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Research Article

The Effect of Nanoparticles of *Piper crocatum* Leaves Ethanolic Extract on Liver Insulin Receptor Expression of Diabetic Rat's Induced by Streptozotocin

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ABSTRACT

Diabetes mellitus is a disease related to hyperglycemia and insulin resistance that can lead to the outcome of chronic liver diseases such as nonalcoholic fatty liver disease (NAFLD). Red betel leaves are known as traditional plants that have anti-hyperglycemic potential. This study aimed to investigate the effect of ethanolic extract of red betel leaves nanoparticle (RbL-Nps) on the liver and hepatic insulin receptor's (INSR) expression in diabetic rats. Thirty rats were included in this study and further divided into five groups containing six rats each. Group I (GI) comprised of the normal rats; while group II (GII), III (GIII), IV (GIV) and V (GV) comprised of diabetic rats induced by streptozotocin (STZ) at dose of 45 mg/kg bw and nicotinamide (NA) at dose of 110 mg/kg bw, intraperitoneally. Group I and II were treated with 0,5% Na-CMC orally. Group III, IV and V were given the oral administration of RbL-Nps at the doses 30, 60, and 90 mg/kg bw diluted in 0,5% Na-CMC, respectively. All groups were treated once daily and subsequently euthanized after 28 days. Liver tissues were collected for immunohistochemistry method to see the INSR expression and haematoxylin-eosin (HE) staining. Result in this study revealed that INSR expression on the GI, GIII and GIV were significantly higher compared to that on the GII ($p < 0.05$). On the other hand, there were no significant differences on the INSR expression between GV and GII ($p > 0.05$). Histologically, liver tissues retrieved from GII showed severe vacuolic and necrotic hepatocytes with dilatated sinusoid. Mild vacuolic and necrotic hepatocytes were observed from GV. There were no pathological changes observed in the liver tissues retrieved from GI, GII, and GIV. We concluded that RbL-Nps improved the liver condition of diabetic rats at doses of 30 and 60 mg/kg bw, but not at doses of 90 mg/kg bw.

INTRODUCTION

Red betel (*Piper crocatum* Ruiz & Pav.) is a plant which belongs to the *Piperaceae* family that can be found in various countries. According to The Plant List (2013), red betel was originally identified in Peru and later found in many countries, including Indonesia. Red betel has been well described to possess anti-inflammatory, anti-microbial, antifungal, antihyperglycemic, and antipro-

liferative potentials (Parfati & Windono 2016). It also has been known to contain active compounds such as flavonoids, polyphenols, and tannins (Dewandari et al. 2013). Recently, there has been growing interest in the study of red betel as a potential source of plant-based antioxidant and also anti-diabetic, anti-inflammatory, and anti-glycative therapeutic agents for some diseases, especially diabetes mellitus (DM).

DM is one of metabolic diseases that affects all of the systems in the body, including liver function. One of the most common features of DM is hyperglycemia which leads to insulin resistance. Insulin resistance, which is exacerbated by oxidative stress and exaggerated inflammatory signals, has been recognized as a dominant contributor to liver injury (Mohamed et al. 2016). Several substances that were released or expressed by the adipose tissues contributed to the development of proinflammatory markers that can be localized in the liver or widely expressed throughout the body. The increase of free fatty acid flow from adipose tissue to non adipose organs as a result of aberrant lipid metabolism, leads to hepatic triglyceride build up and contributes to poor glucose metabolism and insulin sensitivity in muscle and liver as target organs (Bugianesi et al. 2005). In addition, insulin resistance may progress to the chronic and life-threatening diseases such as cirrhosis and end-stage of liver failure (Mohamed et al. 2016).

Previous studies showed that traditional plants such as red betel leaves contain antioxidant and antidiabetic properties such as flavonoids, tannins and polyphenols. Flavonoids have a limited bioavailability but a major health potential that could be explored using various value-added drug delivery technologies. The main limiting factors for flavonoids to bypass the biological barrier and absorbed systematically upon oral administration are water solubility and gastrointestinal stability. Due to these conditions, many promising in vitro bioactivities showed only little or no in vivo activity. On the other hand, flavonoids have substantially improved stability and absorption qualities when administered via nano-sized delivery systems. As an outcome, the activity becomes more intense, noticeable, and prolonged (Bilia et al. 2014).

Thus, this study was conducted to investigate the potential role of red betel leaves nanoparticles (RbL-Nps) as antioxidant and antidiabetic agent targeting the liver and insulin receptor in diabetic rats induced by streptozotocin-nicotinamide (STZ-NA).

