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Antidiabetic effects of *P. macrocarpa* ethanolic fruit extract in streptozotocin-induced diabetic rats

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Abstract

Background: The fruits of *P. macrocarpa* have long been used as a traditional Malay medicinal herb for hundreds of years. Intraperitoneal (i.p.) injection of streptozotocin (STZ) (65 mg/kg) was used to induce diabetes in rats confirmed by an oral glucose tolerance test (OGTT). The ethanol extract of *P. macrocarpa* (EEPM) fruits at 100 and 200 mg/kg were given orally for 35 days, glibenclamide. In total, 0.5 mg/kg served as a positive control.

Results: The present toxicity study suggests that the EEPM fruits are non-toxic. In an OGTT, the EEPM at 50, 100, and 200 mg/kg and glibenclamide (0.5 mg/kg) reduced the blood glucose level (hyperglycemia due to glucose load 2 g/kg p.o.) significantly after 2 h of oral administration, when compared to the diabetic control. Repeated oral administration of EEPM daily for up to 35 days exhibited significant antidiabetic activity in STZ-induced diabetic rats compared to the diabetic control. At the end of 35 days of treatment, the 200 mg/kg (EEPM) dose was found to be more effective than the 100 and 50 mg/kg (EEPM) doses and blood glucose levels decreased from 392.66 ± 3.20 to 174.33 ± 4.32 mg/dl ($p < 0.01$). In contrast, on day 35, the blood glucose levels of the normal control, drug control, and diabetic control were 132.16 ± 5.79, 134.33 ± 7.18 ($p < 0.01$), and 514.83 ± 7.96 respectively. From histology analysis, the pancreases of the diabetic control were granulated and dilated islet cells, whereas in the drug control they appeared granulated, without dilation and important hyper plasticity of islets. The treatment groups (EEPM 100 and 200 mg/kg) also showed granulated pancreatic islets and prominent hyper plasticity islets. Light micrographs in various regions of rat kidney tissue from the treatment groups showed absence of matrix expansion and glomerular basement membrane thickening, suggesting it became normal histoarchitecture of the renal. Biochemical aspects in treating animals' all serum analytic parameters were almost similar to the drug control group with the exception of the 50 mg/kg treatment group.

Conclusion: In this way, it may also serve as a good alternative in the present armamentarium of antidiabetic drugs.

Keywords: *P. macrocarpa*, Toxicity, Histology, Kidney, Pancreas

Background

Past investigations have demonstrated that *P. macrocarpa* contains some optional metabolites that could battle malignant growth or irresistible maladies, yet in addition, incorporate way of life illnesses, for example, diabetes, hypertension, and atherosclerosis [1]. The *P. macrocarpa* extract was obtained using ethyl acetate which showed potential antihyperglycemic effect in alloxan-induced type 2 diabetic rat model. This is expected to be arbitrated by averting the weakening of hepatic associated diabetes [2]. It contained various potential bioactive compounds such as naringin, kaemferol, rutin, myricetin, or flavonoids. These biological lead compounds were shown to be very potent representatives for antidiabetic, antifungal, and

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antibacterial activities [3]. The *P. macrocarpa* leaves' extract showed antioxidant and antibacterial properties in vitro model [4–6]. Studies on mesocarp and pericarp of fruits lead to the separation and structure elucidation of two novel compounds—mangiferin and icariside **C3** [7]. Identification of phalerin, a new benzophenoicglucoside (3,4,5-trihydroxy-4′-methoxy-benzophenone-3-0-β-$_D$-glucoside) was isolated from the methanolic extract of the leaves extract of *P. macrocarpa* [8]. There were several compounds isolated from fruit extracts like mahkoside **A,** phenolic glycoside, mangiferin, palmitic acid, kaempferol-3-0-β-$_D$-glucoside, sucrose, dodecanoic acid, and ethyl stearate [9], matairesinol, lignanspinoresinol and lariciresinol [10, 11]. Pure seed extracts contained desacetylfevicordin **A**, 29-norcucurbitacin, and its derivatives (fevicordin **A**, fevicordin **A** glucoside, and fevicordin **D** glucoside) and their serious cytotoxic effects have been reported in vitro study [12, 13]. Traditionally, crushed seed powder or extract has been used to cure skin disease purposes and as a traditional biopesticide [14, 15]. Based on the previous toxicity studies on *P. macrocarpa*, used doses of 250 mg/kg/b.w and 1000 mg/kg/b.w do not likely to produce any toxicity effect [16]. The present study used 5000 mg/kg/b.w of PM extract following the revised up-and-down method. The objective of the present study is to identify toxicity and antidiabetic activity following treatment with EEPM fruits.

Methods

Chemicals

STZ and glibenclamide were purchased from Sigma Chemicals Co. (Saint Louis, Missouri, USA) and all other reagents and chemicals used in the present study were of analytical grade.

Collection and identification of plant material

The ripe fruits (835 g) of *P. macrocarpa* were collected and identified with Voucher specimen (no.: P-0230). The mesocarp and pericarp of the fruits were sliced and dried in a normal room temperature at 25 °C for 10 days, then it was pulverized to a powdered form (713 g) using Fritsch Universal Cutting Mill-Pulverisette 19, Germany, and kept at 4 °C until further use [3].

Preparation of ethanol extract

The sample (710 g) was extracted by cold maceration with ethanol (4:1, solvent: solid ratio) for 72 h at room temperature, filtered into a sterile round bottom flask using adsorbent cotton wool and filter paper (Whatman No. A-1). The extraction procedure with the solvent was repeated several times to ensure the highest percentage of the yield of ethanol-soluble compounds from the EEPM fruit powder. The ethanol extract was concentrated (temperature at 45 °C, 175 mbar, and rotation 80-85 rpm) by using a rotary vacuum evaporator (BUCHI R-205) to a final corrected volume of 500 ml. This was further frozen at −80 °C and shifted instantly to three week's successive freeze drying at −50 °C using bench-top freeze dryer (ALPHA 1-4LD-2), to give an ultimate yield of 213 g.

