Palm leaf catechins improved insulin-related pathways in diabetic rats

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Abstract. Catechin-rich oil-palm leaf extract (OPLE) (*Elaeis guineensis*) was previously demonstrated to possess benefits for diabetes and cardio metabolic health (vasodilation, antioxidant, cardiovascular, anti-hypertensive, anti-inflammatory, hepatoprotective and nephroprotective properties) in animal models. For insights into OPLE anti-diabetic mode-of-action and possible toxicity, the effects of dietary OPLE on insulin-signaling pathways mRNA expressions in the liver, kidney, pancreas, and spleen of normal and diabetic rats were examined. Type-2-Diabetes Mellitus (T2DM) were induced by chronic high-fat diet and streptozotocin (35 mg/kg) intraperitoneal injection. The OPLE (100 mg/kg body weight) were fed daily to normal and T2DM-induced rats. The OPLE suppressed hyperglycaemia and excessive weight gain in the T2DM rats, and appeared harmless to normal rats. The OPLE supplementation significantly ($p < 0.05$) modulated the mRNA expressions of phosphatidylinositol-3 kinase (PIK3R1); insulin signaling receptor (INSR); insulin-receptor substrates 1 and 2; and ectonucleotide pyrophosphatase-1 (ENPP1) especially in the livers of normal rats and the spleen of diabetic rats. Results suggested the OPLE probably help prevent diabetes in healthy mammals and ameliorate the immune functions of diabetic mammals. The OPLE improved the antioxidant defence responses, insulin-pathways mRNA expressions in the normal and diabetic rats; suppressed hyperglycaemia and excessive weight gain in T2DM rodents without observable liver or kidney toxicity at the dose used.

Keywords: catechins, diabetes, insulin pathways, liver, mRNA, oil-palm leaf

INTRODUCTION

Palm leaves are under-utilized, agro-waste from the palm oil industry. The oil-palm (*Elaeis guineensis*) leaf alcoholic extract (OPLE) contains high amounts of green tea catechins and flavonoids (Jaffri *et al.*, 2011a). The green tea catechins demonstrated benefits against diabetes (Rosalina Tan *et al.*, 2011), inflammation, cancer, arthritis, bacterial/virus infection, oxidative stress, angiogenesis, neuropathy and hypercholesterolaemia (Khan & Mukhtar, 2007; Mohamed, 2014). The oil-palm leaves are known

to be good ruminant feed and have been as such for decades without observable toxicity.

The OPLE has cardiovascular protective properties by maintaining coronary arteriole wall-lumen ratio under hypertension (Jaffri *et al.*, 2011b), inducing vasorelaxation (Runnie *et al.*, 2004), and inhibiting lipoprotein oxidation (Salleh *et al.*, 2002). Animal model studies showed dietary OPLE was protective against inflammation (Anyanji *et al.*, 2013), cardiovascular disorders (Jaffri *et al.*, 2011), hepatopathy (Jaffri *et al.*, 2011),

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neuropathy (Mohamed *et al.*, 2013), oxidative tension and nephropathy (Mohamed, 2014; Rosalina Tan *et al.*, 2011).

Almost 90% of diabetes are linked to insulin resistance or impaired cell response to insulin, caused by prolonged excessive blood insulin levels (Type-2 diabetes or T2DM). The T2DM treatment tries to focus on reducing unbalanced liver glucogenesis, increasing insulin sensitivity, enhancing insulin production, amending insulin signaling pathways, preventing obesity, regulating lipid metabolism and controlling lipotoxicity. The T2DM metabolic disorder is characterized by dyslipidaemia, hyperglycaemia, hypertension, atherosclerosis, inflammation and pro-thrombotic conditions.

