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Original Research Article

The Effect of Red Betel Leaf (*Piper crocatum*) Ethanol Extract on the Histopathological Eye Image of Alloxan-Induced Diabetic Rat (*Rattus norvegicus*)

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ABSTRACT

Diabetes mellitus (DM) is a heterogeneous group of diseases characterized by elevated blood glucose concentrations. Diabetic retinopathy is a complication that DM sufferers almost always experience. Diabetic retinopathy occurs due to hyperglycaemic conditions for a certain period, which cause physiological changes resulting in endothelial damage. The objective of this study was to analyze the impact of red betel leaf extract (*Piper crocatum*) on the histopathological features of alloxan-induced diabetic Wistar rats (*Rattus norvegicus*). The rats were categorized into five groups: the negative control group, positive control group, and three treatment groups with different dosage; 150, 250 and 350 mg/kgBW, respectively. Eye damage was assessed using Roenigk's Spoiled Histopathology scoring. The test results showed significant differences between the histopathological image of the groups treated with red betel leaf extract and those induced with alloxan (p<0.05). Eye damage that occurred could be prevented and repaired by the presence of antioxidants (flavonoids, tannins, saponins, and alkaloids) in red betel leaves. In summary, the administration of red betel leaf extract led to a significant improvement in the histopathology of the eyes in Wistar rats induced with alloxan-induced diabetes.

Keywords: Diabetes, *Piper crocatum*, Betel, Alloxan, Retinopathy

Introduction

Diabetes mellitus (DM) refers to a diverse set of medical conditions marked by increased levels of blood glucose.[1] It is an umbrella term encompassing various metabolic disorders primarily characterized by persistent hyperglycemia. The underlying factors include compromised insulin secretion, insulin-related abnormalities, or a combination of both. The World Health Organization (WHO) has previously posited that DM cannot be described clearly and concisely. Still, it is generally a collection of anatomical and chemical complications resulting from several factors with absolute or relative deficiencies and impaired insulin function.[2] DM is a disease that ranks sixth in causing death in the world, based on the WHO report. The WHO reports that around 150 million people in the world suffer from DM, and these figures will continue to rise. The report suggested that about 1.3 million people die before age 70 (4%) due to DM.[3-4]

Diabetes Mellitus stands as the fourth most prevalent disease in Indonesia, with a continually rising trend. Projections indicate that the number of affected individuals is expected to reach 21.3 million over the next decade. Consequently, the quality of life for those with diabetes mellitus is anticipated to experience a significant decline in the upcoming years. As per the 2018 primary health research data from Indonesia, the prevalence of individuals diagnosed with diabetes mellitus (DM) by a doctor for over 15 years is reported at 2%. However, when assessed through blood sugar checks, the prevalence increases to 8.5%.[5]

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This percentage has increased significantly from 2013 to 6.9%. East Nusa Tenggara reported 0.9% of the prevalence of DM in that province. DM can cause various complications, both microvascular in the form of abnormalities in the retina of the eye, kidney glomeruli, and nerves and macrovascular in the form of blockages in the myocardium, cerebral blood vessels, and peripheral blood vessels.[6]

Diabetic retinopathy is a major complication of DM due to persistent hyperglycaemic, which causes physiological changes resulting in endothelial damage. Endothelial damage results from thickening of the vascular endothelial basement membrane and alterations in retinal blood flow. This causes the formation of microaneurysms in the retinal blood vessels, making the vessels fragile with the tendency of easy breakage and causing visual disturbances. To prevent organ damage due to diabetes mellitus, different strategies are used in the treatment, including pharmacological therapy consisting of oral or injectable hypoglycaemics, exercise, and lifestyle modifications. Oral or injectable hypoglycaemic preparations are helpful in stimulating insulin secretion, increasing insulin sensitivity, inhibiting alpha-glucosidase, inhibiting the dipeptidyl peptidase-4 enzyme, and inhibiting sodium-glucose co-transporter 2. The use of these drugs has been associated with some adverse effects, including hypoglycaemia, urinary and genital tract infections, and weight gain.[7] To prevent side effects from medications, there is a need for alternative treatments for diabetes mellitus: proper dietary control, exercise, and the use of medicinal plants.

