



The Effect of Nanochitosan Preparation of Neem Leaf (*Azadirachta indica*) Ethanol Extract on the Liver Structure of White Rats (*Rattus norvegicus*)

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Abstract

Neem (*Azadirachta indica*) is an herbal plant that has a high antioxidant content and has potential as a medicine. Constraints in oral administration are the low bioavailability of drugs and the distribution of active compounds from herbal plants. One way to overcome this problem is to package herbal plant extracts into smaller nano-sized particles. The purpose of this study was to examine the response of the liver structure of male white rats (*Rattus norvegicus*) to the administration

of nano chitosan preparations of neem (*Azadirachta indica*) leaf ethanol extract. This study used 32 rats aged 2 months which were divided into 4 treatments, each treatment had 8 replications, namely P0 (a group of normal rats induced with 2ml aqua dest), P1 (normal rats induced with NaTPP and 2ml chitosan), P2 (normal mice induced with nano chitosan: neem leaf ethanol extract 1:0.5), P3 (normal mice induced with nano chitosan: neem leaf ethanol extract 1:1). The research design used RAL (Completely Randomized Design). Data analysis used ANOVA with a significance level of 5% with further tests, namely the Kruskal Wallis test and Duncan's test with SPSS software version 24. The results showed that the administration of nano chitosan preparations of neem leaf ethanol extract 1:0.5 and 1:1 had no significant effect on the Hepatosomatic index, diameter hepatocyte, and hepatocyte damage score ($P > 0.05$), so it can be concluded that the administration of nano chitosan preparations of neem leaf ethanol extract (*Azadirachta indica*) can deliver neem leaf bioactive compounds to the liver to minimize liver histological damage due to neem toxic compounds.

Keywords: *Azadirachta indica*, liver, hepatocytes, nanoparticles

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Introduction

Neem (*Azadirachta indica*) is one of the plants used in traditional medicine. Neem leaves have been used in Ayurvedic medicine for more than 4,000 years for their anti-inflammatory, antibacterial, antifungal, and antioxidant effects, lowering blood lipids, lowering blood sugar, stimulating immunity, and protecting the liver. Neem leaves contain several components resulting from the production of secondary metabolites such as phenols, flavonoids, saponins, steroids, alkaloids, amino acids, and tannins (Fitriyani *et al.*, 2020).

Flavonoids are antioxidants due to the presence of a phenolic hydroxy group (-OH) in their molecular structure. Flavonoids have a high ROS capture ability, which can turn ROS into less reactive, regenerate damaged cells and restore tissue to a normal state (Repetto & Lleruy, 2006). Research by Javandira (2016) stated that neem leaves contain a toxic compound, namely Azadirachtin, which inhibits metabolism and protein synthesis and high levels of Azadirachtin in neem interfere with the digestive process in rats. Traditional medicines from plant materials such as neem leaf extract will go through a digestive process, to be further absorbed by the intestines and detoxified in the liver. This causes the liver to be very susceptible to the influence of various chemicals that enter the body through the digestive tract and is often the main target of the toxic effects of chemicals (Hasana *et al.*, 2019).

The liver has a relationship with body weight because the liver metabolizes food substances that will later be used for physical activity or stored as food reserves in the body. Food substances stored in the body will affect the bodyweight of an individual (Niendya *et al.*, 2011). Bodyweight and liver weight are used to determine the value of the hepatosomatic index, related to liver function as an energy reserve and metabolic activity (Ashwini *et al.*, 2016).

Changes in morphology and physiology of injured liver cells begin with increased water absorption, then spread to organelles and cytoplasm. This causes cell swelling, characterized by an increase in cell volume and size (Zachary & McGavin, 2012). The increase in cell size is characterized by a change in the diameter of the hepatocyte cells. Changes in the histological structure of the liver can be influenced by the entry of certain amounts and types of compounds into the organ because the compounds that enter the body will undergo absorption, distribution, metabolism, and excretion in the body (Wahyuningtyas *et al.*, 2018). Kupradinun *et al.* (2012) stated that neem side effects are thought to cause damage to the liver and kidney structures. Sitasiwi *et al.* (2017) have proven that the ethanolic extract of neem leaves causes an increase in liver weight and the value of the Hepatosomatic Index of mice.

