

Subchronic Toxicity Test of Purple Leaves Ethanol Extract (PLEE) on the Histopathological Picture of the Pancreas of Wistar Rats

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Abstract: Medicinal plants are considered a rich source of bioactive metabolites and have the potential for the discovery and development of new drugs, one of which is the purple leaf plant which is known to treat various diseases. The aim of this research was to determine the effect of administering purple leaf ethanol extract over a certain period of time at doses of 500 mg/kgBB, 2.000 mg/kgBB and 5.000 mg/kgBB on the histopathology of the Wistar rat pancreas. In this study, an experimental method was used with a randomized block design, using 20 male Wistar rats which were divided into 4 research groups, 1 control group which was given Na-CMC. and 3 treatment groups were given purple leaf ethanol extract at a dose of 500 mg/kgBB (P₁), dose of 2.000 mg/kgBB (P₂) and dose of 5.000 mg/kgBB (P₃). The test preparation was administered orally, for 28 days. The results of subchronic toxicity testing of purple leaf ethanol extract on Wistar rats carried out for 28 days, up to a dose of 5.000 mg/kg BB, showed that at a dose of 2.000 mg/kg BB - 5.000 mg/kg BB it was toxic to the histopathology of the Wistar rats pancreas, which was characterized by the presence of cell degeneration, increased organ index and the presence of symptoms of toxicity.

Keywords: Pancreas; Purple leaf; Subchronic toxicity

Introduction

Medicinal plants are considered a rich source of bioactive metabolites and have potential for the discovery and development of new medicine (Yusuf et al., 2020). Bioactive secondary metabolites that have pharmacological and toxicological effects on humans such as alkaloids, polyphenols, tannins, flavonoids, steroids, terpenoids, and many others. These bioactive compounds are found in various parts of plants, such as flowers, seeds, stems, bark, fruit, leaves and roots (Bitwell et al., 2023). Apart from plants, bioactive compounds are also found in animals, microbes and other marine organisms (Puspitasari, 2018).

One of the plants used in medicine is the purple leaf plant or wungu. In Indonesia, purple leaves have long

been used in the treatment of tonsillitis, abscesses, rheumatism, breast swelling, breast abscesses, hemorrhoids, and antidiabetic (Alza et al., 2024). In addition, empirically as an anti-inflammatory drug, laxative, hemorrhoid, ulcers, and skin disease (Sartika et al., 2021).

The chemical compounds found in purple leaf plants are flavonoids, alkaloids, tannins, saponins and glycosides (Tahseen et al., 2023). Purple leaves as medicine can be used for external use or drunk, for external use it can be used to treat wounds, swelling, ulcers, boils and skin diseases, while for oral use it is usually used to treat urinary tract infections, gallstones, menstrual flow and liver disease (Salim et al., 2020). In Gribaldo et al. (2021) stated that purple leaf extract is classified as mildly toxic with an LD₅₀ value of 3.890

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mg/kg in accordance with the potential acute toxicity of BPOM RI at toxicity level 4 (oral LD₅₀ 500 – 5.000 mg/kg) (Gribaldo et al., 2021).

Unexpected effects from the use of drugs derived from natural ingredients encourage safety testing. The safety test that can be carried out in accordance with the Organization for Economic Cooperation and Development (OECD) guidelines and the regulations of the head of the Food and Drug Supervisory Authority (PerkaBPOM) is the toxicity test (Putra et al., 2023). Subchronic toxicity tests are tests carried out to provide general information about possible health hazards that may arise after long-term treatment. This information is useful for selecting dose levels for further chronic toxicity testing and establishing appropriate criteria for safe exposure levels in humans (Chiranthan et al., 2022). Toxic effects of the test preparation can occur in the vital organs of the test animal, such as the pancreas. Pancreatic damage often occurs due to the toxic effects of a compound, namely pancreatitis and pancreatic cancer, causing a decrease in pancreatic performance and disruption of the body's metabolism (BPOM, 2022). Clinical symptoms of pancreatic cancer can be clearly seen when pancreatic cancer enters an advanced stage (Puspita et al., 2023). Toxic substances can change the color of organs and changes in the shape and weight of organs, which indicates organ damage or impaired organ function (Herlina et al., 2022).

