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Effect of giving nanochitosan preparations ethanol extract of neem leaves (*Azadirachta indica*) against pancreatic histology of white rat male (*Rattus norvegicus*) Sprague Dawley

SUCI HANDAYANI♥, AGUNG JANIKA SITASIWI, SRI ISDADIYANTO, SITI MUFLICATUN MARDIATI
Department of Biology, Faculty of Science and Mathematics, Universitas Diponegoro. Jl. Prof. H. Soedarto, Tembalang, Semarang 50275, Central Java, Indonesia. Tel.: +62-24-7474754, ♥email: suci5maret@gmail.com

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Abstract. *Handayani S, Sitasiwi AJ, Isdadiyanto S, Mardiati SM. 2022. Effect of giving nanochitosan preparations ethanol extract of neem leaves (*Azadirachta indica*) against pancreatic histology of white rat male (*Rattus norvegicus*) Sprague Dawley. Cell Biol Dev 6: 13-19.* The selection of herbal plants as a treatment in the community is considered safer, more practical, and cheaper than synthetic drugs. Neem (*Azadirachta indica* A.Juss.) is one of the herbal plants that have the potential as an antioxidant. However, the low bioavailability of drugs and the distribution of active compounds in herbal plants are constraints in administering drugs orally. The solution to overcome this problem is to prepare the test material in the form of nanochitosan. This study aimed to analyze the effect of nanochitosan ethanol extract of neem *(A. indica)* leaf extract on the pancreas histology of male Sprague Dawley rats. This study used a completely randomized design (CRD) with 32 rats aged 2 months divided into 4 treatments, and each treatment consisted of 6 replications. P0 (normal rat group treated with 2 mL distilled water, P1 (normal rats induced with *Natrium tripolifosfat "*NaTPP" and 2 mL chitosan), P2 (normal rat group induced by nanochitosan neem leaf ethanol extract 1:0.5), P3 (normal rat group induced nanochitosan ethanol extract of neem leaves 1:1). Data were analyzed by ANOVA test with a significance level of 5%. Data that were not normally distributed were tested by the *Kruskal Wallis* test and Duncan's test. The results showed that the administration of nanochitosan ethanol extract of neem leaves 1:0.5 and 1:1 had no significant effect on the diameter parameter of the islets of Langerhans ($P \geq 0.05$) but had a significant effect on the parameters of pancreatic weight and damage scoring of the islets of Langerhans ($P \leq 0.05$). The administration of nanochitosan preparations of ethanolic extract of neem leaves can deliver bioactive compounds neem to the pancreas and minimize damage to the cells that make up the islets of Langerhans due to toxic neem compounds.

Keywords: Islet of Langerhans, nanoparticles, neem plant, pancreas

INTRODUCTION

The selection of herbal plants as a treatment in the community is considered safer, more practical, and cheaper than synthetic drugs, so they have become an alternative treatment for various diseases. The neem plant (*Azadirachta indica* A.Juss.) is one of the most studied herbal plants because of its properties that can treat various diseases (Pristiani and Astuti 2005; Fathoni et al. 2013). The benefits of the neem plant can be used as an antirheumatic, antioxidant, anti-inflammatory, immuno-potentiation, antifertility, antiviral, anticancer, and antipyretic (Ambarwati 2011). However, the side effects of neem are thought to cause kidney and pancreas damage. Wowiling's research (2013) stated that the advantages of neem include easy cultivation, its use as herbal medicine, relatively safe for humans, and the prevention of various diseases. However, the deficiency of the neem plant can cause liver and pancreas damage. That is presumably due to toxic compounds in neem and the low distribution of the active compound content of neem.