Experimental animals

Experimental procedure of this study was approved by the Institutional Animal Care and Use Committee/IACUC bearing an approval ID: /IACUC Approval/2014/(3) (8). It conformed to the International guidelines for the use and care of laboratory animals (OECD, 425). Efforts were made to minimize the number of animals and their discomfort in the laboratory uses. Healthy adult male Sprague-Dawley (SD) strain rats (8–10 weeks) weighing from 180 to 200 g were obtained from the Laboratory Animal Centre, Universiti Putra Malaysia (UPM), Serdang, Selangor, Malaysia. The current study was performed with a single-sex (male) to reduce variability in the test animals. A pair of animals were housed in a standard propylene cage, acclimatized for 7 days to the animal laboratory conditions at room temperature (22 ± 3 °C), relative humidity (RH) of 46–70%, and 12 h light/dark cycle [17], allowing adequate cross ventilation prior to the test. Moreover, the animals were fed with standard commercial dry pellet (Gold Coin Feed Mills Sdn. Bhd., Kuala Lumpur, Malaysia) diet containing 22% crude protein, 46% fat, 4% fiber, 7.6% ash, 12.0% moisture, 1.2% calcium, 0.73% phosphorus, and water ad libitum. Fasting animals were deprived of food for at least 16 h with free access to drinking water prior to the study [18].

Acute oral toxicity study

Acute oral toxicity of EEPM was performed on male Sprague-Dawley strain rats, according to the procedure described by the revised up-and-down method OECD (Organization of Economic Cooperation and Development) Guideline 425 [19]. Two groups, with six rats each, were used for the study. Group I served as a control and received drinking water. Group II received a single oral dose of EEPM (5000 mg/kg). The rats underwent fasting (16 h) overnight and the body weight (g) of each rat was recorded prior to the test. The test substance was administered in a single dose by oral gavage using a stomach tube and a suitable intubation cannal and observed closely at 4 h initially, then every 6 h intervals for changes in skin, fur, eyes, mucous membranes, behavioral (alertness, restlessness, irritability, recumbence, vomiting, fearfulness), neurological (spontaneous activity, convulsion, gait, bleeding orifices, touch/pain response), autonomic (defecation, micturition) profiles, and/or mortality, and any significant morbidity or mortality within 24–168 h was recorded [20].

Induction of diabetes in experimental animals

Healthy male Sprague-Dawley rats were made to fast overnight (16 h) and their basal weight (g) and fasting blood sugar (mg/dl) were appropriately measured and recorded. Diabetes was induced under light ether anesthesia by a single i.p. injection of STZ (Sigma-Aldrich, USA) at 65 mg/kg, based on our result of the pilot study. STZ was first weighed individually for each animal according to the weight, solubilized with 0.1 ml of freshly prepared cold Na-citrate buffered (NaB- 0.1 M, pH 4.5), and administered within 5 min to prevent degradation. The injection of STZ volume was prepared to contain 1.0 ml/kg. Rats were supplied with 5% glucose solution for 48 h immediately after STZ injection to counteract severe acute hypoglycemic effect. Control rats received an equivalent volume of PBS without STZ interperitoneal [21]. Diabetes induction was confirmed by determination (Life Scan One-touch Ultra Glucose Meter, USA) of high fasting blood glucose level alongside diagnostic triad (polyuria, polydipsia, weight loss) on the 3rd day after STZ administration. Only the rats that displayed the fasting blood sugar (FBS) level ≥ 300 mg/dl were selected for subsequent experiments [22].

OGTT

The rats were made to fast (16 h) overnight and their baseline glucose level was measured and recorded. All the animals were given oral administration of glucose (2 g/kg) 30 min after dosing and their glycemic status was re-evaluated intermittently at 0, 30, 60, 90, and 120 min respectively. Fasting blood sugar levels ≥ 300 mg/dl further confirmed diabetes in these rats. Mild and sub diabetics rats after day 3 of STZ injection were further assessed by the oral glucose challenge test [23, 24].

Experimental design

Six groups of rats, six in each (6 normal; 30 diabetic, total 36) received the following treatment schedule.

Group I: Normal control (drinking water)

Group II: Diabetic control (STZ, 65 mg/kg + drinking water)

Group III: Drug control (STZ, 65 mg/kg + glibenclamide, 0.5 mg/kg)

Group III: Drug control (STZ, 65 mg/kg + EEPM, 50 mg/kg)

Group III: Drug control (STZ, 65 mg/kg + EEPM, 100 mg/kg)

Group III: Drug control (STZ, 65 mg/kg + EEPM, 200 mg/kg)

Blood glucose measurement

The fasting plasma glucose levels were recorded before the induction of STZ and observed very carefully for the first 24 h; 5% glucose solution was prepared in order to prevent any accidental loss of animal life due to hypoglycemia. Then they were kept for another 48 h in a diabetic state for the stability of physiological characteristics. Blood glucose was measured repeatedly in groupwise before starting the extraction treatment and was continued for 35 consecutive days using glucose-oxidase, peroxidase reactive strips, and a glucometer (One Tuch Ultra Life Scan Inc. USA) with a similar dose of 0.5 mg/kg (glibenclamide), 50, 100, 200 (EEPM) mg/kg/day orally using oral gavage.

Biochemical estimation

Blood collection and preparation

At the end of the 7-day period of EEPM fruits oral administration, the rats were humanely sacrificed with an anesthetic agent (diethyl ether). Before the sacrifice, about 3 to 5 ml of blood was collected directly from the heart of each rat via intra cardiac puncture using 23 G syringe. The blood was collected in ethylene diamine tetraacetic acid (EDTA) tubes. Plasma was separated using refrigerated ultra-centrifuge (Allegra X-12R), 4 °C at 2000x*g* for 10 min. The plasma was stored in working aliquots at −80 °C for subsequent biochemical analysis.

Serum biochemistry

The following parameters were determined calorimetrically by employing the standard ready-to-use kits (Human Gesellschaftfür Biochemica and Diagnostica MBH, Germany), creatinine (mg/dl), phosphorus (mg/dl), HDL cholesterol (mg/dl), LDL cholesterol (mg/dl), cholesterol (mg/dl), triglyceride (mg/dl), aspartate aminotransferase (AST-U/L), alanine aminotransferase (ALT- U/L), alkaline phosphatase (ALP-U/L), total bilirubin (T.BIL-mg/dL), albumin (ALB-g/dL), glucose (Glu-mg/dL), and total proteins (T.PROT-g/dL) (Cobas Mira, The automated Thermo Scientific Konelab 20 and 20XT analyzers). The manufacturer's instructions for each biochemical parameter were strictly followed in the course of these investigations.

Statistical analysis

Data were expressed as a mean ± standard error mean (SEM) for six rats in each group, and analyzed using the SPSS software for Windows Version 21.0. Statistical differences were computed using one-way analysis of variance (ANOVA, SPSS, and Version-21), followed by Tukey's honest significant difference (HSD) test. Data were considered statistically significant at $^{*}P < 0.05$ and highly significant at $^{**}P < 0.01$ and 95% confidence interval.