The preparation being tested was administered with a variety of different doses for 28 days orally, then clinical symptoms and symptoms of toxicity were observed for 2 hours after the test preparation was administered. Wistar rats body weight measurements were carried out at least every week. Organ weight measurements and organ macropathology observations were carried out at the end of the study immediately after the test animals were sacrificed (BPOM, 2022).

In several other studies, purple leaves have been confirmed to be useful in treating constipation, rheumatism, scabies, urinary tract infections, hemorrhoids, boils, edema, hepatomegaly, as an anti-inflammatory drug, and as a laxative (Goswami et al., 2021), antibacterial (Syahrani et al., 2024). And in Indonesia, it has long been used in the treatment of tonsillitis, abscesses, rheumatism, breast swelling, breast abscesses (Makkiyah et al., 2021), haemorrhoid (Kusumawati et al., 2023), and antidiabetic (Tahseen et al., 2023). Toxicity tests using the Brine Shrimp Lethality Test (BSLT) method show that the ethanol extract of purple leaves has different toxicity depending on the concentration. The LC₅₀ (Lethal Concentration 50) value obtained in this study was 302.9005 µg/ml, so it can be concluded that the ethanol extract of purple leaves is cytotoxic and has anticancer properties (Saragih et al., 2023).

In research conducted by Gribaldo et al. (2021) regarding the acute toxicity test of purple leaf ethanol extract at doses of 500 mg/kgBB, 2.000 mg/kgBB and 5.000 mg/kgBB. In this study, administration of purple leaves at a dose of 500 mg/kgBB to 5.000 mg/kgBB caused the death of test animals on days 2, 8, and 11. So the results were classified as mild toxic with an LD₅₀ value of 3.890 mg/kg, which is included in the preparation classification criteria. moderate toxic (Gribaldo et al., 2021).

These results are not enough to guarantee whether the ethanol extract of purple leaves is not dangerous and has detrimental effects on the body. So it is necessary to carry out a subchronic toxicity test to obtain information on whether or not there are toxic effects that occur during the induction of the test preparation repeatedly over a certain period of time, information on doses that do not cause toxic effects (No Observed Effect Level NOAEL), as well as studying the cumulative effects and reversibility of test preparations (BPOM, 2022).

Method

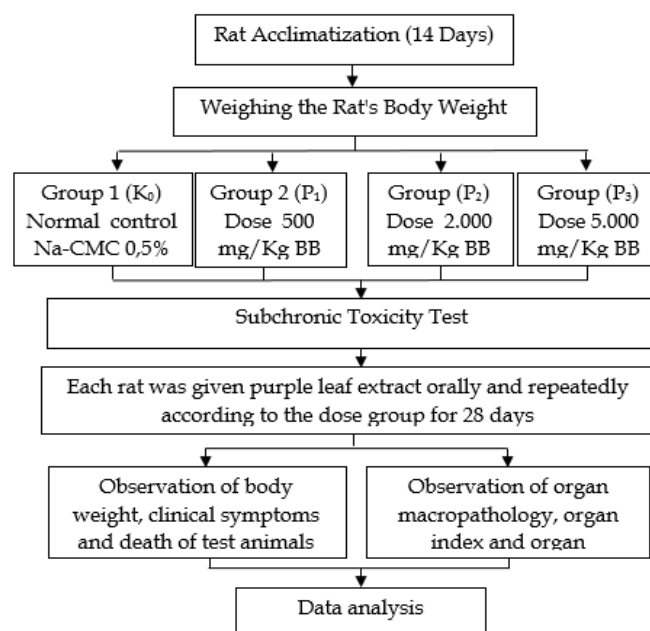


Figure 1. Research work scheme

The study used an experimental method with a randomized block design, using 20 male Wistar rats divided into 4 groups. In this study, 1 control group and 3 test groups were used. In the control group they were only given Na-CMC, in treatment group 1 (P₁) they were given purple leaf ethanol extract at a dose of 500mg/kgBB. Then treatment group 2 (P₂) was given purple leaf ethanol extract at a dose of 2.000 mg/kgBB and treatment group 3 (P₃) was given purple leaf ethanol

extract at a dose of 5.000 mg/kgBB. The test preparation was administered orally, for 28 days (BPOM, 2022).