Genes involved in the insulin signaling pathways include the ENPP1 (Ectonucleotide pyrophosphatase-1) that inhibits the tyrosine phosphorylation at the insulin receptors (INSR); insulin-receptor substrate 1 and 2 (IRS-1 and IRS-2); phosphatidylinositol-3 kinase (PIK3R1), and mitogen-activated protein kinase MAPK8IP1 (Cao *et al.*, 2007). The T2DM changes the insulin receptors (IRS1/IRS2-PI3K) functionality, signaling, protein kinase B and C (PKB and PKC) activities and MAPK signaling (Fröjdö *et al.*, 2009). The glucose metabolism is influenced by the hexosamine biosynthetic pathway genes, namely glycoprotein PC-1, glucose transporters 4 and 2 (GLUT-4 and GLUT-2), and the diabetes-associated peptide; amylin or IAPP (Islet Amyloid Polypeptide) (Cao *et al.*, 2007).

A comparison of four different Type 2 diabetes rat models showed that the high-fat diet with low-dose STZ injections is a suitable and appropriate animal model which is analogous to the human T2DM, with evidences of insulin resistance and similar changes in IRS-1 or Akt phosphorylation using soleus muscle from each model (Chao *et al.*, 2018). For insights into OPLE anti-diabetic mode-of-action and possible toxicity, the effects of dietary OPLE on insulin-signaling pathways mRNA expressions in the liver, kidney, pancreas, and spleen of normal and T2 diabetic rats were examined. This research investigated the influence of catechin-rich OPLE in normal and diabetic rats, for modulating glucose metabolic responses, by monitoring insulin pathways related genes such as PIK3R1, INSR, IRS1, IRS2, ENPP1, IAPP mRNA

expressions, together with its effects on the liver and kidney.

MATERIALS AND METHODS

Plant materials

Oil-palm Leaves from Universiti Putra Malaysia were washed, dried in a 45°C oven, milled and soaked in 100% ethanol (Merck, Germany) (ratio 1:10), overnight. The filtrate, was then rotary evaporated to dryness for producing the oil-palm leaves extract (OPLE) (Jaffri *et al.*, 2011). The hydrolysed OPLE (80 mg in 6M HCl, refluxed for 2 hours at 95°C) were cooled, filtered (0.4µm Whatman nylon membrane) and analysed with reverse-phase-HPLC using reported procedures (Jaffri *et al.*, 2011). The peaks were tentatively identified via the relative retention times of >95% pure epicatechin gallate (ECG), epigallocatechin gallate (EGCG), (+)-catechin, (-)- epicatechin, and epigallocatechin (EGC). Myricetin, kaempferol, quercetin and rutin were used as internal and external standards. Quantitative determination of the detected compounds were calculated based on the total area of the peak in the calibration plot obtained from each standard compounds analyzed via HPLC.

Animal (in vivo) studies

Male rats (*Sprague-Dawley*) weighing 240±20 g, were supplied by Universiti Putra Malaysia (Faculty of Veterinary medicine), and kept in polypropylene cages at ambient temperatures, with 12:12 h light:dark cycle. The rats were acclimatised for 14 days on tap water and rat chow containing 3% fat, 22% protein, 50% carbohydrate, 5% fiber and 3.2 kcal/g (Gold Coin Sdn. Bhd., Klang, Malaysia). The experimental protocol adhered closely to the approved University Putra Malaysia institutional animal ethical guidelines (UPM/FPSK/PADS/BR-UUH/00360). The rats were grouped into five categories (n=6); normal non-treated control, normal+100 mg OPLE /kg body weight, diabetic non-treated control, diabetic+100 mg/kg OPLE dissolved in drinking water and a positive control group (diabetic+250 mg/kg oral metformin). Metformin was used merely as a reference for the comparing the effects on the fasting blood sugar

level. Type-2 diabetes were induced with a high calorie diet containing 21% fat, 19.7% protein and 43.3% carbohydrate (Total 4.3 kcal/g) for a month. They were then intraperitoneally injected with 35 mg/kg Streptozotocin (Sigma-Aldrich, USA) (Motshakeri *et al.*, 2013). After one week induction, rats with 15 hours fasting blood glucose (FBG) of over 150 mg dl⁻¹ (8.3 mmol l⁻¹) were confirmed as hyperglycaemic/diabetic. The 100 mg/kg oral dose is based on the recommended dose from earlier studies (Jaffri *et al.*, 2011; Rosalina Tan *et al.*, 2011). The dose-response study for the effects of OPLE on diabetes have been reported previously (Rosalina Tan *et al.*, 2011), hence was not repeated in this study. After two weeks of treatment, the rats were exsanguinated under anaesthesia (50 mg/kg ketamine and 5 mg/kg xylazine) for tissue mRNA analysis. The livers, pancreas, spleens and kidneys were isolated, weighed and cleaned with cold saline, before being stored in RCl solution at -80°C for mRNA analysis.

