Free Radicals Scavenger Potency of Betel Leaves (*Piper betel* L.) Extract and Various Fractions

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Abstract

The imbalance between free radicals and antioxidants in the biology system can cause various diseases. Betel leaves (*Piper betel* L.), parts of a medicinal plant, are popularly used as a herbal remedy for diseases, but the scientific basis especially of their antioxidant properties remains unknown. To evaluate free radicals scavenger activity of ethanol extract and various fractions (hexane, ethyl acetate, butanol and water fraction), *1,1-diphenyl-2-picryl-hydrazyl* (DPPH) and hydroperoxide (H_2O_2) scavenging activity were determined. To determine the DPPH and H_2O_2 scavenging activity, betel leaves extract and fractions were prepared in 10 concentrations. The result of this research showed that **b**etel leaves extract and fractions had higher H_2O_2 scavenger and lower DPPH scavenger activities than *gallic acid*. The highest DPPH scavenging activity with Inhibitory Concentration (IC_{50}) was found in *gallic acid* 0.732 µg/mL, ethyl acetate fraction 3.156 µg/mL, and ethanol extract 5.489 µg/mL. The highest H_2O_2 scavenging activity with IC_{50} was found in butanol fraction 0.223 µg/mL, *gallic acid* 0.597 µg/mL, and ethyl acetate fraction 0.783 µg/mL. In conclusion, betel leaves extract and fractions were potential free radicals scavenger; they could be potential candidates to inhibit oxidative stress. *Gallic acid* and ethyl acetate fraction were the highest free radicals scavenger both in DPPH and H_2O_2 free radical scavenging activity.

Keywords: free radical, betel leaves, DPPH, H_2O_2, antioxidant

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Potensi Pemerangkapan Radikal Bebas Ekstrak dan Fraksi-fraksi Daun Sirih (Piper betel L.)

Abstrak

Ketidakseimbangan antara radikal bebas dan antioksidan dalam sistem biologi dapat menyebabkan berbagai jenis penyakit. Daun sirih (Piper betel L.) adalah tanaman obat yang secara populer digunakan untuk pengobatan berbagai jenis penyakit, tetapi bukti ilmiahnya, khususnya sebagai antioksidan, masih kurang. Untuk menguji aktivitas pemerangkap radikal bebas dari ekstrak etanol dan berbagai fraksi (heksan, etil asetat, butanol dan fraksi air), dilakukan uji aktivitas pemerangkapan 1,1-diphenyl-2-picryl-hydrazyl (DPPH) dan hidrogen peroksida (H_2O_2). Untuk menentukan akvitas pemerangkapan DPPH dan H_2O_2, ekstrak dan fraksi-fraksi daun sirih disiapkan dalam 10 level konsentrasi. Hasil penelitian menunjukkan bahwa ekstrak dan fraksi-fraksi daun sirih memiliki aktivitas pemerangkap H_2O_2 lebih tinggi dan pemerangkap DPPH lebih rendah dibanding asam galat. Aktivitas pemerangkapan DPPH dengan nilai Inhibitory Concentration (IC_{50}) tertinggi ditemukan dalam asam galat 0,732 µg/mL, fraksi etil asetat 3,156 µg/mL, dan ekstrak etanol 5,489 µg/mL. Aktivitas pemerangkap H_2O_2 dengan nilai IC50 tertinggi ditemukan dalam fraksi butanol sebesar 0,223 µg/mL, asam galat 0,597 µg/mL, fraksi etil asetat 0,783 µg/mL. Sebagai kesimpulan, ekstrak dan fraksi-fraksi daun sirih memiliki potensi pemerangkap radikal bebas dan menghambat stres oksidatif. Asam galat dan fraksi etil asetat menunjukkan aktivitas paling tinggi sebagai pemerangkap radikal bebas dalam memerangkap baik radikal DPPH maupun H_2O_2.