This research was approved by the ethics committee of Tadulako University with ethical approval letter no10581/UN28.1.30/KL/2023. This research was conducted in June-August 2023, carried out in the phytochemistry-pharmacognosy laboratory, STIFA Pelita Mas Palu pharmacology laboratory and at the Biopath Laboratory.

Research Procedure

Preparation of Ethanol Extract of Purple Leaves

Purple leaves obtained from Gunung Sari Village, Pasangkayu Regency, are sorted first to obtain a uniform material and processed into simplicia. Making purple leaf ethanol extract was carried out using the maceration technique by weighing 2,400 grams of purple leaf simplicia powder which was extracted using 8 L of 96% ethanol solvent for 3x24 hours at room temperature while stirring occasionally. Ethanol solvent is used because it is semipolar, making it possible to dissolve polar components and non-polar compounds in simplicia (Siregar et al., 2018). and evaporates easily with a boiling point of 78°C so it can leave a high residue. In addition, ethanol is a solvent with the greatest extraction power for all materials with low molecular weight such as alkaloids, saponins and flavonoids (Jabbar et al., 2019).

Then the extract is filtered using filter paper and a filtrate is obtained. Next, the filtrate was concentrated using a Rotary Vacuum Evaporator at a temperature of 60°C and continued with evaporation using a water bath until a thick extract is obtained (Ulfah et al., 2020). The percent yield of the extract is calculated by dividing the final weight of the extract by the initial weight of the plant sample (Prasad et al., 2024).

Qualitative tests for phytochemical compounds include tests for alkaloids, saponins, tannins and flavonoids. Testing was carried out based on Lawal et al. (2019) with modifications. The alkaloid test was carried out by adding 1 mL of HCl and 3 mL of distilled water to the ethanol extract of purple leaves, heating over a water bath for two minutes, then cooling and filtering. Then 2 drops of Dragendrof reagent were added, the orange color change indicated the presence of alkaloid compounds. The saponin test was carried out with 1 ml of extract plus 10 ml of distilled water in a test tube, then shaken. If the foam that forms lasts for 1 minute, it shows that it is positive for containing saponin. The tannin test is carried out by adding 2 drops of 5% FeCl₃ solution, the formation of a blackish green color indicates the presence of tannin compounds. Then the flavonoid test was carried out by adding a little Mg metal powder and a few drops of concentrated HCl. If an orange-reddish

color forms, it indicates the presence of flavonoid compounds (Lawal et al., 2019).

Preparation of Test Animals

The test animals used in this research were white rats (*Rattus norvegicus*) of the Wistar strain with the criteria being 3-4 months old, body weight 150-250 grams, white fur color, active and healthy rats, male sex and had been adapted for 14 day. In this research, male white rats were used because they are known as good experimental animal models, are easy to handle, and can be obtained in large quantities and the research results are more stable because they are not influenced by the menstrual cycle and pregnancy as in female white rats. The Wistar strain was chosen because Wistar rats have a relatively fast metabolic capacity so they are more sensitive when used in research (Lahamendu et al., 2019). In this study the test animals were divided into 4 test groups. Each test group consisted of 5 wistar rats. The treatment in the test group, namely the control group, was given 0.5% Na-CMC preparation. Then the treatment group was given ethanol extract of purple leaves, in P₁ the dose was 500 mg/kgBB, in P₂ the dose was 2.000 mg/kgBB, and in P₃ the dose was 5.000 mg/kgBB.

