THE PROTECTIVE EFFECTS OF *PHALERIA MACROCARPA* LEAVES METHANOL EXTRACT ON PANCREATIC ISLETS HISTOLOGY IN STREPTOZOTOCIN–INDUCED DIABETIC RATS

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ABSTRACT: Background*:This study investigated the protective effects of Phaleria macrocarpa leaves methanol extract on pancreatic islets in STZ-induced diabetic rats.*
Methods*:The experimental groups rendered diabetic by a chemical combination of STZ (65 mg/kg bw, iv) and NA(230 mg/kg bw, ip) in healthy adult rats. Diabetic rats were orally force-fed with the extract (500 mg/kg bw) daily for 14 days. The reductions in blood glucose levels were determined by evaluating of RBG, FBG, IPGTT, as well as, total body weight, and, subsequently, pancreatic islets of Langerhans were examined histologically.*
Results*:Photomicrographs of pancreatic islets showed the administration of the extract has improved their cellular density, which suggests it was capable of inducing β-cells recovery and/or regeneration, following the destructive effect of STZ.*
Conclusion*:These findings indicate that P.macrocarpaleaves extract could exhibit a protective effect on the pancreatic islets and involved in the correction of altered biological parameters in diabetic rats,therefore, this extract may serve as a candidate for developing a safe, complaisance and promising nutraceutical product for the management of diabetes.*

Keywords: *Phaleriamacrocarpa*, Leaves Extract, Diabetes Mellitus, Islet of Langerhans

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1. INTRODUCTION

Diabetes mellitus is a complex heterogeneous metabolic disorder affecting nearly 4% of the population worldwide.Its prevalence is expected to increase by 5.4% in 2025 [1], and up to 7.7% (439 million adults) by 2030 [2].In experimental diabetes model, chemical induction with streptozotocin(STZ) diminished insulin production and causes high levels of blood circulating glucose, which is similar as found in human diabetics [3]. The altered physiological function of the pancreas from the action of STZ provides the signs of abnormalities in pancreatic islets function and morphology, and is defended by an increase in oxidative stress markers reported in pancreatic islets of diabetic rats [4]. Antidiabetic medicinal plants are generally known to exert their rational means for the treatment of diabetes. Though, their action modes shall depended on the phytochemicals components endowed in the plant [5] which are, the natural bioactive components found in plants that work with nutrients and fibers to form an integrated part of defence system against various diseases and stress conditions [6]. Additionally, medicinal plants have the advantage over synthetic drugs due to the presence of antioxidant compounds, which are important to modulate the level of oxidative stress [7]. Free radical scavenging molecules, such as terpenoids, tannins, lignins, flavonoids, alkaloids, phenols and other metabolites are rich in antioxidant activity [8]. *Phaleria macrocarpa,* is a plant from the family of Thymelaeaceae, commonly known as “God's Crown” or Mahkota Dewa,indigenous to Indonesia and Malaysia [9]. Traditionally, it contributes to human health and vitality , whereas, the extracts is reported for numbers of valuable medicinal properties, such as anti-cancer, anti-diabetic, anti-inflammatory, anti-fungal, anti-oxidant, anti-bacterial, and vasorelaxant activities [10].Recent studies on animal models have confirmed the antidiabetic efficacy of the fruits and some other parts of this plant, in terms of biochemical findings [9-12], yet efforts to define the histological studies and graphic evidences, in this regard, are not very far establish. Therefore, this study has designed to investigate the protective effects of *P.macrocarpa* leaves methanol extract on the histology of the pancreatic islets of Langerhans in STZ-diabetic rats.

2. MATERIALS AND METHODS

2.1 Plant Extract

Fresh leaves of *P. macrocarpa* were collected from the northwestern part of Malaysia (Kedah), and taxonomically identified. The selected plant parts were dried, crushed in an electric grinder and pulverized into a coarse powder. Methanolic extraction was prepared by soaking 100g of the coarse powder in a conical flask with 500 ml of absolute methanol. The mixture was kept for 36 hours and stirred intermittently at 4 hours interval. It was then filtered, and dried under low pressure using rotary evaporator fitted with vacuum pump.At the end of the drying process the paste obtained were dissolved in normal saline to prepare the oral dosage.

