



Original Research Article

Effect of *Pseuduvaria macrophylla* in attenuating hyperglycemia mediated oxidative stress and inflammatory response in STZ-nicotinamide induced diabetic rats by upregulating insulin secretion and glucose transporter-1, 2 and 4 proteins expression

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ABSTRACT

Pseuduvaria macrophylla (Family: Annonaceae) is commonly used as medicinal plant in Malaysia. A recent study with the *Pseuduvaria* species showed antioxidant and antidiabetic effects. This study aimed to ascertain antidiabetic potential of methanolic extract of *Pseuduvaria macrophylla* bark (PM) using streptozotocin-nicotinamide induced diabetic rat models. Various phytochemical and biochemical properties of the plant have been evaluated. The results showed that the extract has potentially normalized the elevated blood glucose levels by upregulating the insulin and C-peptide levels and alleviated oxidative stress by improving glutathione (GSH) and reducing lipid peroxidation (LPO) in the diabetic rats. In addition, PM has drastically downregulated the levels of pro-inflammatory cytokines and transforming growth factor beta-1 (TGF- β 1). Histopathological examination of the pancreas in PM treated diabetic rats showed significant recovery of the pancreatic structural degeneration and thus reflected the protective role of PM against peroxidation damage by a rise in insulin level as evidenced by the immunohistochemistry study. The improved expressions of GLUT-1, GLUT-2 and GLUT-4 further confirmed the restoration of β -cell mass by PM. Interestingly, the findings demonstrated the antioxidant, anti-inflammatory and antihyperglycemic potential of PM which may provide future lead for the management of type-2 diabetes.

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Introduction

Type 2 diabetes mellitus (T2DM) is a combination of resistance to insulin action and an inadequate compensatory insulin secretory response (Genuth et al., 2003). In addition, it also compromised metabolism of carbohydrates, proteins and fats. The release of free radicals associated with T2DM consist of reactive oxygen or nitrogen species, which play crucial role in developing

diabetes and eventually causes damage to the multiple organs leading to diabetic complications (Arya et al., 2012a). Therefore, interference in oxidative stress and inflammatory response pathways has been highlighted as an important means of preventing diabetes and diabetes related complications (Dandona et al., 2004). Synthetic hypoglycemic agents are currently in clinical practice, but due to its side effects, scientists have started looking for alternatives from the nature. A large proportion of people around the globe rely on traditional medicines especially herbal medicine and it has been showed that few naturally existing products demonstrate significant antidiabetic activity by stimulating β -cell and then secrete insulin and recover insulin sensitivity (Lombardo

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and Chicco, 2006). Thus, it is essential to investigate the mechanism involved in the antidiabetic activity of the natural plant products that have been used by traditional healers for the management of T2DM.

Many plant species are used for the treatment of diabetes attributing to the antioxidant, anti-inflammatory and antihyperglycemic nature. In addition, it has been observed that the presence of flavonoids, phenolics, alkaloids and terpenoids are the key components responsible for the biological activities (Taha et al., 2014). One such plant is *Pseuduvaria macrophylla* (PM) which belongs to the Annonaceae family and locally known as “*cagau biasa*” in Malaysia. The antioxidant and antimicrobial properties of the crude extract of the plant were reported previously (Aziz et al., 2016). The indigenous peoples of Malaysia (orang asli) have been using this plant for the treatment of cough and management of fever but little is known about its antidiabetic effect due to lack of documentation. In Southern Thailand, it is considered as medicinal plant known as Kloi khang or Phrik nok, and the woody part of the plant is used for treating nausea and vomiting (Chuakul et al., 2004). Plants from this family and species have been reported to exhibit various interesting pharmacological activities due to the presence of different types of constituents such as alkaloids, flavonoids and polyphenols (Taha et al., 2011).

However, there is not much scientific evidence of extensive chemical or biological studies on *Pseuduvaria macrophylla*. Recently Taha et al. (2014) have found that *Pseuduvaria monticola*, a plant from the same genus has antioxidant and antidiabetic potential. One of the mechanisms of medicinal plants and their phytochemicals for the treatment of diabetes is the capacity to enhance the antioxidant system (Vinayagam and Xu, 2015). In fact, most of medicinal plants with anti-diabetic property possess antioxidant activity (Hulbert et al., 2005; Nasri et al., 2015; Rafieian-Kopaei and Nasri, 2012). Since *Pseuduvaria macrophylla* has shown anti-oxidant property before and it is a plant from the same genus which has anti-diabetic potential, it is worth to investigate its antihyperglycemic effects. In our best knowledge there was no *in vivo* antihyperglycemic study reported or documented previously on the bark methanolic extract of *Pseuduvaria macrophylla*. By keeping the aforesaid points in view, the current study aims to investigate the antioxidant, anti-inflammatory and antihyperglycemic potential of PM on STZ-Nicotinamide induced diabetic rats and the β -cell protective effect of the extract by determining the pancreatic proteins.

Materials and methods

Chemicals

Glibenclamide, Streptozotocin (STZ) and Nicotinamide were obtained from Sigma-Aldrich, USA.

Collection of *Pseuduvaria macrophylla*

Dried stem bark of *Pseuduvaria macrophylla* (1 kg) were collected from Kenong Forest Park, Malaysia. Identification and authentication of specimen was established by the Department of Chemistry, Faculty of Science, University of Malaya. A voucher specimen (PM/M2114) had been deposited at the department herbarium, University of Malaya, 50,603.

Extraction of *Pseuduvaria macrophylla*

The dried stem bark of *Pseuduvaria macrophylla* (500 g) were finely powdered using blender and extracted with Soxhlet extractor for 48 h at 43 °C with 100% n-hexane. Then, the obtained residue was extracted again with methanol and chloroform. To

collect the pure extract of *Pseuduvaria macrophylla*, each crude portion was dried at 40 °C by BUCHI-R-215 (Rotary Evaporator) with reduced pressure. The extracts were preserved in –20 °C refrigerator until used.

Phytochemical characterization of *Pseuduvaria macrophylla*

The phytochemical constituents of PM were analyzed by Liquid Chromatography Mass Spectrometry (LCMS-QTOF, AB Sciex TripleTOF 4600) equipped with HPLC (Spark Holland Symbiosis PICO) system with a C18 Kinetex reverse phase column (2.6 μ m, 100 \times 3.0 mm, Phenomenex, USA). The solvent system consisted of a mixture of 0.1% formic acid in water (Solvent A) and acetonitrile with 0.1% formic acid (Solvent B), which was used as mobile phase by eluting at a flow rate of 0.2 ml/min with an injection volume of 10 μ l, from 10% to 100% gradient system. The complete scans of the mass spectra were recorded from 50 *m/z* to 1000 *m/z* and data was processed by Analyst Software. The wavelengths used were 210 nm and 350 nm. 1 mg/ml of PM methanol extract was fractionated. 15 fractions were collected and pooled together based on their TLC pattern. The presence of alkaloids was detected by exposure to Dragendorff's reagent. The structures of the isolated compounds were identified by combination of MS spectroscopic techniques, UV, ¹H-NMR, ¹³C-NMR (JEOL ECA 500, Japan). For NMR analysis, approximately 5 mg of dried isolated compound was dissolved in a 2 ml deuterated solvent (deuterated methanol or chloroform) and pipetted into NMR tube (5 mm in diameter). Then the NMR tube was inserted into a probe and analyzed for 5 min for nuclear magnetic field.