Results

In vivo safety profile study EEPM fruits

The aim of the preliminary in vivo toxicity study conducted was to determine the appropriate safe dose range

that could be used for subsequent experiments. The method of up- and downregulation in accordance with the guidelines of OECD, 2008 utilizes only six rats, and provides 24 h minimum lethal dose (LD_{50}) value which was adequate for most experimental animal research. The individual body weights of animals increased from the first day of dosing to just before the sacrifice with a normal growth (Table 1).

During the two-week dosing period, all the animals were observed daily for clinical signs and mortality patterns once before dosing, immediately after dosing, and up to 6 h after dosing. At the dosage levels used, there was no evidence of any toxic symptoms or significant behavioral changes in some animals at a single maximum dose of EEPM 5000 mg/kg/b.w and no problematic clinical signs were observed in the surviving rats. Careful observation was recorded every 4 h interval/day for any changes affecting physical behavior (alertness, breathing, restlessness, motor activity), central nervous system (CNS) (convulsions, drowsiness, coma), autonomic nervous system (ANS) (vomiting, urination, defecation), motor activity, salivation, skin coloration, and other general signs of toxicity. Up to the end of the study, there was no sign of toxicity and mortality observed in any of the experimental animals that received 5000 mg/kg/b.w of EEPM fruits orally (Table 2).

As such, it was concluded that the EEPM fruits had no adverse effect in a single large dose up to 5 g/kg b.w. Thus, the minimum lethal dose (LD_{50}) value could be greater than 5000 mg/kg b.w in rats. The in vivo toxicity study data revealed that the EEPM fruits proved to be non-toxic at tested dose levels and were well tolerated by the experimental animals. There were no significant differences between the control and treatment groups. It could be inferred that there is no effect on the body weight after oral administration of 5000 mg/kg EEPM fruits.

Serum biochemistry for safety profile study

The biochemical observations (Table 3) showed that the treated animal serum glucose levels were slightly decreased ($p > 0.05$) when compared to the control group. However, the treated rats showed minimal variations in a few biochemical parameters in comparison to the normal control. From a statistical analysis, it was shown that there were no significant differences between the treated animals and the control group ($p > 0.05$).

Histopathological findings for control and treated animals

The liver tissue of the control rats was examined for structural changes under a light microscope using hemotoxylin coupled with eosin staining. The liver of the rats in the control and treated group appeared to be divided into the classical hepatic lobules; each was formed of cords of hepatocytes radiating from the central vein to the periphery of the lobule. The cell cords were separated by narrow blood sinusoids which received blood from terminal branches of the hepatic artery and portal vein at the periphery of lobules and deliver blood into the central vein. Hepatocytes were in a concentric arrangement around the central vein. The cells are large in size with more or less centrally placed prominent nucleolus (Fig. 1). The current study showed that there was no periportal necrosis of the hepatocytes and no inflammation of lymphocytes and macrophages in both control and treated groups.

The histological preparation of rat kidneys from the control and treated groups showed that the various segments of kidney tubules were well preserved. An abundant glomeruli portion of the nephron segment with interspersed blood capillaries was also clearly seen (Fig. 2).

There were no significant histological alterations in the glomeruli or any other segment of the kidney tubules following extract treatment in the current study. All the constituent structures of the kidney tubule in the extracted treatment groups appeared to be well

Table 1 Results showing the individual weights of animals treated with the EEPM (5000 mg/kg/b.w) fruits

Individual body wt. during dosing (g)		Individual body wt. after treatment (g)				Individual body wt. before sacrifice (g)	
Control group	Test group	Control group		Test group		Control group	Test group
0 h	0 h	6 h	3 days	6 h	3 days	7 days	7 days
195.6	190	196.6	199.3	190.9	198.3	231.1	224.3
190.1	187	190.2	194.5	187.9	194.5	227.2	222.6
187.3	188	187.9	190.3	188.9	196.3	223.5	224
189.6	189.6	190.	194.3	190.1	197	224.8	224.9
183.8	192.5	183.8	188.2	192.9	198.8	228.8	227.5
192	192	192.9	195.2	193	200.5	229.6	228.5
mean ± SEM							
189.7 ± 4	189.7 ± 4	189.7 ± 4	189.7 ± 4	189.7 ± 4	197.5 ± 2	227.5 ± 3	225.3 ± 2

*$P > 0.05$ no significant difference from control; $n = 6$

Table 2 Results showing the time course of onset of signs of toxicity in rats treated with 5000 mg/kg/b.w EEPM fruits

Observations	Control group		Test group		Mortality	
	6 h	7 days	6 h	7 days	Control group	Test group
Physical activity	N	N	N	N	Nil	Nil
CNS activity	N	N	N	N		
ANS profile	N	N	N	N		
Skin and fur	N	N	N	N		
Eyes	N	N	N	N		
Mucous membrane	N	N	N	N		
Salivation	N	N	N	N		
Lethargy	N	N	N	N		
Sleep	N	N	N			
Diarrhea	N	N	N	N		
Coma	N.O*	N.O*	N.O*	N.O*		
Tremors	N.O*	N.O*	N.O*	N.O*		

N normal, *N.O** not observed

maintained. Various regions of the kidney tubules appeared to be normal without any changes in the mesangial matrix.

OGTT

An immediate effect of the EEPM on blood glucose levels of glucose-hyperglycemias (OGTT) and STZ-diabetic rats were observed. The results from the study indicated that the dose of EEPM at 50, 100, and 200 mg/kg, and glibenclamide (0.5 mg/kg) reduced the blood glucose level significantly after oral administration of 2 g/kg p.o. glucose, when compared to the diabetic control group as shown in (Table 4).

Changes in body weight

At the end of 35 days, the body weight (Fig. 3) of normal rats, positive control group and EEPM-200 group were increased significantly ($p < 0.001$) (from 214.3 ± 3.4 g to 384.8 ± 5.2 g, 205 ± 6.3* to 329.8 ± 5.7** g, and 236.5 ± 1.1* to 291.6 ± 5.9**), whereas the body weight of diabetic control group rats was decreased gradually (from 218.5 ± 0.4 to 179.6 ± 3.7 g). In EEPM treated groups, we observed that their body weights increased significantly ($p < 0.001$) because their blood glucose levels were also decreasing gradually. At the doses of EEPM 50, 100, and 200 mg/kg, their body weight patterns were from 233.63 ± 1.3* to 259.33 ± 6.15* g, from 220.17 ± 1.32* to

Table 3 Effects of EEPM fruits extract on hepatic, renal, and lipid function indices in male rats