As an agricultural country rich in natural resources, Indonesia has the potential for herbal medicinal plants to treat diabetes mellitus. Red betel leaf, scientifically known as *Piper crocatum*, is a herbal plant in Indonesia widely utilized in traditional medicine to address elevated blood sugar levels. The leaves are commonly consumed, and extracts are derived for the treatment of various ailments. Red betel leaf is rich in compounds such as flavonoids, saponins, alkaloids, and tannins, all of which are recognized for their potent antioxidant activity. It has been suggested to provide benefits for individuals with diabetes mellitus (DM). Antioxidants in red betel leaves can neutralize excessive free radicals in pancreatic β cells by breaking chain elongation in free

radicals or donating electrons to terminate the aggressiveness of free radical reactions. Antioxidants in Red betel leaf have been associated with the potential to lower blood sugar levels.[8]

Materials and Methods

Ethical Approval
This study received ethical approval from the Health Research Ethics Commission of the Faculty of Medicine, University of Nusa Cendana. The ethical approval certificate number is 043/KEH/SK/VIII/2022.

Animals
The rats were acclimatized for 1 week for proper adaptation and were given standard rodent feed (BR® pallet). Throughout the acclimatization period, the Wistar rats' weights were measured to assess if they met the research criteria, and to determine whether there was a decline in the weight of rats exceeding more than 10% during the adaptation period. A physical examination was also conducted to assess whether the test animals were healthy. The criteria for healthy rats were clean white hair, bright red eyes, no hair loss, no secretions or fluid coming out of the eyes or ears, behaviour or reasonable activity, and a good appetite.

Preparation of Red Betel Leaf Ethanol Extract
Red betel leaves were collected from Kupang City, East Nusa Tenggara, Indonesia, in October 2022. It was identified, and voucher no: PID 411113-20 was assigned. Subsequently, the leaves were washed with running water and left overnight to dry. They were cut into small pieces and dried under shade for 3 days. The material was ground into powder using a blender. Subsequently, 500 grams of the powder underwent extraction through maceration with 96% ethanol (3x1L) for 24 hours, followed by filtration. The resulting filtrate was then concentrated using a rotary evaporator at 50℃ and 40 rpm until a dense extract was obtained. The obtained extract was dissolved in 100 mL of 2% Tween 80 and the concentration was calculated based on the previously determined concentration.

Administration of Alloxan and Red Betel Leaf Ethanol Extract
Prior to the study, the rats were categorized into 5 groups, each consisting of 6 animals, with an average weight of 260 grams. The test subjects underwent a fasting period with access to water for 8-12 hours before being treated with alloxan. Alloxan, administered subcutaneously at a dose of 140 mg/kg BW, was given once on the first day of treatment. Following the injection, normal feeding and water intake were resumed. Blood sugar levels were assessed on the 14th day after alloxan administration, identifying hyperglycemic (>200 mg/dL) in Wistar rats. Subsequent to diabetes induction, the treatment groups received oral doses of red betel leaf extract at 150 mg/kg BW, 250 mg/kg BW, and 350 mg/kg BW.

Examination of Blood Glucose Levels
Blood glucose levels were assessed twice over a span of 4 weeks, specifically on days 22 and 51 post-administration. Rats underwent an 18-hour fasting period before blood glucose measurement. Blood samples were collected from the rat's tail and applied to the glucometer test strip. The concentration of blood glucose was then read in mg/dL.

Organ Harvesting
Rats were anaesthetised after the treatment, eyes were immediately removed, and histological preparations were made using paraffin. Histological preparations were stained using Hematoxylin-eosin staining.

Preparation of eyeball samples for histological examination
The samples were fixed with 10% formalin for 3 to 4 hours. The samples were dehydrated using acetone 3 times, for 2 hours each\, then cleaned using toluene 3 times, each for 1-2 hours. Samples were then soaked in liquid paraffin at 60℃ 3 times for 2 hours each to obtain the paraffin blocks. The paraffin blocks formed were sliced with a microtome tool to create a sheet with a thickness of 2 µm, and the sheets were placed in a water bath at 30℃. The sheets were withdrawn from the water bath, transferred onto glass slides, and subjected to a 2-3 minute drying period in an oven.

Examination of Eyeball histology
Histological preparations of the eye were examined using a light microscope in five distinct fields, each observed at a magnification of x400. Twenty cells were randomly counted in each field of view to observe the eye macula. Then, the average weight score of histopathological changes of the eye from five fields of view of each mouse was calculated using the Histopathology Scoring model.

Data analysis
Histopathological alterations in the eye were assessed through a bivariate test. The One-Way ANOVA test was utilized for result analysis. In instances where the assumptions of the parametric test were not satisfied, the Kruskall-Wallis test was employed, followed by a post hoc test. The post hoc test applied in this study was the LSD (Least Significant Difference) Test.