MATERIALS AND METHODS

Materials

Red betel leaves were obtained from local farmers in Sleman and Bogor areas, chitosan, sodium tripolyphosphate (Na-TPP), STZ (Nacalai Tesque), NA, Mouse/Rabbit Probe HRP Labeling Kit (BIOTNA, Cat. No. TAHC03D-15), insulin receptor rabbit polyclonal antibody (ABCLONAL, Cat. No. A0287), and sodium carboxymethyl cellulose (Na-CMC).

Methods

Plant determination

Plant sample had been determined as *Piper crocatum* Ruiz & Pav. species by Laboratory of Plant Systematic, Faculty of Biology, Universitas Gadjah Mada (number: 0149494/S.Tb./I/2021).

Plant materials and extractions

The fresh red betel leaves were determined by Laboratory of Plant Systematic, Faculty of Biology, Universitas Gadjah Mada (UGM). For the extraction, fresh leaves were washed, cleaned, and dried using oven at the temperature of 60-70 °C. Dried leaves were ground into powder then processed to maceration extraction using ethanol 96% at room temperature for 48 hours. The filtrate was then evaporated by evaporator (Abubakar & Haque 2020).

Preparation of nanoparticle synthesis

Ionic gelation method between tripolyphosphate (TPP) and chitosan was used to prepare the red betel leaf extract-loaded chitosan nanoparticles (Sundari et al. 2014). One percent of chitosan (w/v) suspension was added dropwise into 5% red betel leaf extract solutions under constant stirring until dissolved. Subsequently, under stirred condition, 1% natrium tripolyphosphate (Na-TPP) solutions were added dropwise to this mixed solution until a homogeneous solution was obtained. Thereafter, the homogenous mixed solution was filtered then dried by evaporator.

Particle size

Particle size distributions of RbL-Nps were performed using Malvern Zetasizer nano instrument. The measurements were performed in triplicates. The data were recorded by computer system. In this study the nanoparticles defined as particles with size range from 10-1000 nm.

Animal preparation

Treatment for the animal model was approved by Institutional Animal Care and Use Committee (IACUC) Faculty of Veterinary Medicine, Universitas Gadjah Mada (Number: 0005/EC-FKH/Int/2021). In this study, 30 adults male Wistar rats weighing 180 ± 20 g, age 3-4 months were used as the animal model. The rats were housed in standard single caged at room temperature with a natural dark-light cycle, provided with commercial pellet diet (Japfa Comfeed, AD II pellets) and free access to water. Prior to experiment, the rats were quarantined for a week.

Experimental design of animal treatment

Rats were randomly separated into 5 groups (GI, GII, GIII, GIV, and GV), each group consists of 6 rats and placed in individual caged. Group I (GI) was the normal control group; while group II (GII) was the diabetic control group. Group II (GII), III (GIII), IV (GIV) and V (GV) were groups in-

duced by streptozotocin (STZ) and nicotinamide (NA). Group I (GI) and GII were then treated with 0,5% Na-CMC orally as placebo. Meanwhile Group III, IV and V were treated by oral administration of RbL-Nps.

Prior to diabetes induction for the GII, GIII, GIV and GV, rats were fasted overnight. The rats of GII, GIII, GIV and GV were then injected intraperitoneally with nicotinamide (NA) at the dose of 110 mg/kg body weight (bw) which was diluted in normal saline. After 15 minutes, the rats were injected with STZ at the dose of 45 mg/kg bw which was diluted in buffer citrate (pH 4.5). After 72 hours, animals with fasting blood glucoses level more than 150 mg/dL were considered diabetic (Ghasemi et al. 2014).

Diabetic rats of GIII, GIV, and GV were treated orally with RbL-Nps at the dose of 30, 60, and 90 mg/kg bw, respectively. The RbL-Nps were diluted in Na-CMA 0,5% before given to the animals. The treatments were conducted once daily using intragastric tube every morning, for 28 days.

On the 29th day, all of the rats were anesthetized using ketamine at the dose of 90 mg/kg body weight after overnight fasting. After the rats were deeply anesthetized, the animals were perfused and subsequently proceeded to the fixation process using 10% neutral buffered formalin. The rat's livers, the left lateral lobe (Garcia-Moreno et al. 1994), were collected and immersed in 10% neutral buffered formalin. The fixed livers were trimmed into 4 mm and were processed into paraffin embedding. Livers were cut into 5 µm thickness and mounted on the object glass and coated slides.

