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Original Article

Antioxidant and anti-advanced glycation end products formation properties of palmatine

[Propiedades antioxidantes y contra la formación de productos finales de glicación avanzada de palmatina]

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Abstract

Context: Oxidative stress and formation of advanced glycation end products (AGEs), due to glycation of proteins, lipids and nucleic acids are characteristic in diabetic patients. Palmatine, a protoberberine alkaloid bioactive isolated from *Coscinium fenestratum* (CF) stem extract, which previously has shown to possess antidiabetic and antioxidant properties and to be able to protect the kidney and liver in a STZ-diabetic induced rat model.

Aims: To evaluate the *in vitro* and *in vivo* antioxidant and anti-AGE activity of palmatine.

Methods: *In vitro* and *in vivo* studies were conducted to measure radical scavenging, reducing power, inhibition of lipid peroxidation, carbonyl trapping and metal ion chelation. *In vitro* antiglycation activity was done using bovine serum albumin-methylglyoxal (BSA-MGO), bovine serum albumin – glucose (BSA-GLU) and glycated hemoglobin. *In vivo* antioxidant and antiglycation activity were used to evaluate liver and kidney extracted from STZ-induced diabetic rat after treatment with palmatine.

Results: The results showed palmatine blocked the formation of AGE as shown by the results of BSA-GLU, BSA-MGO, glycated hemoglobin and inhibited the free radicals generated by DPPH, nitric oxide, hydrogen peroxide, lipid peroxidation, FRAP and metal ion chelating. It was able to stimulate *in vivo* the activity of catalase, super oxide dismutase and glutathione peroxidase.

Conclusions: Palmatine possess antioxidant and antiglycation properties, the mechanism of action seems to be via the blockage of free radical formation, decreasing of reactive carbonyl. Further research is ongoing to determine effect of palmatine on glyoxalase 1 and aldose reductase pathway and interaction with receptor for AGE.

Keywords: advanced glycation end products; anti-glycation; antioxidants; diabetes mellitus.

Resumen

Contexto: El estrés oxidativo y la formación de productos finales de glicación avanzada (AGE), debido a la glicación de proteínas, lípidos y ácidos nucleicos, son característicos de los pacientes diabéticos. Palmatine, un alcaloide bioactivo de protoberberina aislado de extracto de tallo de *Coscinium fenestratum* (CF), previamente ha demostrado poseer propiedades antidiabéticas y antioxidantes y ser capaz de proteger el riñón y el hígado en un modelo de rata inducida por STZ – diabetes.

Objetivos: Evaluar la actividad antioxidante y anti-AGE *in vitro* e *in vivo* de la palmatina.

Métodos: Se llevaron a cabo estudios *in vitro* e *in vivo* para medir la captación de radicales, el poder reductor, la inhibición de la peroxidación de lípidos, el atrapamiento de carbonilo y la quelación de iones metálicos. La actividad antiglicación *in vitro* se realizó utilizando albúmina-metilglioxal de suero bovino (BSA-MGO), albúmina de suero bovino-glucosa (BSA-GLU) y hemoglobina glucosilada. La actividad antioxidante y antiglicación *in vivo* evaluó el hígado y el riñón extraídos de ratas diabéticas inducidas por STZ después del tratamiento con palmatina.

Resultados: Los resultados mostraron que la palmatina bloqueó la formación de AGE como lo muestran los resultados de BSA-GLU, BSA-MGO, hemoglobina glucosilada e inhibió los radicales libres generados por DPPH, óxido nítrico, peróxido de hidrógeno, peroxidación lipídica, FRAP y quelación de iones metálicos. Fue capaz de estimular *in vivo* la actividad de catalasa, superóxido dismutasa y glutatión peroxidasa.

Conclusiones: Palmatine posee propiedades antioxidantes y antiglicantes, el mecanismo de acción parece ser a través del bloqueo de la formación de radicales libres, disminuyendo el carbonilo reactivo. Se están realizando más investigaciones para determinar el efecto de la palmatina sobre la vía de la glioxalasa 1 y la aldosa reductasa y la interacción con el receptor para AGE.

Palabras Clave: anti-glicación; antioxidantes; diabetes mellitus; productos finales de glicación avanzada.

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INTRODUCTION

Glycation is a non-enzymatic reaction between a reducing monosaccharide and an amine group in proteins, lipids or nucleic acids forming an unstable Schiff base, a secondary aldimine. This undergoes further rearrangement to form a stable Amadori, product, which then leads to the irreversible formation of an advanced glycation endproduct (AGE) (Gautieri et al., 2017). During these rearrangements highly reactive intermediates, *a*-dicarbonyls, also known as reactive carbonyl species (RCS), accumulate and cause carbonyl stress. Examples of these products are 3-deoxyglucosone (3DG) and methylglyoxal (MGO) (Gautieri et al., 2017). RCS and AGE formation can also occur by glycoxidation or lipid peroxidation (Fu et al., 1996). Glycated stress (e.g., hyperglycemia) or oxidative stress accelerates this reaction and can also affect short-lived substrates (e.g., enzymes and hormones), inducing structural changes (Stirban et al., 2014).

Oxidative stress and protein glycation are closely related and tightly linked under hyperglycemic conditions in diabetic patients (Wan-Ju et al., 2017). Oxidative stress is a condition of an imbalance between free radicals and antioxidant, which can cause cellular damage and activation of various signaling pathway linked to many different diseases (Mateen et al., 2016). Oxidative stress generation and hyperglycemia induce the formation of AGEs through various pathways, including the polyol pathway. The activation of (aldose reductase) ALR2 and the first rate-limiting enzyme of the polyol pathway has been reported to be involved in the increased generation of AGEs (Re et al., 1999). The 3-deoxyglucosone produced as a by-product from the polyol pathway is a major precursor of AGE. Diet, rich in protein and fat, is the external source of AGEs that is proportional to increased serum levels of AGE, which contribute to the crosslinking of proteins, worsening the complications in patients with diabetes (Re et al., 1999). The amount of AGEs is based on the rate of formation, determined by oxidative stress and reducing sugars, and the rate of clearance, determined by the activity of the glyoxalase system, where glyoxalase I (Glo I) is able to detoxify reactive carbonyl compounds (Xue et al., 2011).