Experimental animals and dosing

Sprague Dawley rats (250–280 g) were obtained from the Experimental Animal House, Faculty of Medicine, University of Malaya. The rats were maintained in wire-bottom polypropylene cages under pathogen-free and light controlled (12 h light/dark cycle) room at a temperature (25 \pm 2 °C) with 35–60% humidity and administered with standard rat pellet diet and distilled water. All the experiments were performed according to the institutional ethical guidelines after obtaining an ethical approval (2014-07-01/ PHARM/R/NS). Animals were divided into 5 groups (n=6) as follows; group-1 administered only with distilled water (Normal control, NC); group 2 were treated with STZ-Nicotinamide to induce diabetes (Diabetic Control, DC); group 3 were kept as diabetic rats with STZ-Nicotinamide treatment and 200 mg/kg PM (PMa); group 4 were kept as diabetic rats with STZ-Nicotinamide treatment and 400 mg/kg PM (PMb); and group 5 were kept as diabetic rats with STZ-Nicotinamide treatment and Glibenclamide (2.5 mg/kg) (Positive Control, PC).

The treated rat groups were orally administered with PM (200 mg/kg and 400 mg/kg) and Glibenclamide (2.5 mg/kg) dissolved in distilled water, once a day by intra-gastric tube. After 24 h of last treatment dose, all the experimental rats were sacrificed.

Toxicity study

In line with the OECD (Organisation for Economic Co-Operation and Development) guidelines, the acute toxicity tests were performed on PM treated groups (PMa and PMb) with normal rats. After overnight fasting, the rats were orally administered with three different doses of PM (500, 1000 and 3000 mg/kg body weight) and the normal control group was administered with distilled water alone. Next 2 h, all the rats were continually observed for any abnormalities in behavioral, anatomical and neurological features. This monitoring was further continued until next 24 and 72 h (Turner, 2013).

Induction of T2DM by streptozotocin-nicotinamide

For the purpose of inducing T2DM, the normal rats were fasted overnight (15 h) and then a single dose of nicotinamide 210 mg/kg body weight (bw) was injected intraperitoneally. After 15 min, 1 ml/kg bw of freshly prepared STZ (55 mg/kg bw) in citrate buffer (0.1 M, pH 4.5) was administered intraperitoneally. Type 2 diabetes was confirmed in rats after 72 h of STZ-Nicotinamide injection by measuring the blood glucose levels (Considered as T2DM > 16.7 mmol/l) (Arya et al., 2012b).

Blood samples collection

After 45 days of the treatment period, all the rats were anesthetized by Ketamine 50 mg/kg and Xylazine 5 mg/kg combination and collected 3 ml of blood samples from the inner canthus of the rat's eye. Serum preparation was carried out at ambient temperature by allowing the blood samples to clot in vacutainer tube for 15–30 min. Then, the samples were centrifuged in a refrigerated centrifuge at 4000 rpm for 10 min to collect the serum, which was stored at -80°C for further use.

Biochemical assessment

The body weight and fasting blood sugar of all the rats were measured on 7, 21 and 45th days. The glucometer (Accu-Check Nano Performa) was used to determine the blood glucose level and ELISA Assay (589501, Cayman, USA & Rat ELISA Kit K4757, Bio Vision Corporation, USA) was performed following manufacturer's protocol to quantify the Serum Insulin and C-peptide levels in each group of rats.

Antioxidant parameters

Serum glutathione (GSH) and lipid peroxidation (LPO) levels of diabetic rats were measured using GSH and LPO assay kits according to the manufacturer instruction (Cayman Chemical, USA, 703002, 705,003).

Transforming growth factor-beta 1 (TGF- β 1) assessment

At first, the collected serum samples from experimental rat models were frozen at -20°C before starting the analysis of determining TGF- β 1 using ELISA kit (TGF- β 1 Rat ELISA Kit, ab119558) following manufacturer's protocol. Activation of each 50 μl of the sample was initiated by incubating it with 1 μl of 1.0 N HCl at room temperature for 15 min and then neutralization was done by 1 μl of 1 N NaOH to further activate the serum TGF- β 1 until it reaches the immunoreactive form (Rajavel et al., 2012). Serum TGF- β 1 is expressed as total amount excreted in pg/ml.

Pro-inflammatory cytokines assessment

The levels of pro-inflammatory cytokines (TNF- α , IL-1 β and IL-6) were measured in serum using Rat TNF- α ELISA Kit (Item no: ab46070), Rat IL-1 β ELISA Kit (Item no: ab100767) and Rat IL-6 ELISA Kit (Item no: ab100772) according to the manufacturer protocol (Abcam, USA).

Histology

At the end of the *in vivo* experiments, the animals were scarified and rat's pancreas was washed properly with normal saline. The tissues samples were then preserved for a week in 10% formalin at room temperature. Later the samples were sliced to 5 μm diameters in size, and the tissue sections were stained with

hematoxylin and eosin (H&E) for microscopic examinations after gradual rehydration in a series of graded alcohols.

Immunohistochemistry

Pancreas were fixed in formaldehyde, embedded in paraffin, and sectioned (5 μm thickness). The dewaxed sections were rehydrated with tris-buffered saline (TBS) in a 3% hydrogen peroxide-methanol solution for 30 min to block endogenous peroxidase activity, followed by 30 min incubation at 90°C . Immunolabeling of insulin was performed with a 1:500 dilution of goat-polyclonal-Insulin-A (sc-7839; Santa Cruz,) antibody and slides were incubated for 2 h at 37°C and subsequently rinsed three times with TBS for 3 min per wash. Further, slides were incubated with peroxidase-conjugated protein A (1:100) for 1 h at room temperature (25°C) and washed again with 3,3-diaminobenzidine tetrahydrochloride (DAB Chromogen Kit, Biocare Medical, Concord, USA) for imaging. Next, slides were counterstained with Mayer's hematoxylin and mounted. Images were obtained using a light microscope plus camera (Eclipse 50i, Nikon, Tokyo, Japan) at a magnification of 40 \times .

GLUT-1, -2 and -4 analysis by enzyme linked immunosorbent (ELISA) assay

ELISA kit for Glucose Transporter 1 (GLUT1), Glucose Transporter 2 (GLUT2) and Glucose Transporter 4 (GLUT4) were obtained from USCNLIFE Science Inc, (Wuhan, China). Rat's pancreatic sample were rinsed in ice-cold PBS to remove excess blood thoroughly and weighed before homogenization. Minced the tissues to small pieces and homogenized them in 5–10 ml of PBS with a glass homogenizer on ice. The resulting suspension was sonicated with an ultrasonic cell disrupter. After that, the homogenates were centrifuged for 5 min at 5000 $\times g$. The supernatant was collected and the protein was measured and used for the assay according to the protocol of manufacturer. The concentrations of target proteins were determined at 450 nm immediately in a microplate reader. The optical density of treatment was compared with the optical density of control and the value was expressed as percentage difference compared to control.

Statistical analysis

All Values were reported as mean \pm SD. The Statistical significance of differences between groups was assessed using one-way ANOVA followed by post hoc Tukey's multiple comparison tests. A Value of $P < 0.05$ was considered significant.

Results

Pseuduvaria macrophylla extract yield

Extract yield of *Pseuduvaria macrophylla* stem bark was 2 g for hexane (PH), 3.11 g for chloroform (PC) and 152.35 g for methanol (PM). According to preliminary screening of all extracts, highest percentage yield of *Pseuduvaria macrophylla* (methanolic extract) showed hypoglycemic effects on different biochemical assays (data not shown). Therefore, methanolic extract was chosen for further studies.

