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Effect of Ethanol Extract Red Betel Leaf (*Piper crocatum*) On Creatinine and Ureum Levels of Streptozotosin-Nikotinamid Induced Male Wistar White Rats

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ABSTRACT

Red betel (*Piper crocatum*) is a herbal plant that has various bioactivities including antioxidant activity. Red betel leaf can be used in the treatment of diabetes mellitus and has properties as an antioxidant. This study aims to find the activity of normal levels of creatinine and urea induced by Streptozotosin-Nicotinamide in diabetic rats. Red betel leaves are extracted by soaking in 96% ethanol solvent. Phytochemical screening is used to test the class of compounds in red betel leaf extract using the test tube method. Experimental animals in the study were placed in 6 treatment groups, namely no treatment (CMC 1%), negative control (STZ-NA), positive control (Glibenclamide), dose 1 (125 mg/kg BW), dose 2 (250 mg/kg BW), and dose 3 (500 mg/kg BW). A UV-Vis spectrophotometer was used to measure creatinine and urea on days 24, 31, and 39. One Way ANOVA and Post Hoc Test Multiple Comparisons-Tukey HSD were the data analyzers used. Flavonoids, tannins, saponins and alkaloids were shown from the phytochemical screening. Data analysis showed that the 500 mg/kg BW ethanol extract of red betel leaves was able to produce creatinine and urea better than the 125 mg/kg BW and 250 mg/kg BW ethanol extracts of red betel leaves. The results showed that the ethanol extract of red betel leaves at a dose of 500 mg/kg BW was able to reduce creatinine and urea levels in male white wistar rats.

1. Introduction

Insulin resistance refers to the body cells' inability to use glucose in the blood for energy, resulting in elevated blood glucose levels (American Diabetes Association, 2019). Prolonged hyperglycemia increases angiotensin activity, impairing the glomerular filtration rate, which can cause damage to the glomeruli and tubules. Damaged glomeruli and tubules can result in excess protein secretion, as well as an increase in urea and creatinine levels in the blood, ultimately leading to kidney failure (Sulistyoningrum, 2014).

The medicinal plant known as red betel (*Piper crocatum*) was one of the many species of plants that grew on Kalimantan Island. The red betel plant was a member of the *Piperaceae* family (Fajarwati et al., 2018). Red Betel contained the chemical components such as flavonoids, alkaloids, tannins, and saponins (Craft et al., 2012; Lister et al., 2014). Based on literature studies, bioactive metabolites extracted from red betel leaves has various activities, such as antioxidant, antidiabetic, anticancer, antiseptic and anti-inflammatory (Ramadhan et al., 2019).

The main chemical content of red betel was flavonoids. Flavonoids function as antioxidants which could slow down oxidation which produces excess free radicals to stimulate the release of pro-inflammatory cytokines which result in damage to the structure and function of the kidneys. Damage to the structure and function of the kidney through the help of hydrogen atoms and increase the activity of endogenous antioxidants such as glutathione peroxidase (Sulistyoningrum, 2014).

Based on *in vivo* studies carried by Dewi et al., (2014) and Siregar et al., (2015) which examine the extracts from red betel leaves in rats at doses of 50 and 100 mg/kg BW; and alloxan-induced doses of 100 and 200 mg/kg BW mice has the ability to reduce glucose in the blood. In this case, red betel leaf extract could activate pancreatic beta cells to produce insulin.

Based on the results of previous studies, researchers wanted to test red betel leaf extract on improving kidney function in diabetic nephropathy. The test parameters were seen from the decrease in creatinine and urea levels. Tests were carried out on streptozotocin-nicotinamide-induced test animals and this study has never been studied (Felisia, 2016).

2. Materials and Methods

2.1. Tools

For this study, the maceration method was used, and the following equipment was required: stirring rod (Pyrex®), glass funnel (Pyrex®), rotary evaporator (Dlab®), 3 cc and 5 cc syringes (Terumo®), UV spectrophotometer (Shimadzu®), visible spectrophotometer (Spectrtumlab 22PCS), hematocrit pipette (Marienfeld®), centrifuge (Hettich EBA-21), vortex (Gemmy®), cuvette tube (brand®), and microscope (Oreagon®).

2.2. Materials

The test materials used were red betel leaf (Piper crocatum), 96% ethanol, streptozotocin, nicotinamide, glibenclamide, creatinine reagent (Diasys®), urea reagent (Reiged Diagnostics®), ethanol, xylene, distilled water obtained from PT Brataco Chemika Solo, and alcohol from Merck.