Compounds with AGE inhibiting property also act as natural oxidative stress scavenger. The strategies that prevent AGE formation depend on different mechanisms such as antioxidant ability, scavenging of reactive carbonyl species and inhibition of aldose reductase.

The current study evaluated the *in vitro* and *in vivo* antioxidant and anti- advanced glycation end products (anti-AGE) activity of palmatine. Palmatine is a protoberberine alkaloid bioactive isolated from *Coscinium fenestratum* (CF) stem extract. CF belongs to the plant family name *Menispermaceae* and commonly known as 'tree turmeric'.

Previous report has shown that the stem extract of CF and palmatine possess antidiabetic and antioxidant properties and was able to protect the kidney and liver in STZ-diabetic induced rat model (Okechukwu et al., 2013). Current experiment reports palmatine possess *in vitro* and *in vivo* antioxidant and anti-AGE activities.

MATERIAL AND METHODS

Chemicals

Chloroform (Mallinckrodt Chemicals, USA); Streptozotocin (STZ), tolbutamide, palmatine (Aldrich, USA); thiobarbituric acid (TBA) (Sigma-Aldrich, Germany); trichloroacetic acid (TCA), acetic acid, NaOH, hydrogen peroxide, nitro-blue tetrazolium (NBT), sodium phosphate (R & M, UK); hydroxylamine hydrochloride (PubChem CID: 443297); 5,5'-dithios-nitrobenzoic acid (DTNB) (Sigma, USA); dipotassium hydrogen phosphate, potassium hydrogen phosphate (Merck, Germany); sodium nitrite (Systrem, Malaysia); ethylene diamine tetra acetic acid (EDTA) (Sigma-Aldrich, USA), methanol (Merck, Germany); bovine serum albumin (BSA) (Sigma-Aldrich, USA); sodium chloride (Gene Chemical, France) potassium dichromate (Hopkins and Williams, UK), sodium carbonate, hydrochloric acid (Sinar Scientific, Malaysia) aminoguanidine (Sigma-Aldrich, USA), 2,2-

dihenyl-1-picrylhydrazyl (DPPH) (Sigma-Aldrich USA), methylglyoxal (Sigma-Aldrich, USA), glimepiride (Amaryl Sanofi India. Ltd. India) metformin (Glucophage, EMD Serono Inc., America), butylated hydroxytoluene (Sigma-Aldrich, USA) and quercetin (Sigma-Aldrich, USA).

Equipment

Vortex mixer (LMS Co., Ltd, Japan); Benchtop Centrifuge (Zentrifugen, Germany); pH meter (Mettler Tolledo, Switzerland); UV-Vis spectrophotometer (SECOMAM, France); Water bath (Memmert, Germany); Electronic balance (Mettler Toledo, Switzerland); FLUOstar Omega Plate Reader (Thermofisher, USA).

***In vitro* antioxidant activity**

DPPH radical scavenging activity

The experiment was performed according to the method described by Singh et al. (2002). One milliliter (1 mL) of palmatine, glimepiride, metformin and BHT at concentrations (29.2, 58.4, 87.61, 116.81 and 146.02 µM) was added to the container containing 1 mL of DPPH (0.05 mM) and incubated for 30 min separately in the dark chamber. The absorbance was measured at 517 nm against blank. One mL of absolute ethanol added to 1 mL of DPPH against blank, which was used as negative control, while BHT was used as positive control. Ethanol was used as a blank in this assay. The following equation [1] was used to determine the percentage of radical scavenging activity of each compound.

$$\% \text{ Inhibition} = \frac{(\text{Abs Control} - \text{Abs Sample})}{(\text{Abs Control})} \times 100 \quad [1]$$

Where Inh: Inhibition; Abs: Absorbance.

Nitric oxide radical scavenging activity

The experiment was performed according to method described by Patel et al. (2010). The reaction mixture was made up of 2 mL of 10 mM sodium nitroprusside in 0.5 mL of phosphate buffer saline (pH 7.4). Zero point five (0.5 mL) of palmatine, glimepiride, metformin, and quercetin at concentrations (29.2, 58.4, 87.61, 116.81 and 146.02 µM) was added to the reaction mixture, shaken, and incubated for 2.5 h at room temperature. Zero point five (0.5 mL) of solution was taken from reaction mixture and mixed in 1 mL of 0.33% sulfanilic acid in each test tube and allowed to stand at room temperature for 5 min. After the incubation, 1 mL of 0.1% naphthylene diamine chloride was added and incubated at room temperature for 30 min. Reaction mixture without sample was served as negative control while phosphate buffer saline was used as blank. The absorbance was measured at 540 nm and the percentage (%) of inhibition was calculated using above equation [1].

H_2O_2 radical scavenging activity

The experiment was performed according to method described by Engoor et al. (2013). Sixty microliters (60 µL) of 1 mM ferrous chloride, 90 µL of 0.2 M phosphate buffer (pH 7.8), 150 µL of 0.17 M hydrogen peroxide and 100 µL of palmatine, glimepiride, metformin and BHT at concentrations (29.2, 58.4, 87.61, 116.81 and 146.02 µM) was mixed separately. The mixtures were shaken and incubated at room temperature for 5 min. Negative control was prepared without sample in the reaction mixture and phosphate buffer was used as a blank while BHT was used as a positive control. The absorbance was measured at 240 nm and % inhibition was calculated using above equation in section [1].

