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Original Article

Small intestine histologic neuronal features of type-2 diabetes mellitus rats treated with ethanolic extract red betel leaf (*Piper crocatum*) nanoparticle (EERbLNp)

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Abstract

Objective: This study marked histological features of small intestine neurons in the type-2 diabetes mellitus (T2DM) rat model treated with ethanolic extract red betel leaf nanoparticle (EERbLNp)
Methods: Thirty male Wistar rats were allotted into five groups (six rats each). Group I is nondiabetic control; group II is streptozotocin-nicotinamide (STZ-NA)-induced T2DM; group III-V are the STZ-NA-induced T2DM treated daily per oral with EERbL-Np at the doses 30, 60, and 90 mg/kg, respectively, within 28days. The duodenum, jejunum, and ileum were collected for routine histological staining and cresyl violet special staining to evaluate the feature of neurons in the Meissner (submucosa) and Auerbach (muscular) plexus. Descriptive statistical analysis ANOVA and Tukey HSD were used to compare neuron indexes among groups.
Results: The necrotic neuron index in the duodenal and ileal Auerbach plexus, including the degenerative neuron index in the jejunal Auerbach plexus, were significantly decreased with the EERbL-Np treatment at the dosages 60 and 90mg/kg.
Conclusions: The 60 mg/kg EERbL-Np administration in the T2DM model may provide a neuroprotectant candidate in duodenal and ileal neuropathy. Dosage of 90mg/kg EERbL-Np is also promising in jejunal neuropathy treatment.

Keywords: Nanoparticle; Neuron; Red betel leaf; Small intestine; Type-2 diabetes mellitus

INTRODUCTION

Diabetes mellitus is a metabolic disease with high morbidity and mortality consequences worldwide. Non-insulin-dependent type-2 diabetes mellitus (T2DM) is considered in up to 90% of total diabetes patients [1, 2]. Beta cell dysfunction and insulin resistance lead to persistent hyperglycemia, which characterizes T2DM [3]. Gastrointestinal motility disorder is a common complication in around 75% of cases found in each segment of the digestive system and manifests as nausea, vomitus, abdominal pain, bloating, constipation, or diarrhea [4, 5]. However, information about the intestine histological features of T2DM patients is still lacking.

Diabetes associated-intestinal motility alteration raised the significant role of the enteric nervous system (ENS), which can be damaged by prolonged hyperglycemia and oxidative stress in T2DM [6]. There are two major plexuses of ENS presented throughout the gastrointestinal tracts, Meissner (submucosa) and Auerbach (muscular or myenteric) plexus that play roles in the secretory function and

controlled peristaltic including blood flow by smooth muscle contraction and sensory responses to the substances, microenvironment, antigens, or pathogens in the lumen [7]. Disruption of the ENS consequences functional imbalance such as enteric neuropathy by a metabolic disease, diabetes mellitus [6]. Animal model of T2DM can be induced by streptozotocin (STZ) and nicotinamide [8, 9]. The acute feature of DM at 14th–28th days after the onset of streptozotocin (STZ)-induced diabetic rats showed destruction of villi, crypts, and epithelium of duodenum [10, 11]. Since diabetic conditions can occur due to oxidative stress [6], increasing plasma malondialdehyde is one of the oxidative stress biomarkers [12]. It has been reported that injection of STZ led to increased plasma malondialdehyde levels [13]. The histological profile of ENS in the T2DM animal model is urgently needed due to the ethical issues and limitations in the human study. The antioxidant treatment might improve muscle motility in the diabetes associated-gastrointestinal disorder. Red betel leaves (*Piper crocatum*) contain phytochemical compounds such as alkaloids, flavonoids, and tannins which are effective as antihyperglycemic and antioxidants [14]. The antidiabetic potency of red betel leaf (*Piper crocatum*) is promising for T2DM treatment. We recently reported ethanolic extract red betel leaves nanoparticles (EERbL-Nps) treatment increased the hepatic insulin receptor and improved the liver condition of diabetic rats [15]. The absorption quality and stability of flavonoids were improved by nano-sized administration [16, 17]. Nanotechnology is widely used due to the form of nanoparticles possibly increasing the surface area, thus, also increasing the action of the active compounds. Nanotechnology involves the study of materials that have a dimension range of 1 to 100 nm [18]. Treatment with EERbL-Nps.maybe has a significant effect on the T2DM model. Hence, this study aimed to evaluate the small intestine neuronal features of the T2DM rats model treated with ethanolic extract red betel leaves nanoparticles (EERbL-Nps). Such a profile might support the neuroprotectant potency of EERbL- Nps of the T2DM neurophaty in a rat's small intestine.