Subchronic Toxicity Test

Test animals were given the test preparation orally according to the treatment group. The preparation was given every day, for 28 days. Observations carried out include observing clinical symptoms and death in test animals. The symptoms observed were tremors, weakness, diarrhea, walking backwards and walking using the stomach (Putra et al., 2023). Then body weight monitoring was carried out for 28 days, by weighing the wistar rats one by one. The aim is to detect changes in the weight of test animals before, during and after administration of purple leaf ethanol extract (Rahmatudina et al., 2023). then at the end of the research the test animals are necropsied, namely taking organs from the samples that will be used for the next test process (Rahmatudina et al., 2023). At the end of the study the test animals were necropsied, then their pancreatic organs were taken to be weighed. and fixed in formalin for histopathological examination (BPOM, 2022; Abolfazl et al., 2023).

Organs were harvested from wistar rats that had completed testing and clinical symptoms were observed after anesthesia with chloroform. Rats were dissected and their pancreas organs were removed, then the pancreas were washed with physiological NaCl solution and macroscopic observations were made in terms of color and compared with normal controls (Masykur et al., 2021).

Organ index observation is one of the parameters used to determine the ability of a compound to cause adverse effects. The organ index is also an indicator of the effect of the test compound, where significant differences between the organ index of the control group and the test group may be different and cannot be seen morphologically (Hasibuan et al., 2015). Organ indices were analyzed by dividing the absolute organ weight by the body weight of the test animal (Fatirah et al., 2019). Organs that have been weighed are then fixed with 10% formalin. 10% formalin is used to preserve tissue and maintain tissue structure, this is important to ensure that the tissue remains in good condition for histopathological analysis (Mujimin et al., 2013; Arapahni et al., 2019).

Histopathological observations of the Wistar rat pancreas were carried out using the Hematoxylin-Eosin staining method. Hematoxylin and eosin are dyes that are often used to color tissue so that it is easier to observe with a microscope. The principle of this coloring is that the acidic cell nucleus will attract alkaline substances so that it turns blue. The alkaline cytoplasm will attract acidic substances so that it turns red. Histopathological observations in this study used a microscope with 400x magnification (Nuralifah et al., 2022). And scoring is carried out based on Mordue (2001), namely: score 0, in one field of view there is no degeneration and necrosis found in the part being observed. Score 1, in one field of view there is 1-20% degeneration and necrosis in the part being observed. Score 2, one visual field found 21-50% degeneration and necrosis in the part observed. Score 3, one visual field found 51-75% degeneration and necrosis in the part observed (light damage). And a score of 4, one visual field has more than 75% degeneration and necrosis in the part observed (severe damage).

Degeneration is an endocrine cell disorder in the internal structure of the cell which causes a reduction in the number of cell masses and the endocrine cell structure becomes irregular, becomes smaller and some even disintegrates and disappears, while necrosis is one of the basic patterns of cell death which is characterized by the presence of empty spaces. on Langerhans Island. The histopathological picture of the pancreas of healthy wistar rats shows that the endocrine cells are still intact and dense. The nuclei of endocrine cells appear bluish purple and the cytoplasm (Hermawati et al., 2020). Data obtained from various observed parameters was then analyzed using One-Way ANOVA and Kruskal Wallis statistical tests.

Results and Discussion

In this study, purple leaf test material was used from the district. Pasangkayu, West Sulawesi. The identification is carried out to ensure the correctness of

the test material used. Identification of purple leaves was carried out at the Biological Resources UPT, Tadulako University, Sulawesi. This is done with the aim of ensuring that the type of plant used in research is the correct type of plant in question, so that there are no errors in selecting the type of plant for research (Putri et al., 2018). The identification results prove that the purple leaves used in the research is indeed the *Graptophyllum pictum* (L) Griff, which belongs to the *Acanthaceae* family. 2,400 grams of purple leaf powder were macerated in 3 maceration vessels and used 8 liters of 96% ethanol solvent. The maceration results obtained were then filtered using filter paper and concentrated using rotary vacuum evaporator. The thick extract obtained was 188 grams and the extract yield was 7.8%. The phytochemical screening tests carried out included tests for alkaloids, flavonoids, saponins and tannins. From the test results it was found that the ethanol extract of purple leaves positively contained alkaloids, flavonoids, saponins and tannins.