Another similar set of four groups of rats (n=10); [normal non-treated control, normal+100 mg OPLE /kg body weight, diabetic non-treated control, diabetic+100 mg/kg OPLE dissolved in drinking water] were kept for five weeks to evaluate their kidney and liver histopathological conditions after exsanguination under anaesthesia.

Blood were kept in heparinised tubes, at 0°C before centrifuging at 3000 g for 10 minutes to obtain the plasma. The separated red blood cells (RBC) were rinsed and repeatedly centrifuged thrice with saline. Both RBC and plasma were stored at -80°C before use.

The liver and kidneys were sliced and preserved in 10% neutral buffered formalin. The residual tissues were kept at -80°C and analysed within four months. The antioxidant enzymes and malondialdehyde (MDA the lipid peroxidation biomarker), biochemical analysis and homogenate protein content were analysed as previously described (Matanjun *et al.*, 2010). The liver and kidney injury indicators [blood aspartate transaminase (AST), blood alanine transaminase (ALT), blood urea nitrogen (BUN), plasma creatinine, and plasma triglycerides levels were analysed using the auto-analyzer (Roche/Hitachi, Germany) and commercial kits (Roche Diagnostics). Liver and kidney samples (5 µm)

were treated for hematoxylin-eosin (H&E) staining (Matanjun *et al.*, 2010).

RT-qPCR mRNA analysis

The RNA were isolated using Ribopure RNA extraction kit (Ambion, Austin, TX, US). About 50 mg of the organ tissues were processed to eliminate DNA, proteins and other compounds. The extracted RNA concentrations, purity and integrity were tested using the NanoDrop 2000 UV-Vis Spectrophotometer (Thermo Scientific, Wilmington, USA), then stored at -80°C for the RT-PCR.

The genes studied and the primers constructed using the NCBI blast primer design software, and GenomeLab eXpress Profiler software (GeXPS) are summarised in Table 1. The RNA (50 ng) were reverse transcribed with multiplex universal reverse primers, using GenomeLab™ GeXP Start Kit (Beckman Coulter). The RT reaction was performed in a thermal-cycler at: 48°C for 1 min; 37°C for 5 min; 42°C for 60 min; 95°C for 5 min and then held at 4°C.

The fragment size range was 150-350 nt with a 7-nucleotide minimum separation size between each PCR product. Besides the 11 genes of interest, each panel has an internal control gene (Knr) and five normalization genes (ACTB, CYOA, EF1, GAPDH and ATPSYN). The reverse primers contained 20 nucleotides matching the selected gene and were labelled to a 19-nucleotide general reverse order. The forward primers contained 20 nucleotides conforming to the selected gene and were tagged to a 18 nucleotides universal forward sequence. All primers were from Proligo (France SAS), supplied by Sigma Aldrich from rat (*rattus norvegicus*) gene sequence adopted from the NCBI (National Center for Biotechnology Information) GenBank Database at <http://www.ncbi.nlm.nih.gov>. GeXPS primer stocks were dissolved in nuclease-free water to 500 nM for reverse primer sets, and to 200 nM for forward primer (Table 1).

Table 1. Selected genes and primer sequence of the genes.

