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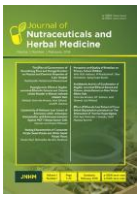

Identification of Flavonoids and Activity Test of Red Betel Leaf Fractions (Piper crocatum Ruiz & Pav) on Reducing Blood Sugar Levels in Diabetic Mice

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Abstract
Diabetes mellitus (DM) is a metabolic disease that can be life-threatening. Treatment for type 2 DM can use oral hypoglycemic drugs, but sometimes they have significant side effects. Red betel leaf extract is an antidiabetic drug derived from nature. The extract still contains impurities that can interfere with its effectiveness. The aim of this study was to determine the presence of flavonoids and the effect of each red betel leaf fraction as a blood sugar lowering agent. The research method used was analytical experimentation using maceration extraction using 70% ethanol solvent and continued with liquid-liquid fractionation using water, ethyl acetate, and n-hexane solvents. Antidiabetic activity was measured using a glucometer and indicated by the % reduction in blood sugar levels in each group. The results of flavonoid identification showed that the extract, water fraction, and ethyl acetate fraction of red betel leaves contained flavonoid compounds, while the n-hexane fraction did not contain flavonoid compounds. The percentage of reduction in blood sugar levels in each group were negative control (9.19%), positive control (50.04%), extract (41.70%), water fraction (47.33%), ethyl acetate fraction (56.40%), and n-hexane fraction of red betel leaves (34.95%). Based on the results, it can be concluded that the research group of ethyl acetate fraction and water fraction of red betel leaves have the potential as antidiabetics because not significantly different from the positive control glibenclamide. The best percentage value of blood sugar reduction is the ethyl acetate fraction group of red betel leaves with an average of 56.40%.

Keywords: Red betel leaf; flavonoids; antidiabetic.

INTRODUCTION

Diabetes mellitus (DM) is a life-threatening metabolic disease. Diabetes occurs due to elevated blood sugar levels (hyperglycemia) above normal levels (Kemenkes RI, 2020). DM is divided into three groups: type 1 DM, type 2 DM, and gestational DM (Mindayani et al., 2019). Generally, in Indonesia, type 2 diabetes mellitus is found, accounting for

approximately 90-95% of cases (Kemenkes RI, 2020). According to data from the International Diabetes Federation (IDF), 19.5 million Indonesians aged 20-79 years suffer from DM (IDF, 2021). One cause of type 2 DM is the presence of free radicals, which can lead to oxidative stress, which can reduce organ function, including the pancreas (Mindayani et al., 2019). Therefore, treatment is needed to suppress the free radicals that cause diabetes mellitus.

Treatment for type 2 diabetes mellitus can use various classes of oral hypoglycemic drugs. These groups include sulfonylureas, glinides, acarbose, biguanides, and thiazolidinediones (Soelistijo et al., 2021). The use of oral hypoglycemic drugs is relatively expensive (Sukandar et al., 2012). Furthermore, these drugs, which are part of pharmacological therapy, can cause more dangerous side effects (Adrianto et al., 2023). Therefore, there is a need to replace alternative treatments using herbal ingredients with antidiabetic properties. Furthermore, the use of herbal medicines can provide reliable efficacy and safety (Kusuma & Andriani, 2019).

Herbal treatments can be used because they contain secondary metabolites. These secondary metabolites provide antioxidant therapeutic properties that can protect the body from free radicals that can cause type 2 diabetes mellitus (Anggraito et al., 2018). One of the secondary metabolites is flavonoids, or polyphenolic compounds, which function as antidiabetic agents with a C6-C3-C6 carbon structure (Cahyana & Adiyanti, 2021).