	Analyte parameter	Control group	Treated group (5000 mg/kg/b.w)	Ref. values
	Creatinine mg/dL	0.4 ± 0.02**	0.46 ± 0.03**	0.2-0.8 mg/dL
Renal profile	Phosphorous mg/dL	6.0 ± 0.2*	.05 ± 0.4**	3.1-11 mg/dL
	HDL cholesterol mg/dL	36.1 ± 2.4*	31.3 ± 1.4*	> 27 mg/dL
	LDL cholesterol mg/dL	32.4 ± 0.8**	30.6 ± 0.3*	< 46.8 mg/dL
Lipid profile	Cholesterol mg/dL	71.0 ± 1.7**	68.2 ± 1.2**	40-130 mg/dL
	Triglyceride mg/dL	75.9 ± 1.4**	76.51 ± 3.3**	60-90 mg/dL
	ASTU/L	62.5 ± 2.3*	63.08 ± 2.6*	45.7-80.8 U/L
	ALTU/L	22.7 ± 0.6*	22.05 ± 0.6*	17.5-30.2 U/L
Liver profile	ALPU/L	78.4 ± 1.4*	79.1 ± 0.8**	56.8-128 U/L
	T.BIL mg/dL	0.3 ± 0.02**	0.3 ± 0.01*	0.20-0.5 mg/dL
	ALB g/dL	3.9 ± 0.06**	3.9 ± 0.07*	3.8-4.8 g/dL
	Glu mg/dL	86.8 ± 1.6*	82.0 ± 1.2*	50-135 mg/dL
	T.Prot g/dL	6.3 ± 0.1**	6.0 ± 0.09**	5.6-7.6 g/dL

AST aspartate aminotransferase, *ALT* alanine aminotransferase, *ALP* alkaline phosphatase, *T.BIL* total bilirubin, *ALB* albumin, *Glu* glucose, *T.PROT* total proteins
*$P < 0.05$
**$P < 0.001$ no significantly different from control; $n = 6$

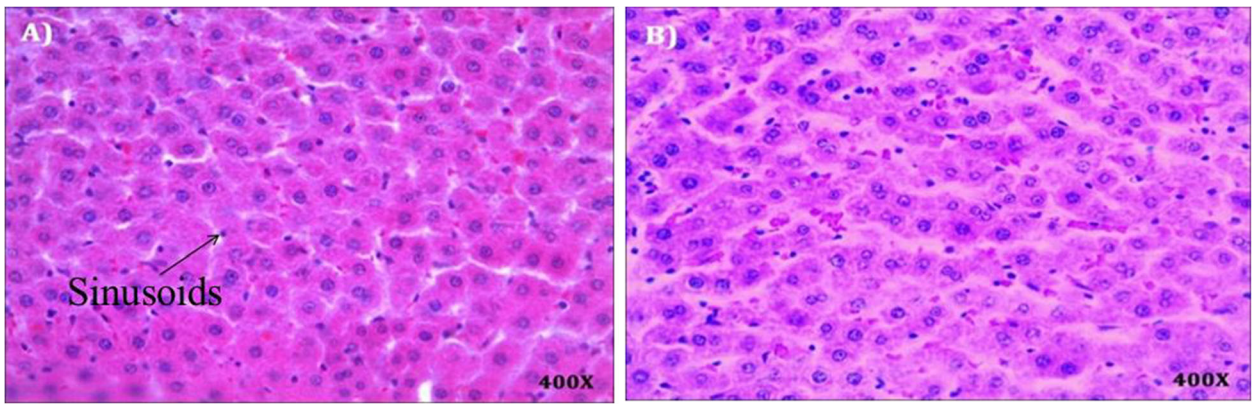

Fig. 1 Light micrographs of rat liver tissue in (**a**) control group and (**b**) treated group

261.33 ± 2.59** g, and from 236.50 ± 1.17* to 291.67 ± 5.92** g respectively (Fig. 3).

Changes in blood glucose level

On repeated oral administration of EEPM fruits daily up to 35 days, STZ-induced diabetic rats exhibited significant antidiabetic activity when compared to the diabetic control. After 35 days of treatment, a dose of (EEPM) 200 mg/kg was more effective than a dose of (EEPM) 100 and 50 mg/kg and the blood glucose levels were decreasing significantly ($p < 0.01$) from 392.6 ± 3.2 to 174.33 ± 4.3 mg/dl. In addition, the dose of (EEPM) 50 mg/kg (395.6 ± 4.4 to 284.6 ± 4.8) and 100 mg/kg (392.5 ± 3.9 to 240.5 ± 9.2) were also shown to be significant ($p < 0.05$) (Fig. 4). The blood glucose levels were decreased in all of the EEPM-treated groups and the results support the hypothesis that EEPM is effective for the treatment of type 2 diabetes.

Histology of pancreas of experimental animals

Microscopic observation of the pancreas in the experimental tissue section is shown in Fig. 5.

Histology of kidney of antihyperglycemic experimental animals

Microscopic observation of the kidney in the experimental tissue section is shown in Fig. 6.

Changes in serum biochemistry in experimental rats

In the serum biochemistry of testing rats, there were significant differences with all tested parameters when comparing the diabetic control group with the normal group. In the serum creatinine and urea level of the EEPM, both the treated groups and the drug control group were almost similar. However, compared to the diabetic control group (creatinine, 0.7 ± 0.9, urea, 42.5 ± 2.6), the EEPM treatment indicated a

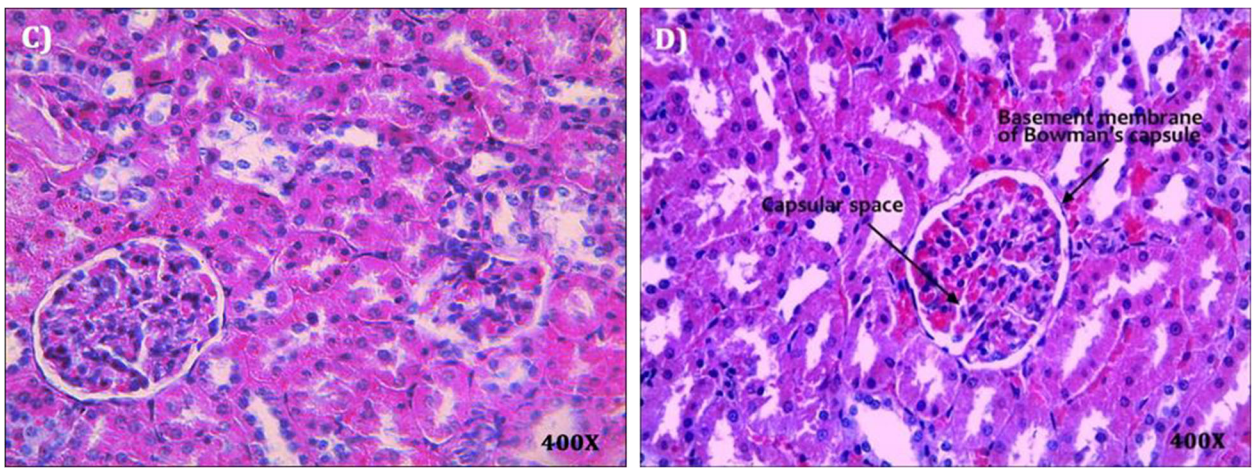

Fig. 2 Light micrographs of rat kidney tissue in (**c**) control group and (**d**) treated group