***Kata kunci**: radikal bebas, daun sirih, DPPH, H_2O_2, antioksidan*

Introduction

Free radicals can cause oxidative damage to all biomolecules and initiate a chain reaction resulting in physiological damage which can be repaired but which may also accumulate over a period of time and cause many degenerative diseases.[1] The large generation of free radicals, particularly reactive oxygen species (ROS) and their high activity, plays an important role in the progression of a great number of pathological disturbances like inflammation, atherosclerosis, stroke, heart disease, diabetes mellitus, multiple sclerosis, cancer, Parkinson's disease, Alzheimer's disease, etc.[2,3] Phenolic compounds, the largest and most widely distributed group of phytochemicals, exhibit a wide range of biological and physiological properties due to their ability to act as antioxidants and free radical scavengers.[4] The structure of phenolic compounds, in particular the position and degree of hydroxylation, is of primary importance in determining their antioxidant activity. In general, polyphenols share the same chemical patterns with one phenolic group or more for which they react as hydrogen donors and in that way neutralize free radicals.[3,5]

Natural antioxidants are favorably accepted and are safer than synthetic antioxidants. Hence, research is needed to obtain safe and economic antioxidants with high activity from natural sources to replace the synthetic antioxidants. Traditionally, Indonesian people often use and consume medicinal plants for preventing and healing many kinds of disease. Empirical data showed that piper betel leaves (*Piper betel* L) can inhibit and heal chronic diseases. In Indonesia, betel leaves (*daun sirih*) are consumed as an infusion and used as an

antibiotic to heal indigestion, constipation, decongestant, nosebleeds. They also help to aid lactation. Many Indonesian women, after giving birth, often put some betel leaves in their bath water to shrink their vaginal canal and to counter unpleasant smell. In India, betel leaves are used to remove living worms from the body or as a remedy for bad breathe. In Malaysia, betel leaves are used to cure headaches, arthritis and joint pain. In Philippines, Thailand, China and Indonesia they are used to cure toothache and to strengthen teeth and gums. The aqueous betel leaves extract has been reported to have antifungal, antibacterial, and antioxidant activities.[6-8] Ethanolic betel leaves extract exhibits anti-inflammatory activity.[9] The leaf extract, fractions and purified compounds are found to play a role in oral hygiene and they have anti-diabetic, cardiovascular, anti-inflammatory/immunomodulatory, anti-ulcer, hepato-protective and anti-infective properties.[10]

However, all the cases are empirical; there are only few scientific data about the extract and fractions of betel leaves, so they should be investigated further. Therefore, this present study was conducted to investigate their antioxidant properties particularly *1,1-diphenyl-2-picryl-hydrazyl* (DPPH) free radical scavenging activity and hydrogen peroxide (H_2O_2) scavenging activity.

Material and Methods

Plant material

Betel leaves (*P. betel* L.) were collected from a plantation located in Bogor, West Java, Indonesia. All material was dried in dry tunnel (40-45^{0}C). When they reached stable water level condition (13-14%), dried material was chopped finely using a blender to produce 60 mesh size.

Preparation of extract and fractions

One kilogram of dried material was extracted with distilled ethanol by maserasi extraction, filtered and evaporated by using rotatory evaporator. Our process resulted ethanol extract of *P. betel* L. 165.13 g (16.513%). The ethanolic extract was partitioned in hexane and water (7:3). The aqueous layer was fractioned respectively with ethyl acetate (1:1) and butanol (1:1). The hexane, ethyl acetate, butanol, water fractions were collected and concentrated with vacuum rotary evaporator at 40^{o}C giving the yields 1.4 g, 33.117 g, 10.6 g, and 35.9 respectively. The ethanolic extract and fractions of betel leaves were stored at 4 ^{o}C.

Sample Preparation

Extract of *P. betel* L. was dissolved with HPLC methanol to reach series of concentrations as followed: 500; 250; 125; 62.5; 31.25; 15.625; 7.813; 3.91; 1.953; 0.977 µg/mL.