The test results of neem plant extracts turned out to show very varied results, even though the extract sources with the same dose and experimental animals were used. Safitri (2014)



suggests that one of the causes of differences in the efficacy of plant extracts is the possibility of changes in the structure of the active compounds contained in medicinal plant extracts. These structural changes can occur due to the degradation of enzymatic reactions during the digestion process. Another reason is that there may be a particle size large enough to penetrate the tissue or blood-tissue barrier so that it cannot reach the target cells. Ohta et al. (2020) stated that one way to suppress the effects caused by the activity of digestive enzymes or the inability to penetrate the tissue is to provide or package them in the form of nanoparticles. The size of the nanoparticles is expected to be able to deliver to the target cell. In addition, the nanosize also causes an increase in the surface area leading to high solubility.

Based on the above background, a study was conducted to see the effect of neem leaf ethanol extract nanoparticles packaged in the form of chitosan on the Hepatosomatic Index, hepatocyte diameter, and hepatocyte damage score of white rats (*Rattus norvegicus L*) Sprague Dawley Male.

Method

Before conducting research, an application for a research permit is submitted first. The permit application is addressed to the Ethics Committee of the Faculty of Medicine, Diponegoro University as a place for ethical feasibility testing. Ethical clearance has been obtained from the Health Research Ethics Commission (KEPK) Faculty of Medicine UNDIP with No. 29/EC/H/FK-UNDIP/III/2021. This research was conducted for 2.5 months in the Lab. BSFH Diponegoro University, Semarang. The design of this study used a completely randomized design (CRD) consisting of 4 treatments and 8 replications.

Laboratory animal

The test animal in this study was a 2-month-old male *Rattus norvegicus* Sprague Dawley weighing 200g. white rats in the rearing cage and acclimatized for 7 days. The process during acclimation rats was given standard feed, with feeding and drinking ad libitum. Preparation of Neem Leaf Ethanol Extract. The neem leaves were dried in an oven at 45°C for 3 days. The dried neem leaves were blended and the simplicia was extracted with 70% solvent. Extraction was carried out until the extractive became colorless and then concentrated by the distillation process until a semi-solid mass was obtained free of solvent (Sitaswi et al., 2016). Preparation of Chitosan-Nanoparticles Extract Ethanone Daum Neem (*Azadirachta indica*). Chitosan solution was made in the Lab. Integrated UNDIP according to the method Pasquoto-Stigliani et al. (2017) by dissolving chitosan is done by diluting 2mg of chitosan with 1% acetic acid. The dissolution



was carried out using a magnetic stirrer. Preparation of NaTPP: Sodium tripolyphosphate as much as 2 mg in every 1 mL of distilled water, using a magnetic stirrer. Furthermore, the preparation of nanoparticle-chitosan preparations of neem leaf ethanol extract was carried out by dissolving the ethanol extract powder into NaTPP, homogenized with a stirrer at a speed of 1200 rpm at a temperature of 60°C for 5 minutes then ultrasonication at 30°C for 30 minutes. Furthermore, it is added by adding a chitosan solution by dripping while homogenizing using a stirrer. The ratio of chitosan: NaTPP is 5:1 (v/v). For example, 80mL (powder + NaTPP) is dropped on 400mL of chitosan so that the final volume is 480mL.

Provision of Nanoparticles-Chitosan Ethanol Extract of Neem Leaf (*Azadirachta indica*)

The administration of nano chitosan preparations of neem leaf ethanol extract was carried out with oral probes in groups P2 and P3 with a ratio of 1:0.5 and 1:1, while in group P0 as a negative control, distilled water was given and P1 as a positive control was given solvents, namely NaTPP and chitosan with a volume of 2 ml. /head/day for 28 days.

Stage of Histologi Hepar of Surgery and Preparation of Liver Histology Preparations

The rats were put in a closed container and anesthetized using cotton soaked in chloroform which had passed out in surgery and the liver was taken and washed with physiological NaCl solution and then weighed. The histological preparations of the liver were carried out using the paraffin method and hematoxylin-eosin dye according to the method of Mardiaty & Saraswati, (2014) then the preparations could be observed under a microscope.

Measurement of Hepatocyte Cell Diameter

Hepatocyte cell diameter was measured using a photomicrograph with a magnification of 400 times in 5 fields of view. Measurements are made by dividing the closest distance and the farthest distance (Wahyuningtyas *et al.* (2018).

