Tropical Life Sciences Research, 25(1), 75–93, 2014

Antihyperglycaemic and Toxicological Evaluations of Extract and Fractions of *Gynura procumbens* Leaves

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Abstrak: Aktiviti antihiperglisemik akut dan subkronik, penilaian jurang keselamatan dan kandungan kimia ekstrak dan fraksi etanol (EE) *Gynura procumbens* (GP) telah dikaji. Tikus diabetik aruhan streptozotosin [(STZ) SDR] dan garis panduan *Organisation for Economic Cooperation and Development* (OECD) 425 dan 407 telah digunakan dalam kajian antidiabetik dan ketoksikan, masing-masing. Data biokimia dan hematologi yang diperolehi daripada kedua-dua prosedur akut dan sub-kronik mencadangkan bahawa ekstrak ini adalah selamat pada ujian had dos (2000 mg/kg); oleh itu, dos maut oral (LD_{50}) ekstrak melebihi 2000 mg/kg. Pengambilan harian boleh terima (ADI) yang telah ditentukan ialah 700 mg/kg/hari. Dalam kajian antihiperglisemik akut, fraksi n-butanol (n-BF) didapati paling berkesan menurunkan paras glukosa secara konsisten, yang juga ditunjukkan dalam kajian 14 hari. Bukan sahaja n-BF menunjukkan penurunan glukosa bergantung kepada dos (51.2% dan 62.0% pada 500 mg/kg dan 1000 mg/kg, masing-masing) paling banyak, kesannya juga adalah yang paling mendekati metformin (63.6%, $p<0.05$). Jumlah flavonoid dalam n-BF dianggarkan sebanyak 62.90% dan 79.68% lebih tinggi daripada dalam fraksi etil asetat (EAF) dan fraksi akueus (AF), masing-masing ($p<0.05$) dan 61.22% dan 49.33% lebih tinggi bagi kandungan fenolik ($p<0.05$). Daun GP mempunyai paras dos tanpa kesan buruk kelihatan (NOAEL) dan aktiviti antihiperglisemik yang bersesuaian dengan kandungan fenol dan flavonoid yang tinggi. Oleh itu, daun GP menjanjikan sumber produk semula jadi antidiabetik baru.

Kata kunci: Ketoksikan, Antihiperglisemik, Glukosa Darah, Fenolik, Flavonoid, *Gynura procumbens*

Abstract: The acute and sub-chronic antihyperglycaemic activity, safety margin evaluations and chemical composition of ethanol extract (EE) and fractions of *Gynura procumbens* (GP) were studied. Streptozotocin (STZ)-induced diabetic rats (SDRs) and the Organisation for Economic Cooperation and Development (OECD) guidelines 425 and 407 were used in the antidiabetic and toxicity studies, respectively. Biochemical and haematological data obtained from both acute and sub-chronic procedures suggest that the extract is safe at the limit test dose (2000 mg/kg); thus, the oral lethal dose (LD_{50}) exceeds 2000 mg/kg. The acceptable daily intake (ADI) was determined to be 700 mg/kg/day. In the acute antihyperglycaemic study, the n-butanol fraction (n-BF) was found to consistently lower glucose levels the most effectively, which was also demonstrated in the 14-day study. Not only did the n-BF show the highest dose-dependent glucose-lowering action (51.2% and 62.0% at 500 mg/kg and 1000 mg/kg, respectively), its effect was the closest to that of metformin (63.6%, $p<0.05$). The estimated amount of flavonoids in n-BF were 62.90% and 79.68% higher than the ethyl acetate fraction (EAF) and aqueous fraction (AF), respectively ($p<0.05$), with a corresponding value of 61.22%

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and 49.33% for phenolics (p<0.05). GP leaves have a no-observed-adverse-effect-level (NOAEL) and antihyperglycaemic activity corresponding to the high content of phenols and flavonoids. Therefore, GP leaves are a promising source of new antidiabetic natural products.