2.3. Sample Collection

Red betel plants were collected from Sepang Simin village, Gunung Mas district, Central Kalimantan and identified at the UPT-Laboratory of Setia Budi University laboratory with identification letter No.207/DET/UPT-LAB/31.03.2021.

2.4. Phytochemical Screening

Phytochemical screening was conducted using the tube test, and chemical compounds tested for included flavonoids, tannins, saponins, and alkaloids.

2.5. Extraction

In the extraction procedure, 96% ethanol was used as a solvent in the maceration method for extract production. Powder from red betel leaves was poured with 96% ethanol at a ratio of 1:10 into the maceration vessel. Afterwards, we putted 800 grams of simplicia into the macerator, and added 8 liters of ethanol. Thereafter, soak a few hours while occasionally shaking, then let stand 1 full day and shake it again. For 3 days, the method was run repeatedly. After the maceration process was carried out, the macerate obtained was filtered. The first filtration uses a flannel cloth followed by filter paper. Filtering may be done repeatedly with a minimum of 2 times filtering. The filtrate obtained was then put into the evaporator at 50ºC to be vaporized so as to get the desired dense liquid extract (FHI, 2017).

2.6. Test Animals

Male Wistar strain white rats (*Ratus norvegicus*), aged 1-2 months and weighing between 150-250 grams, were obtained at the Setia Budi University Laboratory in Surakarta. The test animals were housed in cages maintained at a temperature of 22°C ± 3°C, relative humidity of 30-70%, and with a 12-hour light-dark cycle. Thirty rats were divided into six groups, including a normal group, a negative group, a positive group, and three treatment groups that were given red betel leaf extract at different doses. After taking blood to measure the normal levels of creatinine and urea, STZ induction with a dose of 60 mg/kg BW and NA with a dose of 120 mg/kg BW were administered. The positive group was given Glibenclamide at a dose of 0.45 mg/kg BW, and the three treatment

groups were given red betel leaf extract at a dose of 125 mg/kg BW, 250 mg/kg BW, and 500 mg/kg BW, respectively, for 14 consecutive days. The treatment was conducted after acclimatization for 7 days and STZ-NA induction had been given. Blood was collected 5 times, namely before STZ-NA induction, twice after STZ-NA induction, and twice during the administration of red betel leaf extract, to examine the levels of creatinine and urea. This research was approved by the Health Research Ethics Commission (KEPK) of the Faculty of Health, Muhammadiyah University of Surakarta, and assigned an ethical clearance number of 3513/A.1/KEPK-FKUMS/IV/2021.

2.7. Blood Sampling

Blood sampling was performed using a hematocrit pipette. The test animals were fasted for half a day before blood collection. Blood was collected from the orbital sinus, and immediately centrifuged within 10 minutes at 3000 rpm. The Eppendorf tube containing blood was allowed to stand at room temperature for 10 minutes, and then the serum was stored in a refrigerator at -20°C (BPOM, 2014).

2.8. Creatinine and Urea Examination

The examination of urea and creatinine was carried out at the Nutrition Laboratory of the Center for Food and Nutrition Studies (PSPG), Gadjah Mada University, Yogyakarta. Analyticon reagent kits were used as reagents for measuring creatinine and urea. The levels of creatinine or urea were determined by reacting test serum with monoreagents and homogenizing the mixture. The absorbance was measured with a spectrophotometer for 60 seconds, and then measured again after 120 seconds. The same procedure was done for the blank and standard (reagent + creatinine/urea standard). The method used for checking urea was enzymatic (glutamate dehydrogenase), and the method used for checking creatinine was Jaffe.

2.9. Data Analysis

Data analysis was conducted using SPSS 21.0 for Windows. The Shapiro-Wilk test was used for data normality, and if the significance value (p) was greater than 0.05, one-way ANOVA was used. If p was less than 0.05, Post Hoc-Tukey HSD multiple comparison test was applied.

3. Results and Discussion

3.1. Phytochemical Screening

The results of the phytochemical test of the ethanol extract of red betel leaves can be seen in Table 1.