2.2 Qualitative Phytochemical Analysis

The qualitative phytochemical screening of the extract, to reveal the presence of alkaloids, saponins, flavonoids, tannins,reducing sugar, terpenoids, steroids, cardiac glycosides, and phenolic,were conducted as described previously [14,15].

2.3 Selection of Animal and Animal Care

Male, matured, normoglycemic, Sprague Dawley rats, weighed 250-300 g, were used in present study. The animals were acclimated for a period of 7 days prior to actual experiments, under laboratory standard conditions of 12:12 hour light/dark cycle and fed on standard laboratory pellets and water *ad libitum*. The principles of Laboratory Anima

lthroughout the study were supervised by the internal Animal Ethical Committee.

2.4 Induction of Diabetes

Animal were induced diabetes by combination of Streptozotocin (STZ) (65 mg/kg bw) and Nicotinamide acid (NA) (230 mg/kg, bw). NA was injected in overnight fasted rats 15 minutes prior to STZ administration,dissolved in normal saline and administered by a single intravenous (iv) injection in order to develop moderate and stable non-fasting hyperglycemia [14-16]. Induced diabetes was validated by elevated glucose levels in plasma, determined for 14 days after STZ administration. The rats with uniform Diabetes (FBG>10 mmol/L) for at least a week were forwarded for the proposed study.

2.5 Acute Oral Toxicity Study

Acute toxicity test was conducted as previously describe [15]. The rats (n=28) were divided into two main groups, diabetic and non-diabetic. Each of these groups were further divided into treated and non-treated groups of 7 animals per group. Plant extract was prepared at a dose of 500 mg/kg and feed orally to the respective groups. Animals were then observed at time intervals of 4, 6, 24, and 48 hours after administration of the extract. Furthermore, the LD50 was calculated to be greater than 2000 mg/kg. Therefore, the dose of 500 mg/kg of the extract was subjected to be used in this experimental study.

2.6 Blood Glucose Measurement

Glucose measurements was estimated using an electronic glucometer (AccuCheck Advantage Blood Glucometer-US, Roche Diagnostics). Blood samples were obtained via tail snip for the measurement of glucose readings . A constant reading greater than 10 mmol/L is considered as diabetic. The diabetic rats were monitored, for the following two weeks constantly, by random blood glucose (RBG) and fasting blood glucose (FBG) measurements to ensure their chronic stable hyperglycemia.

2.7 Intraperitoneal Glucose Tolerance Test (IPGTT)

Intraperitoneal glucose tolerance test (IPGTT) was used to measure the effect of insulin production on glucose tolerance. Glucose was measured after a single intraperitoneal bolus of glucose (20% glucose solution,2g/kg) in overnight fasted rats and sampels were collected for the measurement of glucose at 0,30,60 ,90 and 120 minutes.

2.8 Data/Sample Collection

Total bodyweight measurements (using laboratory electrical balance) were determined daily, right from the day after induction of diabetes and subsequently for the next 14 days. At the end of the treatment, animals were deeply anaesthetized, with an i.p injection of a combination of ketamine (100 mg/kg) and xylazine (10 mg/kg) [18]. Then, the abdominal and chest cavities were cut opened, and the bile duct was clamped with a bulldog clamp at the Sphincter of Oddi where it enters the duodenum for the application of in situ ductal perfusion [19]. A 3ml syringe was inserted into the bile duct near the liver and 1–2 ml of distilled water was water slowly injected. The perfusion to all parts of the pancreas causes the inflation of the organ. At this time, it was excised with scissors and placed within a prepared Ringer's solution. The bathed tissues were labelled accordingly and suspended in 10% buffered neutral formalin for histological processing.