Oral administration of drugs is the most widely used method of administering drugs to test animals because the process is easy, safe, inexpensive, and convenient. However, obstacles in oral administering the drug are the low bioavailability and the low distribution of neem compounds to the body. According to research by Ajazuddin and Saraf (2010), the solution to overcome the oral administration problem is to prepare drugs in the form of nanochitosan to facilitate the absorption and distribution of drugs into the body to increase the bioavailability of neem plants. Prasetiowati et al. (2018) stated that the excess use of nanochitosan in medicinal plant extracts can facilitate the absorption and distribution of drugs into the body and reduce the toxic effects of drugs. The polymer used to manufacture nanoparticles is chitosan with a soluble compound in sodium tripolyphosphate (NaTPP) (Kafshgari et al. 2011). Martien et al. (2012) stated that chitosan could reduce the toxic effects of neem exposure, so it is very efficient to be developed as the main ingredient for making nanoparticles. Therefore, nanoparticles can provide an effective solution to overcome the difficulty of drug delivery into the body, facilitate the distribution of bioactive compounds from neem plants into the pancreas, and reduce the toxic effects of neem plants. This study aimed to analyze the effect of neem leaf extracts chitosan nanoparticles on the histological structure of the pancreas of male Sprague Dawley rats.

MATERIALS AND METHODS

This research was conducted for 8 months from March-October 2021 at the Laboratory of Animal Structure and Function Biology, Department of Biology, Faculty of Science and Mathematics (FSM), Universitas Diponegoro (UNDIP), Semarang, Central Java, Indonesia.

Tools and materials

The tools used in this study were 32 sets of rat cages, rat feed, rat drinking bottles, an oven, a grinder, a rotary evaporator, a refrigerator, an analytical balance, a heater, a measuring cup, *thermohygrometer*, 3 mL injection syringe, sonde, a set of surgical instruments, plastic container, gloves, paraffin bath, pins, petri dish, a digital scale with 0.01 g accuracy, sample vial, millimeter block paper, rotary microtome, dropper, object-glass, cover glass, microscopes, photomicrographs, cameras, and stationery.

The materials used were 70% ethanol, aquadest, A594 type chicken feed, drinking water, rice husks, label paper, tissue, latex, masks, cotton, chloroform, physiological saline solution, 10% Neutral Formalin Buffer (BNF) solution, water faucet, NaTPP solution, neem leaves, entellan, paraffin solution, xylol and hematoxylin-eosin (H&E). The test animals used in this study were 32 white male rats *(Rattus norvegicus* Berkenhout, 1769) Sprague Dawley, with no anatomical defects, obtained from the *Unit Pelayayanan Hewan Percobaan (UPHP)*, Universitas Gadjah Mada, Yogyakarta, Indonesia.

Research design

This study used a completely randomized design (CRD) which consisted of 4 treatments, and each treatment consisted of 6 replications. The determination of the dose was carried out according to the study of Sitasiwi et al. (2019). Furthermore, the test animals were grouped into 4 treatment groups, including: (i) P0: Control, normal rats were given 2 mL of distilled water. (ii) P1: Normal rats were given 2 mL of NaTPP + chitosan solution. (iii) P2: Normal rats were given nanochitosan ethanol extract of neem leaves 1:0.5. (iv) P3: Normal rats were given nanochitosan ethanol extract of neem leaves 1:1.

Preparation of neem leaf ethanol extract *(A. indica)*

Neem leaves were obtained from the UNDIP area, Semarang, Central Java, Indonesia. One kilogram of neem leaves was dried in an oven at a temperature of ±45-50°C for 3 days. The dried neem leaves were crushed and ground using a grinder and then sieved to obtain a powder form. According to Abror et al. (2018), the neem extraction step was done by soaking neem leaf powder in 70% ethanol for 3x24 hours. The extract obtained was then filtered. The filtering results were evaporated using a rotary evaporator at a temperature of 50°C to obtain a powdery extract; then, the neem leaf extract was stored in the refrigerator. The making of neem leaf extract was carried out at the Laboratory of the Universitas Semarang, Semarang, Central Java, Indonesia, for ± 2 weeks.