Livers sections of the left lateral lobe were mounted on uncoated slides, deparaffinized, rehydrated and stained following the hematoxylin-eosin protocols. Histological changes in organs were examined under a light microscope and the microscopic images were captured with Optilab. Histopathological findings of the liver hematoxylin-eosin staining were analyzed descriptively.

Immunohistochemistry

Immunohistochemistry procedure for antibody insulin receptor (INSR) was in accordance with Mouse/Rabbit Probe HRP Labeling Kit (BIOTNA, TAHC03D) protocol. Insulin receptors expression was evaluated semi-quantitatively using the modified immunoreactive score (IRS) of Remmele method (Nowak et al. 2007). The method considers both the percentage of positive cells (immunoractive cells) and the intensity of the response colour, and the final result is a product of the parameters, ranging from 0 to 12 points. No reaction = 0 point (-); poor reaction = 1-2 point (+); moderate reaction = 3-4 point (++); severe reaction = 6-12 point (+++). The positive cells (immunoractive cells) are marked by the brown colour on the hepatosite as the result of the use of DAB chromatogen. The images were captured using Optilab and cell counting was processed using imageJ. Immunoreactivity score were then analyzed with Kruskall-Wallis test and Mann-Whitney test using SPSS program ver. 25.

RESULTS AND DISCUSSION

Results

Size distributions of the RbL-Nps

The data presented in the Figure 1a were sorted from the smallest to the largest size of the nanoparticles, then accumulated to the point of 100% and determined the d80 (Figure 1b). Based on the curve the size of samples had d80 = 712.4 nm. This means that 80% of the diameter of the RbL-Nps has a size of 712,4 nm or smaller. However, this size confirmed that the RbL-Nps synthesized for this research were determined as nanoparticles.

The size distributions of RbL-Nps samples were obtained from particle size distribution curve as presented in Figure 1.

Histological feature of liver with hematoxylin-eosin staining

Histological features of rat's liver on group I (GI) showed normal structure of the hepatocytes and the sinusoids. Meanwhile, the group II (GII) showed severe pathological changes of the hepatocytes. Lipid accumulation was seen with expanded vacuoles pushing the nucleus into the periphery of the hepatocyte. Necrosis was also observed in some cells with enlarged sinusoids.