LCMS-QTOF and NMR analysis

The characterisation of the chemical compounds in PM was achieved through LCMS-QTOF and NMR. In this experiment, five major compounds were detected from PM (Fig. 1A). Phytochemical

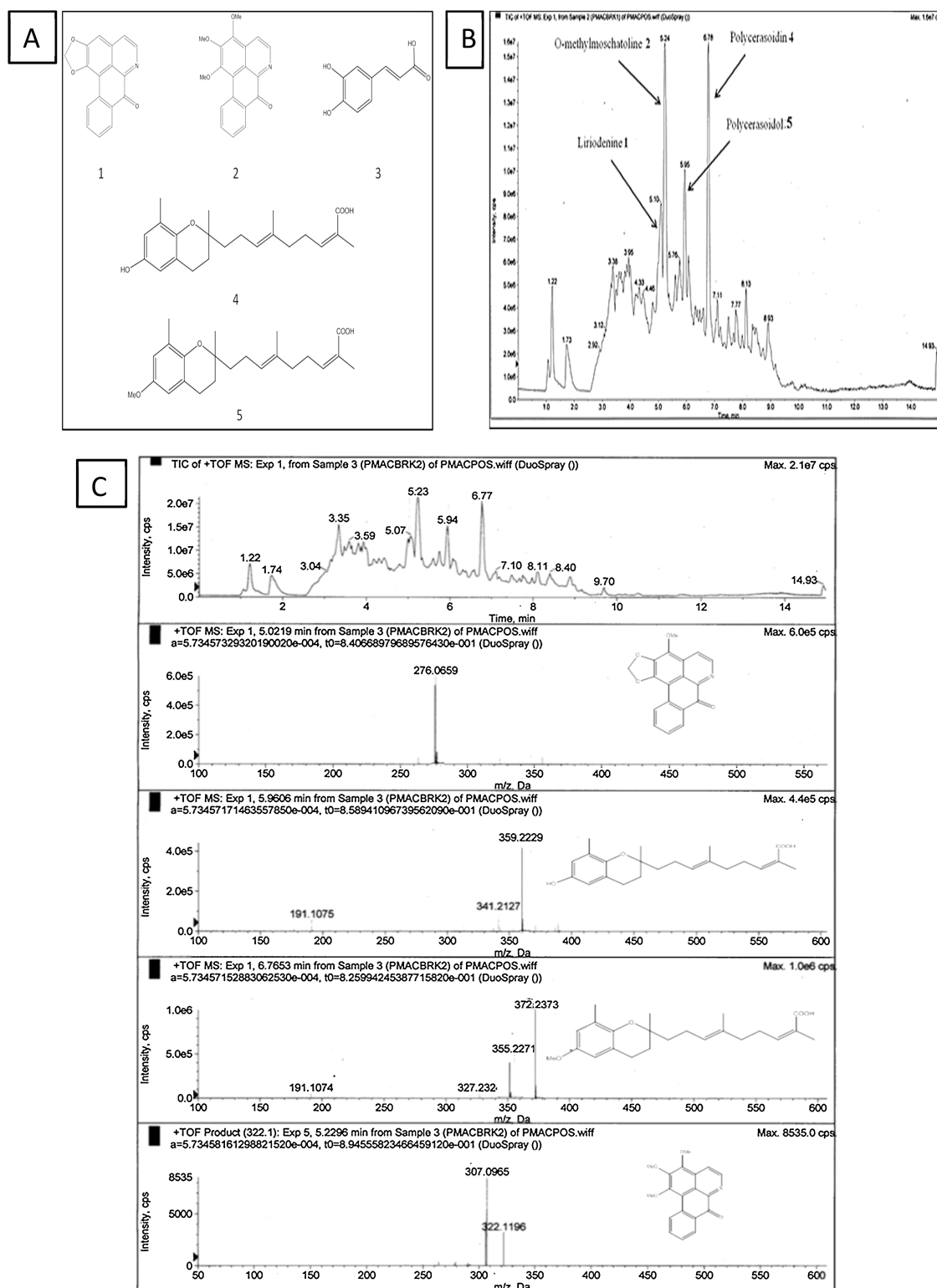


Fig. 1. Major compounds of *Pseuduvaria macrophylla* barks were identified. (A) Molecular structures of five major compounds that were identified and isolated; (B) LCMS-QTOF chromatograms showing four major identified compounds detected in positive ion mode, liriodenine 1, O-methylmoschatoline 2, polycerasoidin 4 and polycerasoidin 5; (C) LCMS-QTOF chromatograms of major peaks detected in negative mode showing caffeic acid 3, polycerasoidin 5 and polycerasoidin 4.

analysis in positive ion mode by LCMS-QTOF has revealed the presence of two oxoaporphine alkaloids such as liriodenine 1 ($[M + H]^+$ ion at m/z 276), O-methylmoschatoline 2 ($[M + H]^+$ ion at m/z 322); and two benzopyran derivatives such as polycerasoidin 4 ($[M + H]^+$ ion at m/z 359 and $[M + H]^+$ ion at m/z 357) and polycerasoidin 5 ($[M + H]^+$ ion at m/z 373 and $[M + H]^+$ ion at m/z 371) based on

mass fragmentations pattern by comparison with reported MS data (Fig. 1B). A second run in negative mode shows the three major peaks tentatively identified as caffeic acid 3 ($[M + H]^-$ ion at m/z 179), polycerasoidin 4 and polycerasoidin 5 (Fig. 1C). All the structures of isolated compounds were confirmed by 1H and ^{13}C NMR experiment and by comparison with those of authentic data.

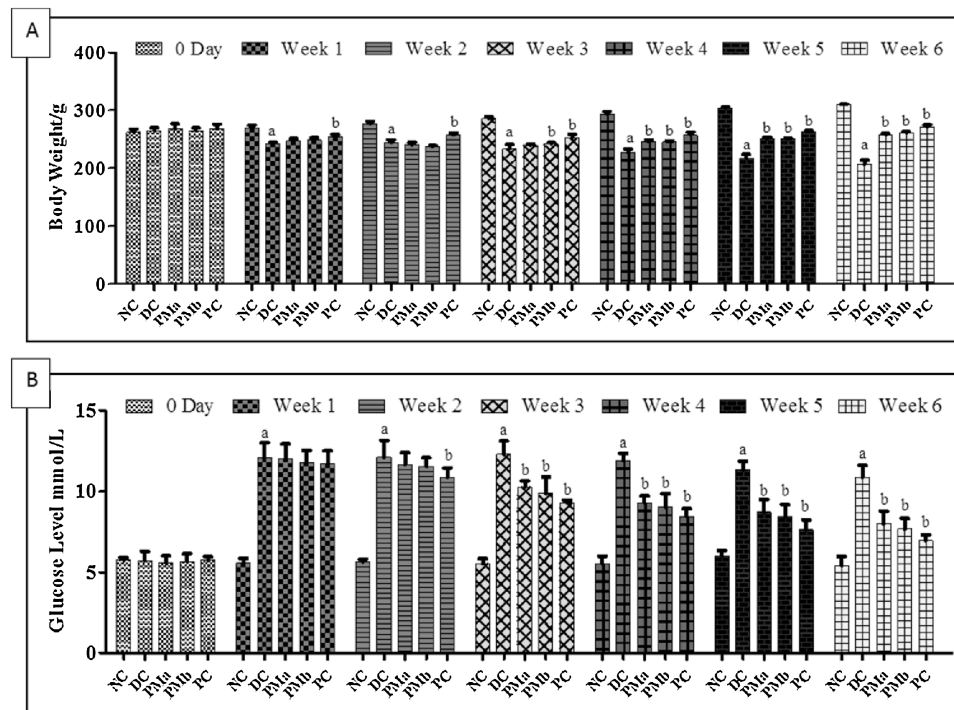


Fig. 2. Effects of PM extract on body weight (A) and glucose level (B) in normal and diabetic rats. All the values are expressed as the mean \pm SD. 'a' indicates the mean difference is significant when compared to the NC group ($P < 0.05$). 'b' indicates the mean difference is significant when compared to the DC group ($P < 0.05$).

Acute toxicity study

Non-toxic nature of PM was represented by acute toxicity study, as no mortality was observed at minimum, medium and maximum doses after 24 and 72 h of treatment. There were no significant changes in the body weight and food consumption against the normal control group.