Nanoparticle preparation

The manufacture of chitosan nanoparticles from neem leaf ethanol extract *(A. indica)* by dissolving 1 mL of neem leaf extract into 35 mL of ethanol and adding 15 mL of distilled water. Then dissolved into 100 mL of chitosan solution using a magnetic stirrer and dissolved in 40 mL of 0.1% sodium tripolyphosphate (NaTPP) solution at room temperature at 3000 rpm for ± 2 hours to form a nanoparticle suspension. Furthermore, measurements were carried out using a Malvern particle size analyzer (Naela et al. 2019). Finally, the characterization of nanoparticles was carried out at the UNDIP Integrated Laboratory.

Test animal preparation

Rats were kept for 28 days in a rat cage and fed and watered ad libitum. The environmental conditions of the cages were checked using a thermohygrometer to measure the temperature and humidity of the cage conditions. Temperature and humidity measurements are carried out every 09.00 (morning) and 16.00 (afternoon). Replacement of husks (bed cage) is done every 3 days.

The final stage of acclimation was weighing the body weight of Sprague Dawley rats. Mice with uniform body weight were then grouped into 4 treatment groups. The lottery method determined the division of rats into treatment groups. Each treatment group had 6 rats as the replication unit.

Administration of test materials to test animals

The administration of neem leaf extract was carried out orally for 28 days every afternoon according to the dose in each treatment, namely P0 (Control) normal rats were given 2 mL of distilled water; P1, normal rats were given a solution of NaTPP and chitosan ethanol extract of neem leaves as much as 2 mL; P2, normal rats were given 2 mL of neem leaf ethanol extract nanochitosan with a ratio of 1:0.5; and P3, normal rats were given 2 mL of neem leaf ethanol extract nanochitosan with a ratio of 1:1.

Animal dissection and tissue collection

The treatment was administered using a cannula syringe or a probe attached to a 3 mL syringe. The provision of test materials begins with handling rats using the scrubbing technique (Darusman et al. 2018).

Pancreatic preparation

The histological preparations of the pancreas were carried out concerning the Berata and Samsuri (2017) method using the paraffin method and hematoxylin-eosin dye with an incision thickness of ± 5-7 microns.

Microscopic observation

Observations of the histology of pancreatic organs were performed using an Olympus BX51 microscope and photomicrograph with magnifications of 200x, 400x, and 1000x randomly in one preparation. In addition, the diameter and damage score calculation was carried out in one preparation with 200x and 400x magnification for each preparation.

Data analysis

Histological observation data in the form of the diameter of the islets of Langerhans, the area of the islets of Langerhans, the volume of the islets of Langerhans, the scoring of damage to the islets of Langerhans, and the weight of the pancreas were tested for normality. The Data is said to be a normal distribution if P≥0.05. Pancreatic weight, diameter, and area of the islets of Langerhans were tested using the One Way ANOVA statistical test at a 95% confidence level. Pancreatic volume data and scoring description of the histological structure of the islets of Langerhans were tested by the *Kruskal Wallis* test, followed by Duncan's test. Statistical testing was carried out using the SPSS version 26 application. The histological structure data of the pancreas were presented descriptively, and the measurement of the islets of the Langerhans area, the volume of the islets of Langerhans, and the weight of the pancreas were analyzed quantitatively.

RESULTS AND DISCUSSION

The morphology of the pancreas of *R. norvegicus* macroscopically looks pale red, elongated vertically with a length ranging from 5 cm from head to tail, has a pancreas weight of 100-200 mg, and visible blood vessels accompanied by indistinct hoops. These results are to the research of Treuting et al. (2018) that the morphology of the pancreas of *R. norvegicus* looks like white to pink grapes and visible blood vessels. A comparison of the morphology of the pancreas of *R. norvegicus* after administration of the test material for 28 days can be seen in Figure 1.