Table 1. Identification results of chemical compounds from red betel leaf ethanol extract

Chemical Compounds	Reactor	Results	Information
Flavonoids	Concentrated HCl	+	Orange color on the amyl alcohol layer
Tannins	FeCl 5%	+	Blackish green color
Saponins	HCl 2N	+	Foam 2 cm high
Alkaloids	Mayer Dragendroff	+	White Precipitate (mayer dragendroff and orange precipitate)

Information :
(+) Positive = Contains chemical compounds
(-) Negative = Does not contain chemical compounds

3.2. Creatinine and Urea

Based on the results of the examination of creatinine and urea levels using One-Way ANOVA on day 0, all treatments were not significantly different (P>0.05). On day 24, the results obtained in the

STZ-NA induced diabetes treatment group ($p<0.05$) for the negative control, positive (glibenclamide), 125, 250 and 500 mg/kg BW extract. From day 31 to day 39, there was a decrease in creatinine levels in the 250 and 500 mg/kg BW extract groups while urea levels decreased in the 125, 250, 500 mg/kg BW extract groups. The test results showed that only the betel leaf extract group of 500 mg/kg BW was not significantly different from the creatinine and urea levels of the positive control. This indicates that betel leaf extract 500 mg/kg BW is able to increase creatinine and urea levels to normal levels for the improvement of kidney function in diabetic nephropathy due to STZ-NA induction (Kishore et al., 2017).

The ethanol extract of red betel leaf has an impact on increasing creatinine and urea levels due to STZ-NA induction. Increased creatinine and urea are forms of diabetic nephropathy (Bamanikar et al., 2016). STZ-NA was administered on the first day to induce diabetic nephropathy in the test animals. Diabetes mellitus can be induced with STZ at a dose of 60 mg/kg BW combined with NA at a dose of 120 mg/kg BW given 15 minutes before STZ administration. Rats experience diabetes for 21 days until they reach a complication condition, namely diabetic nephropathy, which causes an increase in creatinine and urea levels (Ghasemi et al., 2014). This is because STZ induces nephrotoxicity, which clogs the tubule lumen, making it difficult for the levels of urea and creatinine to be excreted through the tubule lumen, resulting in an increase of their levels in the blood (Murnah & Indranila, 2014).

Table 2. Measurement results of creatinine levels (mg/dL) of male white rats from the 0th to the 39th day

Group	D_0	D_{24}	D_{31}	D_{39}
Normal Control	0.70±0.03	$0.778±0.07^{bc}$	$0.81±0.06^{bc}$	$0.83±0.06^{bc}$
Negative Control	0.74±0.03	$3.635±0.08^{ac}$	$3.67±0.07^{ac}$	$3.76±0.07^{ac}$
Positive Control	0.76±0.01	$2.882±0.05^{ab}$	$1.83±0.07^{ab}$	$1.12±0.07^{ab}$
125 mg/kg of extract	0.74±0.26	$2.724±0.08^{abc}$	$3.01±0.52^{abc}$	$2.80±0.10^{abc}$
250 mg/kg of extract	0.74±0.02	$2.825±0.11^{ab}$	$2.67±0.03^{abc}$	$1.74±0.09^{abc}$
500 mg/kg of extract	0.75±0.01	$2.863±0.04^{ab}$	$2.49±0.90^{abc}$	$1.17±0.03^{ab}$

Table 3. Measurement results of urea levels (mg/dL) of male white rats from the 0th to the 39th day

Group	D_0	D_{24}	D_{31}	D_{39}
Normal Control	10.36±0.03	$10.88±0.16^{bc}$	$11.11±0.20^{bc}$	$11.60±0.26^{b}$
Negative Control	10.24±0.03	$47.88±0.91^{ac}$	$48.89±1.04^{ac}$	$49.81±1.21^{ac}$
Positive Control	10.42±0.01	$45.26±0.45^{ab}$	$16.14±0.81^{ab}$	$12.82±0.50^{b}$
125 mg/kg of extract	10±0.26	$44.67±0.84^{ab}$	$38.42±1.67^{abc}$	$25.53±1.08^{abc}$
250 mg/kg of extract	10±0.02	$44.96±1.62^{a}$	$25.03±1.55^{abc}$	$15.90±0.58^{abc}$
500 mg/kg of extract	10±0.01	$44.60±0.60^{ab}$	$21.64±0.92^{abc}$	$13.85±0.69^{ab}$

Description: a = Significantly different from the normal control grup, b = Significantly different from negative control grup, c = Significantly different from the positive control grup.