Effect of PM on body weight

Fig. 2A represents the body weight of the treated groups compared to that of the diabetic control group ($P < 0.05$). There was a significant decrease in the body weight of DC group compared to NC group ($P < 0.05$). During the first two weeks, there were not much changes in body weight against DC group and the change started to show on week 3. However, from week 4, the PMA and PMb treated diabetic rat groups showed significant increase in body weight compared to DC group ($P < 0.05$).

Effect of PM on blood glucose levels

The potential of PM in regulating the blood glucose levels of normal and diabetic rats is demonstrated in Fig. 2B. During the first week of experiment, the blood glucose level was considerably increased ($P < 0.05$) in DC group; whereas, from the second week after treatment, the blood glucose levels was found to be reduced in PMb treated diabetic rats ($P < 0.05$) against DC group. It is noteworthy that, from the third week to six week onwards, both PMA and PMb had considerably decreased the blood glucose level ($P < 0.05$) compared to the untreated diabetic rats (DC group).

Effect of PM on biochemical parameters

The effect of PM on serum insulin levels in diabetic and normal rats are illustrated in Fig. 3A. Without treatment, the serum insulin level was found to be elevated in DC group compared to NC group

at week 2 and 6 ($P < 0.05$). However, during 2nd and 6th weeks, the PMA and PMb treated diabetic rats have shown significant rise in serum insulin levels compared to DC group. On the other hand, Fig. 3B has displayed the effect of PM on C-peptide levels in the serum of normal and treated diabetic rats. Like insulin, the C-peptide level was reduced in DC group during 2nd and 6th weeks when compared to NC group ($P < 0.05$). An improvement in C-peptide level was observed in 2nd week in PMb treated diabetic rats ($P < 0.05$) and from week 6, the PMA and PMb treated diabetic rats showed significant rise in C-peptide levels against the DC group indicating that PM improves glycemic control dose-dependently ($P < 0.05$).

Effect of PM on antioxidant status

Fig. 3 illustrates the antioxidant properties of PM by determining the GSH and LPO levels in normal and diabetic rats, treated with PMA and PMb at week 2 and 6. There was a significant fall of GSH level in DC group when compared with NC group ($P < 0.05$). However, the GSH level was improved in the PMA and PMb treated diabetic rats in 2nd and 6th weeks ($P < 0.05$). The effect of the extract was found to be higher in week 6 compared to previous weeks (Fig. 3C). In contrary to GSH, (Fig. 3D) demonstrates a marked rise in the LPO level in DC group against NC group ($P < 0.05$). Treatment with PMb resulted in decline of LPO level at week 2 compared to DC group. In addition, at week 6, both PMA and PMb significantly inhibited the LPO expression level against the DC group ($P < 0.05$).

Effect of PM on TGF- β 1 level

Fig. 3E reflects a certain increase in the oxidative stress markers (LPO) and TGF- β 1 concentrations after diabetes induction in serum of DC rats compared to NC at 2nd and 6th week. Treatment with PMA and PMb did not show any significant changes in serum TGF- β 1 concentrations for two weeks compared to DC rats

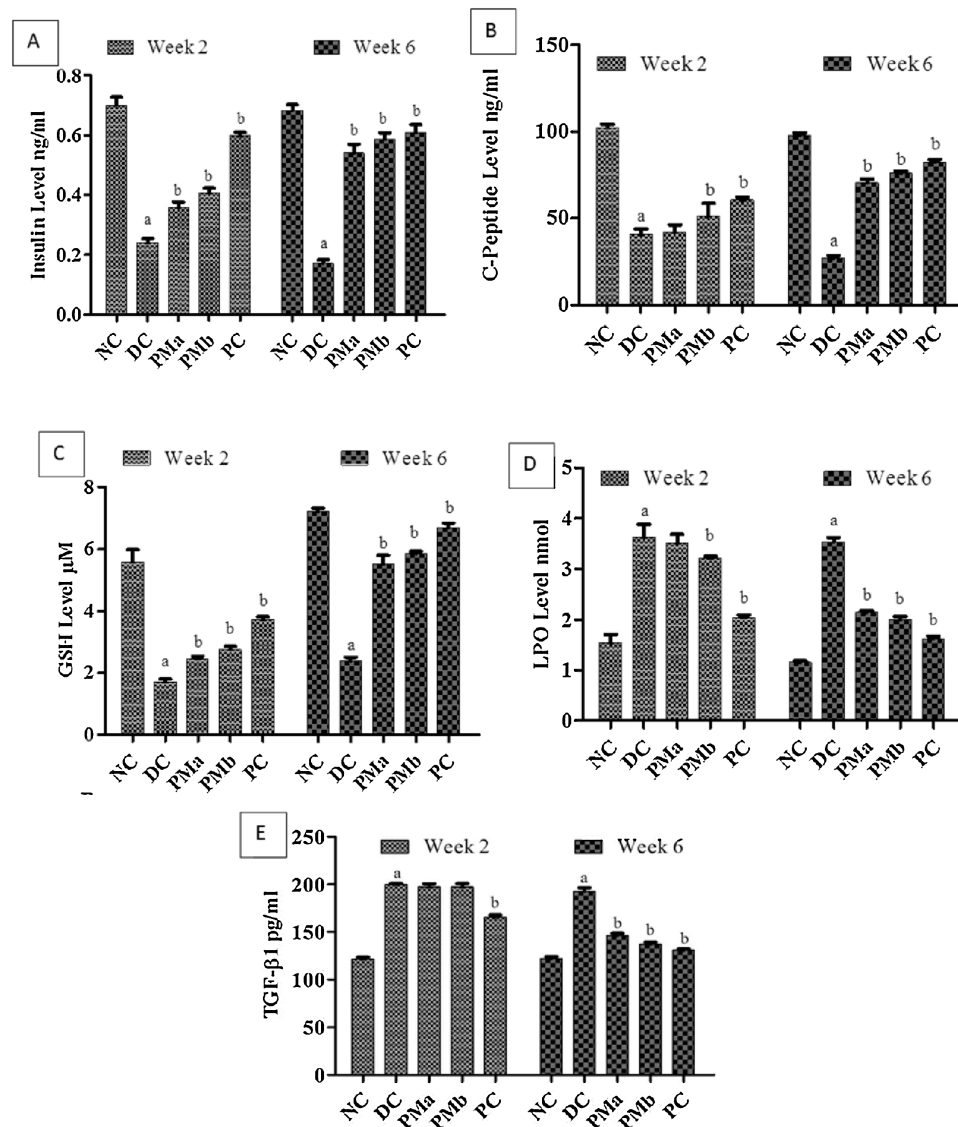


Fig. 3. Effects of PM extract on Insulin (A), C-peptide (B), GSH (C), LPO (D) and TGF-β1 (E) levels in normal and diabetic rats at 2nd and 6th weeks. All the values are expressed as the mean ± SD. 'a' indicates the mean difference is significant when compared to the NC group ($P < 0.05$). 'b' Indicates the mean difference is significant when compared to the DC group ($P < 0.05$).

($P < 0.05$). On the other hand, when both the doses of PM were administered for an extended period i.e. 6 weeks, the concentration of TGF-β1 was found to be decreased against DC rats.

Effect of PM on pro-inflammatory cytokines

Fig. 4 illustrates the effect of two doses of PM on pro-inflammatory cytokines i.e. TNF-α, IL-β1 and IL-6 in normal and diabetic rats. Higher pro-inflammatory cytokines concentrations were detected in DC group compared to NC group during 2nd and 6th weeks ($P < 0.05$). Nevertheless, the administration of PMb had significantly reduced the pro-inflammatory cytokines at week 2 compared to PMa against the DC group ($P < 0.05$). In addition, the level of pro-inflammatory cytokines was completely reduced near to normal level in PMa and PMb treated diabetic rats at week 6 as opposed to DC group ($P < 0.05$).

