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


**A R T I C L E   I N   P R E S S**

**ABSTRACT**

The current study aimed to ascertain the antidiabetic potential of *Pseuduvaria monticola* bark methanolic extract (PMm) using *in vitro* mechanistic study models. In particular, the study determined the effect of PMm on cellular viability, 2-NBDG glucose uptake, insulin secretion, and NF-κB translocation in mouse pancreatic insulinoma cells (NIT-1). Furthermore, *in vivo* acute toxicity and antidiabetic studies were performed using streptozotocin (STZ)-induced type 1 and STZ-nicotinamide-induced type 2 diabetic rat models to evaluate various biochemical parameters and markers of oxidative stress and pro-inflammatory cytokines. Five isoquinoline alkaloids and three phenolic compounds were tentatively identified in the PMm by LC/MS Triple TOF. The study results showed that PMm is non-toxic to NIT-1 cells and significantly increased the glucose uptake and insulin secretion without affecting the translocation of NF-κB. Moreover, the non-toxic effects of PMm were confirmed through an *in vivo* acute toxicity study, which revealed that the serum insulin and C-peptide levels were significantly upregulated in type 2 diabetic rats and that no significant changes were observed in type 1 diabetic rats. Similarly, PMm was found to downregulate the levels of oxidative stress and pro-inflammatory cytokines in type 2 diabetic rats by alleviating hyperglycemia. Therefore, we conclude that PMm may be developed as an antidiabetic agent for the treatment of type 2 diabetes-associated conditions.

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**1. Introduction**

Diabetes mellitus (DM) is a metabolic disorder in the endocrine system characterised by abnormally high levels of glucose in the blood [Arya et al., 2012a]. DM is categorised into type 1 and type 2 diabetes. Type 1 diabetes mellitus (T1DM) is caused by impaired insulin secretion from pancreatic β-cells, whereas type 2 diabetes mellitus (T2DM) is characterised by carbohydrate, lipid, and protein disorders and defects in insulin signalling involving insulin resistance. T2DM’s prevalence is closely related to obesity due to the practice of an unhealthy sedentary lifestyle [Crawford et al., 2010]. It was purported by the International Diabetes Federation (IDF) that the number of diabetic cases will increase from 194 million in 2003 to 333 million by the year 2025 [Zimmet et al., 2003]. Hyperglycaemia, a characteristic of diabetes, has been shown to increase the production of reactive oxygen species (ROS) and promote oxidative stress resulting in the reduction of antioxidant levels. Previous studies have also demonstrated the relationship between oxidative stress and inflammatory cytokines in diabetic nephropathy (Elmarakby and Sullivan, 2012; Vincent et al., 2004).

A variety of plants that were traditionally used in the treatment and management of diabetes mellitus in folk medicine are known to demonstrate antidiabetic properties [Jung et al., 2006a; Paydar et al., 2013a,b]. The active chemical constituents isolated from these plants that are responsible for these hypoglycaemic properties include glycosides, flavonoids, phenolics, steroids, alkaloids, and terpenoids [Sharma et al., 2010; Chung et al., 2011]. Researchers are now attempting to further investigate natural resources for...
2.4. In vitro cell line studies

Pseuduvaria monticola belongs to the Annonaceae family. Plants from the Annonaceae family are mostly studied for their bioactive chemical constituents, which display a variety of pharmacological properties, including anti-diabetic activity (Aminimoghadamfarouj et al., 2011; Leboeuf et al., 1980). For example, Annona squamosa increases the plasma insulin level, and Annona muricata reduces oxidative stress in the pancreatic β-cells of streptozotocin-induced diabetic rats (Patel et al., 2012). In contrast, the chemical constituents from Pseuduvaria species exhibit various biological activities, such as anticancer, antibacterial, antituberculosis, and antimarial activities (Wirasathien et al., 2006; Taha et al., 2011; Uadkla et al., 2013).

Pseuduvaria species are traditionally used to treat fever, nausea, headache, and stomach ailments and have been mostly studied for their alkaloids (Chuakul and Sorinthornchareon, 2003; Sharief, 2007). In Malaysia, the Pseuduvaria species are generally known as Mempisang (Latiff et al., 1999). In this study, we investigated the anti-diabetic activity of this plant because Mempisang is widely used by the natives in East Malaysia as traditional medicine. Thus, the present work was designed to evaluate the hypoglycaemic and anti-diabetic effects of the bark methanolic extract of P. monticola (PMm) using in vitro and in vivo models with the aim of establishing the pharmacological basis for its use for the treatment of diabetes and its associated complications.

2. Materials and methods

2.1. Plant material

P. monticola was collected from the national park located at Cameron Highland, Pahang, Malaysia in October 2011. The plant was botanically identified and classified by the phytochemical group of the Department of Chemistry, Faculty of Science, University of Malaya. A voucher specimen (HER 0009) was deposited at the herbarium, Chemistry Department, University of Malaya.

2.2. Methanolic extract preparation

The dried stem bark of P. monticola (500 g) was ground and macerated with methanol (3 × 1 L) for three days, filtered, and evaporated to dryness under reduced pressure to yield the methanol crude extract (28.5 g).

2.3. LC/MS triple TOF analysis

The chemical compounds in the stem bark were analysed through direct infusion to the ion source delivered by an automated syringe injector at a rate of 10 μL per min (1:99 dilution). The mass spectrometer (AB Sciex TripleTOF 4600) was operated with a Duo Spray source and an electrospray (ESI) probe equipped with an Automated Calibration Delivery system. The turbo ion source settings were recorded from m/z 50 to m/z 1000. The compounds were characterised based on their MS/MS fragmentation spectra data through correlation with previous reports.

The acquisition data were processed using the Analyst software (version 1.5.1).