The red betel plant (Piper croatum Ruiz & Pav) is widely cultivated for its use in traditional ceremonies and its medicinal properties (Mindayani et al., 2019). Medicinally, red betel leaves can treat various diseases, including diabetes, by lowering blood sugar levels (Kusuma & Andriani, 2019). Red betel leaves contain secondary metabolites consisting of flavonoids, terpenoids, vitamin E, and alkaloids (Ramadhan et al., 2019). Previous research has shown a decrease in blood sugar levels with the administration of red betel leaf extract. The dose of red betel leaf extract used to lower blood sugar levels in mice was 2.8 g/kg body weight (Saputra et al., 2018). While extracts can lower blood sugar levels, they still contain impurities, such as minerals, that have no effect on the body and reduce the extract's potency. To address this issue, a separation method called fractionation is needed. Fractionation is a method for separating extracts containing a mixture of compounds into only the active components (BPOM RI, 2023). Research shows that the antioxidant capacity of soursop leaf fractions is higher than that of the ethanol extract (Sari et al., 2015). This study was conducted to identify flavonoids from the fractionated red betel leaf extract and to conduct therapeutic trials to lower blood sugar levels in mice.

METHODS

Determination Sample

The determination process for red betel leaves was conducted at the UPT Herbal Materia Medica Laboratory in Batu, Malang, East Java. The purpose of this determination was to ensure that the red betel plant samples used were accurate and met existing standards (Klau & Hesturini, 2021).

Sample Preparation

Fresh red betel leaves were collected from Sambungmacan District, Sragen Regency, Central Java. The leaves were then oven-dried until they formed a simple substance. The dried leaves were then ground into a powder. The samples were then sieve using a 60-

mesh sieve and then an 80-mesh sieve to obtain a finer powder and optimize flavonoid extraction (Oktavia et al., 2023).

Extract Preparation

Red betel leaf powder weighing 500 grams was subjected to maceration extraction using 5L of 70% ethanol. The first maceration process was carried out using a macerator by soaking 500g powder in 3,750 mL of ethanol (1:7.5) for three days with daily stirring. Then, the extract was re-macerated using 2,500 mL of ethanol (1:2.5) for 2 days. The re-maceration process was carried out in a tightly closed state and stirred occasionally. The material was filtered with filter paper and the extract filtrate was evaporated with a rotary evaporator at 50 o C until the extract was thickened.

Red Betel Leaf Fractionation

The 70% ethanol extract of red betel leaves can be subjected to a modified fractionation process using n-hexane, ethyl acetate, and distilled water in a 1:1 ratio. A 10-gram extract was dissolved in a small amount of ethanol, then 100 mL of distilled water was added. The dissolved extract was placed in a separating funnel and 100 mL of n-hexane solvent was added. The separating funnel was shaken while occasionally opening and closing the tap to prevent it from exploding. This was repeated until a clear fraction was obtained. The fractions were then separated based on their specific gravity, namely the water fraction at the bottom was taken and separated from the n-hexane fraction. The water fraction was separated again by adding 100 mL of ethyl acetate and shaking until a clear fraction was obtained. After obtaining three fractions, namely the n-hexane fraction, the ethyl acetate fraction, and the water fraction, evaporation can be carried out using a rotary evaporator until the fraction thickens (Peratiwi et al., 2023).

Qualitative identification of flavonoids

Reagent Mg powder and concentrated HCl

Testing done with each extract, n-hexane, ethyl acetate, and leaf water fraction of betel red was taken as much as 0.5 grams. Then dissolved with ethanol and water. Solution added reagent Mg powder and concentrated HCl. Positive result as formed color yellow (Januarti et al., 2019).

NaOH reagent

Testing done with each extract, n-hexan, ethyl acetate, and leaf water fraction of betel red was taken as much as 0.5 grams. Then dissolved with ethanol and water. Solution added reagent 2 drops of NaOH to in each sample . The sample contains flavonoids as formed color yellow until yellow brownish (Lindawati & Ni'ma, 2022).

Reagent H_2SO_4

Testing done with each extract, n-hexan, ethyl acetate, and leaf water fraction of betel red was taken as much as 0.5 grams. Then dissolved with ethanol and water. Solution added reagent 2 drops of H_2SO_4 to in each sample. The sample contains flavonoids as formed red brick until chocolate black (Kurnianto et al., 2021).