MATERIALS AND METHODS

Ethanolic extract red betel leaf (*Piper crocatum*) nanoparticle (EERbLNp) preparation

Red betel plant species (*Piper crocatum*) determination was raised in the Laboratory of Plant Systematic, Faculty of Biology, Universitas Gadjah Mada (0149494/S.Tb./I/2021). The leaf extraction was completed according to the previous report [15]. Briefly, fresh, clean leaves were dehydrated at 60-70 °C, then preserved in the form of powder, followed by maceration extraction by employing ethanol 96% at room temperature for 48 hours, then filtered and evaporated. The red betel leaf extract-loaded chitosan nanoparticles were prepared using the ionic gelation method. Nanoparticle size was measured using a Malvern Zetasizer nano instrument within the 10-1000 nm size range.

Tissue samples

Tissue samples (small intestine) were obtained from similar projects (Number: 0005/ EC-FKH/Int/2021). Thirty adult male Wistar rats (*Rattus norvegicus*) 180 ± 20 g weighed from the Laboratory Animal Center of Universitas Gadjah Mada were maintained in a standard condition, housed in single cages at room temperature, fed with commercial pellet diet (AD II pellets, Japfa Comfeed, Indonesia) and water *ad libitum* [9].

Experimental design

Thirty male Wistar rats were divided into five groups (six animals per each). The sample size calculation of this study was obtained by the formula in the previous report [19]. Minimum sample size (n) in each group = 10/number of groups+1. The equation for maximum sample size (n) is 20/ number of groups+1. Based on it, 3-5 animals per group is suitable in this experimental design, however, we add an extra sample (1 animal per group). The control group I (non-T2DM) is administered with natrium carboxymethyl cellulose (Na-CMC) orally. Group II is the type-2 diabetes mellitus (T2DM). Fifteen minutes after nicotinamide (NA, 110 mg/kg, in sodium chloride 0.9%) injection, rats in group II were injected with Streptozotocin (STZ, 45 mg/kg) in citrate buffer. Fasted blood glucose level of the rat was collected in 72 hours after injection. The animal models were designated diabetes mellitus by blood glucose levels >150

mg/dl [15]. Groups III, IV, and V were STZ-NA-induced T2DM and daily treated with the ethanolic extract red betel leaves nanoparticles (EERbL-Nps) in Na-CMC orally at the doses 30, 60, and 90 mg/kg, respectively, within 28 days.

Small intestine sample collection and slides preparation

After 28 days of treatment, the animals were anesthetized intramuscularly ketamine at a dose of 90 mg/kg, then euthanized and fixed in 10% neutral buffer formalin using the appropriate perfusion technique. Duodenum, jejunum, and ileum were cross-section trimmed in 1 cm long and put in the tissue cassettes, washed in running tap water for 30 minutes, then dehydrated in a graded ethanol solution (absolute, 96%, 90%, 80%, and 70%) for 60 minutes each followed by a clearing process in serial xylene for 40 minutes each. The paraffin infiltration process was carried out by inserting the tissue into the liquid paraffin three times, each for 60 minutes, in an incubator at 60°C. Duodenum, jejunum, and ileum were embedded by immersing the tissue in liquid paraffin in the tissue pot cross-section, then kept at room temperature as a paraffin block and stored in the refrigerator overnight. Tissues were sectioned in 5 μm thickness, floated in a water bath, and affixed to the object glass, then placed on a slide warmer at 40°C overnight.