2.4. In vitro cell line studies

2.4.1. Cell culture

The mouse pancreatic β-cell line NIT-1 was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were grown in Dulbecco’s Modified Eagle Medium (DMEM, Life Technologies, Inc. Rockville, MD, USA) supplemented with 10% heat-inactivated foetal bovine serum (FBS, Sigma-Aldrich, St. Louis, MO, USA) and 1% penicillin and streptomycin. The cells were cultured in tissue culture flasks (Corning, USA) and maintained in an incubator at 37 °C in a humidified atmosphere with 5% CO₂. For experimental purposes, cells in the exponential growth phase (approximately 70–80% confluence) were used.

2.4.2. MTT cell viability assay

The influence of PMm on NIT-1 cells was determined through an MTT assay 48 h after treatment (Paydar et al., 2013c). On the first day, 1.0 × 10⁴ cells were seeded into a 96-well plate, and the plate was incubated overnight at 37 °C in 5% CO₂. The following day, the cells were treated with a twofold dilution series of concentrations of PMm and were incubated at 37 °C in 5% CO₂ for 48 h. MTT solution (4,5-dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide) was then added at a concentration of 2 mg/mL, and the mixture was incubated for 2 h at 37 °C in 5% CO₂ to dissolve the formazan crystals in DMSO. The plate was then read at 570 nm absorbance in a Chameleon multitechnology microplate reader (Hidex, Turku, Finland). The viability percentage of the cells was calculated as the ratio of the absorbance of the PMm-treated cells to the absorbance of the DMSO-treated control cells (Looi et al., 2011; Paydar et al., 2013c). IC₅₀ was defined as the concentration of PMm that caused a 50% percent reduction in the absorbance of the treated cells compared with the DMSO-treated control cells. The experiment was performed in triplicate.

2.4.3. Real-time cell proliferation

The in vitro proliferation of PMm-treated and untreated cells was surveyed using an xCELLigence Real-Time Cellular Analysis (RTCA) system (Roche, Mannheim, Germany) as described previously (Looi et al., 2013a). On the first day, 1.0 × 10⁴ cells were seeded in each well of a 16- well plate with electrodes for 18 h to allow the cells to grow to the log phase. Before the cells were seeded, background measurements were performed by adding 100 μL of the culture medium to the wells. The RTCA system monitored the proliferation of the cells every 5 min for a period of approximately 20 h. During the log growth phase, the cells were treated or untreated with different concentrations (3.125, 6.25, 12.5, 25, 50, 100, and 200 μg/mL) of PMm and monitored continuously for 72 h.

2.4.4. 2-NBDG glucose uptake

The effect of PMm al compounds on the uptake of fluorescent hexose 2-NBDG (a glucose analogue) by NIT-1 cells was investigated as previously described (Loaiza et al., 2003; Ayta et al., 2012). In brief, 1.0 × 10⁴ cells were seeded in a 96-well plate and incubated overnight at 37 °C in 5% CO₂. The medium was then discarded, and the cells were washed twice with phosphate-buffered saline (PBS). Then, 100 μL of glucose-free DMEM media supplemented with 1-glutamine and 15% fetal bovine serum was added to each well, and the cells were incubated for 60 min at 37 °C in 5% CO₂. The conditioning medium was then replaced with basal medium containing 1 mM 2-NBDG (Invitrogen, Carlsbad, CA, USA) in the presence or absence of glibenclamide (positive control), PMm, or the compounds. Due to the nonspecific background of 2-NBDG, which could affect the results, different concentrations of 2-NBDG were tested prior to the glucose uptake assay to identify the optimum dose of 2-NBDG with the greatest signal-to-noise ratio. Based on this test, the selected concentration of 2-NBDG was 1 mM, which was the minimum concentration capable of producing a signal-to-noise ratio of 3.0. The cells were incubated for 30 min at 37 °C in 5% CO₂, to permit the endocytosis of 2-NBDG. The 2-NBDG-containing medium was then removed, and the cells were washed with PBS and stained with the nucleic dye Hoechst 33342 for 30 min. The intracellular fluorescence of the cells was observed at Excitation/Emission = 350 nm/461 nm and Excitation/Emission = 475 nm/530 nm for Hoechst 33342 and 2-NBDG, respectively. The plates were then evaluated using the ArrayScan High Content Screening (HCS) system (Cellomics Inc., Pittsburgh, PA, USA), and the data were analysed with the Target Activation BioApplication software (Cellomics Inc.). HCS is a computerised automated fluorescence imaging microscope that automatically identifies stained cells and reports the intensity and distribution of fluorescence in individual cells. The ArrayScan HCS system scans multiple fields in individual wells to acquire and analyse images of single cells according to defined algorithms (Tiong et al., 2013). The images and data regarding the texture of the 2-NBDG fluorescence within each cell and the average fluorescence intensity of the cell population within a well were stored in a Microsoft SQL database for easy retrieval. The data were captured, excluded, and analysed using the ArrayScan II Data Acquisition and Data Viewer (version 3.0, Cellomics).

2.4.5. In vitro insulin secretion

NIT-1 cells (1.0 × 10⁴ cells/ml) were seeded in a 24-well plate and incubated overnight at 37 °C in 5% CO₂. The following day, the cells were washed twice with glucose-free Krebs/Hepes Ringer solution (115 mM NaCl, 24 mM NaHCO₃, 5 mM KCl, 1.1 mM MgCl₂, 2.5 mM CaCl₂, and 25 mM Hepes [pH 7.4]) and pre-incubated at 37 °C in 5% CO₂ for 30 min with the glucose-free Krebs/Hepes Ringer solution. The cells were then incubated for 1 h in Krebs/Hepes Ringer solution containing 1 mg/mL bovine serum albumin and 6.25, 12.5, or 25 mM glucose in the presence or absence of PMm. An aliquot of the supernatant was collected for ELISA. The amount of insulin released was measured with a Mouse Insulin ELISA kit (Merodia, Uppsala, Sweden) according to the manufacturer’s protocol. The results (in pmol) were expressed as the means ± SD of three independent experiments.