2.9 HistologicalExamination

The specimens of the pancreas were fixed in 10% formalin and processed by paraffin techniques prior to microtome sectioning (5µm). Tissue sections were subjected to H&E staining. Histology slides observed under the microscope (Olympus DP 72) and micrographs were captured using Olympus Cell Sens Standard software.

2.10 Statistical Analysis

Results expressed as mean±SEM, the significance of differences of P value were confirmed by one-way ANOVA and multiple Dunnett t-tests, where $P<0.05$ considered statistically significant, and $P<0.001$ highly significant differences in all cases.

3. RESULTS

Various tests conducted, for presence of phytochemicals in this extraction confirmed the presence of secondary metabolites, which includes alkaloid, saponin, flavonoid, tannin, reducing sugar, terpenoids, cardiac glycosides and phenolic compounds.

Toxicity evaluation of the extract did not show any toxicity in rats, even at the highest dose tested. There were no changes in behavioral pattern, and mortality was not observed.The effect of the extract on rats body weight in the control and the experimental groups is highly significant ($P<0.001$) after 14 days of treatment The diabetic treated group showed an increased pattern of weight gain (253.73±5.54 g), which is almost silmilar to the pattern observed in non-diabetic treated group (253.80±5.30 g), compared to diabetic control (239.30±6.11 g) and non-diabetic control (235.94±6.32 g). (Figure 1).

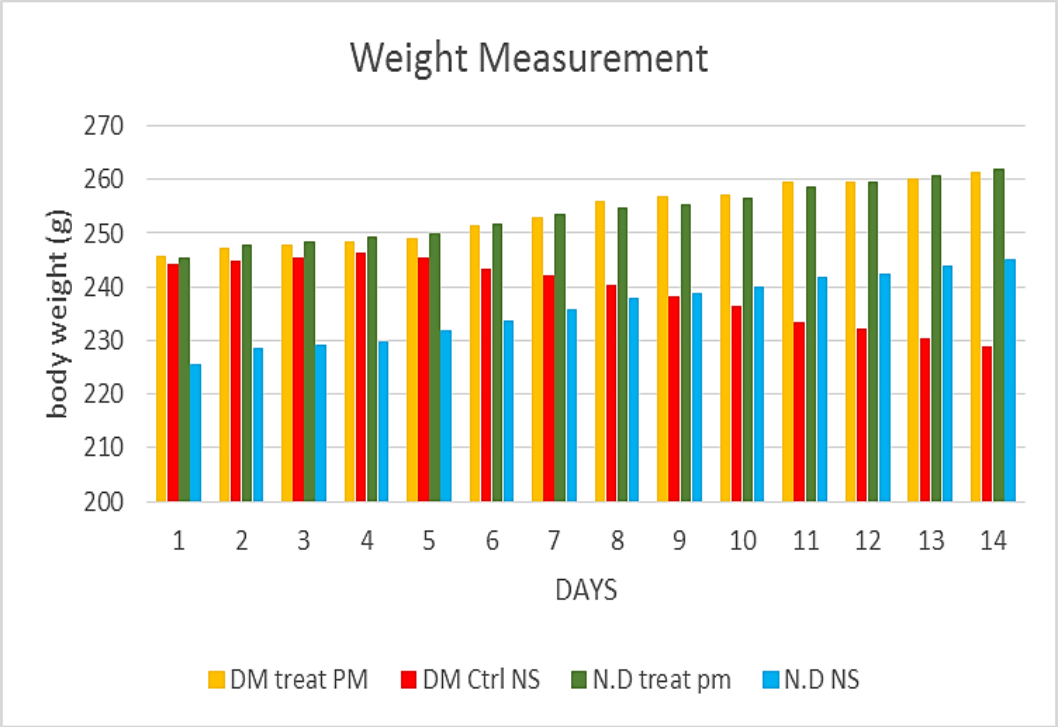

Figure 1: Daily body weight measurement (upto 14 days) in the four groups studies

Table 1: 24 hour glucose measurement of four groups studied for 14 days (Mean±SD)