Cresyl violet staining

Standard histological staining for neurons Cresyl Violet is stained Nissl substance in the cytoplasm of neurons. Sections were deparaf finized in three changes of xylene for 5 minutes each and rehydrated in the serial ethanol (absolute, 96%, 90%, 80%, and 70%) for 3 minutes each. Sections were stained in 0.1% Cresyl Violet (Sigma Aldrich, C5042, USA) for 30 minutes at the temperature of 37°C, then quickly rinsed in tap water to remove excess stain and washed in 70% alcohol. Sections were dehydrated through changes of ethanol (70%, 80%, 90%, 96%, and absolute), cleared in xylene, and mounted in entelan.

Data analysis

Data were analyzed qualitatively based on the histological structure appearance of the neurons in the small intestine of normal nondiabetic rats, diabetic rats, and EERbL-Np-treated diabetic rats. A quantitative histological features assessment denotes a neuron index. The index represents a proportion (percentage) of normal, degenerative, and necrosis neurons among the neuron populations in the Meissner and Auerbach plexus in a systematic random sample area of view (magnification 400x). Descriptive statistical analysis ANOVA and Tukey HSD were employed to compare neuron indexes among groups. A p-value (*P*) less than 0.05 is considered a significant difference.

RESULTS

Histologically, normal, degenerative, and necrotic neurons were observable in all groups. A cresyl violet staining determined the enteric nervous system (ENS) features in two large plexuses, Meissner (submucosal) and Auerbach (muscular) of the rat's small intestine. Degeneration features were seen with ruptured membranes, while necrosis neurons have less cytoplasm without a nucleus, as illustrated in Figures 1, 2, and 3.

Figure 1 exhibits ENS features in the duodenal Meissner plexus and Auerbach plexus. The normal structure was mostly found in the nondiabetic group. However, the normal neuron index at the dosage of 30mg/kg EERbL treatment was significantly lower than the nondiabetic group in both Meissner and Auerbach plexus. Qualitatively, we found the degenerative and necrosis features in all groups (Figure 1, A-E). Degenerative neuron index in treatment groups at the dosage of 30mg/kg and 90 mg/kg were not different from the nondiabetic group of Meissner plexus (Figure 1, F) but higher in Auerbach plexus (Figure 1, G). The necrosis neuron index in the 30mg/kg EERbL-Np treatment group was significantly higher compared to other groups in the Meissner plexus. In Auerbach plexus, the degenerative neuron index of EERbL-Np treated groups (30 and 90mg/kg) were significantly different (higher) than the nondiabetic group but not statistically different in the dosage of 60mg/kg.

Figure 2 illustrates the normal, degenerative, and necrosis structure of neurons in the rat's jejunal Meissner and Auerbach plexus qualita tively (A-E) and quantitatively (F and G). Normal structure neurons of the jejunal Meissner