Effect of PM on pancreas tissue

Fig. 5 represents the impact of PM on pancreas structure in normal and diabetic rats. The pancreas sections were stained with

hematoxylin and eosin and were determined after 45 days of treatment. Panel (A) pancreatic section showed normal islets of Langerhans of pancreatic structure. Panel (B) STZ-Nicotinamide treatment elicited severe injury in pancreatic β-cells, such as, cell damage and necrotic changes. Panel (C) STZ-Nicotinamide induced rats treated with PMa, reflected gradual restoration of pancreatic endocrine cells, degeneration of the pancreatic acini was still observed and moderate expansion of pancreatic islets. Panel (D), treated with PMb had shown reduction in vacuolation and normal architecture of islet cells, such lesions were considerably reduced. After 6 weeks of glibenclamide treatment period, panel (E), demonstrated the most interesting aspect of the examined pancreatic sections by showing normal Langerhans islets, absence of dilation and prominent hyperplastic of islets.

Effect of PM on insulin expression level

Fig. 6 illustrates the changes in insulin expression level in normal and diabetic rats treated with PM. The DC group showed reduced expression of insulin compared to NC group (Fig. 6A–D). However, the insulin expression level was upregulated in PMa and

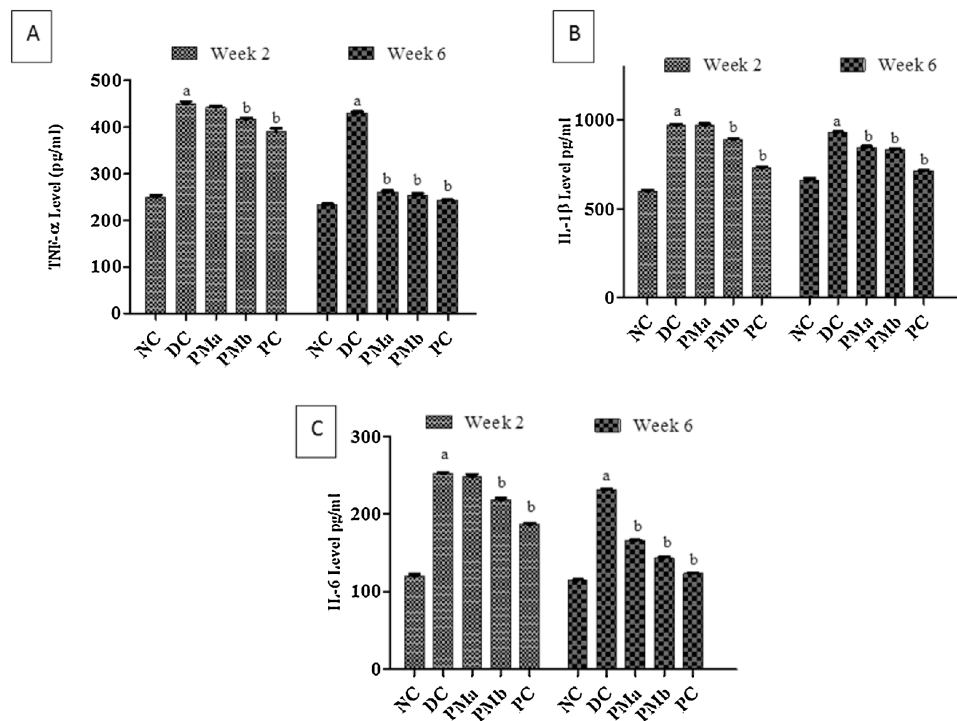


Fig. 4. Effects of PM extract on pro-inflammatory cytokines TNF- α (A), IL-1 β (B) and IL-6 (C) concentrations in normal and diabetic rats at 2nd and 6th week. All the values are expressed as the mean \pm SD. 'a' indicates the mean difference is significant when compared to the NC group ($P < 0.05$). 'b' Indicates the mean difference is significant when compared to the DC group ($P < 0.05$).

PMb treated diabetic rats against the DC group. The results were confirmed by histomorphometric study (Fig. 6E). The DC group showed significant reduction in the insulin expression level against NC group ($P < 0.05$). Nevertheless, both PMa and PMb displayed higher insulin expression concentrations when compared to DC group ($P < 0.05$).

Enzyme linked immunosorbent (ELISA) assay

Fig. 7 demonstrates the expression level of three glucose transporter proteins (GLUT-1, GLUT-2 and GLUT-4) inside the pancreas tissue in normal, PMa and PMb treated diabetic rats. The results revealed that, DC group showed significant decrease of all these proteins against NC group ($P < 0.05$). Conversely, GLUT-1, GLUT-2 and GLUT-4 were significantly increased in the PMa and PMb treated groups compared to DC group ($P < 0.05$).

Discussion

In spite of the tremendous use of oral hypoglycemic agents, plant products play an important role in the management of diabetes although few have been scientifically claimed. The existence of bioactive chemical components or phytochemicals with interesting therapeutic effects in the *Pseudeuvaria* species of the Annonaceae family has led researchers to investigate different aspects of the pharmacological properties that this species possesses. The current study assessed the antihyperglycemic effect of the bark methanolic extract of *Pseudeuvaria macrophylla* (PM) (200 and 400 mg/kg) using STZ-nicotinamide induced diabetic rats in view of the supporting data from the antioxidant property of this extract. Our findings have shown that destruction of the pancreas caused by STZ-nicotinamide was markedly reduced in diabetic rats after receiving PM treatment. This result could be due to the antioxidant, anti-inflammatory, and antihyperglycemic potential of the extract. The major active constituents, such as, alkaloids, polyphenols, and caffeic acid,

which were identified in PM by LCMS-QTOF and NMR after the acute toxicity studies, might have contributed to the downregulation of oxidative stress and inflammatory response in the pancreas during the course of diabetes, and, eventually, recovered the pancreatic degeneration by upregulating the insulin and GLUT protein expressions in the pancreas.

When the biochemical parameters of the diabetic rats were analyzed in this study, we observed a drastic rise in the fasting blood glucose levels accompanied by reduced insulin and C-peptide levels. However, these alterations were reversed significantly by the oral administration of PM dose-dependently because the PMb (400 mg/kg) showed a higher reduction in the blood glucose levels and improved secretion of insulin and C-peptide levels compared to PMa (200 mg/kg). The reason for the recovery of normal blood glucose levels in the PM treated diabetic rats could be directly attributable to the upregulated insulin and C-peptide levels in our study. It is well understood that insulin is the only pancreatic β -cell hormone known to reduce blood glucose levels (Brezar et al., 2011), and the C-peptide released from the β cell is usually used as a marker for the β cell function and an index for insulin secretion (Palmer et al., 2004). The elevated insulin is responsible for bringing the serum and tissue proteins to a normal level by reducing protein catabolism and thus enhancing its synthesis (Arya et al., 2012b). In addition to the antihyperglycemic activity, PM was also able to improve the recovery of normal body weight in diabetic rats due to its similar effect to insulin. The breakdown of structural proteins that usually contribute to body weight due to insulin deficiency leads to a reduction in body weight (Ramesh and Pugalendi, 2006). The observed PM findings of improved insulin and C-peptide levels resemble the reported results of polyphenolic-rich nutraceuticals, quercetin, and quinic acid from *Pseudeuvaria monticola* (Taha et al., 2014).