2.4.6. NF-κB translocation

Briefly, 1.0 × 10⁴ cells were seeded in a 96-well plate and incubated overnight at 37 °C in 5% CO₂. The cells were pretreated with different concentrations of PMm for 3 h and then stimulated with 1 ng/mL TNF-α for 30 min. The medium was removed, and the cells were fixed and stained with the Cellomics nucleus factor-kB (NF-κB) activation kit from Thermo Scientific according to the manufacturer’s instructions. The plate was evaluated using an Array Scan HCS Reader. The
2.5. ROS assay (reactive oxygen species)

A total of 1.0 × 10⁷ NT-1 cells were seeded in each well of a 96-well plate and incubated overnight at 37 °C in 5% CO₂. The cells were exposed to 200 μM H₂O₂ in serum-free RPMI 1640 containing 10% FCS in the presence or absence of quercetin (positive control), PMm, or the compounds. Then, 20 μM dihydroethidium (DHE, Molecular Probes) was added to the medium, and the cells were incubated for 30 min. The DHE dye reagent is converted to fluorescent ethidium and intercalates into DNA in response to intracellular ROS. The cells were fixed and washed with wash buffer as described by the manufacturer’s instructions. The stained cells were visualised and acquired using a Cellomics ArrayScan HCS reader (Thermo Scientific) according to a protocol described previously (Looi et al., 2013b). The target activation bioapplication module was used to quantify the fluorescence intensities of the DHE dye in the nucleus.

2.6. In vivo studies

Healthy mature Sprague Dawley (SD) rats of either sex were procured from the Experimental Animal House, Faculty of Medicine, University of Malaya. The rats, which weighed 200–225 g, were maintained in wire-bottomed cages at 25 ± 2 °C, given tap water and a standard pellet diet, and exposed to a 12-h light/12-h dark cycle. Throughout the experiments, all of the animals received human care according to the criteria outlined in the “Guide for the Care and Use of Laboratory Animals” prepared by the National Academy of Sciences and published by the National Institute of Health (Ethics Number: PAR/10/11/2008/AA[R]).

2.6.1. In vivo acute toxicity study

The oral glucose tolerance test (OGTT) was used to evaluate those group segments that demonstrated the highest glycemc control in the type 1 and type 2 diabetic rat models to determine the effectiveness of PMm. PMm (250 or 500 mg/kg) was administered orally to overnight-fasted rats at a dose of 2 mL/200 g bw. The fasting blood glucose concentrations were measured before the respective PMm administrations and after the oral glucose (3 g/kg) was administered, and the blood glucose levels were measured at 30, 60, 90, and 120 min.

2.6.2. Type 1 diabetes in rats

Type 1 diabetes in rats was induced in normal male rats. The rats were fasted for 15 h (overnight period) and then injected intraperitoneally with 65 mg/kg of freshly prepared STZ (Sigma-Aldrich, St. Louis, MO, USA) in 0.1 M citrate buffer (pH 4.5) in a volume of 1 mL/kg bw (Arya et al., 2012b). These STZ-injected rats were administered a 20% glucose solution for 12 h to prevent hypoglycaemic mortality that may be caused by the STZ injection. Diabetes was confirmed in the rats by measuring their blood glucose levels 96 h after the STZ administration. Rats with a fasting blood glucose level of 19–24 mmol/L were considered to have type 1 diabetes and were used in the study.

2.6.3. Type 2 diabetes in rats

Type 2 diabetes in rats was induced in normal male rats. The rats were fasted for 15 h (overnight period) and then administered nicotinamide intraperitoneally at a dose of 210 mg/kg. After 15 min, 55 mg/kg bw of freshly prepared STZ in 0.1 M citrate buffer (pH 4.5) in a volume of 1 mL/kg bw was injected (Arya et al., 2012b). Diabetes was confirmed in rats by measuring their blood glucose levels 96 h after the STZ-nicotinamide administration. Rats with fasting blood glucose level of 11–14 mmol/L were considered to have type 2 diabetes and subsequently used in the study.

2.6.4. Division of diabetic animals for the study

The type 1 and type 2 diabetic rats were divided into two segments in the present study. The type 1 segment included the following groups: Group 1, normal control rats; Group 2, type 1 diabetic control rats; Group 3, type 1 diabetic rats treated with 6 U/kg insulin (standard positive); Group 4, diabetic rats treated with 250 mg/kg PMm; and Group 5, diabetic rats treated with 500 mg/kg PMm. The type 2 segment was divided into the following groups: Group 1, normal control rats; Group 2, type 2 diabetic control rats; Group 3, type 2 diabetic rats treated with 30 mg/kg glimepiride (standard positive); Group 4, diabetic rats treated with 250 mg/kg PMm; and Group 5, diabetic rats treated with 500 mg/kg PMm. The rats in each group were injected and fed the respective doses of PMm and standard drug once daily every morning for 45 days; the administration was based on volume (2 mL/200 g bw), and PMm was completely dissolved in distilled water and filtered before administration.

2.6.5. Experimental procedure

The fasting blood glucose levels of all the rats in each group were measured on every 11 days after administration through a glucose oxidase-peroxidase enzymatic method using a standardised glucometer (Accu-Check Performa, Roche Diagnostic Germany) and the tail snipping method; the changes in body weight, food intake, and water intake were recorded daily. After 45 days of treatment, all of the groups were fasted for 12 h and then anesthetised using pentobarbital. The blood was collected into heparinised tubes and centrifuged at 2000 rpm for 10 min, and the serum was collected and stored at –80 °C until analysis to determine the biochemical parameters, oxidative stress markers, and pro-inflammatory cytokine levels. The liver, kidney, and pancreas were removed, washed in ice-cold isotonic saline, and blotted individually on ash-free filter paper; the organs were weighed, and the tissues were collected and fixed in 10% formalin for histology estimation.