Based on Table 1, the results of the pancreatic ANOVA test with a significance level of 5% showed a significant difference in the pancreatic weight of rats *(R. norvegicus)* given nanochitosan preparations of neem leaf ethanol extract for 28 days. That is because the administration of the test material in the form of nanochitosan ethanol extract of neem leaves has a smaller size, so it is suspected that it can provide a more effective effect in delivering neem compounds to the target location and can increase the bioavailability of the test material in the body. On the other hand, the P0 and P1 groups experienced a decrease in pancreatic weight which was not significantly different (P≥0.05) in the test animals, which is presumably due to the low bioavailability of the test material. Furthermore, the research of Kakkar et al. (2011) showed that the administration of the test material in the form of nanoparticles could reduce the weight of the pancreas and increase the bioavailability of herbal medicines. Presumably, the test material suppresses the decrease in pancreatic weight compared to the positive control group, induced only by aquadest.

The preparation of nanochitosan was thought to deliver the active compound of the neem plant, as evidenced by the addition of pancreatic weight in groups P2 and P3. That is presumably because the active compound in the neem can protect the cells that make up the islets of Langerhans due to the Azadirachtin compound. Furthermore, Juanda and Jayadi (2015) stated that the higher the concentration of neem leaf extract, the higher the ability of pancreatic cells to minimize damage to the islets of Langerhans. The research of Septiana et al. (2012) showed that pancreatic samples could experience a decrease in the size and weight of the pancreas after being induced by the papaya leaf extract nanoparticle preparation test material. However, the morphology of the pancreas was still relatively the same, and the weight of the pancreas of rats was still relatively normal.

The success of making neem leaf nanoparticles using PSA shows that the smallest nanoparticle size in NaTPP: Chitosan is 202.3 nm, and the largest in NaTPP - Chitosan: SEEDM 1:0.5, which is 324.9 nm (Sitasiwi et al. 2021). The results showed that the particle size formed was still in the nano-size range and effectively delivered the drug to the target location, namely the pancreas, following the opinion of Rawat et al. (2006) that the nanoparticle size is < 300 nm. That proves that the nanoparticle preparation of neem leaf ethanol extract is said to be effective with a nanoparticle size ranging from 300 nm, which can deliver the bioactive content of neem to the pancreas.

Based on the results of the phytochemical test of the neem *(A. indica)* plant showed that the ethanolic extract of neem leaves contained alkaloids, terpenoids, flavonoids, phenolics, saponins, and tannins with positive test results. But negative in the test for steroid content. This result is different from the research of Soraya (2021) explained that neem leaf samples contained tannins, phenols, triterpenoids, saponins, and steroids but were negative in the alkaloid and flavonoid tests. That is because the flavonoid content of neem is not optimally distributed into the pancreas. That is presumably because the compound size is too large, so the compound that enters the body is damaged, which causes neem bioactive compounds cannot to enter the pancreas. Therefore, the administration of nanochitosan can assist in delivering the active compound neem to the cells that make up the pancreas and minimize the damage to the islets of Langerhans, as evidenced by the histological structure of the pancreas, which is getting better in the P3 treatment group. On the other hand, the research of Nugroho et al. (2020) showed that giving the test material in the form of nanoparticles could increase the bioactive content distribution of the binahong plant and repair the damaged cells that make up the islets of the Langerhans. This study proved that the damage to cells that make up the islets of Langerhans in group P1 could be suppressed by the presence of active neem compounds in groups P2 and P3, which showed a decrease in cell damage.

The results of the average diameter of the islets of Langerhans in white rats *(R. norvegicus*) after administration of nanochitosan preparations of neem leaf ethanol extract for 28 days are presented in Table 1. Based on the ANOVA test on the diameter parameters of the islets of Langerhans, the results were not significantly different (P≥ 0.05). The mean value of the diameter of the islets of Langerhans in the treatment groups P0, P1, P2, and P3 was 116.80 m, respectively; 128.05 m; 124.82 m; and 117.44 m. The average diameter of the islets of Langerhans is still

relatively normal, ranging from 100 m to 150 m (Mescher 2016). The results of the analysis showed that the ethanol extract of neem *(A. indica)* leaves in a ratio of 1:0.5 and 1:1 in groups P2 and P3 did not affect the structure of the cells that make up the islets of Langerhans so that the diameters of the cells of the islets of Langerhans were not significantly different. That is presumably because the preparation dosage is still a safe dose or dose. Following the research of Suhendro et al. (2018), the administration of ethanolic extract of neem leaves at a dose of 50-200 mg/g BW in Sprague Dawley rats was still a safe dose. The research of Azmi et al. (2022) showed that the administration of the test material for neem leaf ethanol extract did not affect the diameter of the islets of Langerhans but could improve the cell structure of the islets of Langerhans. That is presumably due to the antioxidant content of the neem plant, which can minimize damage to the structure of the islets of Langerhans.