DPPH free radical scavenging activity assay

DPPH assay was carried out as described by Unlu *et al.* (2003)[6] and Frum and Viljoen (2006)[7]. A 96-well microtitre plate was used to generate a quantitative measure of extract and fractions radical-scavenging activities. Fifty µL of each extract's and fractions's various concentrations were introduced in microtitre plate, followed by the addition of 200 µL of DPPH solution (0.077 mmol/L DPPH in methanol). Mixtures were then mixed gently and kept in the dark for 30 min at room temperature. Absorbance of DPPH was determined by microplate reader at 517 nm. DPPH free

radical scavenging activity of each sample was measured according to the formula below:

$$\text{scavenging } \% = \frac{A_c - A_s}{A_c} \times 100$$

A_s: absorbance of samples, A_c: control absorbance (without sample).

Hydrogen peroxide scavenging activity

To determine the antioxiant activity of extract and fractions according to scavenge hydrogen peroxide, hydrogen peroxide (43 mM) was prepared in phosphate buffered saline (pH 7.4). Standards (gallic acid), extract and fractions solution were prepared at concentrations of 0.977 µg/mL to 100 µg/mL Aliquots of *P. betel* L. extract, ; fractions solutions and gallic acid (200 µL) were added to 800 µL of hydrogen peroxide solution. The reaction mixture was incubated at room temperature for 10 min, and the absorbance was determined at 230 nm. The percentage of scavenging was calculated as follows:

$$\text{scavenging } \% = \frac{A_c - A_s}{A_c} \times 100$$

A_s: absorbance of samples, A_c: control absorbance (without sample).

Results and Discussion

Results

The DPPH free radical scavenging activity of ethanolic extract and fractions of *P. betel* L. and gallic acid antioxidant activity, well known as positive control of various concentrations, were measured to examine the antioxidant activity. Figure 1 shows DPPH scavenging activity of ethanolic extract, hexane fraction, ethyl acetate, butanol, and water fraction of *P. betel* L., and gallic acid. At the highest concentration (100 µg/ml), gallic acid could scavenge DPPH 92.24%, ethanolic extract, hexane fraction and ethyl acetate fraction 89%. The lower DPPH scavenging activity belonged to water fraction and butanol fraction. Figure 1 shows the DPPH scavenging activity of water fraction with the lowest activity.

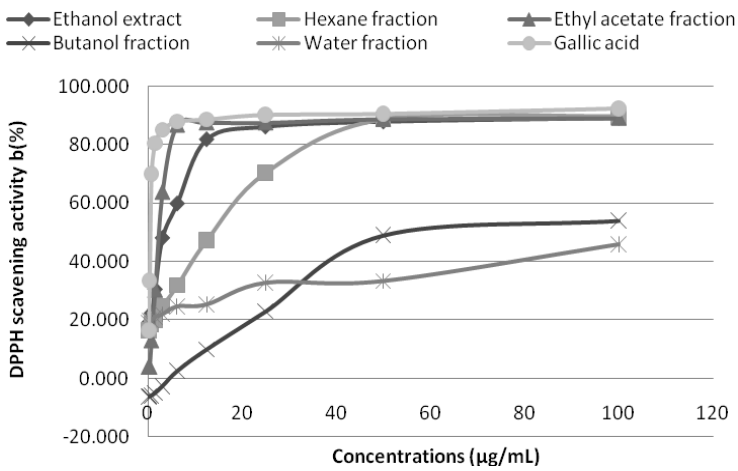


Figure 1. The DPPH Scavenging Activity of *P. Betel* L. Extract and Fractions

The IC50 is the concentration of antioxidants activity to scavenge DPPH free radical 50%. The IC50 values of DPPH scavenging activity among ethanolic extract, hexane fraction, ethyl acetate fraction, butanol fraction, water fraction, and gallic acid can be seen in Table 1. Gallic acid can inhibit 50% DPPH on 0.732 µg/mL, ethyl acetate 3.156 µg/mL and ethanolic extract 5.489 µg/mL. Gallic acid was the strongest antioxidant activity among the extract and fractions of betel leaves.