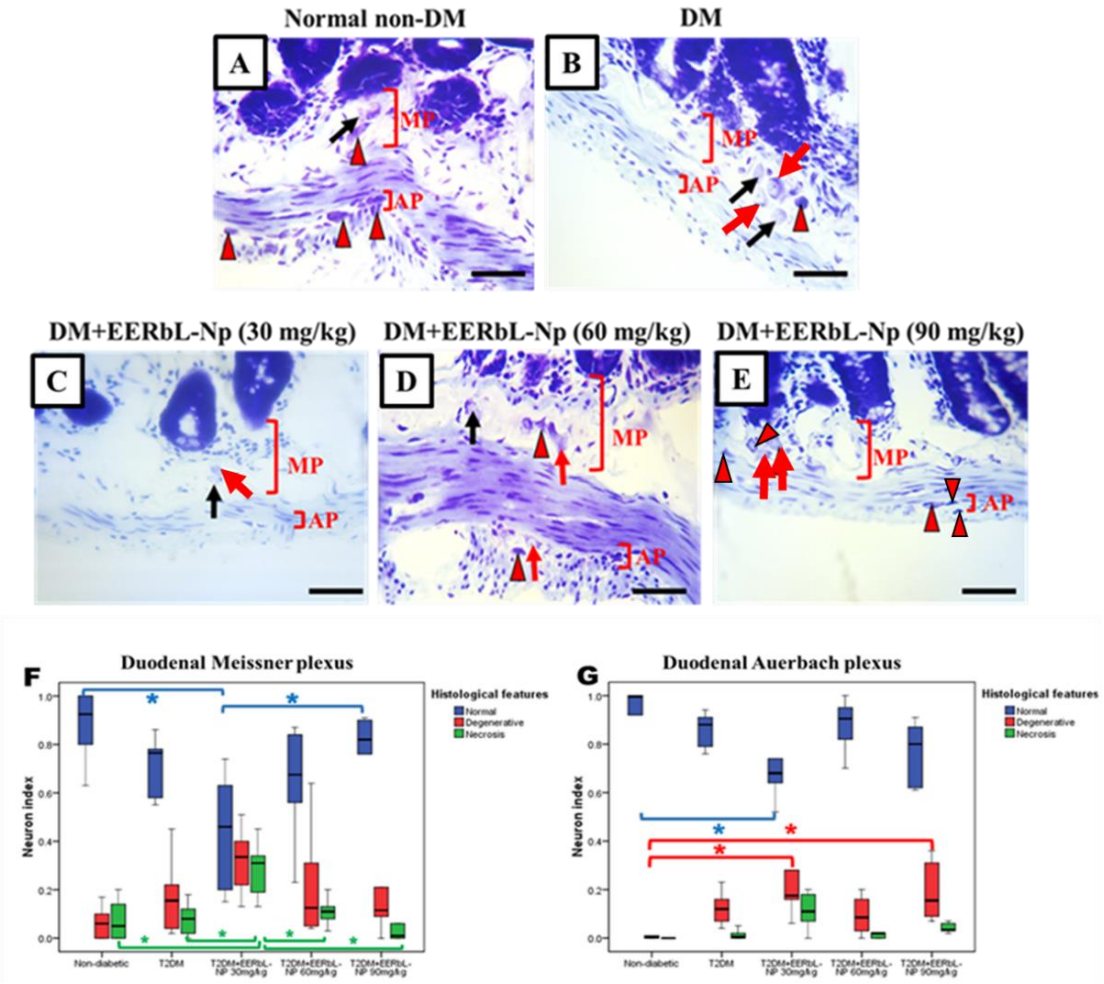

Figure 1. Cresyl violet neuron staining of normal nondiabetic, diabetic, and various dosage of EERbL-NP treatment groups in the duodenal enteric nervous system (**A-E**, bar= 30 µm). Normal structure neurons (red arrowheads) of duodenal Meissner plexus (MP) and Auerbach plexus (AP) are mostly found in the normal nondiabetic rats. Neuron degeneration (red arrow) necrosis (black arrow) features were qualitatively observable in all groups. The graphs showed neuron index in Meissner plexus (**F**) and Auerbach plexus (**G**) of rat's duodenum. Normal neuron index (blue bars) at the dosage of 30mg/kg EERbL treatment was significantly lower than nondiabetic group in both Meissner and Auerbach plexus. Degenerative neuron index (red bars) in treatment groups at dosage of 30mg/kg and 90 mg/kg were not different with the nondiabetic and diabetic groups in Meissner plexus (**F**) but significantly higher than the nondiabetic group in Auerbach plexus (**G**). Necrosis neurons index (green bars) at dose 30mg/kg EERbL-NP treatment was significantly higher compared to other groups in Meissner plexus (**F**). In Auerbach plexus (**G**), degenerative neuron index of EERbL-NP treated groups (30 and 90mg/kg) were significantly higher than the nondiabetic group. Only the dosage of 60mg/kg EERbL-NP treatment showed not different degenerative neuron index with the nondiabetic group (*indicated p-value<0.05).

and Auerbach plexus are prominent in nondiabetic rats. There were no differences in normal, degeneration, and necrosis neuronal indexes among groups in the Meissner plexus (Figure 2, F). However, the normal neurons in the diabetic group were significantly lower than the nondiabetic group and EERbL-Np treatment at the dosage of 90mg/kg group in the jejunal Auerbach plexus (Figure 2, G). Interestingly, in the jejunum, the degenerative neuron index of the EERbL-Np treatment group (dose 90mg/ kg) was significantly lower than the index in the diabetic group, while the necrotic feature is not different among groups.