Table 4 Effect of EEPM fruits on oral glucose tolerance test

Groups, $n = 6$	Treatment	Blood glucose level (mg/dl) at (min)				
		0 min	30 min	60 min	90 min	120 min
Normal control	Distilled water	95 ± 2.1	99 ± 2.2	97 ± 2.1	93 ± 2.1	90 ± 2.1
Diabetic control	STZ + untreated	479 ± 7.1	488 ± 7.0	490 ± 7.1	490 ± 7.1	493 ± 7.2
Positive control	STZ + glibenclamide (0.5 mg/kg)	486 ± 3	482 ± 5.2*	476 ± 5.3*	476 ± 3.8	469 ± 4.2**
EEPM	50 mg/kg	478 ± 4.1	480 ± 4.9	478 ± 3	476 ± 3.7	476 ± 5.0*
	100 mg/kg	482 ± 4.1	482 ± 4.9	481 ± 5.1	478 ± 6.1*	476 ± 6
	200 mg/kg	472 ± 3.1	472 ± 3.1	470 ± 3.5**	469 ± 2.1*	467 ± 3*

*p < 0. 05 represent statistically significant
**p < 0. 01 represent statistically very significant with mean ± SEM, $n = 6$, when compared with diabetic control by using one-way ANOVA followed by Tukey's post hoc HSD multiple comparison test

significant decrease in 100 and 200 mg/kg (p < 0.01) in the creatinine (0.5 ± 0.7 and 0.5 ± 0.6) and urea (p < 0.05) (37.2 ± 2.8 and 34.5 ± 3) level. These results also suggested no kidney failure was caused by the administration of the extracts. In the lipid profile, EEPM-treated experimental rats compared to the diabetic control rats, significant (p < 0.05) reductions of LDL (120.3 ± 4.3* and 112.6 ± 3.7*, 103.3 ± 4.8*) and cholesterol (85.2 ± 4.1*, 77.7 ± 3.8*, and 73.4 ± 3.2**) were found after treatment of EEPM fruits at doses of 50, 100, and 200 mg/kg respectively while triglycerides (88 ± 6.3*, 84 ± 5.6*) showed significant (p < 0.05) values at 100 and 200 mg/kg doses. The HDL cholesterol levels of 200 mg/kg EEPM treated animals (39.7 ± 3*) were significantly (p < 0.05) increased compared to untreated diabetic rats (18.3 ± 3) (Table 5). Thus, it is reasonable to conclude that EEPM fruits could modulate blood lipid abnormalities. The EEPM treated groups exhibited non-significant differences in liver function tests, namely AST, ALT, and ALP activities which fell in the ranges of 77 ± 3.7 to 80.3 ± 2.9, 28.7 ± 3.2 to 30.5 ± 3, and 139 ± 4.6 to 146 ± 5.3 U/L respectively. The total bilirubin of EEPM (200 mg/kg) treated group (0.9 ± 0.6**) was decreased significantly (p < 0.05) when compared to the diabetic control (1.3 ± 0.9), while the value of EEPM (200 mg/kg) became close to the drug control group (0.87 ± 0.5). For total protein, there were significant (p < 0.05) differences in all EEPM-treated groups compared to the diabetic control group. The total protein value (5.9 ± 0.7) of the highest dose (EEPM-200 mg/kg) was nearly equal to the drug control group (6 ±

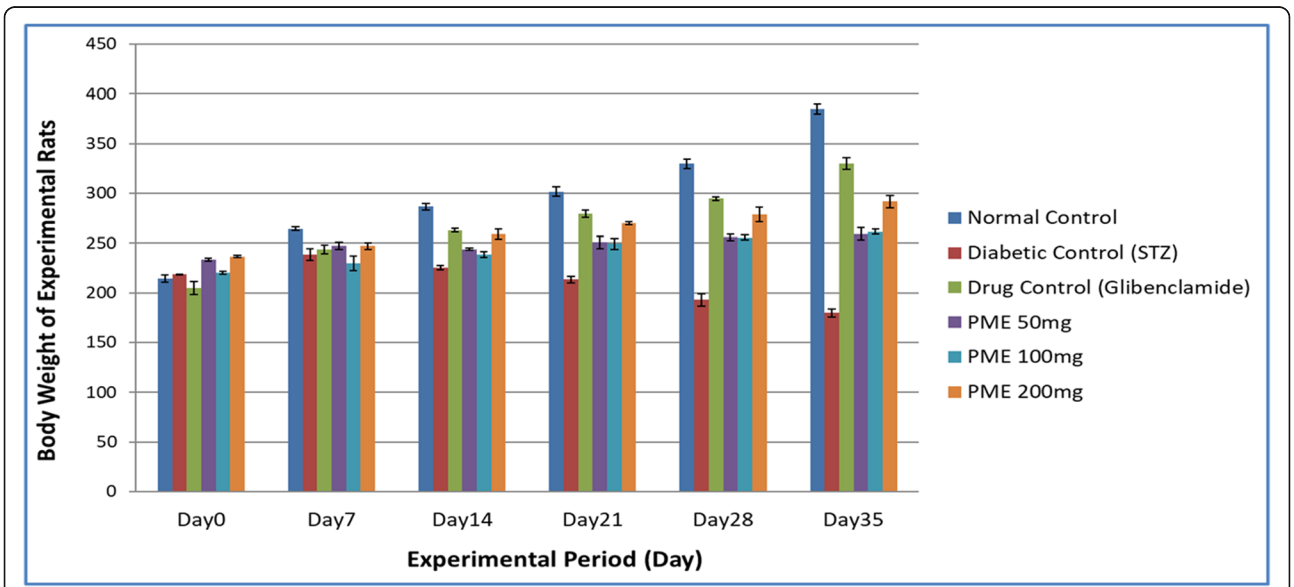

Fig. 3 The body weight of experimental rats after repeated oral administration of EEPM fruits ethanol extract for 35 days (5 weeks). Data are expressed as means ± SEM, $n = 6$ rats per group. *p < 0.5; *p < 0.01, when compared to starting values