Antidiabetic Test

Antidiabetic testing can be performed on the 8th day after the mice have acclimated to their environment. All groups of mice undergo blood sugar testing before being induced with the diabetes agent. The results were then recorded as the mice's blood sugar levels before treatment. Next, all groups of mice were induced intraperitoneally with alloxan at the appropriate dose for the preliminary test. Three days after alloxan administration, blood sugar levels are measured using a glucometer. Before testing, the mice were fasted

for 8-12 hours (Pertiwi et al., 2021). Blood sugar levels are measured by drawing blood from a wound on the tail and smearing the blood onto a glucometer test strip. Mice are diagnosed with diabetes if their blood sugar levels rise above 126 mg/dL (Kodariah et al., 2022).

Animals with diabetes can be tested according to their respective groups. Then labels were given for positive control 1, negative control 2, group 3 extract, group 4 n-hexane fraction, group 5 ethyl acetate fraction, and group 6 air fraction. Each treatment was administered once. Blood sugar levels were then measured on days 0, 3, 7, and 14 (Fadel & Besan, 2020). The results of each treatment were recorded and compared to determine the reduction in blood sugar levels in the mice (Putra et al., 2017).

Data Analysis

The results of the blood sugar reduction effectiveness test of the optimized tablets were expressed by the reduction in blood sugar levels before and after treatment for each test group on days 0, 3, 7, and 14, expressed in mg/dl. In this study, data analysis was performed using SPSS 25 software. Normality was tested using the Shapiro-Wilk test and homogeneity was tested using the Levene test. Furthermore, the One-way ANOVA test was performed on normally distributed and homogeneous data. Meanwhile, the Kruskal-Wallis test can be used on non-normally distributed and non-homogeneous data.

RESULTS AND DISCUSSION

Before the study, a plant identification process was carried out. The identification process of the red betel plant was conducted at the Materia Medica Technical Implementation Unit in Batu, Malang, East Java. Sample identification aims to verify the identity of the plant samples used (Klau & Hesturini, 2021). The results of the red betel plant identification are listed in letter number 000.9.3/716/102.20/2025, which indicates that the samples used in this study are indeed derived from the red betel plant with the Latin name Piper crocatum Luiz & Pav.

After the authenticity of the plant was confirmed, extraction was carried out based on the consideration that the flavonoid compounds to be extracted are heat-resistant. Therefore, the appropriate method is cold extraction by maceration. The yield of the thick extract that has been obtained is calculated. The description of the red betel leaf extract is brown in color with a thick form, has a bitter taste, and has a distinctive aroma of betel leaves. The results obtained are in accordance with the description of the Indonesian herbal pharmacopoeia, namely a thick extract, reddish brown in color, has a distinctive aroma and has a bitter taste. The yield obtained for the red betel leaf extract is 25.34%. The resulting yield is in accordance with the theory because the results show more than the requirements for a good red betel leaf extract yield of > 17% (Depkes RI, 2017). The results obtained are greater than previous research (Moerfiah & Supomo, 2011) with a yield of 14.48%. The method used is maceration with 96% ethanol solvent for 24 hours. The difference in yield is due to the solvent used and the length of maceration time. Using 70% ethanol can produce a higher yield because it can attract more polar compounds.

Table 1. Results of Red Betel Leaf Extract Yield

Simplicia (g)	Extract Yield (g)	Rendemen (%)
500	126,7	25,34

The extract was then fractionated. The results showed that each fraction had a different yield percentage. The n-hexane fraction was 8.33%, ethyl acetate 10.33%, and the water fraction 28.6%. This is consistent with previous research, with the highest yield being the water fraction, at 47.99%. The difference in fractionation results is also due to the polarity of the solvents used. The fractionation process used three solvents, sorted by their polarity: n-hexane (non-polar), ethyl acetate (semi-polar), and water (polar). The highest yield in the water fraction indicates that the compounds contained in red betel leaves are more attracted to solvents that tend to be polar. This is in line with research (Chairunisa et al., 2022) which showed that the yield of the water fraction was 47.99% and the n-hexane fraction was 22.50%. Flavonoid are compounds that will be taken in this research with polar to semipolar properties (Redha, 2010).