Toxicity tests are all tests carried out to detect the toxic effects of a substance on a biological system to obtain typical dose-response data from the test preparation. Subchronic toxicity test research is carried out with the aim of detecting toxic effects that appear after administering the test preparation with repeated doses for 28 or 90 days (BPOM, 2022) in the subchronic toxicity test research, the ethanol extract of purple leaves was carried out orally for 28 days. Using 20 Wistar rats which were divided into 4 groups consisting of 1 control group (Na-CMC) and 3 test groups which were given ethanol extract of purple leaves with varying doses of 500 mg/kgBB, 2.000 mg/kgBB, and 5.000 mg/kgBB.

The results of monitoring the body weight of the test animals can be seen in table 1. The analysis results show that on day 0 the data was normally distributed and homogeneous so it was continued with the One Way ANOVA statistical test. The results showed that there were significant differences in the 4 treatment groups with a p value <0.05 and followed by the Duncane test, the results showed that all treatment groups had different body weights from the control group. On days 7, 14, 21 and 28, the Kruskal Wallis test showed that there were no significant differences between all treatment groups, this was known from the p value > 0.05. Changes in body weight of test animals can be influenced by several factors. Such as, the compound content in the test preparation and internal factors such as genes which are determining factors for traits inherited from the parent and hormones which will regulate all activities in the body as well as external factors from experimental animals such as food, sunlight, activity, temperature and environment (Ubang et al., 2022).

The results of observing clinical symptoms and death can be seen in table 2 and table 3. The results of observing clinical symptoms in the treatment group showed that in the normal control group (K_0), there were no significant symptoms of toxicity and there were no deaths of test animals. Then, when observing a dose of 500 mg/kgBB, there were no significant toxic effects and no deaths occurred in the test animals. Meanwhile, at a dose of 2.000 mg/kgBB, wistar rats experienced tremors

and weakness, and death occurred in 4 test animals. And at a dose of 5.000 mg/kgBB the test animals experienced weakness and tremors and 4 deaths occurred in the test animals. And macroscopic observation of the pancreatic organ showed in table 4 that there was no difference between the control group (Na-CMC) and the treatment group (dose of 500 mg/kgBB, 2.000 mg/kgBB and 5.000 mg/kgBB). The pancreatic organs in the control and treatment groups had a pale gray color.

Table 1. Average Body Weight of Rats for 28 Days (gram) \pm S D

Day	normal control	500 mg/kgBB	2.000 mg/kgBB	5.000 mg/kgBB	P
0	210.4 \pm 7.403	220 \pm 3.209	233 \pm 3.391	241 \pm 7.314	0.000
7	195.8 \pm 12.256	177.2 \pm 6.140	185.5 \pm 14.849	191.5 \pm 2.121	0.129
14	180.6 \pm 18.623	177.6 \pm 10.334	170.5 \pm 16.263	200 \pm 0.000	0.319
21	183.6 \pm 18.461	176.8 \pm 10.183	181 \pm 0.000	200 \pm 0.000	0.451
28	184.4 \pm 15.789	174 \pm 11.247	186 \pm 0.000	194 \pm 0.000	0.379

Table 2. Average Body Weight of Wistar Rats for 28 Days

Clinical Symptoms	Control	500 mg/kgBB	2.000 mg/kgBB	5.000 mg/kgBB
Tremors	-	-	+	+
Diarrhea	-	-	-	-
Weak	-	-	+	+
Walk backwards	-	-	-	-
Walk using your stomach	-	-	-	-

Table 3. Observation of Death of Test Animals

Group	Number of wistar rats (tails)	Number of dead wistar rats (tails)
Control	5	0
500 mg/kgBB	5	0
2.000 mg/kgBB	5	4
5.000 mg/kgBB	5	4

Table 4. Macroscopic Observation of the Pancreas

Group	Color
Control	Pale gray color
500 mg/kgBB	Pale gray color
2.000 mg/kgBB	Pale gray color
5.000 mg/kgBB	Pale gray color

The results of measuring the pancreatic organ index can be seen in figure 1, where there was no significant difference between the normal control and the 500 mg/kgBB, 2.000 mg/kgBB and 5.000 mg/kgBB dose groups. The data is displayed in the form of Mean with SD. A significant increase in organ index is a sign of toxic effects on body organs. However, organ index data cannot be used as an absolute parameter for the presence of toxic effects from the test preparation, this is because there is an unacceptable relationship between the weight of each test animal and the weight of the organs. So it is necessary to carry out a histopathological examination to see in detail the arrangement of cells and tissues in the organ being observed. So that it is known the toxic

effects resulting from administering the test preparation to organs (Putra et al., 2023).