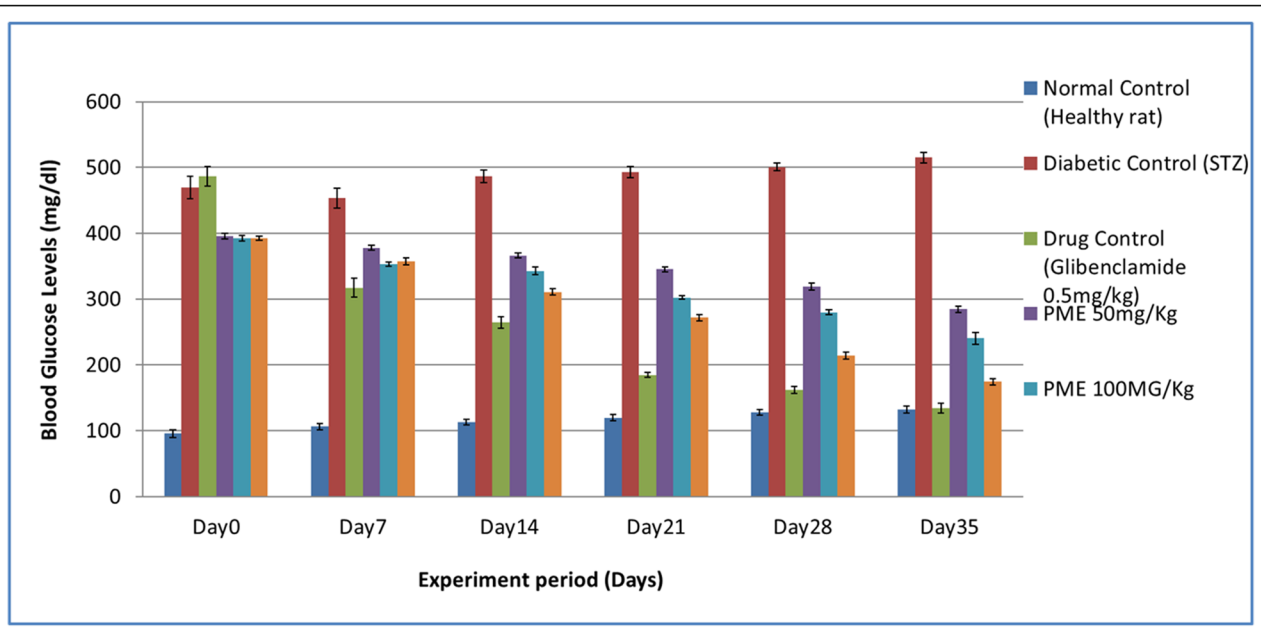

Fig. 4 Fasting plasma glucose levels after repeated oral administration of ethanol extract of EEPM fruits for 35 days (5 weeks) in normal (drinking water), diabetic (untreated), diabetic (glibenclamide), and treatment (50, 100, and 200 mg/kg) rats. Data are expressed as means ± SEM, $n = 6$ rats per group. $p < 0.05$; $p < 0.01$, when compared to starting values

0.7). There was a significant ($p < 0.05$) difference compared to the diabetic control. The liver enzymes and protein profile results indicated that there was no liver damage or dysfunction caused by the administration of the extract.

Discussion

Development of traditional remedies is relatively inexpensive and less time consuming. It is therefore more economically suitable. However, ecotype and genotype variations, seasonal variations, and other factors in

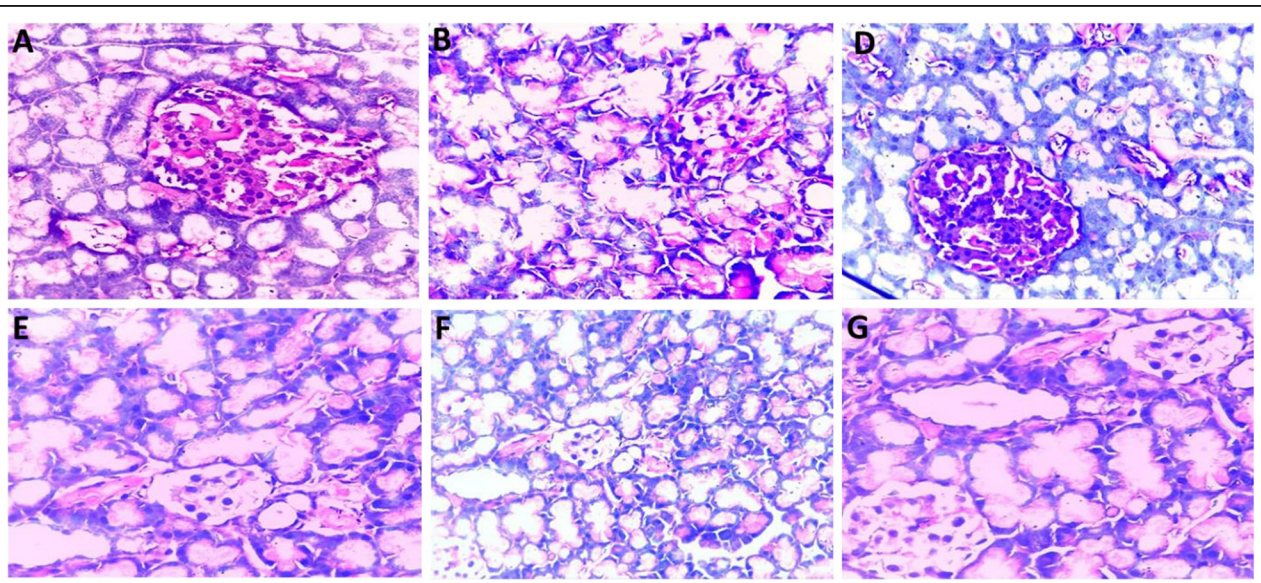

Fig. 5 Microscopic observation of the pancreas in the experimental tissue section. **a** Normal control-presence of normal pancreatic islet cells. **b** Diabetic control-degranulated and dilated islet cell. **c** Diabetic+glibenclamide (0.5 mg/kg)-granulated, nonappearence of dilation, and important hyperplasticity of islets. **d** Diabetic+EEPM (50 mg/kg) pancreas showing islets with endocrine cells showing more cytoplasm and normal endocrine acini. **e** Diabetic+EEPM (100 mg/kg)-granulated pancreatic islets, showing prominent hyper plasticity islets. **f** Diabetic+EEPM (200)-granulated pancreatic islets, showing major hyperplasticity islets