Table 2. Fractionation Yield Results

Extract Weight (g)	Fraction	Fraction Weight (g)	Rendemen (%w/w)
30	N-hexana	2,5	8,33
	Ethyl acetate	3,1	10,33
	Water	8,6	28,7

After obtaining the extract and fractions, flavonoid identification was carried out with the aim of ensuring the presence or absence of flavonoid compounds in the extract, water fraction, ethyl acetate fraction, and n-hexane fraction of red betel leaves. The flavonoid identification test used a qualitative technique by observing the presence or absence of compounds through changes that can be seen directly by the eye. The test parameters were marked by changes in color or the formation of sediment. The results showed that the extract and fraction of red betel leaves, except for the n-hexane fraction, contained compounds, namely flavonoid compounds.

Table 3 Results of Flavonoid Identification Test of Samples

Samples	Test						Interpretation
	NaOH		H_2SO_4		Mg + HCl		
	Control	Result	Control	Result	Control	Result	
Extract	Brownish Green	Red	Brownish Green	Yellow	Brownish Green	Yellow	+
N-hexana Fraction	Brownish Green	Brownish Green	Brownish Green	Brownish Green	Brownish Green	Brownish Green	-
Ethyl acetate Fraction	Brownish Green	Red	Brownish Green	Yellow	Brownish Green	Yellow	+
Water Fraction	Brownish Green	Red	Brownish Green	Yellow	Brownish Green	Yellow	+

The antidiabetic test was then conducted using white mice of the Wistar strain as test animals. The research procedure was reviewed and approved by the Health Research Ethics Committee of the National College of Health Sciences under number 174/EC/KEPK/IV/2025. The mice used for the test were selected to be male to avoid inaccurate data readings due to hormones (Milionis et al., 2008). The criteria for the mice used were that they must meet the requirements, including being 2-3 months old and weighing around 20-40 grams. The mice used in the study were obtained from the Pharmacology Laboratory of the National College of Health Sciences. These animals met the required requirements with an average weight of around 27.94 grams.

The administration of alloxan compounds to mice is a preliminary test to obtain results that increase blood sugar levels above normal. The use of alloxan as a diabetes induction agent because it can provide better diabetes effects and is affordable (Ighodaro et al., 2018). When using alloxan, a preliminary test is necessary first. The purpose of the preliminary test is to obtain results that increase blood sugar levels above normal, indicating that the mice have diabetes. The preliminary test treatment starts from the lowest dose and the effect of increasing blood sugar is observed. If the lowest dose does not work, the next dose can be titrated. Tests are carried out with doses of 150 mg/kgBW (Sadsyam et al., 2025), 175 mg/KgBW (Priyoherianto et al., 2018), and 200 mg/kgBW (Arrafi & Amanatie, 2018) by intraperitoneal injection with a single injection. Intraperitoneal injection is an induction carried out into the abdominal cavity of the test animal. The advantage of this injection is that it achieves a more rapid systemic increase in blood sugar (Lengkong et al., 2023). Based on preliminary test results, an alloxan dose of 200 mg/kg body weight was chosen because this dose was able to induce diabetes mellitus in test animals.

After the preliminary test, the patient proceeds to the antidiabetic test. This test is performed by measuring blood sugar levels using a glucometer. The glucometer operates using a glucose oxidase biosensor. Glucose in the capillary blood sample reacts with the glucose oxidase enzyme present on the test strip. This enzymatic reaction produces electrons, which are captured by the electrodes on the glucometer. The number of electrons captured is proportional to the glucose level in the sample (Yuniwarti et al., 2018).

Antidiabetic testing was conducted by measuring blood sugar levels on day 0, day 3, day 7, and day 14. Antidiabetic testing of mice was conducted for 14 days with the aim of obtaining stable blood sugar reduction results. Measurement on day 0 was the basis for observing the condition of the mice, whether their blood sugar levels before treatment had normal values. This was because mice with diabetes at the beginning could not continue to the testing stage and had to be replaced with new animals that had been adapted to prevent data errors. Measurement on day 3 was the stage to determine the condition of the test animals after diabetes induction using alloxan monohydrate 175 mg/kgBW. The requirement for diabetic mice is to have fasting blood sugar levels >126 mg/dL (Muttaqien & Purnama, 2024). The treatment suspension was administered on days 4 to 6 and again on days 8 to 13, because the mice's blood sugar was measured on days 7 and 14. The 7th-day measurement was used to assess the mice's blood sugar levels and determine whether they were starting to decrease. This was because the mice experienced a drastic decrease in blood sugar levels, indicating antidiabetic activity in the treatment group. The 14th-day

measurement was the final step, confirming that the test animals were free of diabetes and had normal blood sugar levels.