Glomerular filtration which has decreased function was caused by increased levels of urea in the blood so that the cells in the body were damaged which causes kidney damage. Impaired kidney function due to decreased glomerular filtration was not the only reason for increased urea levels. The glomerular filtration rate was a clinical condition that cannot be used as a standard for an increase in urea levels. Vextracellular volume in the body, protein levels in feed, and liver disease were examples of other clinical conditions. Lack of bodily fluids may also contribute to increased urea levels. Serum creatinine was often used in assessing glomerular function. Serum creatinine could assess kidney function that has decreased more specifically. Increased serum creatinine would not be affected by food or fluids (Kee, 2014).

Data from the ethanol extract of red betel leaf treatment showed that the urea level decreased due to STZ-NA induction which could be seen from the average urea level. Comparison of the group

given various doses of the extract with the STZ-NA group showed better urea levels than the STZ-NA group. This result showed that the cells in the kidneys were protected as a sign that the red betel leaf extract has an effect. On day 39, group 4 was 25,53 mg/dL, group 5 was 15,90 mg/dL, group 6 was 13,85 mg/dL and the glibenclamide control was 14,48 mg/dL. The STZ-NA effect increased urea levels by 49,34 mg/dL, which was inversely proportional to the positive control (A. Ghasemi et al., 2014). Blockage of the tubular lumen results in increased creatinine and urea levels which made the damaged cells affect the tubular lumen. Fibronectin protein interactions result in the formation of cylinders that block the tubular lumen. As a result, urea and creatinine were unable to be excreted through the tubular lumen which causes an increase in urea and creatinine (Gound et al., 2015).

The creatinine level measured had the same effect as the urea level, which means the red betel leaf extract that was given caused the creatinine level to decrease compared to STZ-NA. This makes the cells in the kidney protected. On day 39, group 4 was 2,80 mg/dL, group 5 was 1,74 mg/dL, group 6 was 1,17 mg/dL and the glibenclamide control was 1,47 mg/dL. STZ-NA experienced an increase in creatinine levels of 3,76 mg/dL.

The increase in creatinine and urea levels that occur continuously from day to day causes complications in the kidney membranes. Changes in glomerular function led to hyperfiltration, which causes the nephrons to became sclerotic. Chronic hyperglycemia may led to nonenzymatic glycation of proteins and amino acids. Glucose bound to amino acids would cause AGE's (advance glycosylation end-products). Increased AGEs would result in kidney glomeruli being damaged (Dewi et al., 2014).

The reduction in urea and creatinine levels was achieved through a mechanism of secondary metabolites using a test preparation of ethanol extract of red betel leaf, which has antioxidant activity. According to the results of a series of tests to determine the chemical composition, it was found to contain flavonoids, alkaloids, tannins, and saponins. Flavonoids and tannins are compounds that have antioxidant activity. Flavonoid compounds can minimize microvascular complications by reducing creatinine levels. The antioxidant activity of flavonoid compounds can inhibit the rate of formation of AGEs and dicarbonyl compounds and prevent the development of diabetic nephropathy through scavenging effects on mesangial cell mitochondrial ROS. Flavanol compounds inhibit NF-κB activity and reduce lipid peroxides. Flavonoids play a significant role in regenerating damaged pancreatic β-cells, thus overcoming insulin deficiency. Additionally, flavonoids, as strong antioxidants, can inhibit cell damage/cell death (apoptosis) and kidney function damage (Murnah & Indranila, 2014).

Alkaloids effectively act as antioxidants that inhibit the oxidation reaction of linoleic acid and stabilize free radicals through proton donors. Saponins have been proven to possess antioxidant and antiperoxidase activities, enabling them to capture hydroxyl free radicals (El Barky et al., 2017). Given the various compounds mentioned, it is necessary to isolate them in order to identify which ones contribute to stabilizing normal urea and creatinine levels (Dewi et al., 2018; Elekofehinti et al., 2012).

This research needs to be studied more deeply, particularly with the observation of histopathology, especially the level of kidney damage, to further investigate diabetic neuropathy. It is also necessary to proceed with the compound isolation stage to identify the specific contents in the red betel plant.

4. Conclusion

The results of the study concluded that the compounds contained in the ethanol extract of red betel leaves were flavonoids, tannins, saponins, and alkaloids. Red betel leaf extract 500 mg/kg BW is a dose group that can reduce serum creatinine and urea levels in male white rats with Wistar strain which increase due to Streptozotocin-Nicotinamide induction.

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Competing Interests

The authors disclose no conflict.

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