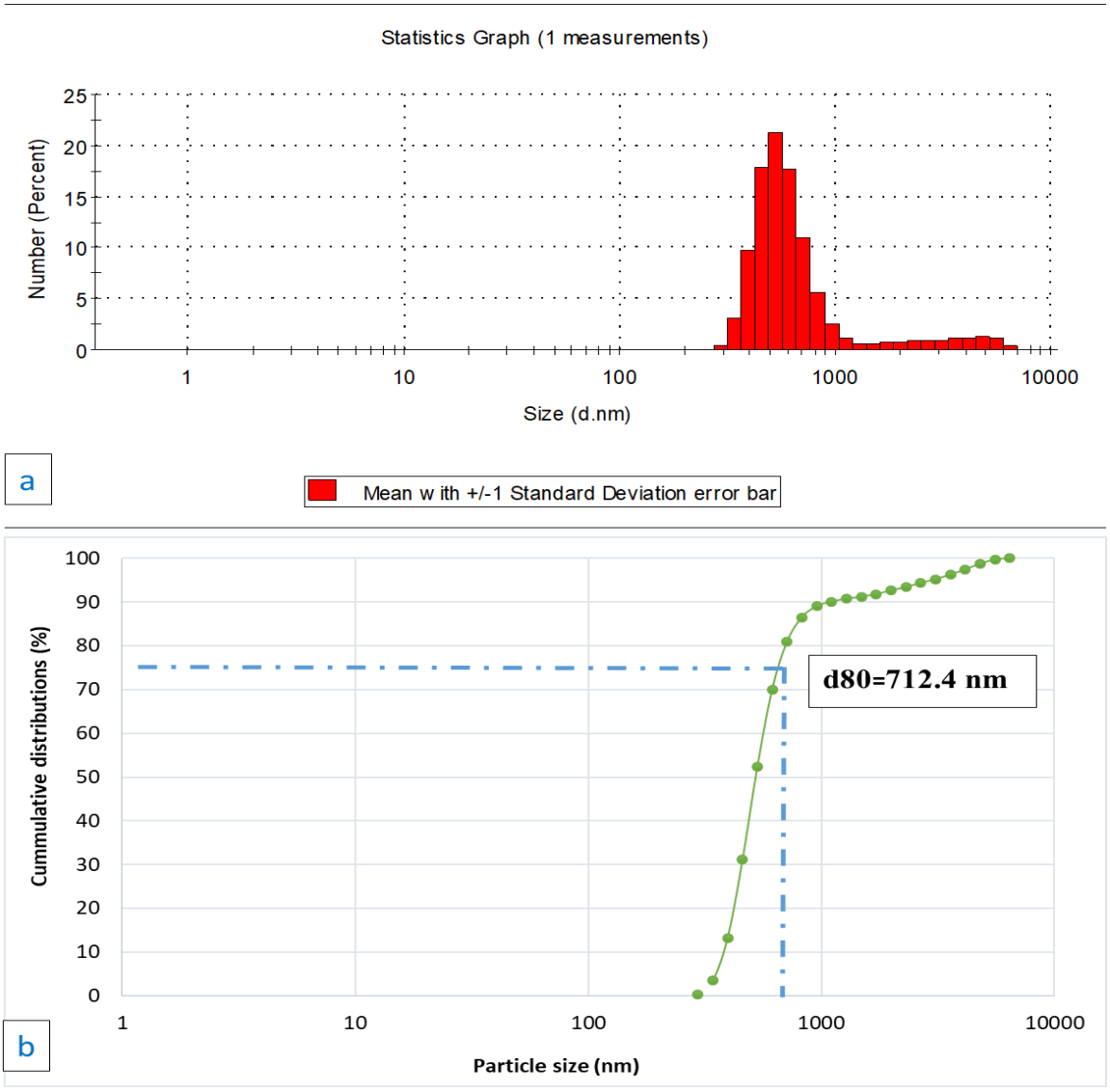

Figure 1. Particle size distribution by number (a) and cumulative distribution curves (b) of RbL-Nps.

All groups that were administered with RbL-Nps showed various histological changes depending on the given doses. Group III (GIII) that were administered with RbL-Nps at the dose of 30 mg/kg bw showed few histological changes of the hepatocytes and the sinusoids. Hepatocytes appeared normal and few of them appeared to have lipid accumulation. The nucleus appeared normal in the center of the hepatocyte and with normal features of sinusoids. Groups that were administered with RbL-Nps at the dose of 60 mg/kg bw (GIV) also showed a similar histological change with GIII.

Compared to GIII and GIV, changes were found on the group V (GV) that were administered with RbL-Nps at the dose of 90 mg/kg bw. Lipid accumulation with small to large vacuoles appeared evenly throughout the hepatocytes and pushed the nuclei to the periphery of the hepatocytes. Widening of the sinusoid were also found in this group. Necrotic area was observed in some hepatocytes with sinusoids filled with red blood cells. Histological changes of the rat's liver were presented on Figure 2.