The H_2O_2 scavenging activity of ethanol extract and fractions of *P. betle* L. and gallic acid of various concentration were measured to examine the antioxidant activity. Figure 2 shows H_2O_2 scavenging activity of ethanolic extract, hexane fraction, ethyl acetate, butanol, and water fraction of *P. betle* L., and gallic acid. At the high concentration (25 µg/mL-100 µg/mL) all samples showed negative antioxidant acitivity. The highest H_2O_2 scavenging activity of each sample showed that hexane fraction 12.5 µg/mL was 46.255 %; ethanolic extract 6.25 µg/mL was 85.249 µg/mL; hexane fraction 6.25 was 86.678 %; ethyl acetate fraction 6.25 µg/mL was 73.128% µg/mL; butanol fraction 0.781 µg/mL was 73.299 ; water fraction 0.781-1.563 µg/mL was 81.48 - 81.99% and gallic acid 0.195 µg/mL was 61.02%.

Table 1. The DPPH Scavenging Activity of *P. Betle* L. Extract and Fractions

Sample	Linear equation	R-squared (R^2)	IC50 (µg/mL)
Ethanolic extract	Y=5.268X + 21.00	0.935	5.489
Hexane fraction	Y=1.506X + 20.93	0.940	19.303
Ethyl acetate fraction	Y=14.12X + 5.439	0.934	3.156
Butanol fraction	Y=0.672X-2.047	0.878	77.451
Water fraction	Y=0.258X+21.25	0.936	111.434
Gallic acid	Y=45.60X+16.62	0.833	0.732

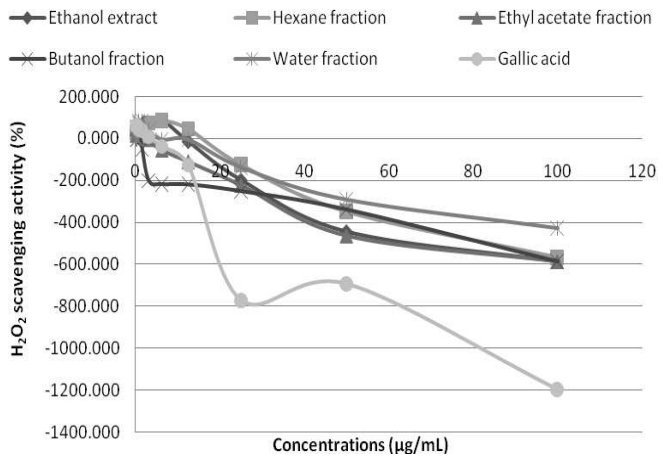


Figure 2. The H_2O_2 Scavenging Activity of *P. Betle* L. Extract and Fractions

Table 2. The H_2O_2 Scavenging Activity of *P. Betle* Extract and Fractions

Sample	Linear equation	R-squared (R^2)	IC50 (µg/mL)
Ethanolic extract	Y=12.71X + 8.967	0.903	3.228
Hexane fraction	Y=9.259X + 34.62	0.91	1.661
Ethyl acetate fraction	Y=34.95X + 22.65	0.822	0.783
Butanol fraction	Y=40.79X + 40.91	0.987	0.223
Water fraction	Y=-20.02X + 103.6	0.898	2.677
Gallic acid	Y=-16.89X + 60.09	0.957	0.597

The IC50 is the concentration of antioxidants activity to scavenge H_2O_2 50%. The IC50 values of H_2O_2 scavenging activity can be seen in Table 2. Ethanolic extract and fractions exhibited high H_2O_2 scavenger activity, whereas the strongest H_2O_2 scavenging activity showed butanol fraction, gallic acid.