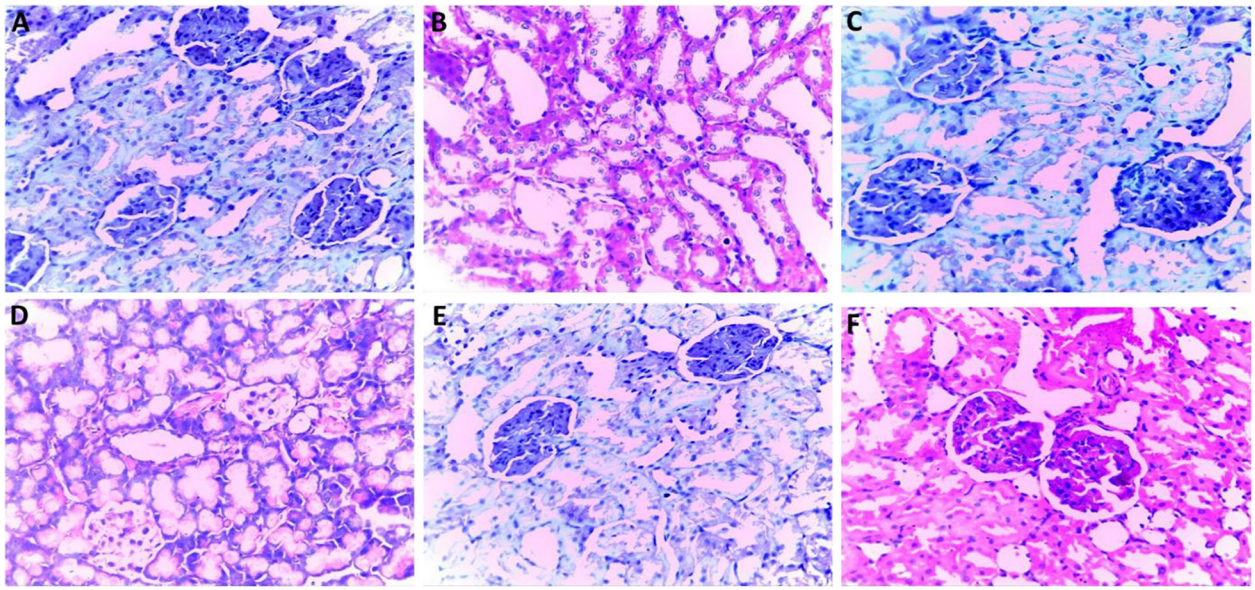

Fig. 6 Microscopic observation of the kidney in the experimental tissue section. **a** Normal control-light micrographs of rat kidney tissue in normal kidney depicted normal renal corpuscle with glomerulus and architecture. **b** Diabetic control-nonappearance of renal corpuscle, glomerulus, and abnormal architecture. **c** Diabetic+glibenclamide-various regions of the kidney tubules appeared to be normal without any changes in the mesangial matrix. **d** Diabetic+EEPM (50 mg/kg) section of kidney showing few changes compares with normal control and less appearance of cells. **e** Diabetic+EEPM (100 mg/kg) and (F) diabetic+EEPM (200 mg/kg) various regions of kidneys of treated animals revealed absence of matrix expansion and glomerular basement membrane thickening; suggesting became normal histoarchitecture of renal

efficacy and safety have to be addressed in phytomedicine development. It has been used to treat diabetes since the ancient era among ASEAN states like Malaysia, Thailand, Indonesia, Philippine, and Singapore. However, there is not sufficient supporting research data on fruit extracts to prove its safety level. The seed extracts have exhibited cytotoxicity effects on breast cancer (MCF-7), Hela cervix cancer (CaSki), colon cancer (HT-29), and malignant brain tumors (CGNH-89 [9]. There have been previous studies that record no toxic effect of EEPM fruits at 1000 mg/kg/b.w [12]. In this study, a fixed-dose of 5000 mg/kg/b.w EEPM was administered orally to each rat and the animals were observed closely, initially at 4 h, then at every 6 h intervals for changes.

Table 5 Effects of repeated oral administration (35 days) of 50, 100, and 200 mg/kg EEPM fruits, normal control, diabetic control, drug control (glibenclamide-0.5 mg/kg) groups on serum creatinine, urea, lipid profile, and liver profile in STZ-induced diabetic rats

Biochemical parameters	Experimental groups, $n = 6$					
	Normal control	Diabetic control	Drug control	EEPM 50 mg/kg	EEPM 100 mg/kg	EEPM 200 mg/kg
Creatinine (mg/dL)	0.2 ± 0.1	0.7 ± 0.9	0.4 ± 0.4**	0.6 ± 0.8*	0.5 ± 0.7	0.5 ± 0.6
Urea (mg/dL)	26.6 ± 0.7	42.5 ± 2.6	32.3 ± 2.9**	38 ± 3.2**	37.2 ± 2.8*	34.5 ± 3*
HDL cholesterol (mg/dL)	55.3 ± 1.4	18.3 ± 3.8	44.6 ± 2.6**	29.1 ± 4.5*	34.2 ± 3.7*	39.7 ± 3*
LDL cholesterol (mg/dL	88 ± 0.7	153.7 ± 4.7	90.7 ± 5.1**	120.3 ± 4.3*	112.6 ± 3.7*	103.3 ± 4.8**
Cholesterol (mg/dL	59.9 ± 1.8	107.3 ± 3.4	69.3 ± 2.6**	85.2 ± 4.1*	77.7 ± 3.8*	73.4 ± 3.2**
Triglyceride (mg/dL	64.2 ± 3.5	100.5 ± 5.7	76.3 ± 3.3*	92 ± 5	88 ± 6.3*	84 ± 5.6*
AST (U/L)	44.8 ± 2.2	93.2 ± 4.9	69.5 ± 3.3**	80.3 ± 2.9*	77.2 ± 4.03*	77 ± 3.7*
ALT (U/L)	21.3 ± 1.8	32.5 ± 4.1	25.9 ± 2.7**	30.5 ± 3**	30 ± 4.4*	28.7 ± 3.2**
ALP (U/L)	89.9 ± 2.8	158 ± 5.3	131.5 ± 6.8**	146 ± 5.3*	144 ± 5.1*	139 ± 4.6*
T.BIL (mg/dL)	0.5 ± 0.4	1.3 ± 0.9	0.8 ± 0.5*	1.2 ± 0.8*	1 ± 0.7*	0.9 ± 0.6**
T.Prot (g/dL)	6.8 ± 0.5	5 ± 1.9	6 ± 0.7**	5.5 ± 1*	5.8 ± 0.9*	5.9 ± 0.7**

$p < 0.05$, $p < 0.01$, values are mean ± SEM, $n = 6$, when compared with diabetic control by using one-way ANOVA followed by Tukey's post hoc HSD multiple comparison test

AST aspartate aminotransferase, *ALT* alanine aminotransferase, *ALP* alkaline phosphatase, *T.BIL* total bilirubin, *T.PROT* total proteins, *HDL* high-density lipoprotein, *LDL* low-density lipoprotein