Lipid peroxidation radical scavenging activity (TBARS)

The experiment was performed according to the method described by Engoor et al. (2013). Lipid peroxidation activity was measured by thiobarbituric acid reactive species (TBRAS). Egg homogenate was used as a rich lipid medium and substrate. Zero point five (0.5 mL) of 10% v/v of egg homogenate and 1 mL of palmatine, glimepiride, metformin and BHT at concentrations (29.2, 58.4, 87.61, 116.81 and 146.02 µM) was mixed. One milliliter (1 mL) distilled water, 0.005 mL of $FeSO_4$ (0.07 M) was added to the mixture to induce lipid peroxidation and incubated for 30 min. Then 1.5 mL of 20% acetic acid and 1.5 mL of 0.8% (w/v) TBA in 1.1% SDS and 0.5 mL 20% TCA were added, and the resulting mixtures was vor-

texed and heated at 95°C for 60 min. Another set of samples were mixed in similar manner but incubated without TBA. After cooling, 5 mL of butanol was added to each tube and centrifuged at 5000 rpm for 10 min. The absorbance of organic layer was measured at 532 nm and % inhibition was calculated using above equation [1].

Chelating capacity

Chelating capacity assay was performed based on the method described by Dinis et al. (1994). One hundred microliters (100 µL) palmatine, glimepiride, metformin and BHT at concentrations (29.2, 58.4, 87.61, 116.81 and 146.02 µM) were mixed with 0.5 mL $FeCl_2$ (2 mM) and 0.2 mL ferrozine (5 mM). The reaction mixture was incubated for 10 min at room temperature. The absorbance was measured at 562 nm and % inhibition was calculated using above equation [1].

Reducing power

Reducing power assay was performed based on the method described by Gülçin et al. (2010). One hundred microliters (100 µL) of palmatine, glimepiride, metformin and BHT at concentrations (29.2, 58.4, 87.61, 116.81 and 146.02 µM) was added to 2.5 mL of sodium phosphate buffer (0.2 M, pH 6.6) and 2.5 mL of potassium ferricyanide (1%) solution. The reaction mixture was vortexed and incubated for 20 min at 50°C. After incubation, 2.5 mL of TCA (10%, w/v) was added to each test tube and centrifuged at 3000 rpm for 10 min at 4°C. After centrifugation, the upper layer (2.5 mL) was transferred into different test tube. Equal amount (2.5 mL) of deionized water was mixed with 2.5 mL of upper layer and 1 mL of $FeCl_3$ was added to the test tube. The reaction mixture was incubated for 10 min at 35°C. The absorbance was measured at 700 nm and % of inhibition was calculated using above equation [1].

In vitro anti-glycation assays

Bovine-serum albumin (BSA-glucose) assay

The anti-AGE assay was done according to the methodology previously reported by Jovanovic et al. (2017). Briefly 3 mL of reaction mixture containing 1 mL of BSA (10 mg/mL), 1 mL of glucose (500 mM) and 1 mL of palmatine, glimepiride, metformin or aminoguanidine at concentrations (29.2, 58.4, 87.61, 116.81 and 146.02 µM) was prepared in 0.2 M sodium phosphate buffer (pH 7.4). The reaction mixture was incubated for 5 min, then 0.5 mL of sodium azide (0.5 mM) was added to each of the tubes. Reaction mixture without sample was served as negative control and phosphate buffer was used as blank. The tubes were incubated for 7 days at 37°C in dark. Aminoguanidine an inhibitor of glycation of proteins was used as a positive control. After 7 days of incubation, the samples were measured at fluorescence intensity (excitation wavelength of 370 nm and emission wavelength of 440 nm) using Omega micro plate reader. The experiment was done in triplicates. The percent inhibition was calculated by using equation [2].

$$\% \text{ Inh} = \frac{\text{FLU Negative control group} - \text{FLU Test group}}{\text{FLU Negative control group}} \times 100 \quad [2]$$

Where Inh: Inhibition; FLU: Fluorescence.

BSA-methylglyoxal (BSA-MGO) assay

Bovine serum albumin (BSA) methylglyoxal assay was performed as described by Jovanovic et al. (2017). BSA (20 mg/mL) and methylglyoxal (60 mM) were dissolved in 0.1 M sodium phosphate buffer (pH 7.4). Three milliliters (3 mL) of reaction mixture containing 1 mL of BSA, 1 mL of methylglyoxal and 1 mL of palmatine, glimepiride, metformin and aminoguanidine at concentrations (29.2, 58.4, 87.61, 116.81 and 146.02 µM) were prepared. One negative control was prepared by reaction mixture with inhibitor and without sample. The reaction mixture was mixed thoroughly and incubated for 5 min at room temperature. After the incubation, 0.5 mL of sodium azide (0.2 g/L) was added to each tube. The tubes were tightly capped and incubated at 37°C for 7 days in the dark. Aminoguanidine an inhibitor of glycation of proteins was used as a reference. After 7 days of incubation, the fluorescence intensity of the samples was measured at an excitation wavelength of 370 nm and an emission wavelength of 440 nm, using an Omega microplate reader. The experiment was

carried out in triplicates. The percentage of inhibition was calculated by using the formula [2].

Glycated hemoglobin

Glycation of hemoglobin was performed according to the methodology described by Gutierrez et al. (2012) with some modifications. Glucose (2 g/dL), hemoglobin (12 g/dL) and 1 mg/mL of palmatine, glimepiride and metformin were dissolved in distilled water. Three milliliters (3 mL) of reaction mixture containing 1 mL of glucose, 1 mL of hemoglobin and 1 mL of palmatine, glimepiride or metformin at concentrations (29.2, 58.4, 87.61, 116.81 and 146.02 µM) were prepared. The negative control was prepared reaction mixture with hemoglobin and without sample and another negative control was prepared without hemoglobin. The reaction tubes were incubated in the dark at 37°C. The concentrations of glycated hemoglobin were measured after the incubation period of 24, 48 and 72 h using an Omega microplate reader at a wavelength of 443 nm. The test was conducted in triplicates and the inhibition of glycated hemoglobin was calculated using above equation [2].

Animal study (*in vivo*)

Animal preparation

Healthy thirty female Sprague Dawley rats (180-200 g) (2 months old) were purchased from the Institute of Medical Research Kuala Lumpur Malaysia. The animals were kept for two weeks in the animal holding room of UCSI University under standard laboratory condition (at a temperature of 22 ± 1°C; humidity 60-70%; 12 h dark/12 h light schedule) and provided with the free access to standard rat feed pellet and water. Animals were acclimatized to the laboratory conditions for 1 week before the initiation of the experiment. The experiment was carried out between 9 am - 5 pm. Experimental protocols were approved by the University Kebangsaan under the ethical code UCSI/2017/PATRICK/20-AUG./801-NOV.-2018-DEC.-2019.