2.6.6. Glucose tolerance test

The oral glucose tolerance test (OGTT) was used to evaluate those group segments that demonstrated the highest glycemc control in the type 1 and type 2 diabetic rat models to determine the effectiveness of PMm. PMm (250 or 500 mg/kg) was administered to overnight-fasted rats at a dose of 2 mL/200 g bw. The fasting blood glucose concentrations were measured before the respective PMm administrations and after the oral glucose (3 g/kg) was administered, and the blood glucose levels were measured at 30, 60, 90, and 120 min.

2.7. Statistical analysis

The results are expressed as the means ± standard deviation (SD). Significant differences between the means of the experimental groups were assessed using the Tukey–Kramer multiple comparisons test (GraphPad version 5.0; GraphPad Software Inc., San Diego, CA, USA).

3. Result

3.1. Characterisation of chemical constituents by LC/MSQTOF

The characteristics of the chemical compounds in PMm was achieved through the direct infusion of the sample into an electrospray ionisation (ESI) source (Table 1 and Fig. S1a–e). This method provides a fast analytical approach for the direct screening and identification of the phytoconstituents and metabolites in plant extracts (Vu et al., 2008); these experiments were conducted in the positive and negative modes to detect protonated [M + H]+ and deprotonated [M – H]− compounds, respectively. Basic or neutral compounds with amino groups, amides or carboxyls are best detected in positive ion mode via the positively charged molecules (protonated), whereas compounds with hydroxyl groups or carbonyl groups that easily lose a proton (deprotonated) can be easily detected in negative ion mode with greater sensitivity (Strege, 1999). Five known oxoaporphine and dioxoaporphine alkaloids were identified: liriodenine (Compound 1, Table 1 and Fig. S1b), N-methylouergidine, ou ergidine (Compounds 2 and 3, respectively; Table 1 and Fig. S1c), oxostephanine, and lysicamine (Compounds 4 and 5, respectively; Table 1 and Fig. S1d). In addition to these alkaloids, we identified three phenolic compounds: Compounds 6, 7, and 8, namely caffeoylquinic acid, quinic acid, and caffeic acid, respectively (Table 1 and Fig. S1e). These compounds were tentatively identified based on fragmentation patterns and comparison with published literature data. In the mass spectra, the key fragmentation patterns of the oxoaporphine alkaloids are...
353 mainly based on M-15 and M-31 (loss of CH₃ and CH₃O). If a methylenedioxy group is present, the loss of formaldehyde (CHO) and CO is observed (Stevigny et al., 2004). The phenolic compounds detected in the negative mode. The aporphine alkaloids identified in the extract were also previously discovered and identified in other *Pseuduvaria* species belonging to the same family (Taha et al., 2011; Wirasathien et al., 2006).

3.2. PMm demonstrated no cytotoxicity on NIT-1 cells

The cytotoxic effect of PMm on NIT-1 cell viability was determined through an MTT assay, and no significant cytotoxicity and cell inhibitory effects were observed in the NIT-1 cells after 48 h of treatment with PMm at a concentration up to 100 μg/ml (Fig. 1a). The results obtained from the real-time cell proliferation assay (RTCA) also indicated no significant difference in the cell proliferation between the control NIT-1 cells and the cells treated with 200, 100, 50, 25, 12.5, 6.25, and 3.125 μg/ml PMm throughout the 72 h of treatment (Fig. 1b).

3.3. PMm reduced H₂O₂-induced oxidative stress in NIT-1 cells

We then assessed the anti-oxidant ability of PMm in reducing H₂O₂-induced oxidative stress. H₂O₂ induced the accumulation of the DHE dye in the nucleus of NIT-1 cells (Fig. 2a). Pre-treatment with 25 μg/ml PMm significantly decreased the fluorescence intensity. We then attempted to identify the potential compounds responsible for this effect. Of the eight above mentioned compounds, caffeic acid, quinic acid, and caffeoylquinic acid showed the highest reduction of the oxidative stress burden, followed by liriodenine, N-methylouregidione, ouregidione, oxostephanine, and lysicamine (Fig. 2b).

3.4. PMm enhanced the 2-NBDG glucose uptake in NIT-1 cells

The uptake of the fluorescent hexose 2-NBDG, a glucose analogue, was assayed on NIT-1 cells. The fluorescence was markedly increased in the PMm-treated cells compared with the untreated cells (Fig. 3a and b). To determine which compounds are responsible for this effect, we repeated the experiments with the eight

Table 1

<table>
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<tr>
<th>Compound</th>
<th>MW</th>
<th>[M + H]+ (positive mode)</th>
<th>[M – H] (negative mode)</th>
<th>ms/ms ions (relative intensity)</th>
<th>Reference</th>
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<td>Liriodenine</td>
<td>275</td>
<td>m/z 276</td>
<td>–</td>
<td>m/z 248, m/z 219</td>
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<tr>
<td>N-methylouregidione</td>
<td>351</td>
<td>m/z 352</td>
<td>–</td>
<td>m/z 337, m/z 308</td>
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<tr>
<td>Ouregidione</td>
<td>337</td>
<td>m/z 338</td>
<td>–</td>
<td>m/z 322</td>
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<tr>
<td>Oxostephanine</td>
<td>305</td>
<td>m/z 306</td>
<td>–</td>
<td>m/z 278</td>
<td>Nik Abdullah Zawawi et al. (2012)</td>
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<tr>
<td>Lysicamine</td>
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<td>–</td>
<td>m/z 264, m/z 233</td>
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<td>Caffeoylquinic acid</td>
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<td>–</td>
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<td>–</td>
<td>m/z 179</td>
<td>m/z 135</td>
<td>Sánchez-Rabaneda et al. (2003)</td>
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Fig. 1. Effect of PMm on cell viability. (a) MTT assay growth curve of mouse NIT-1 cells treated with different concentrations of PMm. (b) Dynamic monitoring of proliferation of NIT-1 cells treated with different concentrations of PMm compared to the untreated cells using RTCA. The cells were seeded in a 16× E-plate device and monitored continuously up to 72 h after treatment. CI values were normalised to the time point of treatment, indicated by the vertical black line.