The elevated serum insulin and C-peptide levels represent the regeneration of the β -cell function that is associated with the

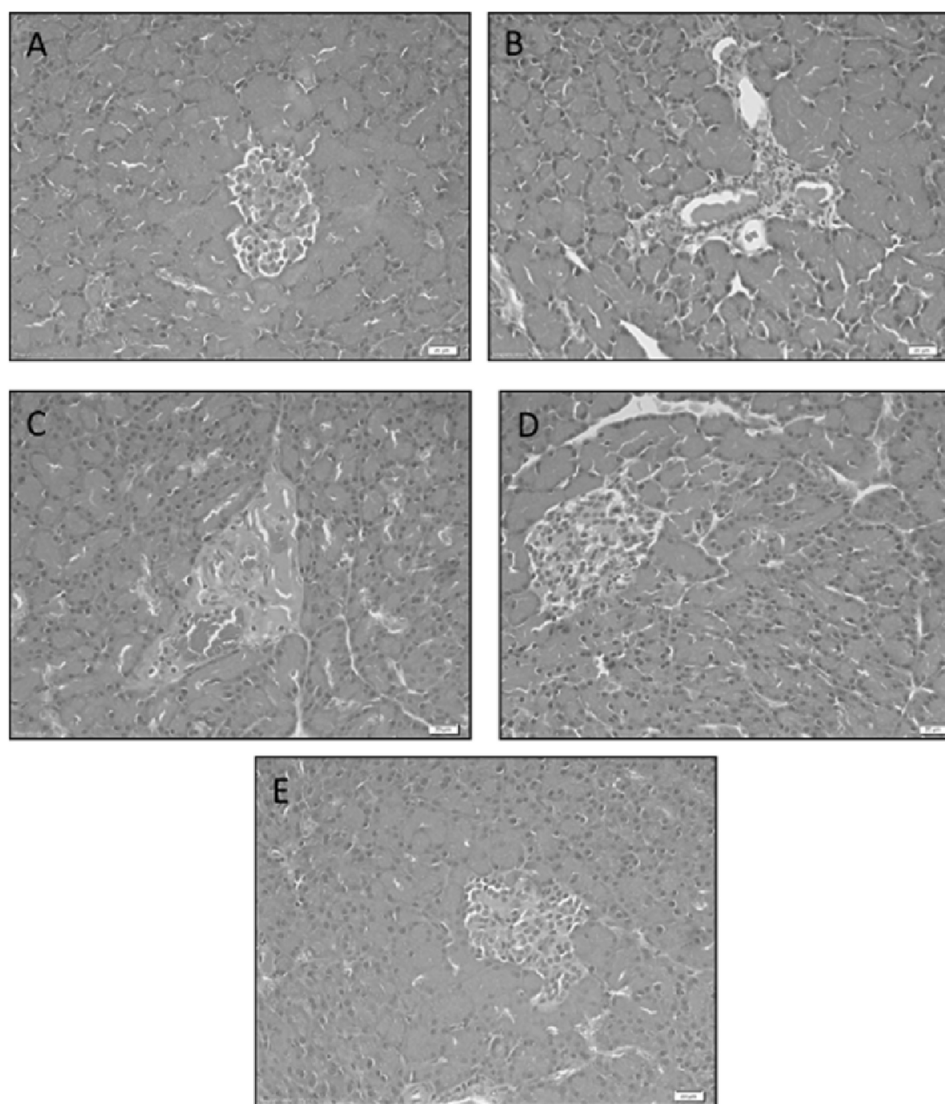


Fig. 5. H & E staining of diabetic rat pancreas (400 x). (A): NC (B) DC (C) PMa (D) PMb (E) PC.

antioxidative and free radical scavenging potential of the extract, as demonstrated in our study by a reduction in the lipid peroxidation level (LPO), oxidative stress markers (TGF- β 1), and an increase in the antioxidant enzyme (GSH) level. The presence of active constituents, such as caffeic acid, a polyphenolic in PM, might have contributed to the reduction of oxidative stress by downregulating the LPO level and upregulating the GSH level. The phenolic compounds, such as resveratrol, and caffeic acid are known to have a potent antioxidant and antihyperglycemic effect by ameliorating oxidative stress in type-2 diabetic mice (Chao et al., 2009; Jung et al., 2006; Ramar et al., 2012). Similarly, the excessive oxidative stress marker (TGF- β 1) in the serum of diabetic rats was potentially reduced by PM confirming its antioxidant and protective nature. The release of transforming growth factor- β 1 (TGF- β 1) by macrophages and impaired tissue between surfaces (interstitium) induced by oxidative stress leads to diabetic glomerular injury (Klahr and Morrissey, 2000), which produces swelling of the neighboring tissue, or a common inflammation, with elevated pro-inflammatory cytokines (Trojanowska, 2012). Thus, our results are in parallel with the study conducted by Rajavel et al. (2012), who demonstrated that the inhibition of TGF- β 1 in palm oil administered diabetic rats was due to the high level of phenolic compounds present in the oil. Moreover, in line with

our antioxidant study, the phenolic compounds identified in the extract have been shown to suppress the secretion of pro-inflammatory cytokines detected in the serum of diabetic rats treated with PM. These associate and correlate the activation of many transcription factors including NF- κ B and pro-inflammatory cytokines (TNF- α , IL-1 β , IL-6) expression generated by T cells or macrophages (Reuter et al., 2010). Based on these findings, it can be suggested that the ability of PM to inhibit oxidative stress might lead to a reduction in pro-inflammatory cytokines. Similarly, besides being antihyperglycemic, the caffeic acid played a protective role in attenuating diabetes-related kidney disease by reducing TNF- α , IL-1 β and IL-6 dose-dependently, thereby supporting our study and indicating that these active components existing in our extract are most likely responsible for the documented effect of this plant extract (Chao et al., 2009).

The impaired state of β -cell degeneration in diabetic rats is caused by the chronic oxidative stress and inflammatory response within the mitochondria due to glucose toxicity and lipotoxicity (Montane et al., 2014). Similarly, our histopathology analysis displayed degranulation of β -cells followed by the damaged structural integrity of the cells, reduction in islet numbers, and necrotic changes in the diabetic rats. Interestingly, the β -cell mass was effectively preserved, and a normal architecture of the islet