Gene	Size	Function	Forward Primer	Reverse Primer
GAPDH	125	Housekeeping genes	AGGTGACACTATAGAATAATGACTC TACCCACGGCAAG	GTACGACTCACTATAGGGAAGCATC ACCCCATTTGATGT
ATPSYN	135	Housekeeping genes	AGGTGACACTATAGAATAAGTGACC ACAGCGTTTCCTT	GTACGACTCACTATAGGGAACAATC ATCCCACCCATGAT
EF1	150	Housekeeping genes	AGGTGACACTATAGAATAAAATGAC CCACCAATGGAA	GTACGACTCACTATAGGGACAGCAT CACCAGACTTCAA
CYOA	157	Housekeeping genes	AGGTGACACTATAGAATACGAGCTG TTTGACAGACAAAG	GTACGACTCACTATAGGGAGATGCC AGGACCTGTATGCT
ACTB	164	Housekeeping genes	AGGTGACACTATAGAATAGTGGGTA TGGGTCAGAAGGA	GTACGACTCACTATAGGGACTGGGT CATCTTTTCACGGT
ENPP1	171	Inhibition of insulin signalling	AGGTGACACTATAGAATACATGACA CCGTACTGCGAAG	GTACGACTCACTATAGGGACAGATT TTCTTGACTGCGGA
GFPT2	178	Hexosamine biosynthesis	AGGTGACACTATAGAATAAGTCATT CAGCAGTTGGAAGG	GTACGACTCACTATAGGGATGTCCG ATATAAGACGGGGA
INSR	185	Insulin signalling receptor	AGGTGACACTATAGAATATCCGGGA GAGGATGTGAGAC	GTACGACTCACTATAGGGATCAATG ACCGAGCAGTTCTCC
IAPP	192	Hormone, glucose uptake pancreas	AGGTGACACTATAGAATACTCGGCC ACTTGAGAGCTAC	GTACGACTCACTATAGGGAATATGT ATTGGATCCCACATTGG
IRS1	199	Insulin signalling transduction	AGGTGACACTATAGAATATCCAGAA GCAACCAGAGGAC	GTACGACTCACTATAGGGAAGGGAT GCATCGTACCATCT

IRS2	211	Insulin signalling transduction	AGGTGACACTATAGAATAACCTACG CAAGCATCGACTT	GTACGACTCACTATAGGGACTCAAA ATGTAGAATTGCTCCC
MAPK8IP1	226	MAPK signal transduction	AGGTGACACTATAGAATACTACCCT TCTGGGCATGAAA	GTACGACTCACTATAGGGAGATCTC CGAAAGGTCTTCATC
PIK3R1	231	Glucose clearance	AGGTGACACTATAGAATATTTTAAT CTCAGCGGAGTGGA	GTACGACTCACTATAGGGACGTACC AGAAAGGTCCCATC
TNF	240	Proinflammatory cytokines	AGGTGACACTATAGAATAAGAACTC CAGGCGGTGTCTG	GTACGACTCACTATAGGGATTGCT ACGACGTGGGCTA
PPARG	250	Lipid and glucose regulation	AGGTGACACTATAGAATACTGTTAT GGGTGAAACTCTGGG	GTACGACTCACTATAGGGACGTGGT AAAGGGTTTGATGTC
RRAD	260	Insulin sensitivity	AGGTGACACTATAGAATAGATGTGC CCATCATCCTAGTG	GTACGACTCACTATAGGGATTGTCTG ACGAGCATTTATCCTC

GAPDH, glyceraldehyde 3-phosphate dehydrogenase; ATPSYN, ATP synthase; EF1, elongation factor 1-alpha; CYOA, ubiquinol oxidase subunit II; ACTB, actin beta; ENPP1, ectonucleotide pyrophosphatase/phosphodiesterase 1; GFPT2, glutamine-fructose-6-phosphate transaminase 2; INSR, insulin receptor; IAPP, islet amyloid polypeptide; IRS1, insulin receptor substrate 1; IRS2, insulin receptor substrate 2; MAPK8IP1, mitogen-activated protein kinase 8 interacting protein 1; MAPK, mitogen activated protein kinase; PIK3R1, phosphoinositide-3-kinase regulatory subunit 1; TNF, tumor necrosis factor; PPARG, peroxisome proliferator-activated receptor gamma; RRAD, Ras related glycolysis inhibitor and calcium channel regulator.

