

The effects of fresh leaf-to-water ratio and heating time on the antifungal and antioxidant activities of betel leaf (*Piper betle* L.) extract

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Submitted: 02-10-2019

Reviewed: 18-11-2019

Accepted: 16-03-2020

ABSTRACT

More than half of the Indonesian people use herbal medicines to maintain their health, including ones that are served immediately after preparation or also known as *jamu gendong*. This plant-based medicine is prepared traditionally by boiling using different ratios of leaves to water and heating time. The study was designed to determine the heating time and the fresh leaf-to-water ratio that exhibited the highest antioxidant and antifungal activities against *Candida albicans*. Fresh betel leaves were extracted by boiling and infusion methods, with the ratio of 1:5 and 1:10 and the heating time of 5 and 10 minutes for boiling and 15 minutes for infusion. The excess water in the resulting extract was removed using a freeze dryer, then the total phenolic content was determined by Folin-Ciocalteu spectrophotometry. Meanwhile, the antioxidant and antifungal activities were tested by the DPPH method and microdilution, respectively. The results showed that betel leaf extract with the highest yield and polyphenol content was obtained by infusion with the sample-to-water ratio of 1:10, and this was positively correlated with antioxidant activity against DPPH (IC₅₀= 17.4 ppm) and antifungal effect against *Candida albicans* (MIC= 0.5%).

Keywords: *Piper betle* L., water extract, DPPH (2,2-diphenyl-1-picrylhydrazyl), *Candida albicans*

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INTRODUCTION

Among the Indonesian population, the leaves of betel (*Piper betle* L.) are known as candidiasis preventive agents. Whether for personal consumption or sale, fresh betel leaves are still processed traditionally by boiling with varying ratios of fresh leaves to water and heating time, producing a beverage called *jamu gendong*. This herbal remedy can be consumed on its own or combined with other traditional medicines.

According to several studies, betel leaves show antioxidant activity (Ali *et al.*, 2018; Chauhan *et al.*, 2016; Sarma *et al.*, 2018), antibacterial activity against *Streptococcus mutans* (Thangavelu, 2013; Nalina and Rahim, 2007; Rahim and Thurairajah, 2011), and antifungal activity against *Candida albicans* (Ali *et al.*, 2010). They contain bioactive components, such as hydroxychavicol, eugenol, isoeugenol, and allyl pyrocatechol 3,4-diacetate (Ali *et al.*, 2018). Temperature and extraction time reportedly affect the resulting extract (Muruganandam *et al.*, 2017). For instance, Soxhlet extraction typically produces ethanol extracts containing hydroxy-chavicol (69.46%), 4-Chromanol (24%), and eugenol (4.86%). Meanwhile, drying at 80°C can cause the main components of the betel leaf to undergo decomposition, and the yield of extraction using water multiplies as temperature and leaf-to-water ratio increase (Pin *et al.*, 2006).

This study has examined the effects of heating time and the ratio of fresh betel leaves to water on the total phenolic content, antioxidant activity, and anti-candidiasis activity of betel leaf-based remedies. The result of this study can be applied to the traditional processing of betel leaves in society or by *jamu gendong* vendors.

MATERIALS AND METHODS

Equipment and materials

The research materials were *Piper betle* leaves (collected from a market in Makassar), distilled water, 2,2-diphenyl-1-picrylhydrazyl (Sigma), Potato Dextrose Broth (Merck), *Candida albicans* ATCC 10231, Folin-Ciocalteu reagent (Merck), gallic acid (Sigma). Meanwhile, the equipment used included infusion pan, incubator (Mettler), Microplate reader (BioTek), and biosafety cabinet.

Methods

Extraction of betel leaves

Fresh betel leaves that had been initially sorted were cut into small sizes of 1-2 cm². These leaves were then extracted using the leaf-to-water ratio of 1:5 and 1:10, and each sample was boiled for 5 and 10 minutes and infused at 90°C for 15 minutes. The extracts obtained were lyophilized, and the yields were calculated.

Determination of total phenolic content

The total phenolic content was analyzed according to the method proposed by Farmakope Herbal Indonesia (Kemenkes RI, 2011), with gallic acid as the standard. The extract weighing 10 mg was dissolved in 25 mL of methanol. One ml of the solution was pipetted, added with 5 mL of 7.5% Folin-Ciocalteu, allowed to stand for 8 minutes, and added with 1% NaOH. After one hour of incubation, the mixture was measured at $\lambda = 755$ m.

Determination of antioxidant activity

The antioxidant activity was determined by the DPPH method using a microplate ELISA reader. The extract was dissolved in analytical-grade methanol to obtain 100 ppm concentration. Samples with varying volumes, namely, 10, 20, 30, 40, 50 μ l, were placed into the well. Then, each well was added with 60 μ l of 40 mM DPPH solution, and the volume was filled up to 200 μ l with DPPH solution. After 30-minute incubation at room temperature, the absorbance of each sample was analyzed at λ of 515 nm.