Groups	24hr Glucose (mmol/L) for 14 days
Diabetic treated with *P. macrocarpa*	9.83±4.98
Diabetic control treated with normal saline	21.05±1.78 *
Non-Diabetic treated with *P. macrocarpa*	4.56±0.22
Non-Diabetic control treated normal saline	4.65±0.24

** highly significant (P<0.001)*

Table 2: IPGTT of diabetic and non-diabetic groups (Mean±SD)

Groups	Minutes (Min)				
	0	30	60	90	120
Diabetic treated with *P. macrocarpa*	18.20±1.50	22.60±1.95	19.90±1.36	15.75±1.71	10.90±1.52*
Diabetic Control with Normal saline	18.50±2.15	23.60±2.22	25.20±1.89	25.70±1.89	26.75±1.89*
Non-Diabetic Control with Normal saline	4.74±0.36	7.74±0.34	6.96±0.59	6.18±0.50	5.20±0.45

** highly significant (P<0.05)*

The antidiabetic properties of the extract was investigated by the 24 hours of glucose measurement. The results indicated highly significant reductions (P<0.001) of fasting blood glucose levels (FBG) of diabetic treated group (9.83±4.98 mmol/L), throughout the 14 days of treatment, compared to diabetic control (21.05±1.78 mmol/L) that showed a constant increase of their blood glucose levels throughout the same duration. Interestingly, the 24 hours blood glucose levels of non–diabetic treated group (4.56±0.22 mmol/L) did not show any signs of hypoglycaemia with the same oral dose used, compared to non-diabetic controls (4.65±0.24mmol/L). Furthermore, the sugar correction ability of the extract showed a constant pattern of blood glucose reduction from the 1stday till 9thday. Signifgicant reduction in glucose readings was observed from the 9th day onwards until the end of the treatment duration. Results of blood glucose measurement recorded at average of 9.83±4.98mmol/L in diabetic treated group, compared to 21.05±1.78mmol/L in diabetic control group, while the non–diabetic treated group did not show any signs of hypoglycemic effect (4.56±0.22 mmol/L) compared to non-diabetic control (4.65±0.24 mmol/L) (Table 1).

Intraperitoneal glucose tolerance test (IPGTT) was used to measure the effect of insulin production on glucose tolerance.

Our results of IPGTT highlighted the sugar lowering ability of the extract on the diabetic treated group, which showed a rapid decrease of glucose levels within 60 minutes and continued after 120 minutes.Glucose levels, administrated intraperitoneally to each group of rats, reduced constantly as compared to diabetic control group(showing increased hyperglycemia), whereas, non-diabetic controls showed a very rapid reduction of glucose right after 30 minutes of glucose administration (Table 2).

The histological changes of the pancreatic islets of Langerhans indicated that these islets of normal controls (non-diabetic) preserved their typical appearance, the normal acini of the pancreas well defined in shape and architecture, the islets were round, clearly seen without cell distortion, sized 33.60µm, and islet cells appeared normal with darker stained nuclei (Figure 2). On the other hand, the diabetic rats (diabetic control) revealed a significant reduction ($P<0.05$) in the size of islet cells, which appeared smaller,with an average of 14.90µm in diameter, distorted pancreatic architecture, mononuclear cellular infiltration, accompanied with majority loss of the islet cells and necrosis with the presence of fibrous tissue and sinusoidal spaces, in comparison with that of the normal group. These changes usually indicates the cytotoxic effects of STZ induction on the pancreatic β-cells populations.

Our results showed that daily oral administration of *P. macrocarpa* leaves extract (500 mg/kg) to the diabetic rats revealed better restoration of the size of Langerhans islets (23.67µm) and increased in number of islet cells along with β–cells repair. There was a huge improvement in the area of Langerhans islets which maintained their appearance closer to what which have been seen in normal control (non-diabetic) group. The exocrine cells appeared normal indicating that the extract has provided antioxidant effects to β–cells and protective activities toward the cytotoxicity of STZ on the pancreatic islets of Langerhans.