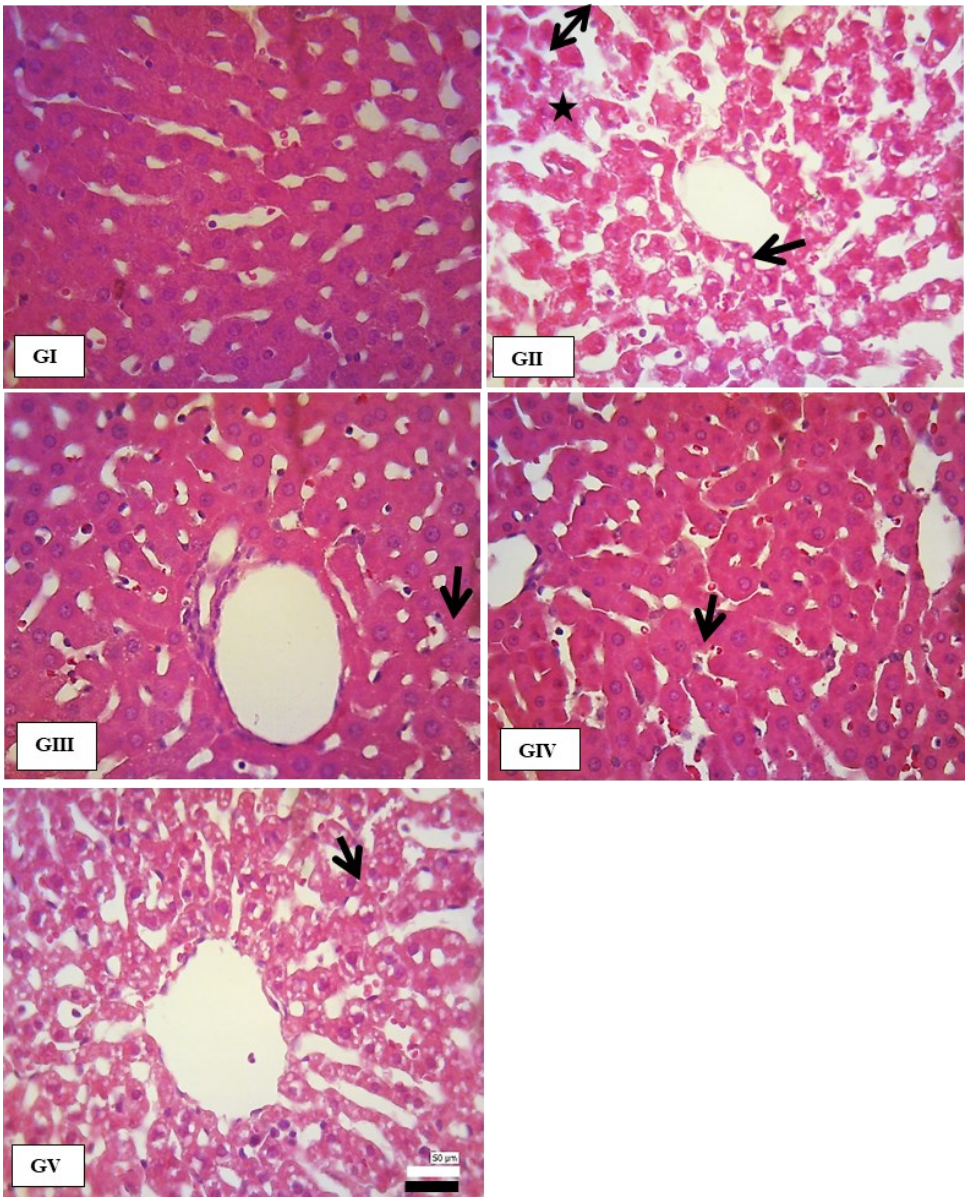

Figure 2. Histological features of the rat's liver, H&E staining. (GI) normal control group; (GII) diabetes control group; (GIII) diabetic control group treated with RbL-

Nps at the dose of 30 mg/kg bw; (GIV) diabetic group treated with RbL-Nps at the dose of 60 mg/kg bw; (GV) diabetic group treated with RbL-Nps at the dose of 90 mg/kg bw. Double headed arrows; star icons; and black arrows represented the widening of sinusoid; necrotic area of the hepatocytes; and fatty degeneration of the hepatocytes, respectively. 50 μm scale bar shown on GV was applied for all images.

Immunohistochemistry Features of Insulin Receptor (INSR) Expression on the Liver

In addition to the histopathological changes, immunohistochemistry staining method was done to determine the expression of INSR on the liver. Insulin receptor can be found on the perivenous area, especially at the cell membrane, cytoplasm, and nucleus of the hepatocyte. Statistical analysis of the insulin receptor's expression on the liver was shown on Table 1 bellow.

Table 1. Average of immunoreactivity score of INSR on the liver.

Treatment's Group	Immunoreactivity Score of INSR (Mean rank ± SD)
GI (normal control)	83.92 ± 0.563^{a}
GII (diabetes control)	60.30 ± 0.450^{b}
GIII (diabetes + RbL-Nps at the dose of 30 mg/kg bw)	83.20 ± 0.498^{ac}
GIV (diabetes + RbL-Nps at the dose of 60 mg/kg bw)	79.05 ± 0.547^{ad}
GV (diabetes + RbL-Nps at the dose of 90 mg/kg bw)	71.03 ± 0.430^{ab}

Note: Different superscript (^{a,b,c,d}) on the different group means significant difference ($p < 0.05$).

Based on the data analysis of the INSR immunoreactivity score shown on the Table 1 there was significant difference ($p < 0.05$) on the INSR immunoreactivity score between GI and GII ($p = 0.013$), GII and GIII ($p = 0.011$) as well as GII and GIV ($p = 0.040$). On the other hand, immunoreactivity score of the INSR among GI, GIII, GIV showed significant differences compared to GII. There was also no significant difference between GII and GV.

According to Table 1, administration of RbL-Nps at doses of 30 and 60 mg/Kg bw to GIII and GIV showed a positive result marked by the increase of insulin receptor expression in hepatocytes compared to the diabetic control group (GII) which were not administered RbL-Nps. There was no statistically significant difference or remained similar mean rank in insulin receptor expression among GI, GIII and GIV.

Immunoreactivity to INSR expression on the liver sections were shown on Figure 3. According to the Figure 3, we found strong expression of INSR on the GI as the nondiabetic control group. Well expressed INSR were shown on the GIII, GIV and GV. The lowest expression of INSR showed on the GII as the diabetic control group.