Histological observations of the pancreas in the P0 group showed a normal histological structure as indicated by the clear boundaries of the islets of Langerhans, round cells, no voids in the islets, no severe damage in the P0 group, and no necrotic and cell degeneration were seen. According to Walean et al. (2020), the aquadest-induced negative control group in normal mice saw the islets of Langerhans under normal conditions. The cell nucleus was clearly visible, surrounded by normal acinar cells. In contrast to the histological observations of the pancreas in the P1 treatment group, the damage was quite severe, indicated by the presence of quite a lot of cell degeneration, necrotic cells started to appear, and parts of the islets of Langerhans cells were not visible, the shape of the cells was abnormal, and the cells underwent degeneration and necrosis. Damage to the P1 group was suspected because the P1 group was not induced by the active compound neem and the low level of solubility of the test material so that to stabilize it, it could be offset by giving a surfactant in the form of neem leaf ethanol extract which can regenerate the cells that make up the islets of Langerhans.

Histological observations of the pancreas in the P2 group showed that the damage was not severe compared to the P1 group, as indicated by the reduced level of necrotic cells. In addition, the distribution of cells looked more regular, and there was a reduction in the empty space in the islets. The following research by Setiadi et al. (2020) showed that the administration of the test material for the ethanol extract of aloe vera leaf at a dose of 120 mg/kgBB was able to improve the structure of the islets of Langerhans but still not like the normal state of the islets of Langerhans. Histological observations of the pancreatic organs of the P3 group showed that the islets of Langerhans in the test animals of the P3 group looked normal with clear boundary characteristics of the islets of Langerhans, parts of the islets of Langerhans cells were seen, the number of cells that were necrotic was slightly, and the shape of the cells was normal. In addition, the islets of Langerhans were more colorful and lighter than the exocrine pancreatic tissue, which is darker in color. The description of the histological structure of the P3 treatment group can be seen in Figure 2.

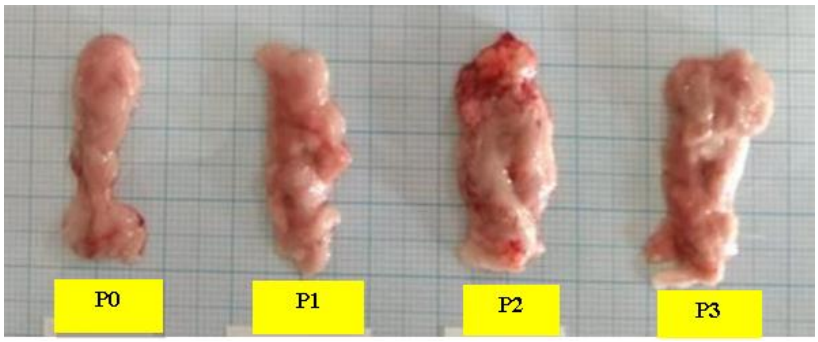

Figure 1. The pancreas of *Rattus norvegicus* Sprague Dawley Male

Table 1. ANOVA test of pancreatic weight, the diameter of the islets of Langerhans, and Duncan's test, the average score of damage to the islets of Langerhans