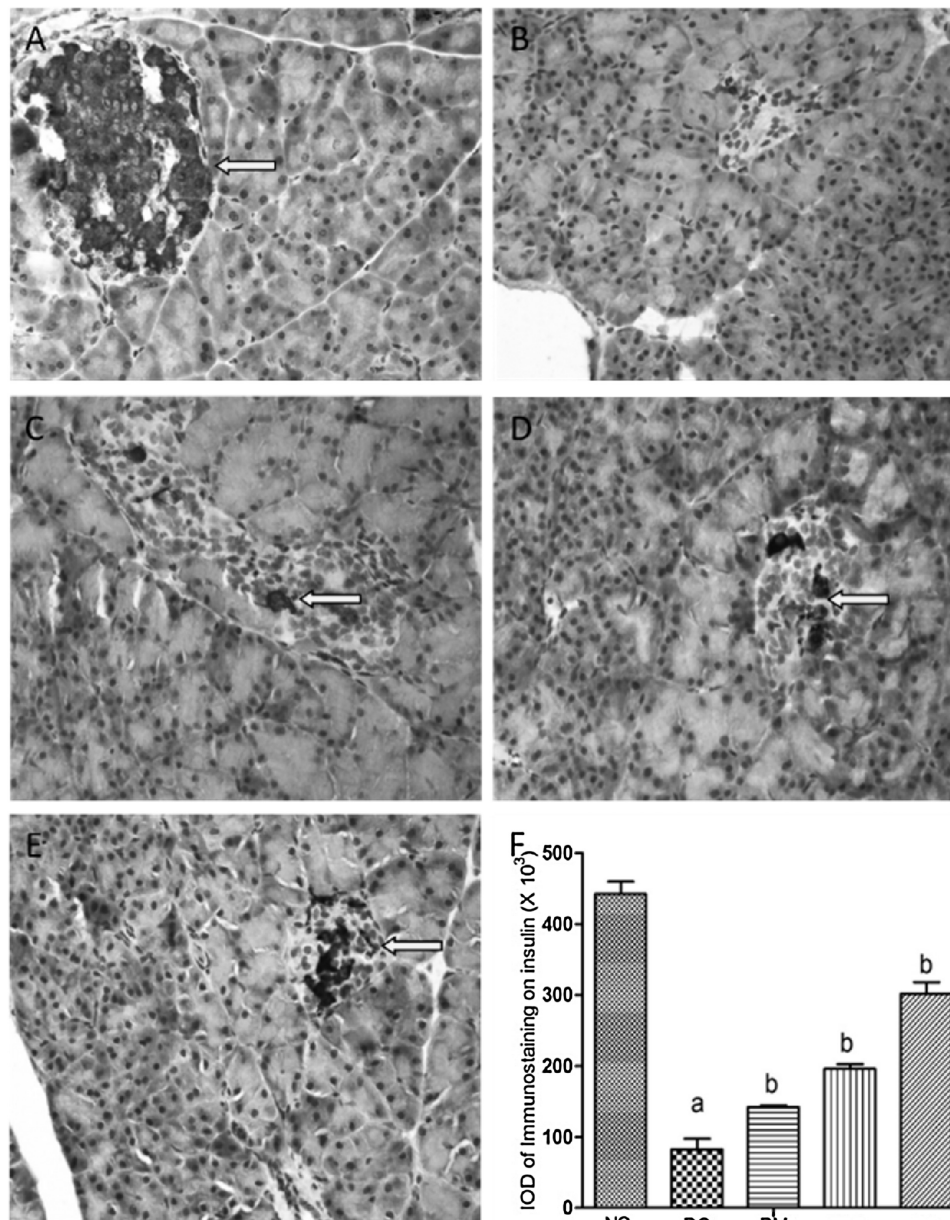


Fig. 6. Effect of PM on insulin expression level in normal and diabetic pancreas rats (400 x) A: NC; B: DC; C: PMa; D: PMb and E: PC. (E) Insulin expression level were quantified by Image J analysis software as mean \pm SD and analyzed by one-way ANOVA (Tucky), 'a' indicates significant difference $P < 0.05$ from NC ($P < 0.05$). 'b' indicates significant difference $P < 0.05$ from DC ($P < 0.05$).

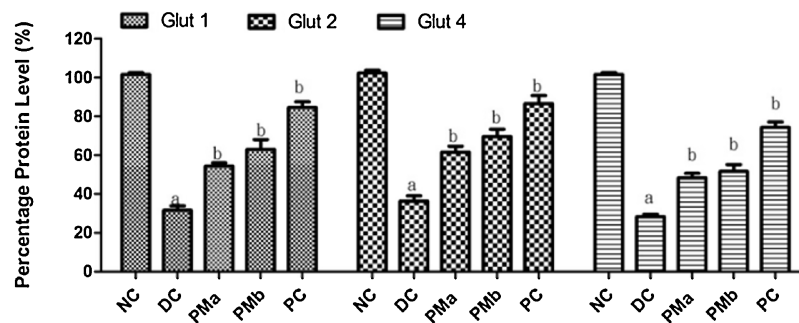


Fig. 7. Pancreas tissue prepared and subjected to enzyme linked immunosorbent (ELISA) assay using an antibody specific for GLUT-1, GLUT-2 and GLUT-4. The percentage protein level was measure and presented as mean \pm SD (B). 'a' indicates significant differences compared with sample of NC ($P < 0.05$). 'b' indicates significant differences compared with sample of DC ($P < 0.05$).

cells was observed with a fairly higher surface area and reduced lesions in the PM treated groups. The present findings are in accordance with the results obtained from previous studies on the caffeic acid and alkaloids co-existing in our extract, where the pancreas histology was restored by reducing the glucose and increasing the insulin level (Gandhi et al., 2011; Karthikesan et al., 2010). The immunohistochemical observation of islets in the present data confirmed the apparent β -cell regeneration in the PM treated diabetic rats by showing increased insulin-immunoreactive expression. The pancreatic-cell disorder associated with insulin resistance is one of the symptoms of type 2 diabetes, which can be recovered by escalating the secretion of insulin (Robertson et al., 1992). Therefore, PM had restored the pancreas histology by alleviating oxidative stress and inflammation resulting in improved insulin secretion from the regenerated β -cell, which is in tune with the antidiabetic study conducted by Chung and Shin (2007).

The restoration of β -cell mass and improved insulin sensitivity in the study was further assessed by means of an enzyme-linked immunosorbent (ELISA) assay, as illustrated by the upregulated expression of GLUT-1, GLUT-2, and GLUT-4 proteins, which is attributed to the insulin-mediated glucose uptake and thereby reduces the blood glucose levels. The enhanced expression of pancreatic insulin from the existing beta cells of islets is responsible for the upregulation of these GLUT proteins, as discussed in the study of Hajiaghaalipour et al. (2015). The PM enriched with alkaloids, flavonoid, and polyphenolic compounds could have caused this elevation of glucose transporter proteins by enhancing the insulin signaling cascade. Our results are in agreement with the study of Chang et al. (2016) and Ong et al. (2011) who stated that alkaloids and phenolic compounds demonstrated an antihyperglycemic effect in STZ-induced diabetic rats by increasing the GLUT-4 expression and thereby enhancing the glucose uptake and insulin-stimulated glucose consumption. Also, caffeic acid has been shown to enhance the GLUT-2 expression in TNF- α induced insulin resistance in mouse liver (FLB83B) cells (Huang et al., 2009). In pancreatic β -cells, glucose is the primary physiological stimulus for insulin secretion and it is ingested into the cell through GLUT-2 and GLUT-4 in the plasma membrane of the cells (Schuit et al., 2001). Interestingly, the findings of Lee et al. in 2009 displayed that genistein derivatives elevated the expressions of GLUT-1 and GLUT-4 in L6 myotubes, indicating the antidiabetic potential of the iso-flavones. Thus, the modulation of these glucose transporter proteins could be one of the mechanisms involved with the anti-diabetic activity of the PM extract.

It is evident from the findings of our present investigation that PM possesses antioxidant and hypoglycemic potential resulting in downregulation of the elevated oxidative stress and pro-inflammatory cytokines. The anti-inflammatory response of PM was further evidenced by a reduction in the transforming growth factor beta-1 (TGF- β 1). Furthermore, the improved regulation of insulin and GLUT-1, GLUT-2, and GLUT-4 account for the protective and regenerative role of PM on the pancreatic β -cell mass of STZ-nicotinamide induced diabetic rats. Nevertheless, the detailed mechanistic approach of PM on insulin signaling pathway is warranted.

Conclusion

The present study has shown potential role of PM in the management of type-2 diabetes by downregulating hyperglycemia and elevated level of pro-inflammatory cytokines. Moreover, PM has ameliorated diabetes-induced pancreatic degeneration by improving antioxidant defense system and pancreatic protein expressions.

Conflict of interests

The authors have no conflict of interests to declare.