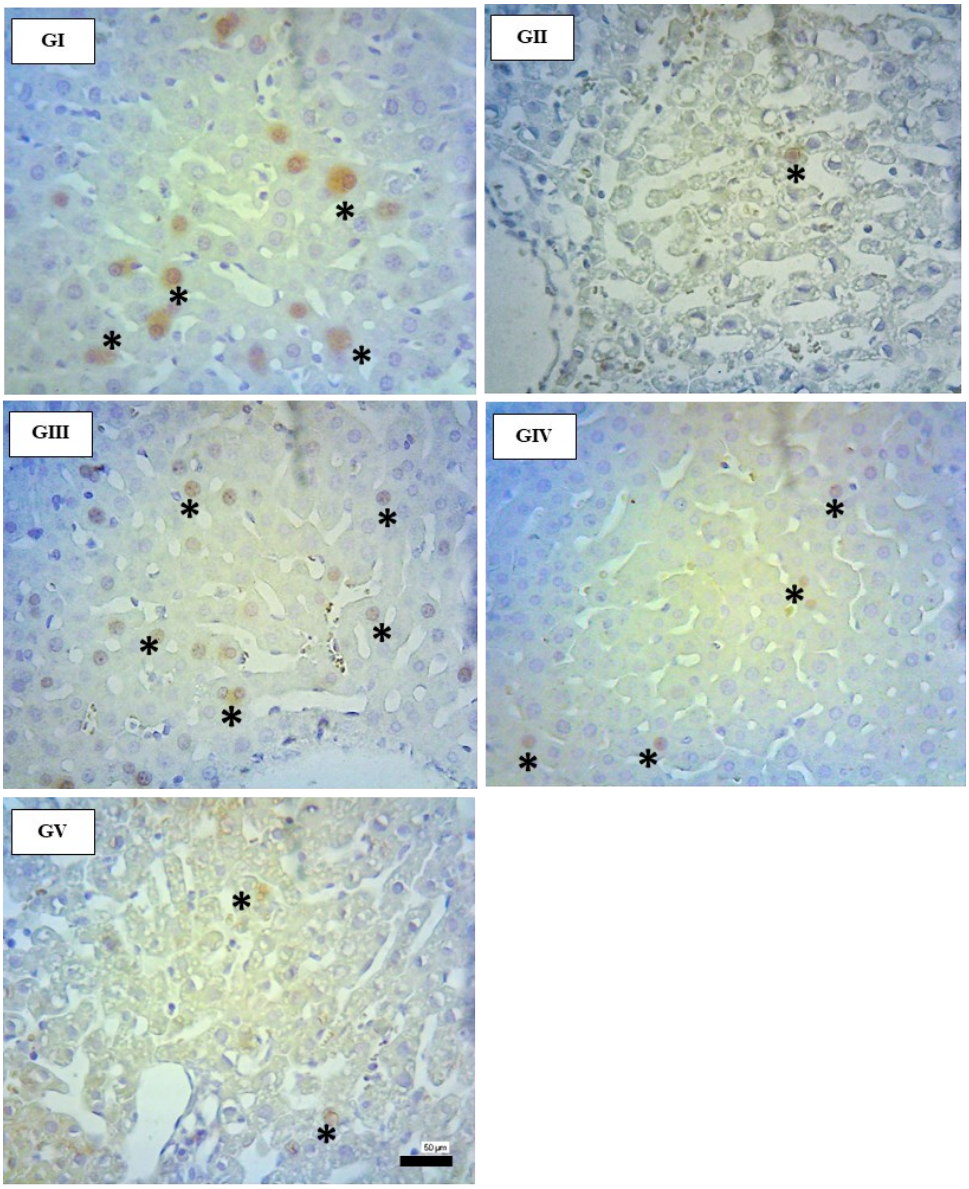

Figure 3. Photomicrograph of insulin receptors immunoreactivity in the rat's liver tissues. (GI) normal control group; (GII) diabetes control group; (GIII) diabetic control group treated with RbL-Nps at the dose of 30 mg/kg bw; (GIV) diabetic group treated with RbL-Nps at the dose of 60 mg/kg bw; (GV) diabetic group treated with RbL-Nps at the dose of 90 mg/kg bw. Immunohistochemistry staining with antibody specific to insulin receptor. 50 µm scale bar shown on figure GV was applied for GI, G II, G III and G IV. Brown colour (*) represented the immunoreactive cells.