The PCR reaction concoction contained the cDNA reverse transcription reaction product (9.3 μ l); the forward universal primer set mix (2 μ l); 25 mM $MgCl_2$ (4 μ l); 0.7 μ l of Thermo Start Taq DNA polymerase (Thermo Fisher Scientific, Pittsburgh, PA) and 4 μ l of 5 $\times$ PCR Master Mix buffer (GenomeLab GeXP Start Kit; Beckman Coulter, inc). The amplification required an initial denaturation step (95°C for 10 min), proceeded by 35 two-step cycles (94°C for 30 s and 55°C for 30 s), and finally an extension cycle (68°C for 1 min), in XP Thermal Cyclers (BIOER; Technology, Germany).

The PCR products from multiplex primer reactions were diluted 1:4 in water, and 1 μ l was added to 38.5 μ l sample loading solution along with 0.5 DNA size standard 400 (GenomeLab GeXP Start Kit; Beckman Coulter, Inc). The GeXPS system separated the PCR products by capillary gel electrophoresis and their dye signal strength were measured in arbitrary units (A.U.) of optical fluorescence minus the background signals. The data were first analyzed using the Fragment Analysis module of the GeXP system and then imported into the analysis module of eXpress Profiler software. The housekeeping genes (Actb, Gapdhs and 18S genes) were evaluated for consistency and stability, and the 18S was most consistently expressed in all the organs, thus was selected for normalizing the other genes (Wu *et al.*, 2019).

Statistics

All statistical analysis were done using Minitab 13 statistical software. The significant differences between the groups were analysed using a two-way analysis of variance (ANOVA), on the mean. This was followed by the Student–Newman–Keuls multiple range test for $p < 0.05$ significance level. Fold change in mRNA expressions (OPLE treated over non-treated groups) of ≥ 1.5 indicate significance at $p \leq 0.05$; Fold change of ≥ 2 indicate significance at $p \leq 0.02$ (Dalman *et al.*, 2012).

RESULTS

The OPLE HPLC quantitative analysis (Figure 1) showed the OPLE contains mainly catechin (3 g kg^{-1}), epigallocatechin gallate (2.8 g kg^{-1}),

epigallocatechin (800 mg kg^{-1}), epicatechin gallate (500 mg kg^{-1}), epicatechin (100 mg kg^{-1}) and their glucosides. The OPLE significantly suppressed the excessive body weight increase (obesity inclination) and hyperglycaemia in the T2DM rats without significantly affecting the body weight or blood glucose levels of normal rats (Figure 2).

Dietary OPLE significantly ($p < 0.05$) upregulated the IRS1 and IRS2 mRNA expressions, and downregulated IAPP expressions in the pancreas of healthy rats (Figure 3a). The OPLE significantly up-regulated the PIK3R1 mRNA expressions in the pancreas and livers of diabetic rats and significantly increased most of the insulin-related mRNA expressions in the livers of normal rats (Figure 3b).

The OPLE downregulated ENPP1 expression in the kidneys and spleens of the healthy rats significantly ($p < 0.05$) (Figure 4). The OPLE upregulated all the studied insulin-pathways related genes in the diabetic rats' spleen and downrelated these genes in the spleen of normal OPLE treated rats (Figure 4b).

The diabetic rats' kidneys showed significant ($p < 0.05$) glomeruli injuries (sclerosis and inflammatory cells infiltration) unlike the normal control rats (Figure 5). These glomerular damages were significantly reduced with the OPLE supplementation. The normal rats on OPLE supplementation showed normal kidney and liver structures. The diabetic rats' livers showed vacuolations, swelling and hepatocytes necrosis and the OPLE treatment attenuated the abnormal hepatocyte numbers in these diabetes-induced rats (Figure 5) towards normal levels.

The control nontreated T2DM rats had 60% mortality after the fifth week of diabetes-induction. The mortality of the OPLE treated T2DM rats after the fifth week was only 10% (Figure 6a). The percentage abnormal hepatocytes, and hepatocellular enzymes (ALT and AST) leakage were highest in the non-treated control diabetic rats (Figure 6b), and the OPLE supplementation mitigated this increase. This suggests that the OPLE was hepatoprotective for T2DM at the daily dose used.

The BUN levels in the control non-treated T2DM rats were significantly ($p < 0.05$) higher than normal (Figure 6c) and the OPLE supplementation insignificantly reduced it. Plasma

creatinine and protein levels were insignificantly different between the groups.