Induction of diabetes and treatment

After two weeks of acclimatization, the animals were divided into six groups (n = 6) Group (i) Normal -; no induction of diabetes and given 1 mL of saline, Group (ii) Negative control -: induction of diabetic and given 1 mL of saline, Group (iii) Positive control 1 induction of diabetic and given 1 mL of 2 mg/kg of glimepiride, Group (iv) Positive control 2 induction of diabetic and given 1 mL 125 mg/kg of metformin, Group (v) Test compound induction of diabetic and given 1 mL of 2 mg/kg of palmatine. To induce diabetes, the animals were fasted overnight at least for 12 h, 0.2 mL of 50 mg/kg of streptozotocin (STZ), dissolved in 0.1 M citrate buffer (pH 4.5) was administered via intraperitoneal (i.p) injection to the animals to induce diabetes mellitus. The plasma glucose level of the rats was checked 72 h post-STZ injection by withdrawing blood from the cut tail region and tested with ACCU-CHEK active blood glucose monitoring system and ACCU-CHEK active glucose strips to confirm the rats were hyperglycemic. Only rats with an elevated plasma glucose level of above 11.0 mmol/L were used for the study of diabetes (Ekeuku et al., 2015). The rats were treated according to the treatment regime above for 12 weeks.

Tissue sample preparation

The animals were sacrificed after 12 weeks. The liver and the kidney were harvested, rinsed with saline for *in vivo* antioxidant and anti-glycation study. To study the *in vivo* antioxidant activity, liver samples were prepared in two ways, for-Tris tissue sample, 1 g of liver tissue was homogenized using mortar and pestle and added to a centrifuge tube containing 10 mL of 0.15 M tris buffer (pH 7.0). The mixture was centrifuged for 30 min at 3000 rpm at 4°C. The supernatant was collected and stored at 4°C. While for phosphate buffer tissue liver sample, 1.8 g of liver sample were homogenized using a mortar and pestle and added to a centrifuge tube containing 6 mL of 0.15 M phosphate buffer (pH 7.4). The mixture was centrifuged for 30 min at 3000 rpm at a temperature 4°C.

The supernatant was collected and stored at 4°C (Ekeuku et al., 2015).

In vivo antioxidant assays

Lipid peroxide (LPO)

Two milliliters (2 mL) of 10% trichloroacetic acid (TCA), 1 mL of 0.9% saline w/v, 1 mL of phosphate buffered sample was mixed in 15 mL of centrifuge tube. The mixture was then centrifuged at 3000 rpm for 10 min at 25°C. From the centrifuged sample, the 2 mL of supernatant was transferred into a new test tube containing 0.5 mL of 1% thiobarbituric acid (TBA). This was then incubated in a water bath for 60 min at 95°C. The absorbance of the solution was read at 532 nm (Ekeuku et al., 2015).

Superoxide dismutase (SOD)

One milliliter (1 mL) of 50 mM sodium carbonate, 0.4 mL of 25 µM of nitro-blue tetrazolium (NBT) and 0.2 mL of 0.1 mM EDTA and 0.5 mL of Tris tissue sample were mixed. Then 0.4 mL of 1 mM hydroxylamine hydrochloride was added. After 2 min of incubation the absorbance was read at 560 nm (Ekeuku et al., 2015).

Catalase (CAT)

Zero point one (0.1 mL) of phosphate buffered liver sample, 0.4 mL of 2 M hydrogen peroxide (H_2O_2) was mixed with 1 mL of 0.01 M phosphate buffer of pH 8 in a beaker. After 3 min, 2 mL of dichromate acetic acid (contain 5% of potassium dichromate and acetic acid with the ratio 1:3). The mixture was heated until it is boiled for 10 min. Then absorbance of the mixture was read at 570 nm (Ekeuku et al., 2015).

Glutathione reductase (GSH)

One milliliter (1 mL) of Tris tissue sample was added in test tube with 0.5 mL of Ellman's reagent (19.8 mg of DTNB mixed with 0.1% $NaNO_2$) and 3 mL of 0.2 M phosphate buffered (pH 8). The mixture was mixed thoroughly, and absorbance was read at 412 nm (Ekeuku et al., 2015).

In vivo *inhibition of advanced glycation end products (AGEs)*

The inhibition of advanced glycation end products (AGE) in extracted liver from treated groups were determined by the method described by Gutierrez and Baez (2014). The extracted liver tissue of treated groups; metformin, glimepiride, palmatine, were dilapidated with chloroform and methanol in the ration 2:1 (v/v) overnight. After washing, the liver tissues were homogenized in 0.1 M sodium hydroxide (NaOH). The homogenized samples were centrifuged at 8000 rpm for 15 min at 4°C. After centrifugation, the supernatant was pipetted out in a new tube. The amount of AGE in the alkali-soluble samples were determined by measuring the fluorescence (emission wavelength of 440 nm and excitation wavelength of 370 nm). BSA (0.1 g/mL of 0.1 M NaOH) was used as standard control, glimepiride and metformin was used as positive drug control. The experiment was carried out in triplicates. The fluorescence values of samples were measured at a protein concentration of 1 mg/mL and expressed in arbitrary units (AU).

Statistical analysis

All data were presented as mean ± standard deviation (SD) in triplicates (n = 6) using the XL. STAT 7.0. One-Way ANOVA using Dunnet's' post hoc test. Values were considered statistically significant when $^{b}p<0.05$, $^{bb}p<0.01$, $^{bbb}p<0.001$ and $^{bb}p<0.0001$ *vs.* BHT/quercetin/aminoguanidine(a); $^{c}p<0.05$, $^{cc}p<0.01$, $^{ccc}p<0.001$ and $^{cccc}p<0.0001$ *vs.* glimepiride; $^{d}p<0.05$, $^{dd}p<0.01$, $^{ddd}p<0.001$ and $^{dddd}p<0.0001$ *vs.* metformin (*in vitro*). Two-way ANOVA using Turkey's HSD post hoc test, when $^{b}p<0.05$ *vs.* normal (non-diabetic) control (a), $^{c}p<0.05$ *vs.* diabetic control group, $^{d}p<0.05$ *vs.* glimepiride, $^{e}p<0.05$ *vs.* metformin (*in vivo*).