Table 4. Blood Sugar Level Measurement Results

Treatment Group	Average Blood Sugar (mg/dL)			
	Day 0	Day 3	Day 7	Day 14
Control Group (+)	78,6	191,6	129,8	95,6
Control Group (-)	83,8	193,4	177,4	175,4
Extract	77,8	187,4	154,4	109
N-hexana Fraction	84,8	188,6	156,8	122,2
Ethyl ace. Fraction	79,2	187,8	144,4	81,8
Water Fraction	75,4	176,6	145,2	92,8

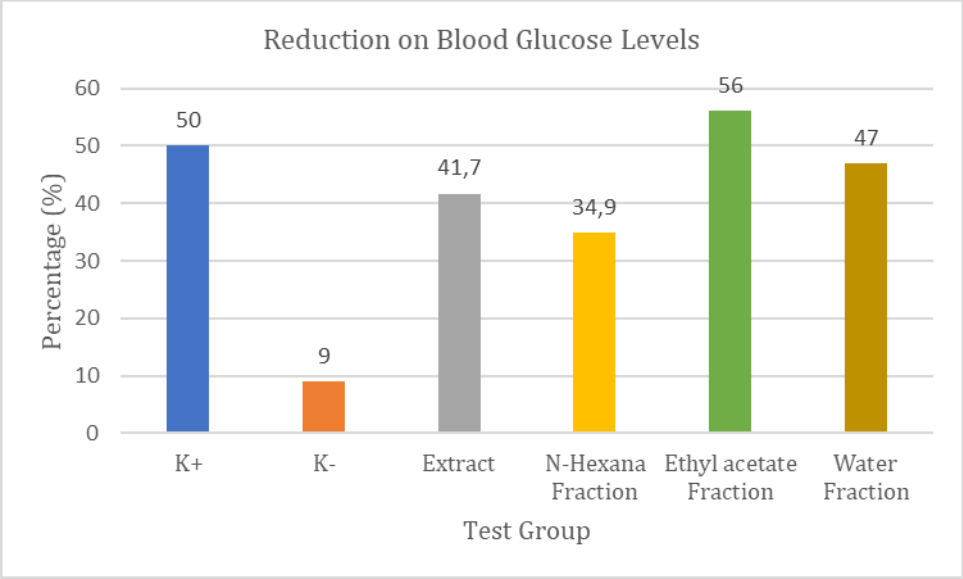
The results showed that on day 0, the test animals from all treatments had normal blood sugar levels, thus meeting the requirements and not developing diabetes. On day 3, the animals began to experience a rapid increase in blood sugar levels, exceeding 126 fasting blood sugar levels (Muttaqien & Purnama, 2024). This increase was due to the effective induction of alloxan, the agent that causes diabetes mellitus. Alloxan's mechanism of action is by reducing the sensitivity of pancreatic beta cells, resulting in reduced insulin production. On days 7 and 14, the mice began to experience a decrease in blood sugar levels toward normal, indicating that several test groups exhibited antidiabetic activity. The positive control group, the water fraction, and the red betel leaf ethyl acetate fraction were all groups capable of lowering blood sugar levels in the mice.

The blood sugar level measurements were then followed by AUC calculations (Table 5). AUC or area under the curve is the amount of drug remaining in systemic circulation. A lower AUC value indicates that the drug can work well in reducing blood sugar (Sa'ad et al., 2024).

