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Phytochemical Profile, Antioxidant and Anti-Methanogen Potential of Red Betel (*Piper crocatum* Ruiz and Pav) Leaf Extracts By Different Solvents

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Abstract. The red betel leaf (*Piper crocatum*) from the island of Sulawesi which is used as a medicinal plant. The red betel leaf contains secondary metabolite compounds that function as bioactive substances. This research aims to determine the phytochemical content and potential antioxidant and anti-methanogen activity of red betel leaf extracts. This research was conducted at the herbal laboratory of Materia Medika, Batu City, East Java. The raw material used was red betel leaf obtained from Materia Medika Garden. The extraction of red betel leaves used 3 types of solvents with different polarities, namely n-hexane, ethyl acetate and 70% ethanol. The content of red betel leaf extracts with different solvents showed total phenols 6.5 - 16.43 mg/g, total tannins 1,05 - 4,31 mg /g, total flavonoids 8.11 - 18.3 mg /g. The antioxidant activity of ethyl acetate red betel leaf extract was IC₅₀ 167.1. Anti-methanogen contained in red betel leaf extracts can reduce the number of methanogens with inhibitory power 13.34% - 55.02%. The conclusion of this study is that red betel leaf extract has the potential to be used as a feed additive and reduce methane gas emissions in ruminants.

Keywords: Antioxidant, Extraction, Red Betel, Methanogen

1. Introduction

The provision of natural ingredients that contain active ingredients that prevent heat stress and are able to lower CH₄ in ruminant cattle is one of the strategies that need to be carried out by farmers [1]. According to [1], mitigation strategies with a decrease in methane gas must synergize with livestock adaptation. Phytochemicals are secondary metabolite compounds that have bioactive compounds that are usually present in plants [2]. Phytochemicals are also reported to play a role as a modifier in fermentation in ruminant livestock rumen [3]. Decrease in methane gas production in rumen by suppressing methanogenic bacteria. According to [4], rumen methanogen bacteria are calibrated in the 10 genera that are the most numerous, namely from 63.2 % Methanobrevibacter, 9.8% Methanosphaera, 7,7 % Methanomicrobium, and 1.2 % Methanobacterium.

Piper crocatum Ruiz and Pav., known by the common names red betel is a plant that originates from the island of Sulawesi in Indonesia and is widely used as a medicinal plant. In general, red



betel leaves contain bioactive compounds, namely flavonoids, glucoside, terpenoids, , alkaloids, isoprenoid, , eugenol, cyanogenic and nonprotein amino acids. Flavonoids are the most abundant component in red betel and they also contain phytochemical such as tannins, alkaloids, , saponins, and triterpenoids [5]. Based on [4] that the content of secondary metabolites in red betel leaves is flavonoids, saponins, alkaloids and tannins. The total content of fresh red betel leaf was 41.29 ± 0.52 and dried red betel leaf was 33.33 ± 1.09 mg GAE/g. Flavonoids, tannins and saponins are compounds that are reported to have properties as antimethanogenic [5]. Tannins can lower methane gas emissions through their performance in reducing the population of rumen methanogens. Meanwhile, the performance of saponins is through the reduction of rumen protozoan populations. The red betel leaf extract and its compounds (eugenol and hydroxychavicol) have antioxidant activity [6]. Antioxidants can be produced from plant secondary metabolite compounds such as flavonoid, tannin, and alkaloid [7].

The medicine plant extraction often uses solvents that are divided based on their polarity, such as polar solvents (such as alcohol), intermediate polar solvents (such as ethyl acetate, acetone, and dichloromethane), as well as nonpolar solvents (such as n-hexane, ether, and chloroform). This extraction process can produce extracts that can be tested for antibacterial, antioxidant, and other pharmacological and biological activities [5]. The purpose of this study is to determine the phytochemical content, antioxidant activity and potential anti-methanogen activity of betel leaf extract extracted with several solvents with different polarities. The results of this study are expected to be used as a reference for further research for the use of this natural material as a feed additive and mitigation agent to reduce greenhouse gases from livestock.

2. Material and method

2.1 Location and source of red betel leaf

This study was conducted at the herbal laboratory of Materia Medika in Batu City, East Java Indonesia. The raw material used is red betel leaf (*P. crocatum*) obtained from Materia Medika Garden. Raw materials are dried using a 40°C oven for 3 days, then ground into flour.

The extraction of red betel leaves used solvents with different polarities, namely 70%, ethyl acetate and ethanol n-hexane. Red betel flour macerated with solvent (1:5/w:v) at 37°C for 2 days. Then the supernatant is filtered with filter paper, then evaporated using a vacuum evaporator.