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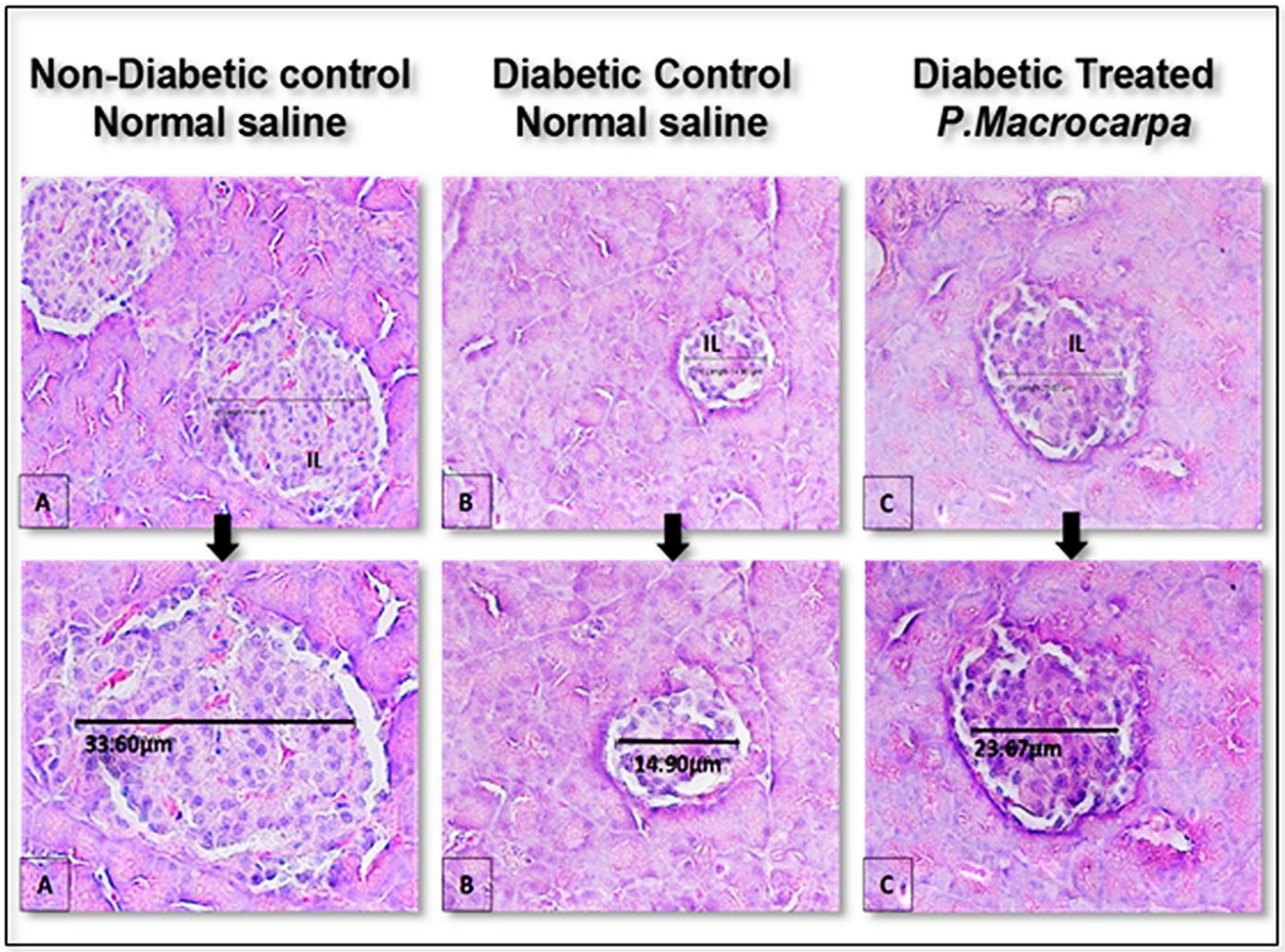