RESULTS

In vitro antioxidants capacity

DPPH radical scavenging capacity

The antioxidant capacity of palmatine, glimepiride, metformin and BHT was investigated using DPPH scavenging assay. Palmatine showed a very strong DPPH free radical scavenging activity with IC_{50} = 45.4 ± 1.45 µM, compared to glimepiride and metformin with IC_{50} = 52.95 ± 2.15 and 50.15 ± 2.25 µM, respectively. However, BHT and palmatine had similar effect on the inhibition of DPPH radicals as shown in Fig. 1A.

Nitric oxide radical scavenging capacity

The scavenging capacity of palmatine, glimepiride, metformin and quercetin was evaluated on the generation of nitric oxide radical. Based on IC_{50}, the result showed that palmatine glimepiride, and metformin had similar effect on the nitric oxide radical scavenging activity with IC_{50} = 2.51 ± 0.35, 2.5 ± 0.47 and 2.58 ± 0.64 µM. Quercetin with IC_{50} = 1.76 ± 0.73 µM had significant ($p<0.0001$) higher nitric oxide radical scavenging activity compared to palmatine, glimepiride and metformin, respectively as shown in Fig. 1B.

Hydrogen peroxide scavenging capacity

Palmatine, gliempiride, metformin and BHT were evaluated for scavenging hydrogen peroxide radical. From the result palmatine, glimepiride, and metformin and showed similar hydrogen peroxide radical scavenging effect with IC_{50} = 3.38 ± 0.39, 3.86 ± 0.22, and 3.5 ± 0.25 µM. Palmatine, glimepiride and metformin had significant ($p<0.0001$) higher hydrogen peroxide inhibitory activity compared to standard drug, BHT respectively as shown in Fig. 1C.

Lipoperoxidation radical scavenging capacity (TBRAS)

The lipid peroxidation activity of palmatine, gliempiride, metformin and BHT was evaluated on MDA (malondialdehyde) formation. From the result of the experiment on IC_{50}, glimepiride, metformin and palmatine had similar effect on the inhibition of MDA formation with IC_{50} = 1.32 ± 0.59, 1.3 ± 0.28 and 1.31 ± 0.59 µM, respectively. Palmatine, glimepiride and metformin had significant ($p<0.0001$) higher lipid peroxidation inhibitory activity compare to standard drug, BHT as shown in Fig. 1D.

Chelating capacity

The effect of palmatine, glimepiride, metformin and BHT on the metal chelating ions was evaluated using chelating capacity activity as shown in Fig. 1E. Comparative IC_{50}, showed that BHT had significant ($p<0.0001$) strong iron cheating capacity with IC_{50} = 9.17 ± 0.97 µM followed by glimepiride, metformin and palmatine with IC_{50} = 20.05 ± 1.09, 22.5 ± 1.1 and 45.44 ± 1.25µM. glimepiride and metformin showed significant ($p<0.001$) higher chelating capacity than palmatine. Palmatine showed least chelating capacity.

Reducing capacity

The reducing power of palmatine, glimepiride, metformin and BHT was evaluated using reducing capacity as shown in Fig. 6. Based on IC_{50}, palmatine had significantly ($p<0.0001$) higher reducing capacity with IC_{50} = 1.35 ± 0.35 µM than glimepiride, metformin and BHT. Glimepiride and metformin had similar reducing capacity activity with IC_{50} = 2.75 ± 0.7 and 2.55 ± 0.5 µM. BHT had least reducing capacity activity with IC_{50} = 3.99 ± 0.94 µM as shown in Fig. 1F.

In vitro antiglycation assays

The inhibitory activity of palmatine, glimepiride, metformin and aminoguanidine on the advanced glycation end products was evaluated using glucose-BSA and MGO-BSA glycation protein model. Palmatine showed a significantly ($p<0.0001$) higher inhibition of AGEs with IC_{50} = 2.0 ± 0.25 µM compared to metformin, glimepiride and aminoguanidine with IC_{50} = 14.2 ± 0.98 µM, 7.78 ± 0.81 µM and 7.72 ± 0.86 µM. Glimepiride and aminoguanidine had similar inhibition effects on the AGEs and metformin had the least antiglycation activity in the glucose-BSA glycation protein model as shown in Fig. 2A. However, Metformin showed significantly ($p<0.000$) higher inhi-

bition of AGEs with IC_{50} = 1.33 ± 0.55 µM, which is followed by aminoguanidine, palmatine and glimepiride with IC_{50} = 2.44 ± 0.5 µM, 10.5 ± 0.75 µM, and 14.42 ± 0.39 µM respectively in MGO-BSA glycation protein model as shown in Fig. 2B.

Hemoglobin assays

The effect of palmatine, glimepiride and metformin on the inhibition of glycation of proteins at different time intervals. As shown in Fig. 2C, glimepiride showed higher inhibition of glycation at 24 h with IC_{50} = 12.55 ± 0.19 µM and showed strong inhibition at 48 h time interval with IC_{50} = 8.45 ± 0.17 µM but the mild inhibition was observed after 72 h time interval with IC_{50} = 18.10 ± 0.06 µM. Metformin showed mild inhibition at 24 h with IC_{50} = 14.74 ± 1.36 µM and weak inhibition after 72 h time interval with IC_{50} = 55.7 ± 3.09 µM but showed higher inhibition at 48 h time interval with IC_{50} = 9.5 ± 0.25 µM. Palmatine showed higher inhibition after 48 h with IC_{50} = 4.27 ± 0.08 µM followed by inhibition after 72 h time interval with IC_{50} = 9.17 ± 0.01 µM. Palmatine showed weak inhibition after 24 h with IC_{50} = 15.38 ± 0.11 µM. However, palmatine and metformin had similar effect on the inhibition of glycation of proteins after 24 h of time interval while palmatine showed higher inhibition of glycation of proteins after 48–72 h time interval compared to metformin and glimepiride.

