100, and 1000 ppm test concentrations. 48 H old of \( A. \text{ salina} \) Leach (10 nauplii) was added to a test tube containing ethyl acetate extract of betel leaves (20 ml) previously prepared.

Experiments were conducted in room temperature (27 - 30°C) Twenty-four hours later, the number of surviving nauplii was calculated and the number of dead was also recorded. DMSO (50 µl) in seawater was used as a control. Experiments were performed in triplicate for each concentration. Lethal Concentration 50% (LC\(_{50}\) value) was calculated using Probit Analysis [12].

3. Results and discussion
3.1. Phytochemical screening of betel leaf
The screening process gives a positive result with \( \text{FeCl}_3 \) reagents showing that the betel leaf sample has a high phenolic content. The phenolic compound having the -OH functional groups interacting with \( \text{Fe}^{3+} \) iron ions from \( \text{FeCl}_3 \) produces a complex compound which gives the greenish-black color of the solution [13]. Green betel leaves have low alkaloid compounds. This is indicated by the negative results of the test using the Meyer, Dragendorf, or Bouchardat reagents.

In contrast to red betel which has high alkaloid levels [14], green betel leaves actually show the low content of these compounds. In fact, alkaloid was not detected by tests that have been performed using the Meyer, Dragendorf, or Bouchardat reagents, in this case. Biologically active compounds in betel, their levels are highly dependent on variety, season, and weather in the growing region [7]. Negative test results with aquadest show that betel leaves have low saponin content.
3.2. Antidiabetic activity of ethyl acetate extract of betel leaf

Extract of ethyl acetate obtained from a series of extraction process as much as 5.36 g (0.089%) in liquid form of dark brown. The effect of ethyl acetate extract of betel leaf as an inhibitor to the activity of α-glucosidase enzyme at various extract concentrations is shown in Figure 1. All doses of extract used indicate its inhibitory power, which in the range of 50-1000 ppm, the higher the extract concentration the greater the inhibition power to the activity of the α-glucosidase enzyme. Linear regression between the concentration of ethyl acetate extract of betel leaf and its inhibition power to α-glucosidase enzyme activity is shown in Figure 2. The linear regression equation between extract concentration and its inhibition power to α-glycosidase showed by equation (2).

\[ y = 0.0214x + 48.961 \]  

where, \( y \): percentage of inhibition  
\( x \): concentration of ethyl acetate betel leaf extract

Based on the regression equation (2) obtained, with the coefficient of determination, \( R^2 \) of 0.7232 shows that the concentration of extract has a relatively large influence on the activity of α-glucosidase enzyme, meaning 72.32% percent values of inhibition in this experiment is influenced by the concentration of betel leaf ethyl acetate extract.

\( IC_{50} \) is a concentration of betel leaf ethyl acetate extract that inhibits 50\% of α-glucosidase enzymes. The result of calculation based on equation (2) found that \( IC_{50} \) ethyl acetate extract of betel leaf is 48.42 ppm. This relatively low \( IC_{50} \) value indicates that the betel leaf ethyl acetate extract has great potential to be developed as a natural antidiabetic agent, especially when compared with some other sources from previous reports, such as the phenolic compound of Britewell Blueberry Anthocyanin Extract (BAEs) having \( IC_{50} = 57.5 \) ppm [14] and even much stronger than a positive acarbose control having an \( IC_{50} \) value of 188.5 ppm [15].

![Figure 1](image)

**Figure 1.** Inhibition of α-glucosidase by ethyl acetate extract of betel leaves at various concentrations.
This fact indicates that the betel leaf ethyl acetate extract may contain high levels of phenolic compounds. The betel leaf extracts with high levels of phenolic compounds appeared as the best inhibitors for the $\alpha$-amylase enzyme at various concentration levels. Phenolic content in an extract is also proportional to its antioxidant activity [16]. Cofactor metal ions in an enzyme such as the $\alpha$-amylase and/or $\alpha$-glucosidase enzymes are likely to form complexes with the $-\text{OH}$ groups that are present in the phenolic compounds resulting in the action of the enzyme being blocked and even lost.