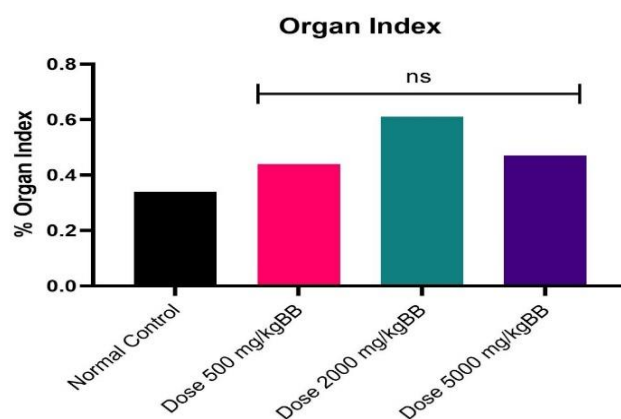


Figure 2. Results of pancreatic organ index measurement

The results of the histopathological examination of the pancreas which had been given the test preparation for 28 days can be seen in Figure 2. The results of the observations showed that the condition of the pancreas in the normal control group (Na-CMC) had no damage to the tissue or the condition of the pancreas was healthy (figure a), this was proven by the damage scoring value of 0, where a value of 0 indicated there was no damage to the islets of Langerhans. Then in the test group with a dose of 500 mg/kgBB damage occurred (image b) with a score of 1 and the endocrine cells were still within

normal limits. then in the test group with a dose of 2.000 mg/kgBB there was mild damage to the pancreas (figure c) with a scoring value of 2, many cells in the islets of Langerhans experienced degeneration, characterized by unclear cell boundaries and pale nuclei, and in the test

group with a dose of 5.000 mg/kgBB there was also mild damage to the pancreas (figure d) with a score of 2. Many of the cells in the islets of Langerhans experienced degeneration which can be seen from the unclear cell boundaries and pale nuclei (Mordue et al., 2001).