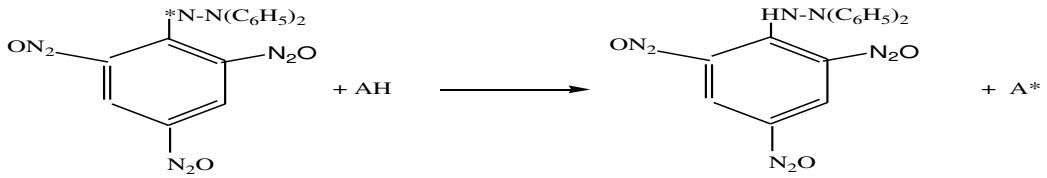
Discussion

In the DPPH scavenging activity test, a sample which contains an antioxidant will donate hydrogen (H) and will be captured by 1,1- diphenyl-2-picrylhydrazyl (DPPH) free radical and will become 1,1- diphenyl-2-picrylhydrazyn.[15,16] The changes in color (from deep–violet to light–yellow) were measured at 517nm.[17] The DPPH assay showed that the sample having the highest antioxidant activity would present the fastest color change compared to the other samples or the progressive decrease in the absorbance. The sample having the lowest antioxidant activity may not change its color for several hours; even the color of the sample may remain purple.[16]

Ethanolic extract and fractions of betel leaves exhibited high hydrogen peroxide scavenging activity. While hydrogen peroxide itself is not very reactive, it can generate the highly reactive hydroxyl radical (OH) through the Fenton reaction.

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + {}^{*}OH + OH^{-}$$

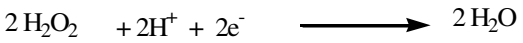
Thus, the scavenging of H_2O_2 is an important antioxidant defense mechanism. The decomposition of H2O2 to water involves the transfer of electrons.



1,1- diphenyl-2-picrylhydrazyl (purple colour)

1,1- diphenyl-2-picrylhydrazyn (yellow colour)

Figure 3. Chemical Reaction at the DPPH Scavenging Activity Test



The scavenging of H_2O_2 by phenolic compounds has been attributed to their electron-donating ability.[13]

These results suggested that ethyl acetate fraction had the highest DPPH and H_2O_2 scavenging activites because of its phenolic compounds. The DPPH and H_2O_2 scavenging activites are related to the presence of bioactive compounds such as phenolic compounds in fraction.[18] Betel leaves extract and fractions exhibited high antioxidan activity because its contains high polyphenol compounds. Many biological function of polyphenols act as antioxidants in vitro by scavenging reactive oxygen and nitrogen species an chelating redox-active transition metal ions.[19]

In phenolic compounds using kaempferol as standard, *P. betle* L. extract contains high polyphenol 548.667 µg KE/mg[9]. A previous research by Risdian *et al*. (2010) showed that *P. betle* L. contained high phenolic; when using EGCG as standard, ethyl acetate fraction contained 568.19 µg EGCGE/mg, ethanol extract 269.97 µg EGCGE/mg, hexane fraction 215.58 µg EGCGE/mg, butanol fraction 199.06 µg EGCGE/mg, and water fraction 138.34 µg EGCGE/mg respectively[10]. Ethyl acetate fraction contained higher concentration of phenolic compound than ethanolic extract and the other fractions.

The –OH groups in phenolic compounds were thought to have a significant role in antioxidant activity[11]. The antioxidant activity of phenolic compounds is reported to be mainly due to their redox properties[12,13], which can play an important role in adsorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides. These results showed that ethyl acetate fraction exhibited the highest DPPH free radical and H_2O_2 scavenging activities because of its phenolic compounds. This research was validated by previous research in which the -OH groups in phenolic compounds were thought to have a significant role in antioxidant activity[22] and a strong correlation between both antioxidant activity and total phenolic content.[9]

Conclusion

Ethyl acetate fraction of *P. betle* L. leaves exhibit high antioxidant activities both in DPPH and H_2O_2 free radicals scavenging activities. These results also suggested that *P. betle* L. ethyl acetate fractions may be used to protect the damage of human body because of their free radicals agents.

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