Discussion

In this study, red betel leaves or "daun sirih merah" that were obtained from Sleman and Bogor area were used and confirmed as *Piper crocatum* species. Morphological features of the leaves included the dark green color in upper side with silvery color around the leaf bone and purple color on the bottom (Parfati & Windono 2016). Based on previous study, the young and adult leaves had cordate shapes, and when it went into flowering season, the plant formed hanging branches and the leaves underwent shape changes into elliptical (Astuti et al. 2011).

The RbL-Nps particle size distribution in this study had d80 = 712.4

nm, which means that 80% of the samples RbL-Nps were smaller than 712.4 nm. According to the theory, nanoparticles were defined as particles with a size between 1-1000 nm (Duncan & Gaspar 2011). The particles sizes of RbL -Nps in current study were larger than previous study (Dewandari et al. 2013) probably due to the difference of NaTPP and chitosan concentration. High concentrations of chitosan (1%) and NaTPP (1%) were used in this study. It was demonstrated before that the high concentrations of TPP and chitosan could increase the particle sizes and when lowering both concentrations decreased particle sizes (Mudhakir et al. 2014). Crosslinking between NaTPP and chitosan resulted in the increase of viscosity and homogenization capabilities which lead to aggregation forming larger nanoparticles (Rodrigues et al. 2012).

Nanoparticles have been described to be able to increase bioavailability of drugs or herbal. Particles in nano size have a larger surface area so that the distribution of particles expand and their solubility increase. Therefore, the use of nano-sized particles would increase the contact of the particles with the surrounding materials (Abdullah et al. 2008; Dizaj et al. 2015), which in this case, increased the contact of RbL-Nps with gastric and intestinal mucosa. Chitosan as a nanocarrier has the ability to open the tight junction reversibly, thereby increasing paracellular permeability, aside from that it also has mucoadhesive property (Yeh et al. 2011). Those mucoadhesive and tight junctions opening property may slow and sustain the release of bioactive compound from nanoparticles into the circulation system via paracellular pathway (Sung et al. 2012). It has been reported that the release of total phenol from red betel leaf-chitosan nanoparticles was slowed and sustained in gastric condition (Dewandari et al. 2013).

Observation of the diabetes rat's liver induced by streptozotocin on GII showed the damaged of the liver characterized by the presence of fatty accumulation on the hepatocytes and the occurrence of tissue necrosis. Diabetes mellitus (DM) condition due to insulin reduction could cause hyperglycemia and the upregulation of the hormone-sensitive lipase on the adipose tissue resulting in lipid breakdown into free fatty acid. Free fatty acid in the blood circulation then accumulated in the liver causing fatty liver. Liver damage could be exacerbated by the release of tumor necrosis factor α and leptin which resulting in the mitochondrial oxidative stress on the hepatocytes (Mohamed et al. 2016).

The improvement of the liver damage due to DM condition could be seen on the groups that were administered with RbL-Nps. Observation on the histological changes of the liver tissue showed an improvement in liver structure indicated by the reduction of the fat vacuoles in the group administered with RbL-Nps at the doses of 30 (GIII) and 60 mg/kg bw (GIV) compared with diabetes control group (GII). Group administered with RbL-Nps at the dose of 90 mg/kg bw (GV) also showed an improvement on the liver histological structure but not as good as GIII and GIV. Findings in this study demonstrated the potential role of RbL-Nps in repairing liver damage caused

by free fatty acid accumulation and oxidative stress on the liver.

Red betel leaf extract has been widely known to contain active compounds in the form of polyphenols, tannins, and flavonoids (Dewandari et al. 2013). Study conducted by Lister et al. (2019) had shown the main compounds of the red betel leaf ethanolic extract were eugenol and hydroxychavichol which demonstrated an antioxidant activity indicated by the result of 1,1-diphenyl--2-picrylhydrazyl (DPPH) scavenging test, scavenging of H_2O_2, 2,2'-Azinobus-(3-ethylbenzo thiazoline-6sulfonic acid) (ABTS) reduction and the ferric reducing antioxidant power (FRAP) assay.

Lister et al. (2020) also showed that antioxidant properties of red betel leaves extract at low concentration could diminish TNF-α level, reduce the necrotic cell percentage and reactive oxygen species level; as well as gaining the glutathione peroxidase (GPX) gene expression in H_2O_2-induced liver injury model. This was also explained by Dinda et al. (2019) that flavonoids could affect the genes related to inflammation such as TNF-α, IL-6, IL-1β, MCP-1, TLR-2/4, TGF-β, INOS, ICAM-1, and NOX by decreased their activation.