Histological features of normal, degenerative, and necrosis neurons among nondiabetic, diabetic

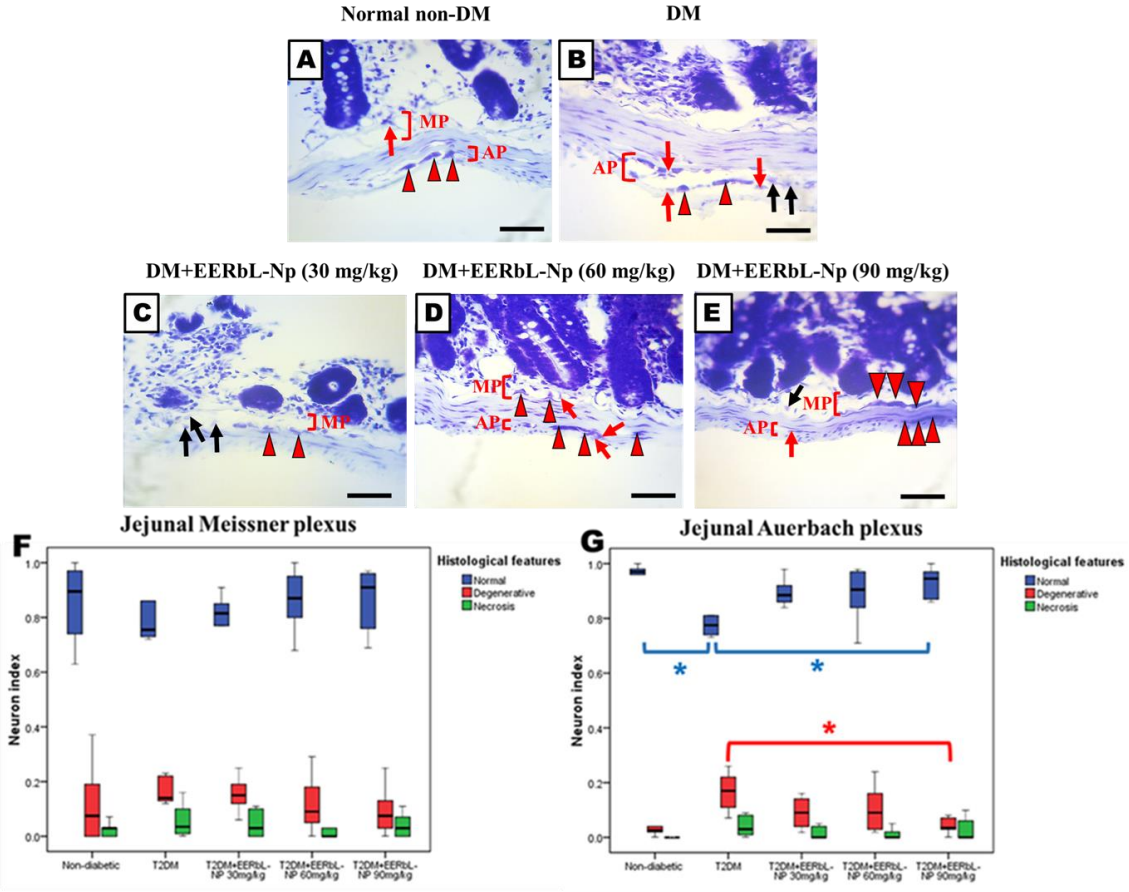

Figure 2. Cresyl violet neuron staining of normal nondiabetic, diabetic, and various dosage of EERbL-NP treatment groups in the jejunal enteric nervous system (**A-E**, bar= 30 µm). Normal structure neurons (red arrowheads) of the jejunal Meissner plexus (MP) and Auerbach plexus (AP) are found in all groups. Degenerative and necrosis neurons were indicated qualitatively in red and black arrow, respectively. In Aurbach plexus of dosage 90mg/kg (E) showed a lower number of degenerative neurons compared to T2DM group (B). The graphs showed neuron index in Meissner plexus (**F**) and Auerbach plexus (**G**) of rat's jejunum. There were no differences of normal, degeneration and necrosis neuronal indexes in Meissner plexus between groups. Normal neuron (blue bars) in jejunal Auerbach plexus of diabetic group were significantly lower than the nondiabetic group and EERbL-NP treatment at the dosage of 90mg/kg. The degenerative neuron index (red bars) in EERbL-NP treatment group (dose 90mg/kg) was significantly lower than the index in diabetic group. The necrotic neuron feature is not different among groups (* indicated p-value<0.05).