Hepatocyte Cell Damage

Each visual field observed degeneration of cloudy swelling that occurred in hepatocytes. Each field of view is counted as 100 cells (Restuati & Nasution., 2019):

1. Score 0: no hepatocytes experience cloudy swelling
2. Score 1: 1-10% hepatocytes experience cloudy swelling degeneration
3. Score 2: 11-33% hepatocytes experience degeneration of cloudy swelling
4. Score 3: 34-66% of hepatocytes have degeneration of cloudy swelling
5. Score 4: 67-100% hepatocytes have degeneration of cloudy swelling



HSI Value Calculation

The HSI value is calculated according to the formula according to Ogunji et al. (2008) as follows.

$$\text{HSI} = \text{BW/LW} \times 100$$

Information:

HSI = hepatosomatic index (%)

LW = liver weight (g)

BW = body weight (g)

Data Analysis

Hepatosomatic index data, hepatocyte diameter, and hepatocyte damage were analyzed using the ANOVA test with a significance level of 95%. Data analysis using SPSS software version.24.0.

Result and Discussion

The results of the analysis of the effect of giving a preparation of neem leaf ethanol extract on the Hepatosomatic index gave no significant effect ($P > 0.05$). This shows that the administration of nano chitosan preparations of neem leaf ethanol extract for 28 days did not have a significant effect on energy availability in the test in all treatment groups.

Table 1. Hepatosomatic index, hepatocyte diameter, and hepatocyte damage score after administration of nano chitosan preparations of neem (*Azadirachta indica*) leaf ethanol extract to the liver of male white rats (*Rattus noervegicus*) for 28 days.

Treatments	Variables		
	Hepatosomatic index	Hepatocyte Diameter (μm)	Hepatocyte Damage Score
P0	3.650 ^a $\pm$ 0.078	15.243 ^a $\pm$ 1.181	1.800 ^a $\pm$ 0.715
P1	3.990 ^a $\pm$ 0.426	16.186 ^a $\pm$ 0.931	3.133 ^b $\pm$ 0.413
P2	3.814 ^a $\pm$ 0.285	15.852 ^a $\pm$ 0.885	1.633 ^a $\pm$ 0.577
P3	3.676 ^a $\pm$ 0.197	16.209 ^a $\pm$ 0.740	2.867 ^b $\pm$ 0.640

Description : Numbers followed by the same superscript in the same column show no significant difference ($P > 0.05$) P0 = Normal control (normal rats were given distilled water), P1 = Positive control (rats were given NaTPP-chitosan), P2 = Treatment (normal rats) given NaTPP-chitosan and extract in a ratio of 1:0.05), P3 = Treatment (normal mice were given NaTPP-chitosan and extract in a ratio of 1:1)

Based on Table 1 shows that the administration of nano chitosan preparations of neem leaf ethanol extract did not cause damage to the number of energy reserves in the body and the



condition of the animal. The administration of nano chitosan preparations of neem leaf ethanol extract with a ratio of 1:0.5 and 1:1 did not affect the Hepatosomatic index value. The results of this study can be interpreted that the administration of nano chitosan preparations of neem leaf ethanol extract does not cause a significant increase or decrease in energy availability. The research that has been done by Hasana et al. (2019) explained that the administration of neem leaf ethanol extract at a dose of 8.4 mg/kg BW, 11.2 mg/kg BW, and 14 mg/kg BW did not affect the Hepatosomatic index value of the test animals. The results of this study are in line with this study so it can be assumed that the content in the ethanolic extract of neem leaves can be metabolized and detoxified properly in the liver.

Factors that cause an insignificant effect on the Hepatosomatic index are thought to be due to feeding and exposure to nano chitosan preparations of neem leaf ethanol extract (*Azadirachta indica A.Juss*) until that dose can still be metabolized properly in the liver and has not had a disruptive effect on nutrient metabolism so that the value of the Hepatosomatic index showed no significantly different results. It is also suspected that the reserves of metabolic activity of the test animals are not different. Ashwini et al. (2016) stated that the Hepatosomatic index value is closely related to liver function as a reserve and metabolic activity.

Administration of toxic compounds can induce ROS (Reactive Oxygen Species), ROS will cause oxidative stress in hepatocyte cells which can cause loss of plasma membranes and disruption of membrane permeability. (Guyton & Hall, 2007). Liver tissue damage caused by the administration of toxic compounds from neem leaves is known to have no significant effect on the Hepatosomatic index. This is because the administration of the test material in the form of nano chitosan 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. Permana (2010) added that the average white rat feed during growth was 15-20g where the rat's feed requirement was 10% of the rat's body weight and this study was given ad libitum so that the availability of energy for growth was relatively safe.