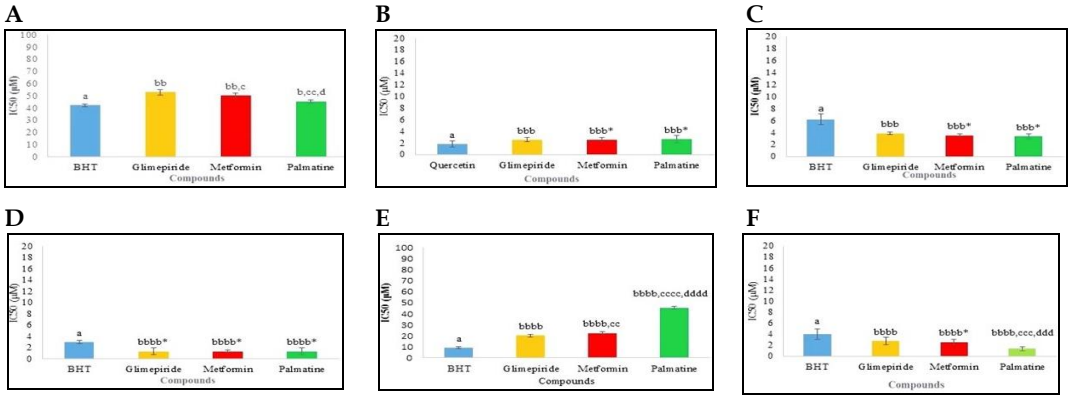

Figure 1. Antioxidant profile of palmatine, glimepiride, metformin, and standard drug (BHT and quercetin).

(A) DPPH radical scavenging capacity; **(B)** NO radical scavenging capacity; **(C)** H_2O_2 radical scavenging capacity; **(D)** Lipoperoxidation radical scavenging capacity; **(E)** Chelating capacity; **(F)** Reducing power capacity. Results are showed as mean ± standard deviation (n = 3). The data were statistically analyzed by One-way ANOVA followed by Dunnet's' post hoc test. Different letter **(a-d)** denotes significant differences (p<0.05, p<0.01, p<0.001 and p<0.0001); (*): No significant differences among the groups.

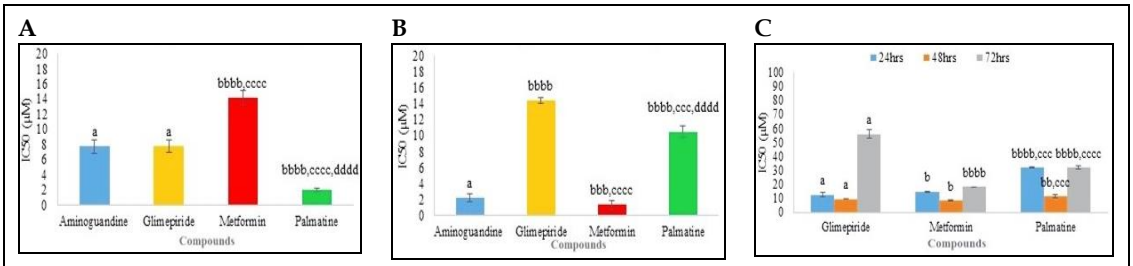

Figure 2. *In vitro* anti-glycation activity of palmatine, glimepiride, metformin, and standard drug (aminoguanidine).

(A) Glucose-BSA protein glycation inhibition activity; **(B)** MGO-BSA protein glycation inhibition activity and **(C)** Hemoglobin glycation inhibition activity. Results are showed as mean ± standard deviation (n = 3). The data were statistically analyzed by One-way ANOVA followed by Dunnet's' post hoc test. Different letter **(a-d)** denotes significant differences (p<0.05, p<0.01, p<0.001 and p<0.0001).

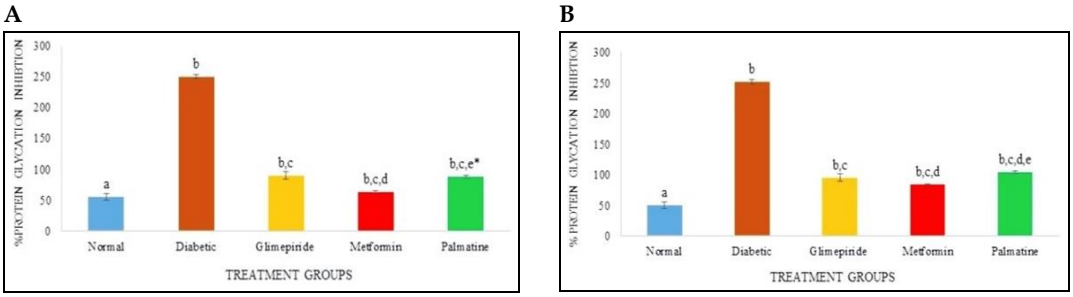

Figure 3. *In vivo* protein glycation inhibition activities in liver **(A)** and kidney **(B)**.

Values were expressed as mean ± SD (n = 6). The data were statistically analyzed by two-way ANOVA followed by Turkey's HSD test. Different letter **(a-e)** denotes significant differences among the groups when ($p<0.05$); (*): No significant.

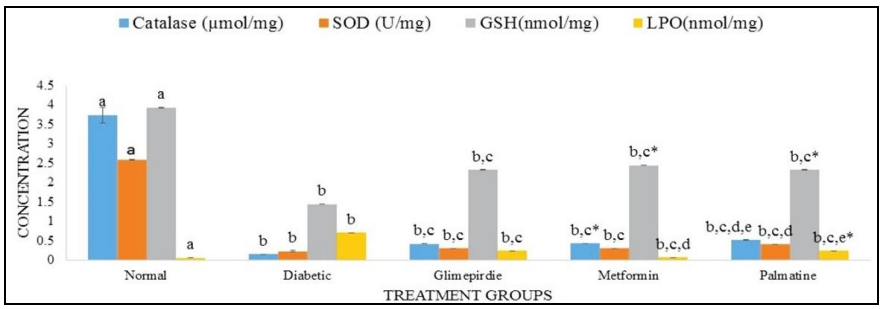

Figure 4. *In vivo* antioxidant activities.