model, and EERbL-Np treatment at different doses in rat's ileum were demonstrated in Figure 3. Normal structure neurons are obviously found in the ileal Meissner and Auerbach plexus of all groups. Degenerative and necrosis neurons were indicated mostly in the diabetic group. There is statistically no difference in normal, degeneration, and necrosis neuronal indexes among groups in the ileal Meissner plexus (Figure 3, F). Noteworthily, in the ileal Auerbach plexus, the degree of necrosis neuron index in EERbL-Np treatment group (dose 60mg/kg) was significantly lower compared to the diabetic group (* indicated p-value<0.05).

DISCUSSION

The proportion of normal structure of an enteric nervous system (ENS) is more dominant in the nondiabetic control group as illustrated in Figure 1-3 (A, F, and G). However, degenerated and necrotic neurons were also observable in the normal control group, as neurons in ENS exhibit degeneration and necrosis under nondiabetic

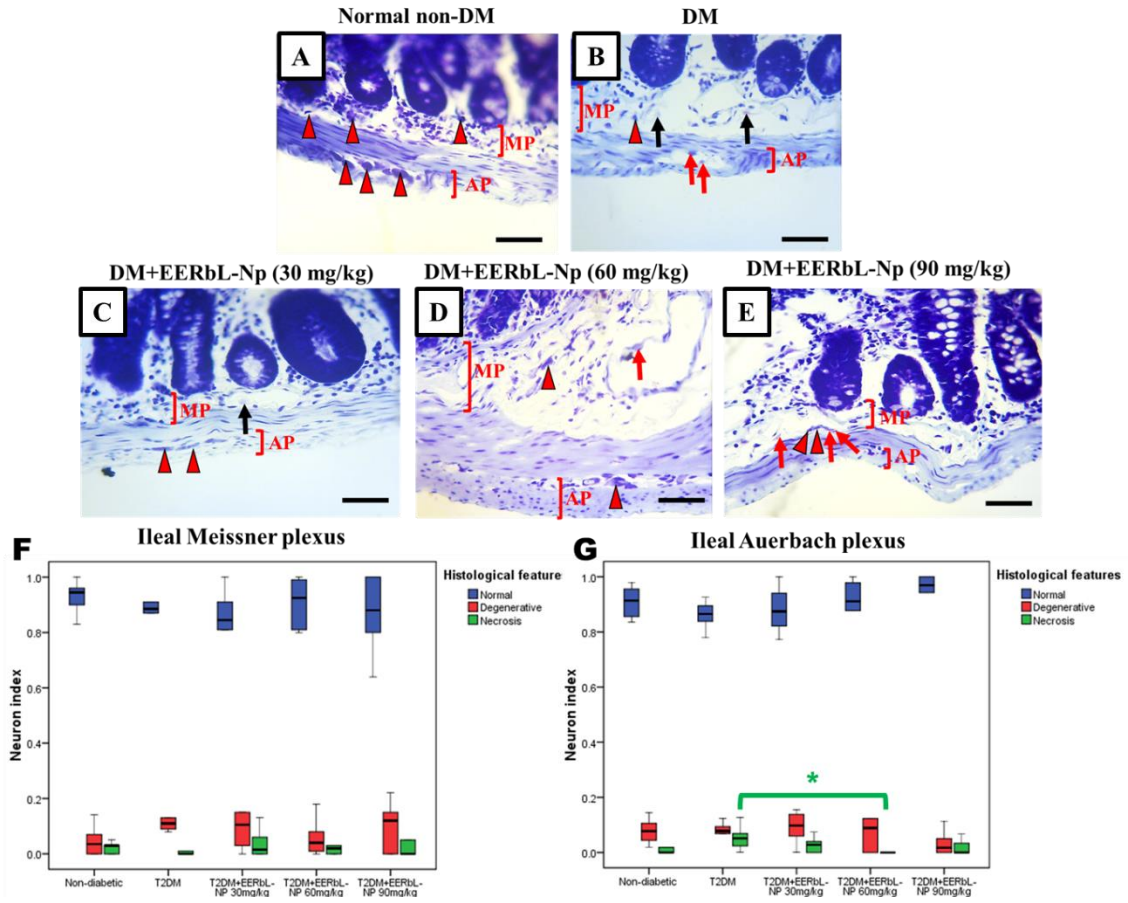

Figure 3. Cresyl violet neuron staining of normal nondiabetic, diabetic, and various dosage of EERbL-NP treatment groups in the enteric nervous system of rat's ileum (**A-E**, bar= 30 μm). Normal structure neurons of Meissner plexus and Auerbach plexus (red arrowheads) were pronounce in all groups. Necrosis neurons (black arrow) and degenerative neurons (red arrow) were indicated mostly in the diabetic group (**B**). The graphs showed neuron index in Meissner plexus (**F**) and Auerbach plexus (**G**) of rat's ileum. There were no differences of normal, degeneration and necrosis neuronal indexes among groups in Meissner plexus. In ileal Auerbach plexus, degree of necrosis neuron index in EERbL-NP treatment group (dose 60mg/kg) was significantly lower compared to the diabetic group (* indicated p-value<0.05).

conditions. Such degenerated and necrosis neurons assume as an apoptotic feature which is normally found in the maintenance process of a healthy adult gut [20]. Again, enteric neurons apoptosis was characteristic in diabetic male rats [21]. Neuropathy in the diabetic group is indicated (Figures 1-3: B, F, and G). The protective properties of NA maintained the cytotoxic effect of streptozotocin (STZ) on pancreatic β cells (T1DM) to demonstrate T2DM [22]. Adminis tration of streptozotocin at a dose of 45 – 200 mg/kg in rats can cause changes in the number and size of neurons. In addition, the degeneration feature was pronounced in diabetic-induced neuropathy [6]. Similarly, the streptozotocin-induced