The results of the analysis of the effect of nano chitosan preparations of neem leaf ethanol extract on hepatocyte diameter showed that the results were not significantly different ($P>0.05$). The results of this study showed that there was no significant effect of the administration of nano chitosan preparations of neem leaf ethanol extract on hepatocyte diameter.

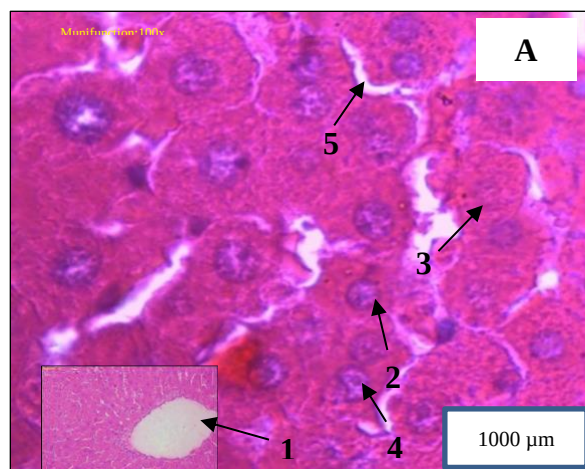
Based on Table 1, shows that the preparation of nano chitosan ethanol extract of neem leaf did not cause damage to hepatocyte diameter. The preparation of nanoparticles is thought to be able to deliver the active compounds of neem leaves as evidenced by the hepatocyte diameter



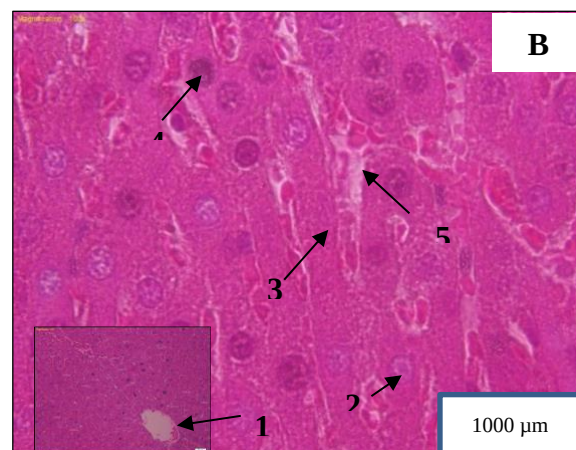
size in P2 and P3 treatments which are still at normal sizes. The results of observations of hepatocyte structure can be seen in Figure 1.

The microscopic description of rat liver that was given the nano chitosan preparation of neem leaf ethanol extract is as follows. Treatment in negative control (P0) hepatocyte diameter averaged 15.24 m. This follows the research of Yuniwanti & djaelani (2016) which states that the diameter of normal hepatocytes ranges from 8-to 17 m. This is following this study which is still classified as normal. The P2 group had the highest average hepatocyte diameter at $16,209 \pm 0.740$ the average was still considered normal. The results of the analysis showed that the administration of nano chitosan preparations of neem leaf ethanol extract with a ratio of 1:0.5 and 1:1 in groups P2 and P3 did not affect the structure of hepatocyte cells so the diameter of hepatocyte cells did not differ significantly. This is presumably because the dosages in the preparations used are still in safe doses .

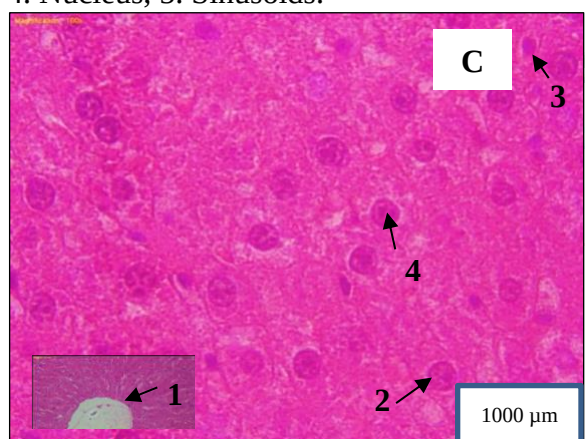
Figure 1 A represents the control group (P0) showing the hepatocyte damage score is at number 1, which means that 1-10% of hepatocyte cells have degenerated. The histological structure of the liver tissue showed a healthy state. Hepatocytes are arranged radially, there is no swelling and cloudiness in the cytoplasm. Sinusoids look normal without any enlargement and the cell nucleus is still clearly visible. Figure 1 B is the P1 treatment group that was given NaTPP and chitosan. The mean score of hepatocyte damage in the P1 group was 4, which means that 67-100% of hepatocyte cells have degenerated, necrotic, and many of the nuclei were black. The structure of the liver tissue in the P1 group showed an abnormal condition, the hepatocyte cells swelled and the cytoplasm was cloudy, the sinusoids were seen to widen so that the distance between hepatocytes was enlarged. Sonin et al. (2020) stated that the hydrophobic nature of the nanoparticles causes no stable suspension to form in the test material so clumps will form on the test material which causes damage to hepatocyte cells. Figure 1 C induced nano chitosan preparation of neem leaf ethanol extract with a ratio of 1:0.5. The mean score of the hepatocyte damage parameter in the P2 group was at number 1, which means it was at 1-10%. underwent cell degeneration, there are abnormal hepatocyte diameters, and sinusoids that start to become unclear but the damage has not reached necrosis. This damage is a characteristic of reversible damage according to the research of Mardana et al. (2019) stated that the administration of flavonoid antioxidant test materials was able to repair damaged cell structures but not yet like the normal state.