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References

- Arya, A., Cheah, S.C., Looi, C.Y., Taha, H., Mustafa, M.R., Mohd, M.A., 2012a. The methanolic fraction of *Centratherum anthelminticum* seed downregulates pro-inflammatory cytokines, oxidative stress, and hyperglycemia in STZ-nicotinamide-induced type 2 diabetic rats. *Food Chem. Toxicol.* 50, 4209–4220.
- Arya, A., Looi, C.Y., Cheah, S.C., Mustafa, M.R., Mohd, M.A., 2012b. Anti-diabetic effects of *Centratherum anthelminticum* seeds methanolic fraction on pancreatic cells, β -TCG and its alleviating role in type 2 diabetic rats. *J. Ethnopharmacol.* 144, 22–32.
- Aziz, A., Taha, H., Ismail, N.H., Yusof, F.Z.M., Bakar, M.Z.A., 2016. Therapeutic potential of plant species derived from some Annonaceae genus. *Int. J. Agric. For.* 6, 214–221.
- Brezar, V., Carel, J.-C., Boitard, C., Mallone, R., 2011. Beyond the hormone: insulin as an autoimmune target in type 1 diabetes. *Endocr. Rev.* 32, 623–669.
- Chang, W., Chen, L., Hatch, G.M., 2016. Berberine treatment attenuates the palmitate-mediated inhibition of glucose uptake and consumption through increased 1, 2, 3-triacyl-sn-glycerol synthesis and accumulation in H9c2 cardiomyocytes. *Biochim. et Biophys. Acta* 1861, 352–362.
- Chao, P.-C., Hsu, C.-C., Yin, M.-C., 2009. Anti-inflammatory and anti-coagulatory activities of caffeic acid and ellagic acid in cardiac tissue of diabetic mice. *Nutr. Metab.* 6, 33.
- Chuakul, W., Soonthornchareonnon, N., Boonjaras, T., Boonpleng, A., 2004. Survey on medicinal plants in Southern Thailand. *Thai J. Phytopharm.* 11, 29–52.
- Chung, H.S., Shin, J.C., 2007. Characterization of antioxidant alkaloids and phenolic acids from anthocyanin-pigmented rice (*Oryza sativa* cv. Heugjinjubyeo). *Food Chem.* 104, 1670–1677.
- Dandona, P., Aljada, A., Bandyopadhyay, A., 2004. Inflammation: the link between insulin resistance, obesity and diabetes. *Trends Immunol.* 25, 4–7.
- Gandhi, G.R., Ignacimuthu, S., Paulraj, M.G., 2011. *Solanum torvum* Swartz. Fruit containing phenolic compounds shows antidiabetic and antioxidant effects in streptozotocin induced diabetic rats. *Food Chem. Toxicol.* 49, 2725–2733.
- Genuth, S., Alberti, K.G., Bennett, P., Buse, J., Defronzo, R., Kahn, R., et al., 2003. Expert committee on the diagnosis and classification of diabetes mellitus. Follow-up report on the diagnosis of diabetes mellitus. *Diabetes Care* 26, 3160–3167.
- Hajiaghaalipour, F., Khalilpourfarshbafi, M., Arya, A., 2015. Modulation of glucose transporter protein by dietary flavonoids in type 2 diabetes mellitus. *Int. J. Biol. Sci.* 11, 508.
- Huang, D.-W., Shen, S.-C., Wu, J.S.-B., 2009. Effects of caffeic acid and cinnamic acid on glucose uptake in insulin-resistant mouse hepatocytes. *J. Agric. Food. Chem.* 57, 7687–7692.
- Hulbert, A.J., Turner, N., Storlien, L., Else, P., 2005. Dietary fats and membrane function: implications for metabolism and disease. *Biol. Rev.* 80, 155–169.
- Jung, M., Park, M., Lee, H.C., Kang, Y.-H., Kang, E.S., Kim, S.K., 2006. Antidiabetic agents from medicinal plants. *Curr. Med. Chem.* 13, 1203–1218.
- Karthikesan, K., Pari, L., Menon, V.P., 2010. Protective effect of tetrahydrocurcumin and chlorogenic acid against streptozotocin–nicotinamide generated oxidative stress induced diabetes. *J. Funct. Foods* 2, 134–142.
- Klahr, S., Morrissey, J.J., 2000. The role of vasoactive compounds, growth factors and cytokines in the progression of renal disease. *Kidney Int.* 57, S7–S14.
- Lee, M.S., Kim, C.H., Hoang, D.M., Kim, B.Y., Sohn, C.B., Kim, M.R., Ahn, J.S., 2009. Genistein-derivatives from *Tetracera scandens* stimulate glucose-uptake in L6 myotubes. *Biol. Pharm. Bull.* 32, 504–508.
- Lombardo, Y.B., Chicco, A.G., 2006. Effects of dietary polyunsaturated n-3 fatty acids on dyslipidemia and insulin resistance in rodents and humans. A review. *The J. Nutr. Biochem.* 17, 1–13.
- Montane, J., Cadavez, L., Novials, A., 2014. Stress and the inflammatory process: a major cause of pancreatic cell death in type 2 diabetes. *Diabetes Metab. Syndr. Obes.* 7, 25–34.
- Nasri, H., Shirzad, H., Baradaran, A., Rafieian-kopaei, M., 2015. Antioxidant plants and diabetes mellitus. *J. Res. Med. Sci.* 20, 491–502.
- Ong, K.W., Hsu, A., Song, L., Huang, D., Tan, B.K.H., 2011. Polyphenols-rich *Vernonia amygdalina* shows anti-diabetic effects in streptozotocin-induced diabetic rats. *J. Ethnopharmacol.* 133, 598–607.
- Palmer, J.P., Fleming, G.A., Greenbaum, C.J., Herold, K.C., Jansa, L.D., Kolb, H., et al., 2004. C-peptide is the appropriate outcome measure for type 1 diabetes clinical trials to preserve β -cell function. *Diabetes* 53, 250–264.
- Rafieian-Kopaei, M., Nasri, H., 2012. Silymarin and diabetic nephropathy. *J. Ren. Injury Prev.* 1, 3–5.
- Rajavel, V., Abdul Sattar, M.Z., Abdulla, M.A., Kassim, N.M., Abdullah, N.A., 2012. Chronic administration of oil palm (*Elaeis guineensis*) leaves extract attenuates

- hyperglycaemic-induced oxidative stress and improves renal histopathology and function in experimental diabetes. Evidence-Based Complementary Altern. Med. 2012.
- Ramar, M., Manikandan, B., Raman, T., Priyadarsini, A., Palanisamy, S., Velayudam, M., et al., 2012. Protective effect of ferulic acid and resveratrol against alloxan-induced diabetes in mice. Eur. J. Pharmacol. 690, 226–235.
- Ramesh, B., Pugalendi, K., 2006. Antihyperglycemic effect of umbelliferone in streptozotocin-diabetic rats. J. Med. Food 9, 562–566.
- Reuter, S., Gupta, S.C., Chaturvedi, M.M., Aggarwal, B.B., 2010. Oxidative stress, inflammation, and cancer: how are they linked? Free Radical Biol. Med. 49, 1603–1616.
- Robertson, R.P., Zhang, H.-J., Pyzdrowski, K.L., Walseth, T.F., 1992. Preservation of insulin mRNA levels and insulin secretion in HIT cells by avoidance of chronic exposure to high glucose concentrations. J. Clin. Invest. 90, 320.
- Schuit, F.C., Huypens, P., Heimberg, H., Pipeleers, D.G., 2001. Glucose sensing in pancreatic β -cells. Diabetes 50, 1–11.
- Taha, H., Arya, A., Paydar, M., Looi, C.Y., Wong, W.F., Murthy, C.V., et al., 2014. Upregulation of insulin secretion and downregulation of pro-inflammatory cytokines, oxidative stress and hyperglycemia in STZ-nicotinamide-induced type 2 diabetic rats by *Pseuduvaria monticola* bark extract. Food Chem. Toxicol. 66, 295–306.
- Taha, H., Hadi, A.H.A., Nordin, N., Najmuldeen, I.A., Mohamad, K., Shiota, O., et al., 2011. Pseuduvarines A and B, two new cytotoxic dioxoaporphine alkaloids from *Pseuduvaria rugosa*. Chem. Pharm. Bull. 59, 896–897.
- Trojanowska, M., 2012. Mediators of fibrosis (Suppl. 1). Open Rheumatol. J. 6, 70.
- Turner, R.A., 2013. Screening Methods in Pharmacology. Elsevier.
- Vinayagam, R., Xu, B., 2015. Antidiabetic properties of dietary flavonoids: a cellular mechanism review. Nutr. Metab. 12, 60.