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isolated compounds. Caffeic acid, quinic acid, and caffeoylquinic acid were found to enhance glucose uptake in NIT-1 cells, whereas liriodenine, N-methylouregidione, oureidione, oxostephanine, and lycsinamne showed no significant changes compared with the control cells. In general, the fluorescence was restricted to the cytoplasm, which indicates that the cells retained their heterogeneous glucose uptake activity (Fig. 4a and b).

3.5. Effect of PMm on insulin secretion

PMm markedly increased insulin secretion in a dose-dependent manner at glucose concentrations of 6.25, 12.5, and 25 mM (Fig. 5). A significant induction of insulin secretion was observed in NIT-1 cells treated with PMm at a concentration as low as 12.5 μg/ml, and the induction level obtained with the higher concentrations remained unchanged compared with that obtained with 12.5 μg/ml.

3.6. PMm did not inhibit NF-κB nuclear translocation

Nuclear factor kappa B (NF-κB) is a transcription factor that is critical for cytokine gene expression. The activation of NF-κB in response to inflammatory cytokines, such as tumour necrosis factor α (TNF-α), mediates nuclear migration to enable DNA binding and facilitate target gene expression. PMm exhibited no inhibitory effect against TNF-α-stimulated nuclear NF-κB translocation. In addition, in both control and treated cells, TNF-α stimulation led to NF-κB translocation from the cytoplasm to the nucleus (Fig. 6a and b).

Fig. 2. PMm or its chemical constituents alleviated H2O2-induced ROS production. (a) Fluorescence images of mouse NIT-1 cells exposed to 200 μM H2O2 in serum-free DMEM for 1 h in presence or absence of quercetin (positive control), PMm or the compounds. The cells were stained with 20 μM of dihydroethidium for 30 min. PMm, caffeic acid, quinic acid and caffeoylquinic acid exhibited significant anti-oxidative activity against H2O2-induced ROS production in the treated NIT-1 cells. (b) Bar chart indicating average fluorescence intensities of DHE dye in the nucleus. Data were mean ± SD of fluorescence intensity readings representative of three independent experiments. Significant differences compared to 200 mM H2O2-stimulated untreated control (* P < 0.001; Student’s t-test).
3.7. Acute toxicity study

Different concentrations (100, 200, 400, 800, and 2000 mg/kg) were used to evaluate whether PMm is cytotoxic in type 1 and type 2 diabetic rats. The acute oral toxicity studies revealed that PMm is non-toxic in nature; in fact, no lethal or toxic reactions were observed at any of the doses tested.

3.8. Effect of PMm on blood glucose levels of type 1 and type 2 diabetic rats

The fasting blood glucose levels of all of the groups in each of the segments (the type 1 and type 2 diabetic rat model segments) were measured starting on day 11 to the end of the 45-day treatment period. The results of the type 2 diabetic model segments revealed a significant reduction in the elevated blood glucose levels of type 2 diabetic rats treated with PMm (500 and 250 mg/kg) compared with that obtained for the untreated diabetic rats (Table 2a). In contrast, the type 1 diabetic rats did not show any significant reduction in their blood glucose level after treatment with PMm at different doses (500 and 250 mg/kg) compared with the untreated diabetic rats. At the end of the study period, the percentage inhibition effected by 500 and 250 mg/kg bw PMm was 55.47% and 36.49%, respectively, compared with that effected by glibenclamide (62.04%) in the type 2 diabetic model segment (Table 3a).

3.9. Oral glucose tolerance test of type 2 diabetic rats

Table 4 displays the results of the oral glucose tolerance test on type 2 diabetic rats. Upon treatment with 500 and 250 mg/kg PMm, the diabetic rats showed significant decreases in their blood glucose levels after 60 min of glucose load compared with
untreated diabetic rats. Similarly, after 90 min, the blood glucose levels were consistently reduced to 56.64% and 46.82% in the PMm-treated group, whereas the glibenclamide-treated group exhibited a reduction of 61.27%. This initial reduction was followed by a reduction of 66.12% and 56.83% 120 min after the administration of the PMm and glibenclamide doses, respectively, whereas glibenclamide displayed a 69.94% reduction in the blood glucose levels.

### 3.10. Effect of PMm on Insulin and C-peptide level of type 1 and type 2 diabetic rats

Tables 2b and 3b demonstrate the insulin and C-peptide levels in the type 1 and type 2 diabetic segment groups. The untreated diabetic rats in both segments exhibited significant reduction in their insulin and C-peptide levels in the serum compared to the normal control rats. The daily treatment of the type 2 diabetic rats with the positive standard or 500 or 250 mg/kg bw PMm for 45 days showed significant increase in the insulin and C-peptide levels compared with the untreated diabetic rats. In contrast, the insulin and C-peptide levels of the type 1 diabetic rats did not present any significant sign of improvement.

### 3.11. Effect of PMm on oxidative stress markers of type 1 and type 2 diabetes in rats

In both segments groups, the serum GSH levels were reduced, whereas the MDA levels were significantly elevated in the untreated diabetic rats. Similarly, after 90 min, the blood glucose levels were consistently reduced to 56.64% and 46.82% in the PMm-treated group, whereas the glibenclamide-treated group exhibited a reduction of 61.27%. This initial reduction was followed by a reduction of 66.12% and 56.83% 120 min after the administration of the PMm and glibenclamide doses, respectively, whereas glibenclamide displayed a 69.94% reduction in the blood glucose levels.