	Parameter		
Treatment	**Pancreas weight (mg) (Mean ± SD)**	**Diameter of the islets Langerhans (μm²) (Mean ± SD)**	**Average damage score (Mean ± SD)**
P0	101^{a} ± 47,273	$116,80^{a}$ ± 12,740	0^{a} ± 0
P1	104^{a} ± 35,265	$128,05^{b}$ ± 16,599	$3,12^{b}$ ± 0,94
P2	132^{b} ± 31,365	$124,82^{c}$ ± 15,515	$1,45^{c}$ ± 0,83
P3	168^{c} ± 33,391	$117,44^{a}$ ± 8,647	$1,29^{c}$ ± 0,85

Note: Different superscripts showed significant differences (P≤0.05). P0 = Normal control (normal rats were given 2 mL of distilled water), P1 Positive control normal rats were induced with 2 mL NaTPP and chitosan solution), P2 (Normal mice were induced with nanochitosan ethanol extract of neem leaves 1:0.5), P3 (Normal mice were induced with nanochitosan ethanol extract of neem leaves 1:1)

Table 1 shows the average value of the Langerhans Island damage score. The negative control treatment group (P0) got an average score of 0 with score of 0, which showed the normal pancreatic histological structure in the form of clear islets of Langerhans, the structure and size of the cells looked normal, and there was no hypertrophy of the cells or cell degeneration. That indicates that the islets of Langerhans are in normal condition or there is no necrosis. According to Tandi et al. (2017), normal islets of Langerhans were given a score of 0 with normal cell shape, absence of necrotic cells and cell degeneration, and normal cell shape. The P1 group rats induced with NaTPP and chitosan had an average score of 3.12 with a score of 3, meaning that the damage was quite severe. The damage shown in the histological preparations was pancreatic cells that underwent necrosis, abnormal cell shape, and degenerated cell nuclei, which causes the structure and shape of the islet of Langerhans to be irregular and different from the shape of the islet of Langerhans in general. The P2 group had an average damage score of 1.45 (Table 1), with some of the islets of Langerhans being given a score of 2.

The state of the islets of Langerhans, which was given a score of 2, was the boundary condition of the islets of Langerhans that started to become unclear, the number of cells was reduced, and some of the cells degenerated. There are abnormal cell shapes, and there are no necrotic cells visible. This damage is a characteristic of reversible damage. Tandi et al. (2017) stated that the characteristics of the islets of Langerhans, which were given a score of 2, were the boundaries of the islets of Langerhans, which looked unclear; the cells were irregular in shape. Some had cell degeneration, and the cells did not appear necrotic. The P3 group had an average damage score of 1.29 (Table 1) with a score of 1. The condition of the islets of Langerhans, which was given a score of 1, showed clear boundaries on the islets of Langerhans; no necrotic cells were seen, normal cell shape, and only degeneration. A comparison of the histological structure of the pancreas in all treatments can be seen in Figure 3.

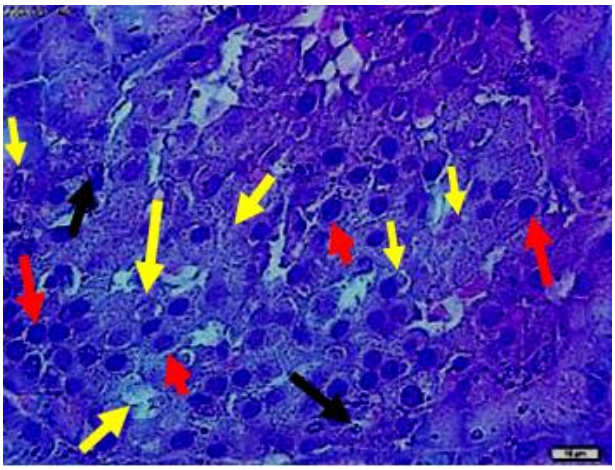

Figure 2. Histology of the islets of Langerhans *Rattus norvegicus* in group P3 at 1000x magnification with *Hematoxylin-Eosin* staining. Description: red arrows: normal cells, yellow arrows: cell degeneration, abnormal cell shape, and black arrows: necrotic cells