Values were expressed as mean ± SD (n = 6). The data were statistically analyzed by two-way ANOVA followed by Turkey's HSD test. Different letter **(a-e)** denotes significant differences among the groups when ($p<0.05$); (*): No significant.

*Renal and liver function test (*in vivo*)*

The *in vivo* anti-glycation activity was evaluated in the diabetic and treated groups glimepiride, metformin and palmatine in liver and kidney. There was a significant ($p<0.0001$) increase in the glycated proteins in the diabetic rat compared to the normal group. All treated groups showed a significant ($p<0.0001$) reduction in the glycated protein level compared to diabetic control group in the liver and kidney as shown in Fig. 3. Metformin treated groups showed more significant ($p<0.001$) inhibition of glycated proteins compared to glimepiride and palmatine in both liver and kidney as shown in Fig. 3A-B. Glimepiride and palmatine treated groups had similar effect on the inhibition of the glycated proteins in the liver organ while glimepiride treated groups was more effective on the glycated proteins inhibition in the kidney.

In vivo *antioxidant assays*

The effect of palmatine, glimepiride and metformin on oxidative stress markers was presented in Fig. 4. The result showed that palmatine, metformin and glimepiride treated groups showed a significant ($p<0.001$) increase in the activity of catalase, SOD, GSH and LPO compared to the diabetic control groups. The effect of glimepiride, metformin and palmatine were a par in the activation of catalase and SOD activity, while the activity of GSH and LPO was higher in metformin treated compared to glimepiride and palmatine treated group.

DISCUSSION

Several studies have shown that oxidative stress increases the rate of formation of advanced glycation end products (AGE). AGEs have the propensity to generate reactive oxygen species and further accelerate the protein glycation under hyperglycemic condition in diabetic patients (Yaribeygi et al., 2018).

Palmatine presented a very strong *in vitro* and *in vivo* antioxidant activity in this experiment. Because of the strong antioxidant property of palmatine, it may have inhibited the generation of free radicals that play a vital role in autoxidation of glucose and non-enzymatic protein glycation,

which results in the weak antioxidant defense system and insulin resistant state. Previous research reports that increased production of free radical via increased glycolysis, activated sorbitol, autoxidation of glucose and non-enzymatic protein glycation, results in the weak antioxidant defense system and insulin resistant state (Phaniendra et al., 2014).

Antioxidant activity plays an important role in cellular physiology as it can neutralize and prevent free radicals, including reactive oxygen species (ROS) and its derivatives from damaging cells. Antioxidants control the autoxidation by interrupting the propagation of free radicals or by inhibiting the formation of free radicals via different mechanisms and help in scavenging the species that initiate the peroxidation, breaking the autoxidative chain reaction, quenching $\bullet O_2^-$, and preventing the formation of peroxides (Gaschler and Stockwell, 2017). The inhibition of intracellular free radical formation would provide a therapeutic strategy to prevent oxidative stress and the related diabetic vascular complications. Antioxidants may act at different levels inhibiting the formation of ROS or scavenge free radicals. Supplementation with antioxidants potentially improve endothelial dysfunction in type 2 DM by re-coupling the mitochondrial function as well as decreasing the vascular NADPH oxidase activity (Oyenihi et al., 2015).

AGEs are substances composed of amino groups of proteins, lipids nucleic acids and reducing sugars. The initial and propagation phases of the glycation process are accompanied by the production of a large number of free radicals, carbonyl species, and reactive dicarbonyl species, of which, methylglyoxal (MG) is the most reactive and can cause dicarbonyl stress, influencing normal physiological functions (Wan-Ju et al., 2017). Antioxidant activities are highly important in preventing oxidative stress and acceleration of the formation of AGE. Many studies have reported various mechanism through, which most compounds with antioxidant potentials inhibits AGEs. Suggested mechanisms include radical scavenging, reducing power, inhibition of lipid peroxidation, carbonyl trapping and metal ion chelation (Jariyapamornkoon et al., 2013). The anti-AGE of palmatine maybe through similar mechanism as reported by because palmatine showed a very strong inhibition of BSA-glucose and MGO-BSA glycation *in vitro* and *in vivo.* This effect maybe through the blockage of free radicals to reduce oxidative stress and decreasing the production of reactive carbonyl and dicarbonyl groups can inhibit the glycation function. It also showed a reducing power, inhibition of lipid peroxidation and metal ion chelation properties, which has been reported to be effective in anti-AGE effect.

Palmatine inhibited the formation of methylglyoxal derived advanced glycation end-products in a bovine serum-albumin-glycation end-products. Furthermore, the results show that palmatine could react with carbonyl groups from reducing sugars, Amadori adducts and dicarbonyl intermediates therefore blocking their conversion to advanced glycation end-products. The anti-glycation activities of palmatine were attributed to their antioxidant property and its abilities to scavenge reactive carbonyls. The ability of palmatine to react with carbonyls was the major mechanism for protein glycation inhibition. Palmatine alleviated oxidative stress under diabetic conditions through the inhibition of lipid peroxidation, prevent and/or delay the onset of renal damage, methylglyoxal system, and may also act by blocking conversion of dicarbonyl intermediates to advanced.

Previous studies have reported seed extract of fenugreek had antioxidant activity via multiple mechanisms and such mechanisms include radical scavenging, reducing power, inhibition of lipid peroxidation, carbonyl trapping and metal ion chelation (Yeh et al., 2017).

The anti-glycation capacity of rutin, along with its metabolites, has been previously reported to be through the inhibition of glucose autoxidation, free radical scavenging, and chelating metal ions properties (Palsamy and Subramanian, 2011).