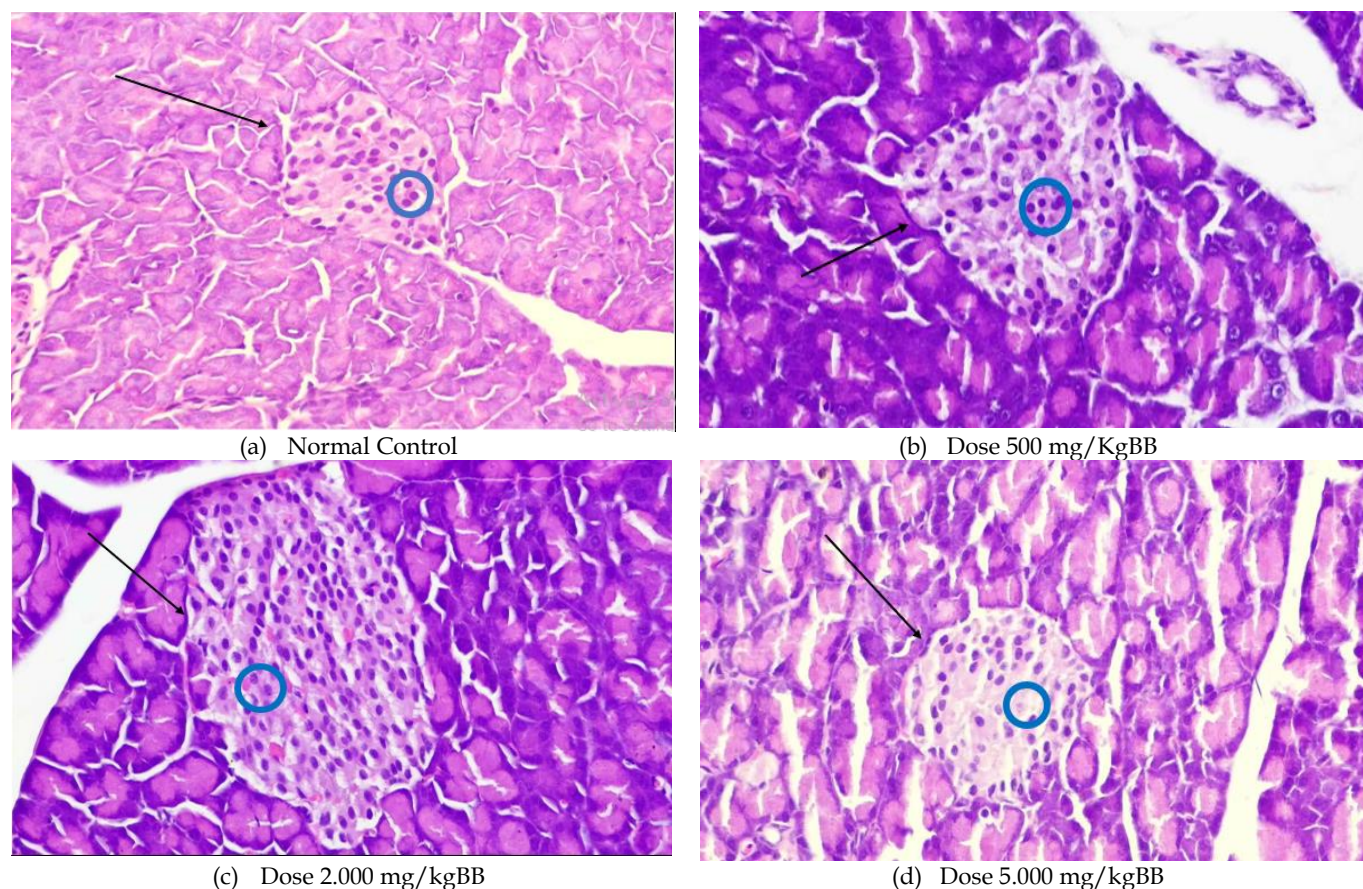


Figure 2. Histopathological picture of the islets of langerhans in the pancreas of test group wistar rats after treatment for 28 days at 400x magnification with HE staining and the endocrine cells in the islets of langerhans were still within normal limits (→), namely their morphology was polygonal with eosinophilic cytoplasm and round, basophilic nuclei (○)

The occurrence of cell degeneration is an early sign of cell damage caused by toxins. If the toxic compound that enters is too large to be carcinogenic, it will cause tissue degeneration (Sijid et al., 2020). Toxicity can occur depending on the amount of toxic substances that enter the body and how long the body is exposed to these toxic substances, so that it can cause problems with the body's organs (Tandanu et al., 2022). Purple leaves contain compounds such as flavonoids, alkaloids, saponins, steroids and glycosides. Apart from having anti-inflammatory and anticancer effects, it can also potentially be a toxic agent if the concentration is too high.

Conclusion

Sub-chronic toxicity testing of ethanol extract of purple leaves on Wistar rats carried out for 28 days, up

to a dose of 5.000 mg/kgBB, showed that at a dose of 2.000 mg/kgBB - 5.000 mg/kgBB it was toxic to the histopathology of the Wistar rat pancreas organ, which was characterized by degeneration cell, increased organ index and the presence of symptoms of toxicity.

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Author Contributions

Conceptualization, Y.W., and N.P.D.; methodology, Y.W., N.P.D., and S.A.; validation, N.P.D.; data analysis, S.A., and M.R.; investigation, Y.W.; resources, J.T.; data curation, S.A., and M.R.; writing—original draft preparation, Y.W.; writing—review and editing, Y.W., S.A., and N.P.D.; supervision, N.P.D. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest

The authors declare there is no conflict of interest.

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