Results and Discussion

The outcomes of the phytochemical screening of ethanolic extracts from Piper crocatum demonstrated the existence of significant secondary plant metabolites such as alkaloids, tannins, saponins, and flavonoids, as illustrated in Table 1. These bioactive compounds present in the leaf extract of the plant could potentially account for the observed pharmacological activity. Regarding the blood glucose study, a noteworthy reduction in blood glucose levels was noted in both the positive control group and the treatment groups of 1, 2, and 3. Conversely, the negative control group exhibited an increase in blood glucose levels as a result of alloxan induction.
Similarly, in the histopathological investigation, the animals' eyeball sections were observed to determine the extent of damage to eye cells. The negative control group, which received no treatment, showed no damage to the eye cells. The positive control group, on the other hand, had 4 out of 6 samples with necrosis and 2 samples with hydropic degeneration. In treatment group I, with a dose of 150 mg/kgBW, there were changes observed in one normal sample. Two samples experienced changes in retinal blood flow (Figure 1a); one sample had thickening of the basal endothelium (Figure 1b), and one sample had necrosis (Figure 1c). Treatment group 2, with a dose of 250 mg/kgBW, showed changes in three normal samples: one sample with changes in retinal blood flow and one sample with necrosis. Treatment group 3, with a dose of 350 mg/kgBW, presented changes in 4 normal samples and 1 sample with thickening of the basal endothelium of blood vessels. This study examines the histopathological changes in hepatocyte cells in the eye using microscopy. The normal histological structure of hepatocyte cells is described as having round and oval-shaped cells with a dense round nucleus in the center. The positive control group was subjected to alloxan induction three times with a dose of 140 mg/kgBW. The histopathological results revealed parenchymal degeneration and water accumulation in the cells, leading to cell swelling and granular cytoplasm. Additionally, hydropic degeneration, characterized by a vacuolated cytoplasm, was observed. These vacuoles appeared clear and were a result of increased water entry into the cell. The negative control group exhibited necrosis or cell death, with cells undergoing karyorrhexis, where the nucleus is fragmented or destroyed, leaving chromatin substance fragments scattered in the cell.[11-12]

Table 1: Phytochemical Test Results of *Piper crocatum* ethanolic extract

Alkaloids	Positive
Saponins	Positive
Tannins	Positive
Flavonoids	Positive

Table 2: Mean Total Blood Glucose Levels (mg/dL)

Treatment Group	7th day	11th Day	18th Day	54th Day
Negative Control	83.66667	77	87.83333	89.5
Positive Control	98.66667	84.83333	138.5	156.8333
Treatment 1	88	82.8	184.6	100
Treatment 2	89	90.8	368.6	118.4
Treatment 3	105.6	76.6	330.8	167
Total	92.98667	82.40667	222.0667	126.3467

Table 3: Changes in the Degree of Eye Damage in Each Sample

Animal test group	Sample size	Classification of changes for each sample			
		Normal	changes in retinal blood flow	thickening of the basal m. blood p. endothelium	Necrosis
Negative Control	6	6	-	-	-
Positive control	6	-	-	2	4
Treatment 1	5	1	2	1	1
Treatment 2	6	3	2	-	1
Treatment 3	5	4	1	-	-

In treatment group 1, with a dose of 150 mg/kgBW, the results showed a decrease in hydropic and parenchymal degeneration compared to the positive control group, as well as a decrease in necrosis in the eye. The decrease in cell damage is due to the antioxidant properties of betel leaves that can counteract free radicals caused by alloxan. In treatment 3, with a dose of 250 mg/kgBW, histopathological results showed an increase in normal cells compared to treatment 1. However, there was still water accumulation around clear cells, which is characteristic of parenchymal degeneration, although not as severe as in treatment 1. In addition, all hepatocyte cells in treatment 3 were normal, and there was no evidence of parenchymal degeneration, hydropic degeneration, or necrosis.

The objective of the bivariate data testing was to assess if red betel leaf extract influenced the histopathological features of the rat liver. Since the normality test indicated a non-normal distribution of the data, a non-parametric test, specifically the Kruskal-Wallis test, was employed for analysis.

The Kruskal-Wallis test yielded noteworthy results ($p < 0.05$), with a specific p-value of 0.02. Consequently, the rejection of the null hypothesis (H0) and acceptance of the alternative hypothesis (H1) can be inferred, suggesting a significant influence of administering red betel leaf extract on the histopathological characteristics of the rat liver. Subsequent to these significant Kruskal-Wallis findings, additional scrutiny was carried out using the Tukey HSD (Honestly Significant Difference) Post Hoc test.