diabetic model of group II in this study represents a lower number of normal neuron, increased degeneration and necrosis neurons in the Meissner and Auerbach plexus of the small intestine (duodenum, jejunum, ileum). The neuronal abnormalities in a diabetic group might correlate with the increased number of serotonin receptors similarly in the small intestine of a pig. The upregulation of serotonin receptors in the diabetic pig model affected the secretory and sensory-motor function of the gut [23]. Thus, the neuron alteration in the muscular plexus may explain the sensory-motor function changes. Intestinal function alteration in the strepto zotocin- induced diabetic rat is also associated

with a declined level of Angiotensin II, which controls small intestine contraction [4]. Necrotic features of neurons in the Meissner plexus were lesser than degenerated form in the T2DM group (Figure 1-3: F) and may be addressed to neuro degenerative complications in the diabetic patient [24]. Necrosis neurons have less cytoplasm without a nucleus, while degeneration features were seen with ruptured membranes (Figure 1-3: A-E). Progressive degeneration might be reasonable for the unmyelinated feature in diabetic neuropathy [25].

The different responses of intestinum tenue to a treatment of EERbL-Np were suggested due to location and function [21]. We illustrated in Figure 1, EERbL-Np treatment at a low dose of 30 mg/kg in duodenum significantly increased necrotic neurons in the Meissner plexus. It is suggested that the duodenum, as the proximal organ of the intestinum tenue, need an adaptation process to absorb nutrient [26]. It is related to the neurons located in the submucosal (Meissner plexus) area of the duodenum to facilitate the absorbtion process. In this submucosal area we also indicated there is no degeneration alteration of the neuron as our results showed degenerative neuron index in 30mg/kg, 60mg/kg, and 90 mg/kg doses of EERbL-Np treatment groups were not different from the nondiabetic group as shown in Figure 1F. However, there was an increased number of degenerative neurons in the Auerbach plexus of the rat's duodenum by EERbL-NP treatment at the dosage of 30mg/kg and 90 mg/kg (Figure 1G). The degenerative neuron index in both groups is significantly higher than the nondiabetic group but not statistically different at the dosage of 60mg/kg. By this evidence, we suggested that EERbL-NP treatment at the dosage of 60mg/kg is a potential agent for ENS neuroprotection, particularly in the duodenum.