The plasma MDA levels were very high in the control non-treated T2DM rats and were significantly reduced by the OPLE supplementation (Figure 6b). The red blood cells (RBC) SOD and catalase activities were lowest in the control non-treated T2DM rats and were amended to normal levels with the OPLE supplementation (Figure 6c). The high blood triglyceride levels in the control non-treated T2DM rats were significantly reduced by the OPLE treatment. All groups exhibited similar glutathione peroxidase GPx activities.

DISCUSSION

The OPLE HPLC profile showed significant catechin (3 g kg^{-1}), epigallocatechin gallate (2.8 g kg^{-1}), epigallocatechin (800 mg kg^{-1}), epicatechin (100 mg kg^{-1}), epicatechin gallate (500 mg kg^{-1}) contents. These are most likely the main bioactive compounds responsible for the anti-hyperglycaemic effects. Catechins reduced FBG in hyperglycaemic rats by either promoting cells glucose uptake /metabolism or inhibiting hepatic gluconeogenesis (Kim, 2008).

Flavonoids affect insulin secretion, insulin resistance, apoptosis and pancreatic β -cells proliferation. Flavonoids amended hyperglycaemia via hepatocytes glucose metabolism; inflammation, tissues oxidation and glucose utilisation in the muscles and white adipose tissues (Babu *et al.*, 2013). Catechins also inhibit the carbohydrates hydrolysing enzymes to delay glucose absorption hence help lower food glycaemic index (Lochocka *et al.*, 2015). EGCG significantly reduced glucose absorption, inhibit the sodium-dependent glucose transporter, and enhanced insulin activity especially in T2DM by increasing insulin sensitivity (Hajiaghaalipour *et al.*, 2015). Similarly, epicatechin gallate (ECG) potently suppressed glucose utilisation and the sodium-dependent glucose transporter, in Caco-2 cells to significantly reduce blood glucose levels (Brown *et al.*, 2011). Here, the OPLE caused no significant changes in the relative expressions of

any of the other monitored genes in any of these organs (e.g. TNF-proinflammatory cytokines; PPAR γ - involved in lipid and glucose regulation).

The IAPP is co-secreted with insulin (ratio 1:10 for IAPP:insulin) by the pancreatic islets β -cells, and may be an indicator of pancreatic β -cells health status and insulin secretion (Andrich & Bieschke, 2015). The OPLE supplementation upregulated IAPP mRNA expressions (although insignificantly) in the diabetic rats' pancreas suggesting increased co-secreted insulin production (Jaikaran & Clark, 2001). This is supported by the improved blood glucose levels (Figure 2). The OPLE downregulated IAPP expressions in healthy rats, signifying improved insulin efficiency, and protection against amyloidogenicity and cytotoxicity caused by high IAPP levels (Lopes *et al.*, 2004). The IAPP slows gastric emptying, promotes satiety, and retards post-prandial spikes in blood glucose levels, reduces digestive juice secretions, food intake and obesity inclination (Andrich & Bieschke, 2015). This is supported by the lower rate of body weight increase in the T2DM rats consuming OPLE (Figure 3a) as compared to the control non-treated T2DM rats. A small percentage of healthy volunteers in an earlier human studies on the effects of dietary OPLE on cognitive functions (Mohamed *et al.*, 2013), reported slight weight reduction.

The ENPP1 is a negative modulator for insulin signaling, and chronic up-regulation of ENPP1 inhibits the cells' insulin receptor tyrosine kinase leading to insulin resistance. The ENPP1 (PC-1) down-regulation in diabetic mice' livers reportedly improved the blood glucose levels and glucose tolerance (Maddux *et al.*, 2006). Here the OPLE down-regulated ENPP1 expression in the kidneys and spleens in the healthy control (Figure 4), and in the diabetic rats' pancreas, suggesting improved insulin-induced glucose transport in these organs, and improved feedback to the diabetic pancreas. The ENPP1 mRNA up-regulation in the healthy rats' livers and diabetic rats' spleen is positively associated with the insulin receptor (INSR), IRS1 and IRS2 augmentations in these organs, and may indicate amended homeostasis responses (Zhou *et al.*, 2009).