Flavonoids could also act as an antidiabetic property due its function to improve the insulin sensitivity on the target organ. Flavonoids have major therapeutic targets on various genes that are metabolically related to target tissues. Flavonoids could increase adiponectin secretion that leads to activation of p-AMPK and SIRT1 that could diminish FoxO1 activation. This condition could lead to the decrease of gluconeogenesis activity, G6Pase and PEPCK on the liver. Increased p-AMPK could also affect the increase of fatty acid oxidation and the increase of p-PPARKγ which caused the decrease of the lipogenesis activity (Dinda et al. 2019).

In our research, the rat model for DM was treated by nicotinamide injection and then followed by injection of streptozotocin. Nicotinamide plays a role to protect the pancreas from severe damages by streptozotocin that make a chronic response of DM. The chronic of DM caused DM type 2 characterized by the disturbance of INSR. Samuel and Shulman (2016) reported that INSR activation plays an important role in initiating a complex of insulin signaling pathways. According to Wang et al. (2019) malfunction of INS thought to be the cause of type 2 diabetes mellitus which is characterized by insulin resistance. Our current study showed that RbL-Nps at the doses of 30 and 60 mg/kg bw increased insulin receptor expression on the group of diabetic rats. Also, in line with our previous study, diabetic rats given RbL-Nps at same doses had significantly increased plasma insulin levels (Nuraniyati 2021) and increased cardiac INSR expressions (Pramesti 2021) than the diabetes control group. The flavonoids compounds contained in RbL-Nps could increase the insulin action on the liver. The increased insulin action on the liver caused the increased of p-IRS1/2 activations which is a substrate formed due to the binding between insulin and insulin receptor (Dinda et al. 2019). Group V that was administered with RbL-Nps at the dose of 90 mg/kg bw showed slightly increased of insulin receptor expres-

sion compared to the diabetes control group, although statistically non significantly different.

In insulin resistance condition, insulin's ability to suppress glycogenolysis or gluconeogenesis is diminished, but it continues to promote hepatic lipogenesis, which contributes to the development of hepatic steatosis (Perry et al. 2014). The latter is characterized by hepatic lipid accumulation (steatosis) in accordance with overt inflammation, which results in hepatocyte death, fibrosis, and impaired hepatic function (Michelotti et al. 2013). This was proven in our study by the accumulation of vacuolic hepatocytes on the control diabetic group. Vacuolic hepatocytes also were found on group administered with RbL-Nps at the dose of 90 mg/kg BB. We assume that at highest dose of RbL-Nps can exacerbate the liver function. It has been known that herbal supplement can induce liver injury (Lin et al. 2019).

Based on our findings, administration of RbL-Nps to the diabetic rats could repair the liver damage due to fatty acid accumulation and oxidative stress. Thus, administration of RbL-Nps at the doses of 30, 60 and 90 mg/kg bw can repair liver damage due to fat accumulation and oxidative stress and has the potential to increase insulin sensitivity by increasing insulin receptor expression on the diabetic rat's liver. However, the outcomes at the dose of 90 mg/kg bw were not as favorable as in the two lower dose groups. RbL-Nps at the dose of 90 mg/kg bw showed induce disturbance in the livers of rats.

Futher research is needed to study the effect of administration of RbL-Nps at the dose of 30 and 60 mg/kg bw of normal control group on liver, for safety reason to healthy people, since it is possible people will consume nanoparticle of red betel leaves extract in normal situations. The expression levels of genes related to inflammation and antioxidant genes in liver tissues also need to study further which could give a better understanding of the molecular mechanisms involved.

CONCLUSION

Administration of RbL-Nps at the doses of 30, 60 and 90 mg/kg bw could improve the repairment of the liver damage and improve the expression of the insulin receptor (INSR) on the diabetic rats induced by streptozotocin. RbL-Nps administration at the doses of 30 and 60 mg/kg bw resulting the best effect at repairing the liver damage and improve the INSR expression better than RbL-Nps at the dose of 90 mg/kg bw. These findings indicated that RbL-Nps at the dose of 30 and 60 mg/kg bw improved diabetic rats' liver condition, however at the dose of 90 mg/kg bw, it did not ameliorate liver damaged of diabetic rats.

AUTHORS CONTRIBUTION

Tri Wahyu Pangestiningsih designed the research and supervised all the process, Citra Ayu Pramesti collected, prepared, and stained the sample, Nu-

saibah Nuraniyati analyzed the data, Bambang Sutrisno examined microscopic finding and Agus Purnomo maintained and cared animal treatment.

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CONFLICT OF INTEREST

The authors declare that they have no known competing personal interest and relationship that could have appeared to influence the work reported in this paper.

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