As can be seen in Table 5, the negative control group exhibits a significant difference from the positive control group while not showing a notable contrast with treatment group 1, which received red betel leaf extract at a dose of 150 mg/kgBW. Similarly, there was no significant difference between treatment group 2 (red betel leaf extract at 250 mg/kgBW) and treatment group 3 (red betel leaf extract at 350 mg/kgBW). The positive control group demonstrates a significant divergence from both the positive control itself, treatment group 2 (250 mg/kgBW), and treatment group 3 (350 mg/kgBW), while showing no significant distinction from treatment group 1 (150 mg/kgBW).

Additionally, treatment group 1 (150 mg/kgBW) does not show a significant difference from the negative control group, positive control group, treatment group 2 (250 mg/kgBW), and treatment group 3 (350 mg/kgBW). Treatment group 2 (250 mg/kgBW) exhibits a significant difference from the positive control group but not from the negative control group, treatment group 1 (150 mg/kgBW), and treatment group 3 (350 mg/kgBW).

In addition, treatment group 3 (350 mg/kg body weight) shows a distinct difference from the positive control group, with no significant difference from the negative control group, treatment group 1 (150 mg/kg body weight), treatment group 2 (250 mg/kg body weight) and treatment group 3 (350 mg/kg body weight).

The statistical outcomes of this study reveal significance with a p-value of 0.002. The observed ocular damage can be mitigated and restored due to the presence of antioxidants found in red betel leaves, such as flavonoids, tannins, saponins, and alkaloids.

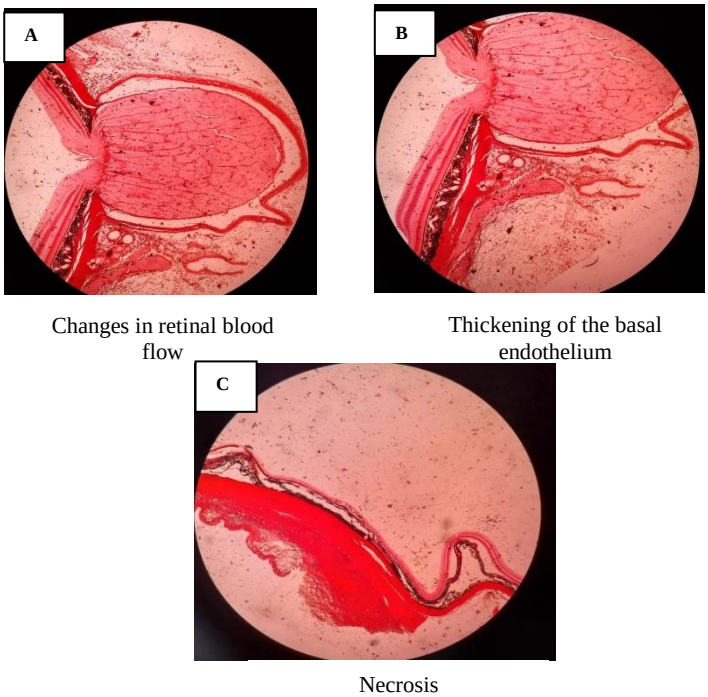

Figure 1: The histological studies of the eye

Conclusion

The research findings regarding the impact of red betel leaf extract (Piper crocatum) on the histopathological features of the eyes of white rats (Rattus norvegicus) induced by alloxan lead to the conclusion that there is a significant difference in the effectiveness of red betel leaf extract in improving eye histopathology under alloxan induction. Moreover, variations were noted in the histopathological presentation of the eyes in treatment group 2 (250 mg/kgBW) and treatment group 3 (350 mg/kgBW), indicating a dose-dependent response. However, no significant distinction was observed in the histopathological appearance between the negative control group and treatment group 1 (150 mg/kgBW). The study identifies 350 mg/kgBW as the most effective dose of red betel leaf extract in mitigating alloxan-induced eye damage.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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Table 4: Kruskal-Wallis Test Results of Red Betel Leaf Extract Administration on Histopathology of White Rat Hepar of All Treatments.

	Asym. Sig	Notes
Degree of Hepatic Cell Damage	0.002	There was a significant mean

Description: Kruskal-Wallis test
Value: $p < 0.05$

Table 5: Results of Post Hoc Mann-Whitney Test of Red Betel Leaf Extract on Histopathology of White Rat

Test Group I	Test Group II				
	Negative Control	Positive Control	Treatment 1	Treatment 2	Treatment 3
Negative Control		0.000*	0.51	0.377	0.993
Positive Control	0.000*		0.091	0.004*	0.000*
Treatment 1	0.51	0.91		0.756	0.146
Treatment 2	0.377	0.004*	0.756		0.674
Treatment 3	0.993	0.000*	0.146	0.674	

Notes: *: there is a significant difference significant
P value <0.05: Significant

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