3.3. Cytotoxic activity of ethyl acetate extract of betel leaf

The cytotoxic activity test of phenolic compounds was performed using BSLT method, an easy and inexpensive method [16]. By using probit analysis and linear regression, the relationship of log concentration with probit mortality can be seen as in Figure 3. The linear regression equation between the log concentration of the extract and its inhibitory power to $\alpha$-glucosidase showed by equation (3).

$$y = 1.175x + 4.303 \quad \ldots \ldots \text{(3)}$$

where, $y$: probit mortality  
$x$: log concentration

The coefficient of determination, $R^2$ of 0.870 from equation (3) shows the relationship between the variables is good enough, where the variable concentration of extract affects the mortality of larvae with the intensity of 87% and there is only 13% the influence of other factors.

The result of calculation of ethyl acetate extract from betel leaf obtained Lethal Concentration, $LC_{50}$ value of 3.92 ± 0.42 ppm. These results indicate that the cytotoxic activity of ethyl acetate extract of betel leaf to shrimp larvae is very high based on the literature [16], which classifies extracts and pure compounds as toxic ($LC_{50} < 1000 \mu g / ml$) and non-toxic (value $LC_{50} > 1000 \mu g / ml$). However, it is still less toxic than the methanol extract of red betel leaf, with $LC_{50}$ as low as 1.34 ppm (Emrizal et al., 2014), and the phaleria macrocarpa seed methanol extract with $LC_{50}$ value of only 0.16 μg / ml [17].

![Figure 2. Regression between percentage of inhibition with ethyl acetate leaf extract concentration of betel leaves.](image-url)
Figure 3. The linear regression between log concentration with probit value of mortality.

As mentioned above that the cofactor metal ions in an enzyme can form complex compounds with phenolic compounds, enzymes contained in the body system *A. salina* Leach is likely to gradually interact with phenolic compounds contained in the ethyl acetate extract of betel leaf so that within 24 hours there are a certain number of *A. salina* Leach that are dead.

4. Conclusions and recommendations
Green betel leaf ethyl acetate extract had high antidiabetic activity with LC$_{50}$ of 48.42 ppm and cytotoxic activity was also quite high with LC$_{50}$ values of 3.92 and standard error of 0.42. This value can be used as a reference to make ethyl acetate extract of betel leaf as an alternative source of herbal medicine by first tested its bioactivity such as antimicrobial, antioxidant, anticancer, antidiabetic, and others. Fractionation of phenolic compounds and their molecular formulas is required. Further studies to find out the biologically active compounds need to be done.

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References
[1] Pradhan D *et al* 2013 *Journal of Pharmacognosy and Phytochemistry* **1** 147
[2] Makpol S *et al* 2013 *Complementary and Alternative Medicine* **13** 210
[3] Emriza *et al* 2014, *Chemistry*. **13** 79
[4] Arambewela L S R *et al* 2005 *Journal of Ethnopharmacology* **102** 239
[5] Dinesh M D *et al* 2016 *Global Journal of Advanced Research* 3(5): 341-344
[6] Guha P 2006 *J. Hum. Ecol* **19** 87
[7] Sripradha S 2014 *J. Pharm. Sci. & Rev* **6** 36
[8] Valle D L et al 2015 Asian Pac J Trop Biomed 5 532
[9] Malik A 2016 Prosiding Seminar Nasional Kimia dan Pendidikan Kimia (Medan) (I) (Indonesia: Sumatera Utara) pp 1
[10] Erwin et al 2013 Prosiding Seminar Nasional Kimia (S) vol 1 (Indonesia: Kalimantan Timur) pp 52-58
[11] Riris I D et al 2014 International Journal of Chemistry 6 15
[12] Vincent K 2008 Probit analysis Preprint gr-qc/13032017
[13] Artini P E U et al 2013 Jurnal Farmasi Udayana 2 1
[14] Wu Y et al 2017 Food Research International 5 86
[15] Johnson M H et al 2011 Journal Agriculture Food Chemistry 59 8923
[16] Meyer B N et al 1982 Medicine 45 31
[17] Lisdawati V et al 2006 Buletin Penelitian Kesehatan 34 111