2.2 Phytochemical measurement of red betel leaf extracts

The Folin-Ciocalteu (FC) colorimetric method was used to determine the total phenolic content in the extract. It is based on chemical reduction in reagents, which is a mixture of tungsten and molybdenum oxide. Determination of total flavonoid content (TFC) in extract using $AlCl_3$ colorimetric method. The modified Folin-Ciocalteu method was used to measure the total tannin content (TTC) with the results expressed in the form of tannic acid equivalent (mg TA/g extract). This measurement uses spectrophotometry. The sample extract of 10 mg is dissolved in 10 mL of solvent then taken 500 μ L to be put into the test tube. The solution was mixed with 2.5 mL of 10% Folin-Ciocalteu reagent, then 2.5 mL of 7% Na_2CO_3 solution (0.7g/10ml) was added, and incubated at room temperature for one hour. Absorbance measurement using a UV-Vis spectrophotometer (745 nm). Tannic acid is used as standard, with a solution at a concentration between 10-50 ppm measured for absorbance at a wavelength of 745 nm. Then a calibration curve is made with the absorbance value on the y-axis against the concentration value on the x-axis.

2.3 Measurement of antioxidant activity of red betel (*P. crocatum*) leaf extracts

A stock solution is made from DPPH 1.9716 mg in 50 ml of ethanol. Then it is incubated in a dark room for 30 minutes. The solution is put into the cuvette and its absorption is measured at λ_{max}

520 nm. Pad the other part, prepare a sample solution from the extract to be tested. Maisng-each with a variation in concentration as shown in the appendix. Then from each sample, DPPH 0.1 mM was added with a ratio of 1:1. This mixture was incubated in a dark room for 30 minutes, and then its absorption value was measured at $\lambda_{\max}$ 520 nm. The measurement of inhibition (% of inhibition) is obtained from the following equation:

$$\% \text{ inhibition} = \frac{\text{Abs.control A} - \text{abs.sampel}}{\text{Abs.Control A}} \times 100$$

The IC₅₀ value is obtained from the the linear curve equation of % of inhibition to the concentration that is able to inhibit DPPH radicals by 50%.

2.4. Measurement of inhibitory potential against methanogen bacteria of red betel leaf extract

The inhibition test against methanogens was carried out using inoculum isolate derived from bovine rumen fluid obtained from the slaughterhouse. The inhibition test was carried out using the liquid culture method, modified from the Weimar (2016) method [8]. The medium used in the inhibition test is the selective media of methanogens [9].

Red betel leaf extract was tested using 70% ethanol extract, ethyl acetate extract, and n-hexane extract. Each extract was evaluated at two concentrations, 25 mg/ml and 50 mg/ml, with Tween as the diluent. Chloroform 100 M served as the positive control, and Tween 20 as the negative control. The samples were incubated anaerobically at 38°C for 24-48 hours in an anaerobic jar. The growth of each sample was assessed by measuring the optical density at 610 nm with a UV-vis spectrophotometer, and the amount of methanogen was estimated using McFarland's standard.

3. Result and discussion

3.1 Phytochemical profile of red bethel leaf extracts

Selecting the right solvent is essential in maceration, as it determines the efficiency of phytochemical extraction from the sample and facilitates the extraction of heat-sensitive compounds. Solvents used in medicinal plant extraction are categorized based on their polarity: polar (e.g., alcohol), interpolar (e.g., ethyl acetate, dichloromethane, acetone), and nonpolar (e.g., n-hexane, chloroform, ether)[8].

The total content of phenolic, total tannins and total flavonoids of red betel leaf extract is proposed in Table 1.

Table 1. Yield, TPC, TFC and TTC of red bethel leaf extracts with different solvent^a

Solvent of Extract	Yield (%)	TPC (mg GAE/g)	TTC (mg GAE/g)	TFC (mg QE/g)
Ethanol 70 %	10,0	16,43 ±0,03	43.07	11,65 ±0,40
Ethyl acetate	3,96	6,50 ±0,07	20.06	18,30±0,38
n-hexane	1,28	6,67 ±0,13	10.47	8,11±1,13

^asource: primary data

Based on Tab 1 that the highest yield extract of red betel leaf was by ethanolic solvent follow by ethyl acetate and n-hexane. The polar solvents (ethanol) are more capable of dissolving phenolic compounds including tannins. While semi-polar (ethyl acetate) dissolves flavonoid compounds. As indicated in [11] the type and polarity of the solvent significantly impact the Total Phenolic Content (TPC). Additionally, various plant parts contain differing levels of TPC.

Based on Tab. 1 that TPC in red betel leaf extract is between 6.50 – 16.43 mg GAE/g, where the TPC of ethanol extract is the highest compared to others. Phenolic compounds are important components in plants because they have redox properties that contribute to their antioxidant activity. The presence of phenol compounds in plants can be beneficial for preventing disease [11].