Polyphenols a group of secondary metabolites with diversified phytochemical classes such as flavones, flavanones, flavones and proanthocyanidins, has been reported to possess anti-glycation activity radical scavenging, carbonyl trapping, metal iron chelation, protein interaction and block-

ing the receptor for advanced glycation end products (RAGE) (Khangholi et al., 2016; Yeh et al., 2017).

The combat of oxidative stress is one of the therapeutic approaches in treating diabetic patients. It is reported that AGEs associated pathologies of diabetes complications can be treated effectively using synergistic action of compounds offering both anti-glycation and antioxidant properties (Ramkissoon et al., 2013). Antiglycation activity of natural products including spices and condiments has strong correlation with antioxidant activity (Ramkissoon et al., 2013).

AGE-inhibition capacity of palmatine may be attributed to the plasma glucose lowering activity, through the activation of glyoxalase 1 pathway and inhibition of the aldose reductase (ALR2). Many reports have associated the formation of AGE to activation of ALR2 and hyperglycemia and inhibition of AGE formation through the activation of glyoxalase 1 pathway. Apart from RAGE activation, reactive oxidative stress (ROS) generation and generation of AGE cause activation of the polyol pathway by the induction of ALR2 and the rate-limiting enzyme of the pathway. The 3-deoxyglucosone produced as a by-product from the polyol pathway is a major precursor of AGE. Hyperglycemia-induced activation of ALR2 increases AGE production that in turn stimulate further activation ALR2.

Previously report have shown plasma glucose lowering activity of palmatine and its effect on liver, kidney, and antioxidant enzymes parameters in STZ induced diabetic rat model (Ekeuku et al., 2015). The plasma glucose reducing and antioxidant property of palmatine may have contributed to the activation of glyoxalase 1 pathway and inhibition of the ALR2. Many reports have shown many agents that possesses plasma glucose lowering and antioxidant properties shows inhibition of AGE through activation of glyoxalase 1 pathway and inhibition of the ALR2 (Dixit et al., 2005). Compounds with AGE inhibiting property also act as natural ROS scavengers. Polyol pathway induced AGE formation might be controlled by inhibiting the activity of ALR2. Natural ALR2 inhibitors with AGE suppressing potency and antioxidant properties would be a three-sided useful approach to control diabetic complications (Palsamy and Subramanian, 2011).

Several small studies on metformin, glucose-lowering medication, showed some beneficial effects on glycation measures, such as a decrease of MGO and lower AGEs and oxidative stress in patients with (type 2 diabetes mellitus) T2DM (Rabbani et al., 2010). AGE-inhibition capacity of metformin might be attributed merely to improved glycemic control instead of dicarbonyl quenching (Rabbani et al., 2010). Pyridoxamine can inhibit the conversion of Amadori products to AGEs and is able to scavenge reactive oxygen species and the reactive carbonyl intermediates that are products of sugar and lipid degradation (Voziyan and Hudson, 2005). Lipid-lowering medication might inhibit AGE formation as well due to anti-oxidative properties, which partly reduces lipid peroxidation. Atorvastatin showed a decrease in serum AGEs, in addition to a decrease of RAGE expression in carotid artery plaques, by inhibition of AGE formation (Cuccurullo et al., 2006).

Many nutraceuticals are rich in polyphenols that possess anti-glycation activity through various mechanisms, such as regulation of glucose metabolism, antioxidant effects and inhibition of the ALR2 pathway (Cuccurullo et al., 2006). Rutin have been reported to reduce plasma glucose level in STZ-induced diabetic rats, protect the kidney and liver, antioxidant and anti-AGE properties and the mechanism of action is reported to be through the inhibition of ALR2 (Palsamy and Subramanian, 2011). Resveratrol has been reported to exert its beneficial effects on diabetic complications by inhibition of aldose reductase (AR), resulting in a decrease of AGE formation in the kidney and improvement of the glomerular filtration rate and renal function in diabetic rats (Palsamy and Subramanian, 2011). The normalization effect of AR activity resveratrol has been shown to support additional effect on polyol pathway enzymes such as sorbitol dehydrogenase and glyoxalase-I (Glo-I), which limits AGE formation and glycation damage to the kidneys (Xue et al., 2016). In a clinical study the effect of resveratrol and hesperetin on

vascular function was observed. These dietary bioactive compounds demonstrated to be strong inducers of glyoxalase I, that is responsible for the detoxification of RCS compound MGO (Konda et al., 2019). Co-therapy of resveratrol and hesperetin, instead of individual administration, was able to improve fasting plasma glucose, oral glucose insulin sensitivity and arterial and renal function in obese subject (Xue et al., 2016). Several researchers have shown that the increased antioxidant activity results in the decreased glycemic index level in the STZ-induced diabetic rat model (Okechukwu et al., 2019).

CONCLUSIONS

Palmatine possess antioxidant and anti-glycation properties, the mechanism of action seems to be via the blockage of free radical generation and activation of antioxidant enzymes and decrease of reactive carbonyl, therefore blocking their conversion to AGE. Palmatine action may be attributed to the plasma glucose lowering and antioxidant properties, and its abilities to scavenge reactive carbonyls. Further research is ongoing to determine effect of palmatine on glyoxalase 1 and aldose reductase pathway and interaction with receptor for AGE.

CONFLICT OF INTEREST

The authors declare no conflicts of interests.

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AUTHOR CONTRIBUTION:

Contribution	Mridula S	Masroor WS	Mellinnda X	Hui TW	Chan HK	Chirara K	Okechukwu PN
Concepts or ideas							x
Design	x						x
Definition of intellectual content					x	x	x
Literature search	x	x	x	x			x
Experimental studies	x	x	x	x			
Data acquisition	x	x	x	x			x
Data analysis	x	x	x	x	x	x	x
Statistical analysis	x	x	x	x	x	x	x
Manuscript preparation	x	x	x	x	x	x	x
Manuscript editing	x	x	x	x	x	x	x
Manuscript review	x	x	x	x	x	x	x

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