Figure 2: Sections of the pancreatic islets of Langerhans of the diabetic and non-diabetic rats. (A) Normal architecture of islets cells of the pancreas. (B) Complete depletion of the islets, with smaller size and distorted pancreatic cells. (C) Section of the pancreatic islets of diabetic rat treated with the extract showed improvement in islets size and evenly distributed β-cells and an increased number of β-cells. *Modified H&E, 40x.*

4. DISCUSSION

This study revealed the micrographs of healthy rat's pancreas maintaining the islets area with a normal lobular architecture of the pancreas, embedded within the acinar cells,with intact interlobular connective tissue. Conversely, the pancreatic sections of STZ-induced diabetic rats showed marked histological and morphological changes, the border between the endocrine and exocrine region became indistinct. The diabetic islets showed retraction from their classical round-shaped and atrophied, compared to normal islets. These may corresponded to the effects of circulating reactive oxygen species generated by STZ on β-cells [20-22].The histological examination also revealed that the administration of *P. macrocarpa* leaves extract showed an improvement compared to non-treated animals, the border between exocrine and endocrine portions became distinct and many islets showed an increase in their total volume, suggesting that the extract could be capable of β-cells recovery and/or regeneration following the destructive effects of STZ, that is known to impair glucose oxidation and decreases insulin biosynthesis and secretion, STZ also generates reactive oxygen species that contribute to DNA fragmentation and evoke other deleterious changes in β-cells [20,21].Other morphological changes noticed were increase of total volume of cell populations, which maybe due to the increase of cells numbers, increase of cellular volume, or a combination of both. It has been postulated previously[22] that the increase in total islet volume can be caused entirely by the growth of existing islets, and β-cells are primary sources for the new cells. This could be achieved by intra-islet β-cell mitosis, an event that definitely occurs based on the presence of mitotic figures in intra-islet β-cells. These new cells could be derived from intra-islet stem/progenitor cells.Ideally, there were preservation of β-cell mass together with its insulin secretory granules in diabetic rats treated with the extract, and this shall even attribute to the potentiated efficacy of the extraction. For this reason, the improved architecture of β-cells was primarily due to the remained β-cells mass protected by *P. macrocarpa* extract. These finding supports the histological report of retaining islets and few degranulations of pancreatic β-cells.

On the other hand, the increased generation of circulating reactive oxygen species and the metabolism of excessive glucose and/or free fatty acids have been identified as one of the major contributor to the deterioration of pancreatic β-cells function and structure [23,24], due to the relatively low expression of antioxidant enzymes, such as superoxide dismutase, catalase, and glutathione peroxidase in the pancreas [25]. The pancreatic β-cells may be highly vulnerable to damage when exposed to oxidative stress, for this reason, oxidative stress may play a major role in β-cell deterioration, frequently observed in human type II diabetes mellitus [26]. Therefore, the mechanism for the suggested β-cells recovery or regeneration may be made available from *P. macrocarpa* extract treatment, and the protection of the pancreas against oxidative stress will consequently contribute to its hypoglycemic effect.

Antioxidants have been suggested to afford protection to the pancreas against oxidative stress in diabetes mellitus [27]. The abundance phytomolecules possessed within the extract were hypothesized as a potential antioxidant and may exhibit an anti-inflammatory activity. Hence, they were the candidates in the protection from progressive STZ-induced damages on β-cells of the islets, by clearing up circulating reactive oxygen species generated by STZ to destroy β-cells, and then allowing other phytochemicals of the plant to induce recovery process and regenerative activities.

CONCLUSION

The overall histological studies have demonstrated a convincing outcome of *P. macrocarpa* leaves methanol extract to have functional protective effects on the pancreatic islets of Langerhans in STZ diabetic rat models. It advocates that the protective effects of the extract may be due to the scavenging of free radicals by its antioxidant nature. Though, the exact mechanism underlying this affair still needs further validation.

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