The highest TTC was also in red betel leaf ethanol extract (43.07) followed by ethyl acetate extract (20.06) and hexane extract (10.47) mg GAE/g. Meanwhile, the highest TFC was in ethyl acetate extract of 18.30 followed by ethanol and n-hexane extracts. Tannins are compounds of the phenolic group that are in the form of condensed tannins. The condensed tannins consist of flavan-3-ol units and have antioxidant, anti-inflammatory, antiviral, antibacterial properties in both in vivo and in vitro tests [12].

3.2 Antioxidant of betel leaf extracts

An oxidizing radical called DPPH can be suppressed by antioxidants through the removal of hydrogen atoms from antioxidant molecules. The purple colour of DPPH is changed to a pale-yellow colour. The amount of DPPH decrease will increase with the number of antioxidants present in the extract. High sample scavenger activity correlates with a high decrease in DPPH [13]. The amount of antioxidant needed to reduce the initial DPPH concentration by half is referred to as IC_{50} . Higher antioxidant activity is indicated by a lower IC_{50} value. Antioxidant activity of red betel leaf extracts by different solvents is shown in Fig. 1.

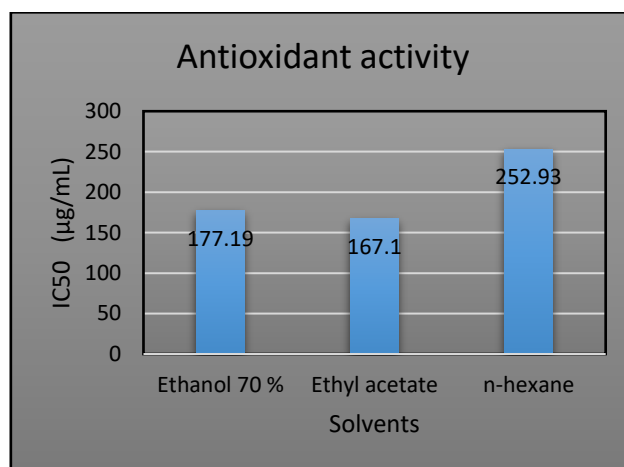


Figure 1. Antioxidant activity of red betel leaves extracts (IC_{50})

Based on Fig 1. that the antioxidant activity of red betel leaf extract was with IC_{50} values of 167.10 (ethyl acetate extract), 177.19 (ethanol extract) and 259.93 ($\mu\text{g/mL}$) (n-hexane extract). The value of antioxidant activity is relatively weak, the higher the IC_{50} value, the lower the antioxidant activity. Previous studies have also reported that red betel leaf extract has antioxidant activity of $IC_{50}186.33 \mu\text{g/mL}$ [6].

The % inhibition of red betel leaf extracts each solvent types is in Figure 2 (ethanolic extract), Figure 3 (ethyl acetate extract) and Figure 4 hexane extract. Antioxidant activity is calculated from the concentration value to obtain 50% DPPH inhibition.