Fig. 4. Glucose uptake evaluation after treatment with PMm’s chemical constituents. (a) Fluorescence images of PMm/compound-treated and untreated mouse NIT-1 cells exposed to 1 mM of the fluorescent hexose, 2-NBDG, for 30 min revealing heterogeneous 2-NBDG uptake and metabolic activity of PMm, caffeic acid, quinic acid and caffeoylquinic acid. (b) Bar chart representing glucose uptake in PMm/compound-treated and untreated NIT-1 cells after 30 min exposure to 1 mM 2-NBDG. Data were mean ± SD of fluorescence intensity readings representative of three independent experiments. Significant differences compared to negative control (’P < 0.001; Student’s t-test).
untreated diabetic rats compared with the normal control rats (Tables 2b and 3b). Upon administration of PMm at a dose of 500 mg/kg, we observed a significant increase in the serum GSH levels of type 2 diabetic rats compared with that found in the untreated diabetic rats. In contrast, the type 1 diabetic rats treated with different doses of PMm did not exhibit any significant increase in their GSH levels. However, the administration of PMm at a dose of 500 mg/kg significant decreased the serum MDA levels of both type 1 and type 2 diabetic rats (Tables 2b and 3b).

3.12. Effect of PMm on pro-inflammatory cytokine levels in type 1 and type 2 diabetic rats

The untreated diabetic rats in the type 1 and type 2 diabetic model segment groups exhibited significantly elevated levels of the pro-inflammatory cytokines TNF-α, IL-1β, and IL-6 in the serum compared with those of normal control rats (Tables 2b and 3b). The daily administration of PMm (500 mg/kg) for 45 days resulted in a significant downregulation of TNF-α and IL-6 in the serum of untreated diabetic rats compared with the normal control rats (Tables 2b and 3b). Upon administration of PMm at a dose of 500 mg/kg, we observed a significant increase in the serum GSH levels of type 2 diabetic rats compared with that found in the untreated diabetic rats. In contrast, the type 1 diabetic rats treated with different doses of PMm did not exhibit any significant increase in their GSH levels. However, the administration of PMm at a dose of 500 mg/kg significant decreased the serum MDA levels of both type 1 and type 2 diabetic rats (Tables 2b and 3b).
479 in the serum.

478 regulation of the pro-inflammatory cytokines TNF-

476 doses of PMm (500 and 250 mg/kg). In contrast, the type 1 diabetic

475 diabetic rats, whereas IL-1

474 Significant difference compared to normal control group (P < 0.05).

473 Significant difference compared to diabetic control (P < 0.05).

472 Effects of PMm on serum Insulin, C-Peptide, GSH, MDA, TNF-

471 Effects of PMm on fasting blood glucose level of type 2 diabetic rats.

470 Parenthesis values shows, percentage decrease of blood glucose level in the treatment groups, compared to diabetic control within same day.

469 Mean values ± SD, n = 6.

468 Parenthesis values shows, percentage decrease of blood glucose level in the treatment groups, compared to diabetic control within same day.

467 Significant difference compared to diabetic control (P < 0.05).

466 Significant difference compared to diabetic control (P < 0.05).

465 Table 2a

464 Effects of PMm on fasting blood glucose level of type 1 diabetic rats.

463

462 Group

461 Fasting blood glucose level (mmol/L)

460 No treatment Treatment Days

460 Day 0 Day 11 Day 22 Day 33 Day 44

460 Normal control

461 5.1 ± 0.23 4.9 ± 0.33 5.3 ± 0.27 5.0 ± 0.31 5.6 ± 0.44

462 Diabetic control

463 19.4 ± 2.06 22.8 ± 2.47 23.5 ± 1.92 24.1 ± 2.10 25.7 ± 1.32

464 Insulin (6 U/kg)

465 20.3 ± 0.93 6.9 ± 0.64 (71.05) 5.9 ± 0.26 (74.89) 7.1 ± 1.57 (76.53) 5.3 ± 0.81 (79.17)

466 PMm (500 mg/kg)

467 21.4 ± 0.76 18.5 ± 1.32 (18.85) 17.2 ± 0.89 (20.50) 16.7 ± 1.66 (30.70) 17.6 ± 0.54 (31.51)

468 PMm (250 mg/kg)

469 19.7 ± 1.13 20.3 ± 1.43 (10.96) 19.2 ± 1.25 (18.20) 18.8 ± 0.98 (21.99) 19.5 ± 1.67 (24.12)

460 Mean values ± SD, n = 6.

459 Parenthesis values shows, percentage decrease of blood glucose level in the treatment groups, compared to diabetic control within same day.

458 * Significant difference compared to diabetic control (P < 0.05).

457 Table 2b

456 Effects of PMm on serum Insulin, C-Peptide, GSH, MDA, TNF-α, IL-6, and IL-1β levels of type 1 diabetic rats when compared to diabetic and normal control rats.

455

454 Group

455 Normal control 169.4 ± 8.3 263.6 ± 9.1 67.4 ± 9.6 75.4 ± 9.5 66.4 ± 11.1

455 Diabetic control 267.3 ± 16.4 57.8 ± 22.4 112.6 ± 23.7 106.2 ± 17.8 76.4 ± 13.5

455 Insulin (mmol/mL)

456 GSH (μmol GSH/g) 276.8 ± 15.2 56.4 ± 21.5 167.4 ± 21.5 99.1 ± 17.9 79.3 ± 28.3

455 MDA (nmoles MDA/g) 56.4 ± 15.2 164.8 ± 21.5 102.7 ± 21.5 116.4 ± 17.9 163.9 ± 28.3

455 TNF-α (pg/mL) 16.4 ± 4.78 89.5 ± 11.4 31.3 ± 6.43 67.5 ± 8.76 75.9 ± 9.56

455 IL-6 (pg/mL) 22.4 ± 4.65 95.4 ± 11.76 36.3 ± 6.23 68.9 ± 5.65 83.4 ± 9.87

455 IL-1β (pg/mL) 21.3 ± 3.78 92.4 ± 4.78 41.3 ± 4.87 64.8 ± 7.98 78.9 ± 9.34

454 Data are presented as means ± SD (n = 6).

453 * Significant difference compared to normal control group (P < 0.05).