On the other hand, shown in Figure 2, in the jejunal Auerbach plexus a normal neuron index in the diabetic group was at a low level compared to the nondiabetic and EERbL-NP dose 90mg/kg groups significantly. Moreover, EERbL-NP treatment at the dosage of 90mg/kg might also reduce degenerative features in diabetic-induced neuropathy. This presumption meets with the statistical evidence that showed a significant lower degenerative neuron index in EERbL-Np treatment group at the dosage of 90mg/kg compared to the diabetic group. We suggest this model answer our hypothesis.

In the ileum, different responses to various dosages of EERbL-NP in Meissner and Auerbach plexuses were indicated (Figure 3). We identified no differences among normal, degenerative, and necrosis neuron index by various dosages of EERbL-NP treatment compared to the nondiabetic and diabetic groups in the Meissner plexus. Noteworthily, in the Auerbach plexus, showed a low necrosis neuron index compared to the diabetic group by EERbL-NP treatment at the dosage of 60mg/kg. A low amount of necrotic neurons was a good indicator of a healthy intestine since neuron is the type of cell that is difficult to regenerate when the cells are damaged.

Histological alteration in the Auerbach plexus correlates to their function in controlling muscular contraction and intestine motility. It also may be associated with most cases in T2DM patients that manifest, abdominal pain, bloating, constipation, or diarrhea [4, 5]. Damaged ENS by prolonged hyperglicemia and oxidative stress in T2DM denotes diabetes-associated-intestinal motility alteration [6]. The dosage of 60 mg/kg EERbL-Np administration in the T2DM model may provide a neuroprotectant candidate in duodenal (Figure 1F, G) and ileal neuropathy cases of T2DM (Figure 3G). EERbL-Np at the dosage of 90mg/kg also showed a promising treatment in the jejunum (Figure 2G). However, further investigation in histochemistry and immunohistochemistry of damaged neuron markers is still needed.

The red betel leaf (*Piper crocatum*) has been reported as a useful herbal medicine in some diseases, particularly diabetes mellitus [27]. It contained phytochemical compounds of alkaloid, flavonoid, and tannin groups, which have anti-hyperglycemic, antioxidant, and antidiabetic properties [28]. The previous study also demonstrated that boiled red betel leaf administration was effectively reduced the blood sugar levels of T2DM patients without complications [28]. Reactive Oxygen Species (ROS) produced from streptozotocin-nicotinamide induction cause oxidative stress including protein denaturation, lipid peroxidation, and DNA damage [6]. The nanoparticles are defined as particles with a size ranging from 10-1000 nm.

In nano-sized, active compound particles are more easily absorbed by the small intestine wall thereby increasing their bioavailability.

The absorption of active compounds was raised due to the increased solubility as a result of the larger surface area of the particles. Nano particles also have a longer residence time because they are adsorbed in the intestinal mucosal layer. According to the previous study, the synthesis of nanoparticles of red betel extract with a chitosan concentration of 0.2% was potential developed as an alternative to diabetes treatment [29]. Changing the shape to nanoparticles down to <200nm can improve functional properties and potential as an anti-hyperglycemic in terms of its inhibitory activity against the α-alphagluco sidase enzyme and increase its availability in the body. With this, the nanoparticles proved to be more resistant to gastric conditions at the acidic pH than in extract form. In the form of nanoparticles, the amount of extract is lesser, with functional properties that can still be maintained. Jusuf [13] has been reported a higher plasma malondialdehid level by a treatment of Red betel leaf ethanolic extract in T2DM rat model compared to Red betel leaf ethanolic extract administration in nanoparticle preparation [30]. An increasing level of plasma malondialdehyde represented rising oxidative stress. Plasma malondialdehyde level is one of the biomarker of oxidative stress [12].

CONCLUSION

The dosage of 60 mg/kg EERbL-Np administration in the T2DM model may provide a neuroprotectant candidate in the duodenal and ileal neuropathy case of T2DM. The dosage of 90mg/kg EERbL-Np is a promising treatment for jejunal neuropathy. However, further investigation in histochemistry and immuno histochemistry of damaged neuron markers is still needed.

CONFLICT OF INTEREST

The authors declare there are no conflicts of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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