Figure 1. HPLC profile of oil-palm leaf extract used.

Figure 2. (a) Body weight gains (Normal healthy animals on Oil-palm Leaf Extract showed similar weight gain to normal healthy control rats; diabetic animals on Oil-palm Leaf Extract also showed weight gains similar to normal healthy control rats); (b) Fasting Blood glucose profile of healthy and diabetic rodents on normal diet and oil-palm leaf extract supplementation. Normal healthy animals on Oil-palm Leaf Extract showed similar blood glucose to normal healthy control rats; Diabetic rats on metformin, and Oil-palm Leaf Extract showed reduced blood sugar.

Figure 3. (a) Fold change in mRNA expressions (between Oil-palm Leaf Extract treated and control non-treated groups) in the pancreas of diabetic rats; (b) Fold change in mRNA expressions (between OPLE treated and control non-treated groups) in livers of normal and diabetic rats. Fold change of ≥ 1.5 indicate significance at $p \leq 0.05$; Fold change of ≥ 2 indicate significance at $p \leq 0.02$ (Dalman *et al.*, 2012).

Figure 4. Fold change in mRNA expressions (between Oil-palm Leaf Extract treated and control non-treated groups) in the (a) kidneys and (b) spleens of normal and diabetic rats. Fold change of ≥ 1.5 indicate significance at $p \leq 0.05$; Fold change of ≥ 2 indicate significance at $p \leq 0.02$ (Dalman *et al.*, 2012).

Figure 5. Effects of Oil-palm Leaf Extract on normal and diabetic rats' kidney and liver architectures under 400 X magnification.

DC= Diabetic control;

NC: Normal control

Figure 6. Effects of Oil-palm Leaf Extract on percentage mortality, normal and diabetic rats hepatocytes, nephrons and blood biochemical parameters.

The OPLE significantly ($p < 0.05$) up-regulated INSR, IRS1 and IRS2 mRNA expressions in normal and diabetic rats' livers, suggesting improved insulin sensitivity, or insulin signaling transduction. This would subsequently ameliorate glucose metabolism, translocation and insulin efficiency (Hanhineva *et al.*, 2010). The INSR mRNA expressions were significantly correlated with lower FBG, HbA1c, triglyceride, and insulin levels in clinical T2DM patients, pre-clinically and *in vitro* (Fröjdö *et al.*, 2009). The IRS1 and IRS2 are linked to insulin secretion regulation and insulin sensitivity. The insulin receptor substrates (IRS-1 to IRS-4) are activated by phosphorylation at the tyrosine residues by insulin receptor tyrosine kinase to support glucose transport by glucose transporter (GLUT4) into the tissue. The IRS phosphorylation activate the phosphatidylinositol-3 kinase (PI3K) and the Ras-MAPK pathways which help reduce hepatic glucose production (via gluconeogenesis and glycogenesis), and activate various anabolic signaling pathways that favour glucose homeostasis (Fröjdö *et al.*, 2009).

The IRS-2 is also actuated by IL-4 for mediating glucose transport through the PI3-Kinase signaling pathway (Fröjdö *et al.*, 2009). The PI3K catalyses the phosphatidylinositol phosphorylation to give phosphatidylinositol-3-phosphates which activate other signaling molecules, including serine kinase, GSK-3, the fork head transcription factors and the cAMP response element-binding protein. The OPLE significantly ($p < 0.05$) upregulated the PIK3R mRNA expressions in the all the analysed organs (liver, pancreas, kidney and spleen) of diabetic rats, suggesting that the OPLE may also improve glucose transport (Cao *et al.*, 2007). The PIK3R1 is a p85 regulatory subunit which encode the PI3K, and increases glucose uptake or glycogen synthesis in insulin-sensitive tissues (Cao *et al.*, 2007). Dysfunctional PIK3R1 causes insulin resistance via the PI3K hexosamine regulation (Suzuki *et al.*, 2013). The significantly ($p < 0.05$) upregulated PIK3R1 mRNA expressions in the diabetic rats' organs were supported by the ameliorated blood glucose levels and may help ameliorate kidney pressure.