There was no mortality or any sign of toxicity. This safety data also correlated with no changes in biochemical and histological aspects in treated animals when compared to the normal control group. It did not produce significant changes in rat behavior. At the same time, neither adverse effect, nor mortality was recorded throughout the period in the study. The current study showed that the EEPM fruits are practically nontoxic with a high margin of safety profile estimated. Previous studies reported that the middle dose of STZ (65 mg/kg/b.w) can induce diabetes by partially destroying β-cells, and the experimental animals were able to maintain the disease state without supplying insulin. These findings correspond positively with those studies of the diabetogenic action of streptozotocin [25, 26]. Mangiferin showed potent α-glucosidase inhibitory activity in vitro model [27]. A previous study was obtained 33.30%, 22.50%, and 9.52% mangiferin from n-butanol fractions, sub-fractions, and methanol extracts respectively. It was inhibited intestinal glucose transport at 49.55% and 61.38%, whereas the compound showed a 66.67% reduction in glucose level in the 12-day in vivo antidiabetic study. Other researchers have also reported that flavonoids have potent antidiabetic activities similar to mangiferin [28, 29]. There were two short-term (12-day) animal studies, using different solvent extracts of *P. macrocarpa* fruits at 1 g/kg/b.w daily, but researchers reported that petroleum ether and water extracts did not have a significant effect on blood glucose levels in diabetic rats [16]. However, methanol extracts of *P. Macrocarpa* fruits showed lower blood glucose level activity compared to diabetic rats. Most probably, the responsible compounds that give the antidiabetic effect were not extracted due to the polarity of the solvent. From previous experience, in present studies, the plant sample was extracted by ethanol. While previous studies used a much higher dose daily at 1000 mg/kg/b.w and glibenclamide 10 mg/kg/b.w of *P. macrocarpa* fruit extracts (methanol, chloroform, ethyl acetate, n-butanol, and petroleum ether) in in vivo animal study (12-day), the blood glucose-lowering activity was not significant. It could be due to extraction, solvent system, improper experimental design, or other factors. The EEPM fruits at 50, 100, and 200 mg/kg daily showed a potent blood glucose-lowering activity in STZ-induced Sprague-Dawley rats after 35 days of treatment. In addition, the current study used a standard drug glibenclamide at 0.5 mg/kg. After oral administration of extract and glibenclamide, a steady reduction of blood glucose level was noted for 3 weeks and the blood glucose-lowering trend continued decreasing until the 5th week. However, this study used 200 mg/kg as an optimal dose to decrease the blood glucose level comparable to drug control groups using glibenclamide (0.5 mg/kg). The previous researcher reported that the flavanoids and phenolics compounds, 6-dihydroxy-4-methoxybenzophenone-2-o-β-$_D$-glucoside and gallic acid of fruit and leaves extract were possessed a potent antioxidant effect [30, 31]. There are three types of enzymes, SOD-1, SOD-2, and SOD-3 which act as an antioxidant catalyst, the extract has been found to increase the superoxide dismutase (SOD) level. A significant antidiabetic activity of EEPM was observed with a minimum effective dose of 50 mg/kg/day achieving reduction in blood glucose levels after 35 days of treatment. Antioxidant and antidiabetic activities are closely related to each other, such as facilitating the normal functioning of the pancreas, which lead to insulin secretion and avoidance of diabetic complications. The present study also showed a significant blood glucose-lowering activity in in vivo experiments. In this case, *P. macrocarpa* might inhibit carbohydrate metabolism or increase insulin secretion. These findings could be used to support consumption of EEPM fruits as a dietary supplement for the management of diabetes mellitus. It may also serve as a good alternative in the present armamentarium of antidiabetic drugs.

Conclusion

Oral administration of the (mesocarp and pericarp) EEPM fruits in a single dose of 5000 mg/kg/day did not produce significant changes in behaviors in rats. Similarly, neither adverse effect nor mortality was recorded throughout the period of observation of the study, indicating that the extract is practically nontoxic with a high margin of safety profile estimated. A significant antidiabetic activity for EEPM was observed, with a minimum effective dose of 50 mg/kg/day achieving reduction in blood glucose level although it is not similar to that of glibenclamide after 35 days of the experiment but gradually it was reducing the blood glucose. In this way, could be supporting the traditional system for including EEPM fruits (mesocarp and pericarp) in the dietary management of diabetes mellitus and possible prevention of its complications. It may also serve as a good alternative in the present armamentarium of antidiabetic drugs. Further studies are required to fractionate, purify, and identify the active principle(s) present in the fruits (mesocarp and pericarp) extract of *P. macrocarpa*. There is a need to identify the exact mechanism of antihyperglycaemic activity, and to standardize the effective minimum dosage in rats. Furthermore, our study not similar to other previous studies, serve as the benchmark for venturing into high primates and possibly the clinical trial phase.

Abbreviations

P. macrocarpa: *Phaleria macrocarpa*; EEPM: Ethanol extract of *P. macrocarpa*; SOD: Superoxide dismutase; STZ: Streptozotocin; LD: Lethal dose; N: Normal; N.O*: Not observed; OGTT: Oral glucose tolerance test; ASEAN: Association of

Southeast Asian Nations; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; ALP: Alkaline phosphatase; T.BIL: Total bilirubin; T.PROT: Total proteins; HDL: High-density lipoprotein; LDL: Low-density lipoprotein; Glu: Glucose; ALB: Albumin; CNS : Central nervous system; ANS: Autonomic nervous system; OECD: Organization of Economic Cooperation and Development; HSD: Honest significant difference; SEM: Standard error mean; ANOVA: One-way analysis of variance; EDTA: Ethylene diamine tetraacetic acid; MCF-7: Breast cancer cell line; HeLa: Cervical cancer cell line; CaSki: Cervix cancer; CGNH-89 and CGNH: Malignant brain tumors

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Plant authentication
The fruit of this plant was verified by a taxonomic expert with an authentication number (P-0230).

Authors' contributions
A. K. A, W. A. S. conception and design of the work; A. K. A. acquisition, analysis, interpretation of data; the creation of new software used in the work; provided funding acquisition, project administration, and resources and A. K. A. wrote the paper. All authors have read and approved the manuscript.

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Availability of data and materials
All data and materials should be available upon request.

Ethics approval and consent to participate
Experimental animals and procedure of this study were approved by the Institutional Animal Care and Use Committee (IACUC) bearing a protocol approval number IACUC Approval/2014/ (3) (8). It conformed to the International guidelines for the use and care of laboratory animals (OECD, 425).

Consent for publication
Not applicable

Competing interests
The authors declare that there is no conflict of interest.

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