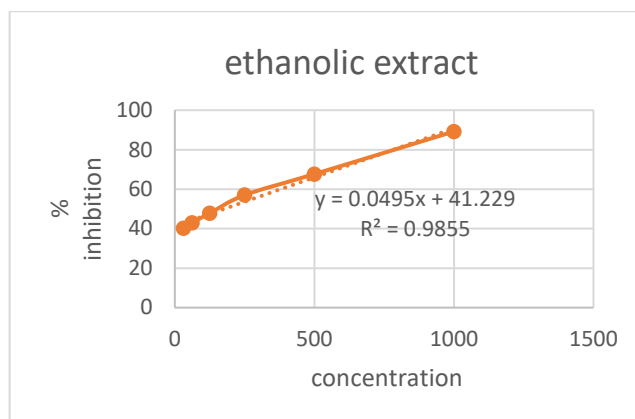


Figure 2. The % inhibition DPPH of ethanolic extracts red betel leaf

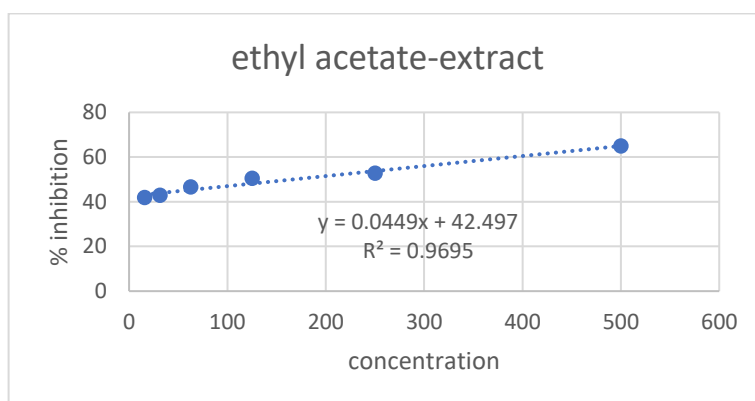


Figure 3. The % inhibition DPPH of ethyl acetate extracts red betel leaf

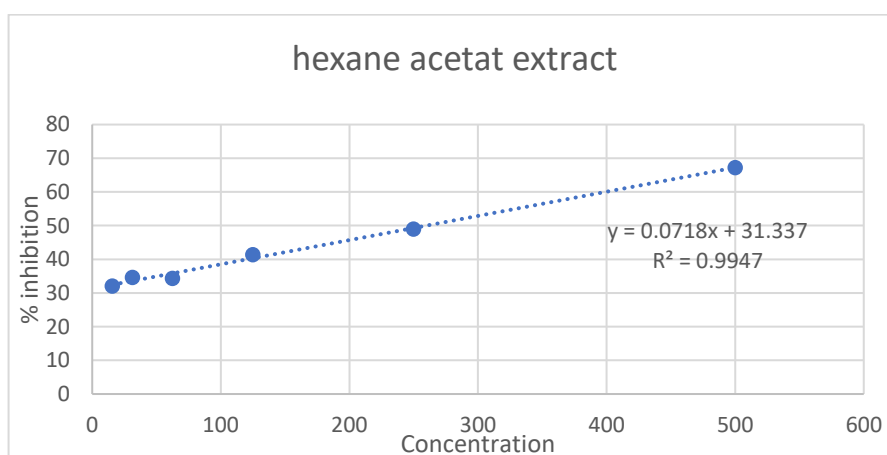


Figure 4. The % inhibition DPPH of hexane extracts of red betel leaf

Antioxidant activity is related to the content of phytochemical compounds in red betel leaf extract. Phenolic compounds that are widely contained in red betel leaf ethanol extract. According to [14] that phenolic compounds work as antioxidants by reacting with various free radicals. The way antioxidants work is involved is through single electron transfer, hydrogen atom transfer, sequential proton loss electron transfer, and transition metal chelation.

3.3 Anti methanogen- bacterial of betel leaf extracts

Tabel 2. The inhibition of Red Betel leaf extracts against methanogen isolated^d

		Concentration	Absorbance 610 nm	Estimated number of bacteria 10 ^x	% inhibition
Control	Media		0.0527	0	
	Media +inoculum		1.2061	18.96	0.00
	tween 15%		1.1440	17.84	5.89
	Chloroform	100 M	0.3952	4.44	76.56
Red Betel leaves extracts	Media+ ethanolic	25 mg/ML	0.8491	12.57	33.72
	extract +inoculum	50 mg/ML	0.62346	8.53	55.02
	Media extract ethyl	25 mg/ML	1.06506	16.43	13.34
	acetate + inoculum	50 mg/ML	0.65174	9.03	52.35
	Media extract	25 mg/ML	0.9948	15.17	19.97
	n-hexane +inoculum	50 mg/ML	0.94538	14.29	24.63

^a Source : primary data

The results of the inhibition test of several red betel leaf extracts against methanol showed a strong inhibition > 50% by ethanol and ethyl acetate extracts with a concentration of 50 mg/mL. This result is thought to be related to the active ingredients contained in the extract.

Flavonoids compounds in this extract are suspected as the cause of the decrease in the amount of methanogen. Flavonoids inhibit bacterial growth by disrupting energy metabolism, inhibiting nucleic acid synthesis, damaging the cytoplasmic membrane, reducing cell and biofilm attachment, inhibiting cell membrane porin, and disrupting cell walls and reducing membrane permeability thus causing bacterial cell lysis. [15]. Flavonoids reported in suppressing methane production in ruminants [4] [16]. So that the administration of red betel leaf extract has a positive impact on DM/OM digestibility, decreased gas and methane production and increased volatile fatty acid (VFA) in vitro [17].

Tannins have been widely reported to interfere with methanogens and degrade methane gas in ruminants [18],[19][20]. Tannins exert their antibacterial effect by aggregating protoplasm, causing protein precipitation, and binding to proteins, which in turn inhibits the formation of bacterial cell walls [15].

4. Conclusions

Ethyl acetate extract of red betel leaf has more potential as an antioxidant than extracts with ethanol and n-hexane solvents. Ethanol and ethyl acetate extracts have the potential to be anti-methanogenic agents. Further research is suggested to test the use of red betel leaf extract for

rumen fermentation in vitro and in vivo. The results of the study are expected to be useful as information on the use of natural ingredients as additional feed for antioxidants and to reduce methane emissions from ruminants.

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