Keywords: Toxicity, Antihyperglycaemic, Blood Glucose, Phenolics, Flavonoids, *Gynura procumbens*

INTRODUCTION

Gynura procumbens (Merr) (GP), a member of the Compositae family, is an herbaceous plant that is widely distributed in Borneo, the Philippines, Java and Peninsular Malaysia (Hoe *et al*. 2011). In Southeast Asia, particularly Indonesia, Malaysia and Thailand, the plant is traditionally known for the treatment of several ailments including fevers, kidney disease, migraines, constipation, hypertension, diabetes mellitus and cancer. Several of these indications have been validated with pharmacological studies (Kim *et al*. 2011).

In previous studies, the glucose response activities of GP were reported (Rosidah *et al*. 2009; Akowuah *et al*. 2001; Zhang & Tan 2000). However, because the findings of the prior studies were inconsistent, a systematic investigation was carried out that was targeted at natural product discovery and/or production of standardised herbal forms. The data from this study were striking and indicated that the 1:3 ethanol-water extract of GP was most responsive in lowering glucose levels in diabetic animal models as well as in glucose tolerance tests (Algariri *et al*. 2013).

To produce GP extracts on an industrial scale, which was the long term goal of the previous study, it is required that the active extracts be biologically effective and safe. Therefore, the present investigation evaluated the safety and/or possible toxicity of the active extract [25% ethanol extract (EE)] of GP using standard procedures. The study also attempted fractionation of the extract for biological activity to enhance future characterisation of active glucose-lowering compounds in the extract to pave the way for standardisation.

MATERIALS AND METHODS

Chemicals and Drugs Used

Metformin (500 mg) was obtained from Glucophage®, Lipha Pharma Ltd. (Bristol, UK) and streptozotocin (STZ) was obtained from Sigma Aldrich Chemical Co. (St. Louis, MO, USA). All chemicals used for extraction, including ethanol, n-hexane, chloroform, ethyl acetate and n-butanol were obtained from R & M Chemicals (Dundee City, UK).

Plant Material and Preparation of Extract

The fresh leaves from GP collected from Herbagus Sdn. Bhd. (Kepala Batas, Pulau Pinang, Malaysia) were authenticated by Mr. V. Shunmugam a/l Vellosamy of the herbarium unit, School of Biology, Universiti Sains Malaysia (USM), and a

voucher specimen (No. 11432) was deposited in the herbarium for future reference. The leaves were washed with water, dried in an oven at 45°C and milled into powder (1200 g). The powdered leaves were extracted by maceration (45°C) in 25% ethanol (2 litres) with the solvent being replenished every 6 hours. The extract was pooled, filtered, and then concentrated at 60°C using a rotary evaporator (Buchi Labortechnik AG, Flawil, Switzerland). The concentrated extract was then freeze-dried (Lebconco Corporation, Missouri, USA). The dried powdered extract was kept in a freezer at –40°C until it was used for animal treatments.

Liquid-Liquid Fractionation of the Extract

Approximately 250 g of the extract suspended in distilled water was first treated with ethyl acetate and, after vigorous shaking, was allowed to separate in a separating funnel. The ethyl acetate portion was collected and the process was repeated until the soluble ethyl acetate portion was completely collected from the extract. The residue was then similarly treated with n-butanol until the entire n-butanol-soluble portion was collected. Finally, the residue (aqueous portion) was also collected. The fractions collected were ethyl acetate, n-butanol and the aqueous residue. These fractions were filtered, concentrated and freeze-dried as described earlier to yield 2%, 23% and 33%, respectively, of the starting amount of extract. These were also preserved in the freezer (–40°C) until being used for animal treatments.

Animals

Sprague Dawley rats (200–250 g) obtained from the Animal Research and Service Centre (ARSC), USM, were used in this study. The rats were acclimatised for a period of 7 days in the Animal Transit Room, School of Pharmaceutical Sciences, USM where the experiments were carried out. They were allowed access to food (Gold Moher, Lipton India Ltd., Hyderabad, India) and tap water *ad libitium*. The temperature of the facility was 22±3°C, and light/darkness was alternated 12 hours apart. The experimental procedures were approved by the Animal Ethics Committee, USM [USM/Animal Ethics Approval/2010/(56)(211)].