The OPLE significantly ($p < 0.05$) upregulated the INSR, IRS1, IRS2, PIK3R1, IAPP AND ENPP1 mRNA expressions in the diabetic rats'

spleen. This suggested enhanced insulin signaling pathways /glucose translocation, which would subsequently improve immune responses for the T-helper cells cytokines production against tissue injuries, allergic reactions and infection (Andrich & Bieschke, 2015). Spleen is the main immune system centre for synthesizing antibodies in the white blood cells, besides filtering blood, removing old blood cells throughout the liver and replacing them back by new RBC produced by the bone marrow, in the hematopoiesis process (Lim *et al.*, 2016).

The OPLE caused significant ($p < 0.05$) down-regulation of ENPP1, INSR, IRS1 and PIK3R1 mRNA expressions in control normal rats' spleen, which may be due to improved energy utilisation in the spleen.

Diabetes related liver tissue architecture disruption and vacuolation, indicates hepatocellular injury, which induces low-grade inflammation, dyslipidemia and insulin resistance. The OPLE influenced the endothelium for regulating vascular tone, homeostasis (Abeywardena *et al.*, 2002) and suppressed inflammation (Abeywardena *et al.*, 2002; Anyanji *et al.*, 2013) thus was probably hepatoprotective through these mechanisms. Plasma ALT is a better indicator of liver inflammation than AST, since it is found mainly in the liver, and AST may increase in acute liver damage or other diseases involving liver parenchymal cells, red blood cells, cardiac muscle, skeletal muscle, kidney and brain tissue injuries (Jaffri *et al.*, 2011b).

The significantly elevated BUN levels in the diabetic rats indicated glomerular damages that was verified by the dilated tubules and increased peritubular space. This was not significantly reduced by OPLE supplementation. Excess protein infiltration, and inflammation in diabetes, cause tubular damage. The tubular structural abnormalities would cause diuresis, impair urine concentrating ability and eventually polydipsia as observed in all the T2DM rats. Catechins with its anti-hyperglycaemia and anti-hypertension properties, may help inhibit kidney damages. Significant plasma BUN or creatinine levels elevations are only observed, when the nephrons are over 70% dysfunctional. The normal rats supplemented with OPLE showed normal tubular cells.

The improved MDA, SOD and catalase levels, indicated ameliorated endogenous antioxidant defence with the OPLE supplementation in T2DM rats. Excessive lipid peroxidation is associated with antioxidant deficiency, that produces toxic aldehydes that can damage membrane proteins, leading to the cytoplasmic enzymes leakage (Jaffri *et al.*, 2011b). Green tea catechins are similarly known to protect against various tissue damage (Burckhardt *et al.*, 2008), insulin sensitivity (Prasain *et al.*, 2010), inflammation, endothelial apoptosis (Curin & Andriantsitohaina, 2005), vascular health (Galleano *et al.*, 2012), and vascular homeostasis (Moore *et al.*, 2009).

CONCLUSION

The dietary OPLE significantly ($p < 0.05$) modulated insulin associated pathways (INSR, IRS1, IRS2, PIK3R1, IAPP, and ENPP1) mRNA expressions especially in the normal rats' livers and the diabetic rats' spleen to increased insulin sensitivity, insulin signaling transduction and glucose translocation into these tissues. The mRNA expressions advocated that OPLE may help prevent T2DM in healthy rats and improve the metabolic and immune functions of diabetic rats. The OPLE up-regulated IAPP expressions suggest improved insulin production, satiety, weight-control and blood glucose homeostasis in these animals. The OPLE demonstrated anti-hyperglycaemic and anti-obesity effects on T2DM rat models. These findings may be confirmed or expanded by further studies on protein expressions in animal diabetic models or in controlled clinical intervention studies.

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collection, and manuscript preparation. Suhaila Mohamed is the grant recipient, main supervisor, project planning coordinator and manuscript writing/editing. All authors read and approved the final version of the manuscript.

CONFLICT OF INTEREST

The authors have declared that no conflict of interest exists.

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