452 * Significant difference compared to diabetic control (P < 0.05).

451 Table 3a

450 Effects of PMm on fasting blood glucose level of type 2 diabetic rats.

449

448 Group

448 Fasting blood glucose level (mmol/L)

448 No treatment Treatment Days

448 Day 0 Day 11 Day 22 Day 33 Day 44

448 Normal control

449 5.6 ± 0.65 5.3 ± 0.71 5.9 ± 0.54 6.2 ± 0.81 5.1 ± 0.25

450 Diabetic control

451 10.4 ± 0.97 11.9 ± 0.76 12.6 ± 0.39 12.9 ± 0.71 13.7 ± 0.66

452 Glibenclamide (50 mg/kg)

453 9.9 ± 0.65 6.4 ± 0.54 (40.21) 5.7 ± 0.32 (60.76) 5.5 ± 0.87 (57.36) 5.2 ± 0.54 (52.04)

454 PMm (500 mg/kg)

455 11.8 ± 0.88 7.4 ± 0.33 6.5 ± 0.39 (46.41) 5.9 ± 0.76 (54.28) 6.1 ± 0.35 (55.47)

456 PMm (250 mg/kg)

457 10.8 ± 1.03 9.5 ± 0.70 (20.15) 9.4 ± 0.43 (25.30) 8.8 ± 0.67 (25.17) 8.7 ± 0.44 (26.46)

450 Mean values ± SD, n = 6.

449 Parenthesis values shows, percentage decrease of blood glucose level in the treatment groups, compared to diabetic control within same day.

448 * Significant compared to diabetic control (P < 0.05).

447 Table 3b

446 Effects of PMm on serum Insulin, C-Peptide, GSH, MDA, TNF-α, IL-6, and IL-1β levels of type 2 diabetic rats when compared to diabetic and normal control rats.

445

444 Group

444 Normal control 164.5 ± 7.5 767 ± 8.6 153.6 ± 8.9 148.3 ± 9.1 131.6 ± 11.8

445 Diabetic control 279.2 ± 12.5 102.4 ± 10.1 234.7 ± 9.8 262.1 ± 11.1 204.7 ± 9.5

446 Glibenclamide (50 mg/kg)

447 265.7 ± 17.6 145.7 ± 34.2 234.5 ± 18.4 212.5 ± 32.7 165.2 ± 35.7

448 PMm (500 mg/kg)

449 52.6 ± 17.6 99.6 ± 22.8 54.8 ± 18.4 61.9 ± 23.6 85.7 ± 19.6

450 IL-6 (pg/mL)

451 18.3 ± 2.23 53.5 ± 5.67 (68.91) 22.7 ± 2.21 26.4 ± 3.35 43.5 ± 4.78

452 IL-1β (pg/mL)

453 25.4 ± 2.23 55.3 ± 5.12 29.4 ± 3.56 31.5 ± 4.43 44.2 ± 6.23

454 23.3 ± 2.47 59.5 ± 6.45 25.5 ± 3.76 28.6 ± 4.12 34.2 ± 5.56

453 Data are presented as means ± SD (n = 6).

452 * Significant difference compared to normal control group (P < 0.05).

451 * Significant difference compared to diabetic control (P < 0.05).

450 4. Discussion

451 In recent years, Pseuduvaria species were mostly studied for their bioactive chemical constituents, which were found to display a variety of pharmacological properties. This study provides the...
first demonstration of the antidiabetic potential of PMm on pancreatic NIT-1 cells and in vivo animal models. Moreover, we provided evidence that PMm is not toxic even at a high dosage (2000 mg/kg) and exerts a positive effect on type 2 diabetic rats by downregulating hyperglycemia, oxidative stress, and pro-inflammatory cytokines.

The antioxidant activity of PMm may be attributed to its rich concentration of polyphenolic active constituents. It is thought that phenolic acids, such as flavonoids, provide free radical scavenging activity and enhance the antioxidant status of the body (Karuna et al., 2009). In this study, five o xoaporphine alkaloids and three phenolic compounds were tentatively identified by LC/MS TripleTOF based on previous mass fragmentation data of known compounds. A previous study of medicinal plants used in the treatment of diabetes revealed that the presence of phenols, flavonoids, terpenoids, and alkaloids contributed to the antidiabetic activity of the plants. Caffeic acid and quinic acid, which are two of the major compounds in PMm, have been previously reported as active compounds with significant antioxidant and anti-hyperglycemic properties (Jung et al., 2006b; Pero et al., 2009). Caffeoylquinic acid also possess higher anti-oxidant and glucose uptake activity and has been shown to be a potent antidiabetic agent (Matsui et al., 2004). The results of this study demonstrated that the antidiabetic activity of PMm may be due to the presence of these compounds (caffeic acid, quinic acid, and caffeoylquinic acid).

We further investigated the antidiabetic effect of PMm on glucose uptake and insulin secretion by NIT-1 cells. Our results revealed that PMm dose-dependently stimulates glucose uptake and enhances insulin secretion in NIT-1 cells, which are a mouse insulinoma β-cell line and are commonly used for evaluating insulin secretion activity (Xia et al., 2011). Our data revealed that PMm significantly elevates insulin secretion in response to higher glucose levels, which suggests that PMm may enhance the sensitivity of NIT-1 cells to exogenous glucose by increasing the insulin level (Palmer and Brogden, 1993). The activation of the insulin signalling pathway may stimulate the glucose uptake of 2-NBDG through a positive feedback mechanism. In target cells, insulin initiates a signal transduction, which has the effect of increasing glucose uptake and storage. Activation of insulin receptors leads to internal cellular mechanisms that directly affect glucose uptake by regulating the number and operation of protein molecules in the cell membrane that transport glucose into the cell and thus glucose enters the β-cells through the glucose transporter, GLUT2. Previous study showed that the activation of Akt and p70 S6 kinase can increase glycogen, lipid, and protein synthesis and in turn promote glucose uptake (Salhieh and Kahn, 2001).