Acute Toxicity Study

Five healthy, adult, female Sprague Dawley rats (200–225 g) were selected for this experiment. The acute toxicity study was carried out according to the up-and-down dosing procedure for testing of chemicals of the Organisation for Economic Cooperation and Development (OECD) guideline 425 (OECD 2008a). A dose of extract at 2000 mg/kg was selected as the limit test dose and given orally (single dose in a 2 ml volume) to the first female rat after an overnight fast. The animal was closely observed for signs of possible toxicity for the first 1, 4, 12 and 24 hours. If the rat survived, four additional rats were similarly and sequentially dosed so that a total of five rats were tested. The weight of the animals was determined on days 1, 7 and 14. Signs or symptoms of treatment-related toxicity and/or mortality were monitored for up to 14 days. Food and water were provided *ad libitium.* The visual observations included changes in skin and fur, eyes and

mucous membranes, respiratory, circulatory, and autonomic and central nervous systems as well as somatomotor activity and behavioural pattern. Animals were euthanised on the last day of the experiment, and blood was collected for haematological and biochemical assays.

Sub-Chronic Toxicity Study

This experiment was also carried out according to the protocol described in OECD guidelines 407 (OECD 2008b) with minor modifications. Forty eight rats (24 males and 24 females) were randomly assigned into 4 groups of 12 rats each: a control group and 3 treatment groups (n = 12; 6 males and 6 females). The 3 treatment groups were administered 250, 500 or 1000 mg/kg extract of GP daily for 28 days (single dose in a 2 ml volume daily). The fourth group, the control, received an equivalent volume of distilled water (vehicle). The extract was freshly reconstituted every week. The rats were observed daily for behavioural changes, and their weight was monitored once a week. At the end of treatment (28 days), the rats were anesthetised under CO_2 inhalation, and blood samples collected via cardiac puncture into non-heparinised and EDTA-containing tubes for biochemical and haematological analyses, respectively. Additionally, the rats were dissected and organs (brain, heart, liver, thymus, spleen, kidneys, adrenal glands, sex organs, lungs, stomach and gut) collected and examined. The relative organ weight (ROW) of each organ was then calculated as follows: ROW (%) = weight of organ / body weight of rat × 100. Haematological and biochemical analyses were performed at Gribbles Pathology (M) Sdn. Bhd. (Pulau Pinang, Malaysia), using an automated haematology analyser (Sysmex-XT-1800, Kobe, Japan) and an automated chemistry analyser (Olympus 640 Biochemistry Analyser, Tokyo), respectively.

Induction of Diabetes

Diabetes was induced in rats by intraperitoneal injection of 55 mg/kg of STZ (Sigma Aldrich Chemical Co, St. Louis, MO, USA) reconstituted in 0.1 M cold citrate buffer (pH 4.5) after an overnight fast. Seventy two hours after STZ administration, blood glucose levels were measured in blood collected by tail vein puncture using Accu-check Advantage II clinical glucose meter (Roche Diagnostics Co., Florida, USA). Rats with a fasting blood glucose ≥15 mmol/l (270 mg/dl) were considered diabetic and included in the study. The percentage change in blood glucose was calculated by % glycaemic change = $(G_x–G_i) / G_x \times 100$; where G_x is the glycaemia at time x and G_i is the glycaemia at the initial time (i).

Acute Antihyperglycaemic Activity Study

In this test, 36 STZ-induced diabetic rats (SDRs) were randomly categorised into 6 groups (n = 6). After an overnight fast, groups 1 and 2, which consisted of the diabetic and positive controls, were treated with 1% carboxymethyl cellulose (vehicle) and metformin (500 mg/kg body weight), respectively. Groups 3–6 accordingly received single oral doses (500 mg/kg) of 25% EEs, ethyl acetate fraction (EAF), n-butanol fraction (n-BF) and aqueous fraction (AF) of GP, respectively. Blood was collected from the tail vein before (0 min), and at 1, 2, 3,

5 and 7 hours post-treatment for glucose measurement. This whole procedure was repeated for a second and third time using 1000 mg/kg and 2000 mg/kg of extract and fractions respectively.