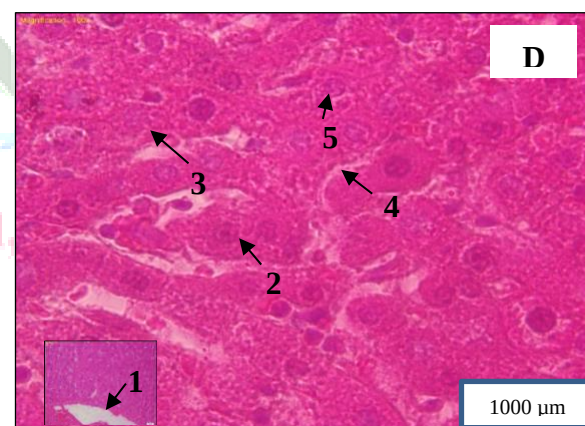
Description: 1. Central vein, 2. Normal hepatocytes, 3. Cloudy swollen hepatocytes, 4. Nucleus, 5. Sinusoids.



Description: 1. Central vein, 2. Normal hepatocytes, 3. Cloudy swollen hepatocytes, 4. Necrosis, 5. Dilated sinusoids.



Description: 1. Central vein, 2. Normal hepatocytes, 3. Cloudy swollen hepatocytes, 4. Binucleic cells



Description: 1. Central vein, 2. Normal hepatocytes, 3. Cloudy swollen hepatocytes, 4. Sinusoids, 5. Binucleic cells

Figure: 1 Histological Microscopic Structure of Male White Mice Liver, HE staining (Personal Document), Description: A (treatment P0), B (treatment P1), C (treatment P2), and D (treatment P3).

Figure D was induced with nano chitosan preparations of neem leaf ethanol extract with a ratio of 1:1, the mean score of hepatocyte damage in the P3 group was at number 2, which means 11-33% of hepatocyte cells have degenerated. The hepatocyte cell structure shows the state of



the tissue that has successfully gone through the repair and regeneration process as indicated by the presence of binucleate cells. Guidotti et al. (2003) suggested that binucleate cells in liver tissue cells often undergo a process of cell division, which can be interpreted as a process of cell regeneration in this tissue. Hepatocyte cells begin to be arranged in an orderly manner. The cloudy swelling cells are reduced, and the sinusoids are seen in a normal size without any enlargement. It is suspected that the nanoparticles can deliver the bioactive compounds of neem leaves to the target and can increase the bioavailability of the test material, reduce the toxicity effect of the test material which is able minimize damage to hepatocyte cells. The test material given in nanosize causes an increase in the number of bioactive compounds contained in the ethanolic extract of neem leaves that reach the target organ. The flavonoids in neem leaves can inhibit the oxidation reaction with a radical scavenging mechanism. The reduced free radicals in the tissues help the cells to regenerate to their original state. (Pokorny et al., 2011).

Conclusion

The results of this study can be concluded that the use of neem leaf (*Azadirachta indica*) ethanol extract in the form of nano chitosan has the potential to deliver neem leaf bioactive compounds into liver cells, and neem leaf bioactive compounds can minimize hepatocyte cell damage due to toxic compounds contained in the ingredients.

Suggestion

Further tests can be carried out to determine whether the bioactive compounds of neem leaf ethanol extract reach the liver or not by testing the MDA activity of the liver.



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