The percentage of DPPH scavenging was calculated using the formula below:

$$\% \text{ DPPH Radical Scavenging} = (A_{\text{blank}} - A_{\text{sample}}) / A_{\text{blank}} \times 100\%$$

Meanwhile, the IC_{50} was computed based on the regression equation $y = ax + b$.

Determination of antifungal activity against *Candida albicans*

The antifungal activity against *C. albicans* was evaluated based on the minimum inhibitory concentration (MIC) by microdilution, in which a 48-well plate containing potato dextrose broth with turbidity equivalent to 1% of the McFarland standards was incubated for 2 x 24 hours.

Data Analysis

The average yield of the extract and the total phenolic content (TPC) of the lyophilized aqueous betel leaf extract were first expressed in mean \pm SD. These data were then statistically analyzed by the Student's *t*-test, and the results were considered significant when $P < 0.05$.

RESULTS AND DISCUSSION

Extraction yield and total phenolic content

This study analyzed the fresh betel leaves used commonly by the community, including *jamu gendong* vendors. A hundred leaves with a length of 10-15 cm and width in the range of 7.5 and 11 cm were sorted and placed on a scale. These leaves, which were averagely 232.12 g in weight, were then washed, drained, and cut into small sizes of 1-2 cm². Afterward, the leaves were grouped based on treatments and the independent variable, i.e., the ratio of leaf to water (1:5 and 1:10). The extraction yield and total phenolic content of the aqueous betel leaf extract obtained by boiling (5 and 10 minutes) were compared with those produced by 15 minutes of infusion. The results of both techniques were then filtered and lyophilized, and the characteristics of the extract obtained by boiling and infusion were evaluated (Table I).

Table I. The average yield (%) and total phenolic content (TPC) of lyophilized aqueous extract from fresh betel leaves

Samples	Yield (%) of lyophilized aqueous betel leaf extract	TPC (%w/w; in gallic acid equivalent-GAE)
A	2.16	12.58 \pm 0.65
B	2.34	26.49 \pm 0.56
C	1.72	25.06 \pm 0.90
D	4.72	26.53 \pm 0.22
E	4.92	23.93 \pm 0.14
F	9.02	27.28 \pm 0.22

Note:

A= Lyophilizate extracted by 5-minute boiling, with leaf-to-water ratio 1:5

B = Lyophilizate extracted by 10-minute boiling, with leaf-to-water ratio 1:5

C = Lyophilizate extracted by 15-minute infusion, with leaf-to-water ratio 1:5

D = Lyophilizate extracted by 5-minute boiling, with leaf-to-water ratio 1:10

E = Lyophilizate extracted by 10-minute boiling, with leaf-to-water ratio 1:10

F = Lyophilizate extracted by 15-minute infusion, with leaf-to-water ratio 1:10

Compared to 1:5, the 1:10 ratio created a higher yield of extract potentially due to the low or insoluble chemical components in water, indicating that a higher water volume is needed. Also, with this 1:10 ratio, 15 minutes of heating at 90°C in the infusion method resulted in higher yields. The highest phenolic content (27.28%) was found in Sample F, which was extracted by infusion with the

1:10 ratio. However, this result does not differ significantly from that of Sample B (extraction by 10 minutes of boiling with 1:5 ratio) and Sample D (extraction by 5 minutes of boiling, with 1:10 ratio). Among the above treatments, infusion with the leaf-to-water ratio of 1:10 provided the highest yield. Multilevel extraction of betel leaves using hexane, ethyl acetate, methanol, and water has proven that the highest total phenolic content is found in the ethyl acetate extract, followed by the hexane, methanol, and lastly water extracts (Abraham *et al.*, 2012). A study of the nutraceuticals properties of *Piper betel* (Paan) (Chauhan *et al.*, 2016) also confirms that, in the order of highest to lowest, phenols can be found in methanol, ethanol, ethanol-water, and water extracts. Another study reports that the water extract of dried betel leaves that have been macerated for 4 hours with hot water has a total phenolic content of 128.89 mg/g extract or approximately 12.9% w/w (Taukoorah *et al.*, 2016). Furthermore, boiling fresh betel leaves with the leaf-to-water ratio of 1:50 for 30 seconds produces extracts containing a total phenolic compound of 676 mg per 100 g of fresh leaves (0.68%) (Tan *et al.*, 2014). In other words, the total phenolic content of betel leaf extract varies depending on the extraction method, the solvent used, and the leaf-to-water ratio. Despite the use of water as a solvent, this study obtained relatively high levels of phenolics. This finding is potentially caused by the manner of heating and the use of fresh leaves. The content of the hydroxy catechol in betel leaf can be increased by raising the extraction temperature to 60°C with an equilibrium point of 30 minutes; drying at 50-60°C is found to be optimum, whereas rehydration at 40°C is claimed to produce the best or most acceptable product (Pin *et al.*, 2006; Balasubramanian *et al.*, 2011).