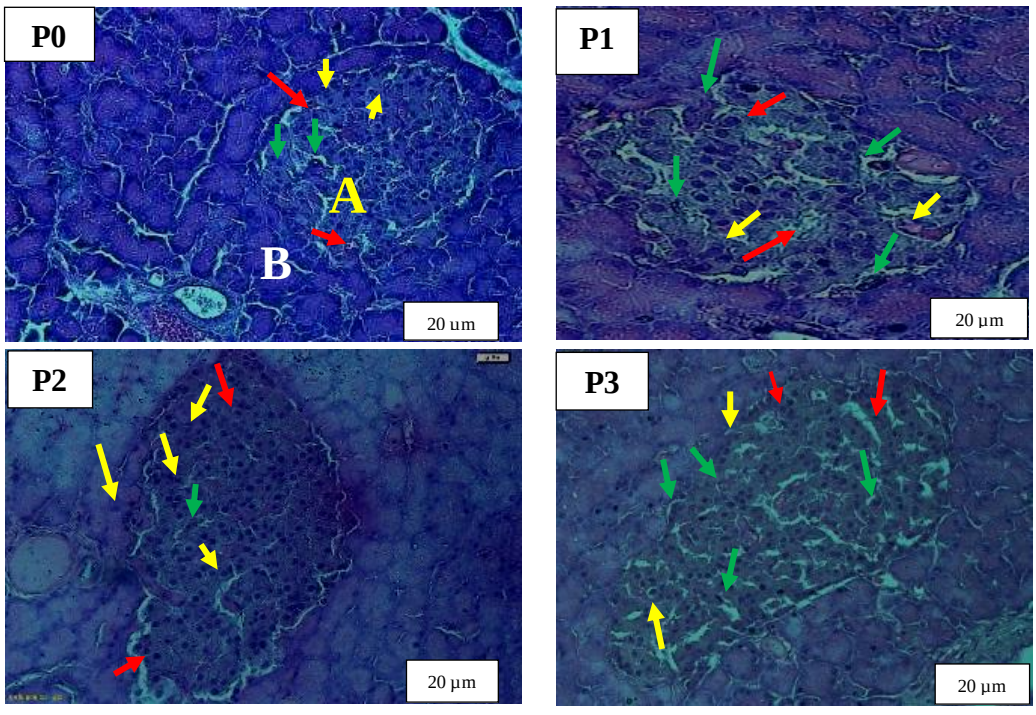

Figure 3. Histological structure of the islets of Langerhans *Rattus norvegicus* (HE,400x). A. Islets of Langerhans, B. Exocrine pancreas. Red arrows: unclear boundaries, yellow arrows: cell degeneration and abnormal cell shape, green arrows: necrotic cells

Islets of Langerhans improved when ethanol extract of neem leaf nanochitosan was given in a ratio of 1:0.5 and 1:1, which was able to repair the damage to the pancreas of rats, indicated by the level of pancreatic cell damage that was seen to be reduced compared to the P1 group. Furthermore, the pancreatic structure in groups P2 and P3 improved in the islets of Langerhans space. The distribution of cells looked more regular than in group P1, with normal cell shape, and the histological structure of the islets Langerhans began to improve. That improved the histological structure of the islets of Langerhans in the P3 group, followed by cell regeneration in the islets of Langerhans. The Prameswari and Widjanarko (2014) research showed that the absence of cell degeneration characterized the regeneration of Langerhans islet cells, and the normal cell shape and structure of Langerhans islets looked close to normal groups. Improvements in the histological structure of the islets of Langerhans in the P3 treatment group were thought to be influenced by the increased bioavailability of neem and the distribution of the antioxidant content of neem, which was able to be well absorbed by the pancreas along with the increase in the dose given in nano size so that an increase in the dose given was thought to cause an increase in the number of bioactive compounds contained in the ethanolic extract of neem leaves that reach the target organs.

In conclusion, neem leaf ethanol extract in the form of nanochitosan has the potential to deliver neem bioactive compounds to the pancreas and minimize damage to cells that make up the islets of Langerhans due to toxic neem compounds.

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