We then evaluated the effects of PMm on STZ-induced type 1 and STZ-nicotinamide-induced type 2 diabetic rat models. These diabetic rats showed significant reduction in their insulin and C-peptide levels, most likely due to the destruction of the pancreatic β-cells and excessive ROS production (Schein et al., 1973). The type 2 diabetic animals treated daily with PMm for 45 days exhibited upregulated insulin and C-peptide levels through the downregulation of hyperglycemia, oxidative stress, and elevated cytokines. C-peptide is the by-product of the enzymatic cleavage of proinsulin in pancreatic β-cells and is secreted into the circulation in equimolar concentrations (Doda, 1996). The measurement of C-peptide provides a sensitive, well accepted, and clinically validated assessment of β-cell function and is the most reliable primary outcome for the investigations of therapies aimed at preserving or improving endogenous insulin secretion in diabetic patients (Palmer et al., 2004). The significant increase in the levels of serum insulin and C-peptide observed in the PMm-treated diabetic rats may be due to the increased secretion of insulin from the remnant β-cells. It is also possible that PMm treatment resulted in a significant increase in hexokinase activity, which controls the blood glucose level (Saravanan et al., 2002). The elevated activity of hexokinase suggests a shift toward carbohydrate metabolism, which promotes the utilisation of glucose at peripheral sites, such as adipocytes or muscle cells (Saravanan et al., 2002).

In the present study, we observed that GSH was decreased and MDA was increased significantly in the serum of type 1 and type 2 diabetic animals. Thus, the decrease in GSH level might reflect a direct reaction between GSH and free radicals generated by hyperglycemia in DM. Of note, we observed a significant increase in the GSH (gluthatione) content in the serum of type 2 diabetic rats compared with untreated diabetic rats. GSH can act as an antioxidant by scavenging free radicals in the body. It has been described that a low GSH level in diabetes is an indicator of increased oxidative stress (Parveen et al., 2011). A decreased level of GSH in diabetic rats may increase their susceptibility to tissue oxidative damage due to the lower activity of the GST antioxidant enzyme (GSH is a substrate for GST activity) (Arya et al., 2012c,d). Thus, PMm treatment may increase the GSH content, leading to higher GST activity, reduced production of free radicals, and protection of the cell constituents from oxidative damage.

MDA is often used as an index of oxidative tissue damage, which causes free radical damage to membrane components of the cell and results in cell necrosis and inflammation (Arya et al., 2012b; Mittal et al., 2009). It has been reported that STZ induces severe oxidative stress in diabetic animals may induce the peroxidation of polyunsaturated fatty acids and lead to the formation of TBARS and MDA as by-products of LPO (lipid peroxidation) (Mahesh and Menon, 2004).

Table 4

<table>
<thead>
<tr>
<th>Group</th>
<th>0 min</th>
<th>30 min</th>
<th>60 min</th>
<th>90 min</th>
<th>120 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control</td>
<td>5.7 ± 0.45</td>
<td>6.5 ± 0.76</td>
<td>6.1 ± 0.81</td>
<td>5.2 ± 0.55</td>
<td>5.5 ± 0.66</td>
</tr>
<tr>
<td>Diabetic control</td>
<td>14.1 ± 0.76</td>
<td>18.9 ± 0.73</td>
<td>19.9 ± 0.82</td>
<td>17.3 ± 0.49</td>
<td>18.3 ± 0.97</td>
</tr>
<tr>
<td>Glibenclamide (50 mg/kg)</td>
<td>5.8 ± 0.65</td>
<td>8.9 ± 0.38(52.01)</td>
<td>7.6 ± 0.61(50.10)</td>
<td>6.7 ± 0.77(51.27)</td>
<td>5.5 ± 0.28(50.94)</td>
</tr>
<tr>
<td>PMm (500 mg/kg)</td>
<td>6.5 ± 0.75</td>
<td>9.1 ± 0.57(51.85)</td>
<td>6.3 ± 0.68(58.20)</td>
<td>7.5 ± 0.92(50.64)</td>
<td>6.2 ± 0.85(56.12)</td>
</tr>
<tr>
<td>PMm (250 mg/kg)</td>
<td>8.9 ± 1.08</td>
<td>10.7 ± 0.78(41.18)</td>
<td>9.9 ± 0.67(50.25)</td>
<td>9.2 ± 0.37(46.82)</td>
<td>7.9 ± 0.92(56.61)</td>
</tr>
</tbody>
</table>

Mean values ± SD, n = 6. Parentheses values shows, percentage decrease of blood glucose level in the treatment groups, compared to diabetic control within same time period.

* Significant compared to diabetic control (P < 0.05).
PMm demonstrated no significant inhibitory activity against TNF-α-stimulated NF-kB translocation in NIT-1 cells, indicating that PMm alleviates oxidative stress through another mechanism. Further, we assessed the anti-oxidant ability of PMm in H2O2-induced NIT-1 cells and observed that oxidative stress developed due to H2O2 in the cells were accumulated in the nucleus of NIT-1 cells and PMm, caffeic acid, quinic acid, and caffeoylquinic acid were capable of reducing the oxidative stress burden in the cells (Saliet and Kahn, 2001). In conclusion, our results exhibited that PMm is a potent antidiabetic agent that is beneficial for the control of diabetes-related abnormalities, including oxidative damage and pro-inflammatory cytokines. Further studies using bioassay-guided isolation may lead to the identification of novel compounds that would be useful for the management of type 2 diabetes.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version.

5. Acknowledgments

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.fct.2014.01.054.

References