Antioxidant activity

The *in vitro* test by the DPPH method revealed that the highest antioxidant activity was exhibited by Sample F (extracted by infusion with the 1:10 ratio). A smaller IC_{50} value can lead to a stronger antioxidant property, which is associated with total phenolic compounds. Sample F showed a relatively high total phenolic level, and the lowest IC_{50} value against DPPH was compared to the other samples.

Table II. The IC_{50} of the lyophilized aqueous extract of fresh betel leaves against DPPH

Samples	IC_{50} ($\mu\text{g/mL}$) against DPPH					
	A	B	C	D	E	F
Average	40.63	23.36	22.24	18.54	19.97	17.14

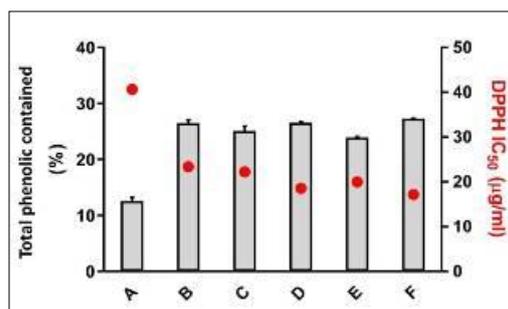


Figure 1. Histogram of the correlation between the total phenolic content (%) and the IC_{50} against DPPH based on the test samples

Several studies have also confirmed the high antioxidant activity of betel leaf extract. For instance, betel leaves are found to exhibit antioxidant activity with the IC_{50} of 179.5 ± 93.1 ppm against DPPH (Nur Sazwi *et al.*, 2013), and the water extract of a selected variety of betel leaves

indicates antioxidant effects against DPPH (Sharma *et al.*, 2010). Furthermore, betel leaf extract has a proton-donating ability and can serve as a free radical inhibitor or scavenger (Umar *et al.*, 2018).

Minimum inhibitory concentration (MIC)

Based on the results of the antifungal activity test against *C. albicans* (microdilution), the lyophilized aqueous betel leaf extract has the MIC of 0.5%-1%, as shown in Table III and Figure 2.

Table III. The MIC values (%) of the lyophilized aqueous extract of fresh betel leaves
MIC values (%) of the lyophilizates of the fresh betel leaf extract

Sample	A	B	C	D	E	F
	1	1	1	0.5	0.5	0.5

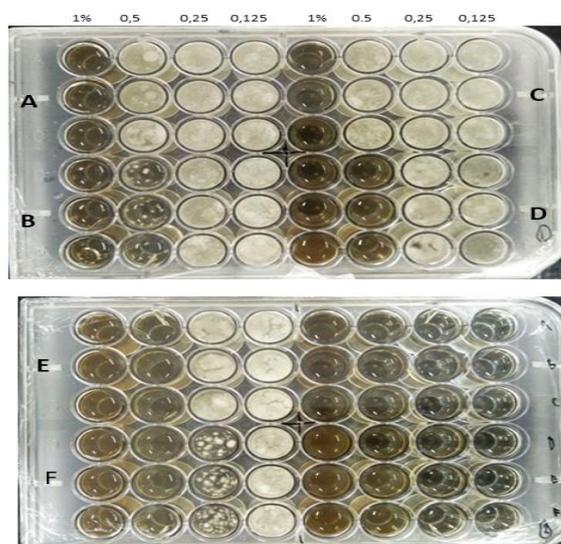


Figure 2. Determination of minimum inhibitory concentration by microdilution (Mueller-Hinton broth media, incubation time at 37°C for 48 hours)

Boiling and infusion using the leaf-to-water ratio of 1:10 exhibited higher antifungal activities against *Candida albicans*, with the MIC of 0.5% (Figure 2 and Table III). Although, Sample F (infusion with a 1:10 ratio) has the same MIC as Samples D and E, at a concentration of 0.25%, the growth of *C. albicans* in Samples D and E was higher than in Sample F (Figure 2); therefore, this study recommends infusion for optimal fresh betel leaf extraction. On the other hand, boiling dried betel leaves using distilled water with the leaf-to-water ratio of 1:10 for 5-6 hours produces extracts with a higher MIC, that is, 12 mg/mL (1.2%) (Himratul-Aznita *et al.*, 2011). Ali *et al.* (2010) suggest that hydroxychavicol isolated from aqueous betel leaf extracts inhibit the biofilm formation by several *Candida albicans* isolates, with an MIC of around 125-500 ppm.

CONCLUSION

With 1:10 leaf-to-water ratio and 15 minutes of heating, extraction by infusion produces betel leaf extracts that have the highest antioxidant and antifungal activities against *Candida albicans*.

RECOMMENDATION

Because aqueous betel leaf extract has been proven to have a strong antioxidant activity against DPPH (IC₅₀ 17.14 ppm), it is recommended as an anti-tumor candidate in cell lines or anti-aging materials and as a gel dosage form for oral mucosal candidiasis in future research.

ACKNOWLEDGMENT

This research was funded by the internal research funding of UNHAS, namely Academic Assistant Lecturer Research Grant, with the contract number 2319 - UN4 / KU / 02.01 / 2019.

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