Table 5. AUC Measurement Results

Treatment Group	Mean AUC Day-		
	0 - 3	4-7	8-14
Control Group (+)	405,3	642,8	788,9
Control Group (-)	415,8	741,6	1234,8
Extract	397,8	683,6	921,9
N-hexana Fraction	410,1	690,8	976,5
Ethyl ace. Fraction	400,5	676	801,5
Water Fraction	378	643,6	833

In Picture 1, the results of the % Reduction in Blood Glucose Levels (%RBGL) of the Antidiabetic Test show that the AUC value of the positive control group has the lowest value with an average AUC value of 1837; in contrast to the negative control group which has the highest AUC value with an average of 2392.2. The treatment groups of the ethyl acetate fraction and the red betel leaf water fraction have moderate AUC values with an average of 1856.6 and 1854.6, respectively. Then, for the high AUC value in the extract and n-hexane fraction groups of red betel leaves with an average of 2077.4 and 2003.3. The AUC result is inversely proportional to the %RBGL, or percentage of antidiabetic activity. The higher the %RBGL value, the better the drug works.



Picture 1. % Reduction in Blood Glucose Levels (%RBGL) of the Antidiabetic Test

The negative control group was treated only with CMC Na suspension. This group served as a comparison group, with CMC Na as the solvent for glibenclamide. This meant that the negative control group did not exhibit a decrease in blood sugar levels in mice due to the non-diabetic nature of CMC Na (Sinata et al., 2023). Blood sugar monitoring results obtained on the 7th day after administration averaged 177.4 mg/dL, and on the 14th day, 175.4 mg/dL. These results align with the theoretical framework, as research (Meilina et al., 2022) showed that the negative control group with 0.5% CMC Na had average blood sugar levels of 263 mg/dL on day 3 and 209.8 mg/dL on day 6.

The positive control group was treated only with glibenclamide suspension. It is clear that this group served as a comparison group, as glibenclamide is an oral antidiabetic drug in the sulfonylurea class. This means that the expected results for the positive control group are lower blood sugar levels in mice. Glibenclamide's mechanism of action is to repair liver damage, allowing pancreatic beta cells to produce more insulin. The use of glibenclamide is also consistent with the treatment group, where red betel leaf contains flavonoid compounds with a similar mechanism of action (Sinata et al., 2023).

Blood sugar monitoring results obtained on the 7th day after administration averaged 129.8 mg/dL, and on the 14th day, it was 95.6 mg/dL. The results obtained were in accordance with the theoretical expectation of having blood sugar levels <126 mg/dL. Research (Meilina et al., 2022) showed that the glibenclamide-positive group had an average blood sugar level of 184.2 mg/dL on day 3 and 94 mg/dL on day 6.

Both the water and ethyl acetate fractions exhibited antidiabetic activity, but the ethyl acetate fraction exhibited the highest antidiabetic activity. The ethyl acetate fraction contains more flavonoid compounds than the water fraction (Saputri & Sa'ad, 2023). This suggests potential as an antidiabetic treatment. The mechanism of flavonoids is to reduce glucose absorption by inhibiting the alpha-glucoxidase enzyme (Meilina et al., 2022). Flavonoids act as antioxidants capable of lowering blood sugar levels by scavenging or neutralizing free radicals such as reactive oxygen species (ROS) or reactive nitrogen species (RNS) associated with phenolic OH groups, thereby repairing damaged tissue. This

allows for the healing of pancreatic tissue damaged by alloxan, thereby increasing insulin production and lowering blood sugar levels (Afsari et al., 2016).
Statistical analysis was performed on the antidiabetic test data. The results showed significant differences between the positive and negative groups, the red betel leaf extract group, and the red betel leaf n-hexane fraction group. The significance value was 0.00, indicating a difference <0.05. This indicates that the different treatments administered to each group significantly reduced blood sugar levels in diabetic mice. The water and ethyl acetate fraction groups did not differ significantly from the positive control group, suggesting potential as an antidiabetic drug.

CONCLUSION

There were no flavonoid compounds in the n-Hexane fraction, but there were flavonoid compounds in the extract, ethyl acetate fraction, and water fraction of red betel leaves. In the antidiabetic test on mice, the results showed that the ethyl acetate fraction and the water fraction of red betel leaves had the potential as antidiabetics because they were not significantly different from the positive control glibenclamide. The ethyl acetate fraction of red betel leaves was the best fraction in reducing blood sugar levels with an average % Reduction on Blood Glucose Levels value of 56.40%.

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