Fourteen-day Antihyperglycaemic Activity Study

Thirty six SDRs were evenly assigned to 6 groups, and 6 non-diabetic rats belonging to the normal control group were used in this study. Following an overnight fast (but with free access to water), 4 groups of the diabetic rats were treated with 500 mg/kg body weight each of the 25% EE, EAF, n-BF or AF. The other two groups of diabetic rats, which served as the negative and positive controls, received 1% carboxymethyl cellulose (in equivalent volume) and 500 mg/kg body weight of metformin. The normal control group also received an equivalent volume of 1% carboxymethyl cellulose. The treatment was administered consecutively for 14 days, and the fasting blood glucose (FBG) levels and the body weight of the rats were measured at the outset of the experiment (baseline fasting blood glucose), on day 7 and at the end of the study (day 14). This procedure was repeated using double amount of the previous dose (1000 mg/kg body weight) of the extract and fractions.

Phytochemical Analyses

Determination of total phenolics in GP fractions

Total phenolic content of the 3 fractions (EAF, n-BF and AF) of 25% EE of GP was determined using the Folin-Ciocalteu reagent method. Briefly, 0.4 ml (1 mg/ml) of each fraction was pipetted into test tubes and 2 ml (10% v/v) of Folin-Ciocalteu reagent was added to the extract sample. Five minutes later, 1.6 ml (7.5%) of sodium carbonate solution was added into the sample. The sample mixture was then incubated for one hour at room temperature, and the absorbance was measured using Perkins Elmer UV-Visible spectrometer (California, USA) at 760 nm. A series of standard gallic acid solutions (20–200 µg/ml) were prepared, absorbance was measured at the same wavelength and these data were used to plot the calibration curve. The total phenolic content was calculated as µg/ml of gallic acid equivalent of extracts. All samples were analysed in triplicates.

Determination of total flavonoids in *GP* fractions

Total flavonoids of the 3 fractions (EAF, n-BF and AF) of 25% EE of GP was determined using the aluminium chloride colorimetric method. Briefly, 1.5 ml of each fraction solution was added to a test tube and was mixed with 1.5 ml of 2% aluminium chloride solution prepared in methanol. The absorbance was measured at 415 nm after 10 minutes of incubation at room temperature using a double beam Perkins Elmer UV/Visible spectrophotometer (California, USA). The flavonoid content of the extracts was calculated in µg/ml as quercetin equivalents by using the equation obtained from the quercetin calibration curve. The calibration curve was constructed using six different concentrations (3.125–100 µg/ml) of quercetin solution prepared in methanol. All samples were analysed in triplicates.

Statistical Analyses

The results were expressed as the mean±SE. Statistical significance determined by a one-way analysis of variance (ANOVA) and the post hoc test – least square difference (LSD) test. Differences were considered significant at $p<0.05$.

RESULTS

Acute Toxicity Effect

The results obtained from the acute toxicity study conducted according to OECD guidelines 425 revised up-and-down procedure indicated no treatment-related mortality at 2000 mg/kg or throughout the 14-day observation period. Both treated and control rats successively increased their weight, and the increases in the test groups at each point were not statistically significant when compared to the control (data not shown), indicating that the extract did not adversely affect the growth of the animals.

Additionally, there were no significant changes observed in the behaviour of the animals, such as apathy, hyperactivity and cramps. In addition, there were no significant changes in the nervous system or brain impairment, breathing or eye movement, within the study duration. Serum biochemical markers of liver and kidney functions and haematological indices measured at the end of the 14 days (data not shown) also indicated that a maximum dose of 2000 mg/kg of GP did not cause any evident toxic signs of necropsy in the liver, kidneys or haematopoietic system of the test rats. Hence, the extract can be considered non-hepatotoxic, non-nephrotoxic and non-haematotoxic at an acute dose.

From these results, it can be concluded that 25% EE of GP is safe with no observed acute toxicity effects. Therefore, the oral lethal dose (LD_{50}) for the female rats is greater than 2000 mg/kg.

Sub-chronic Toxicity Effects

The 28-day administration of graded doses of 25% EE of GP (250, 500 and 1000 mg/kg) were intended to assess the possible cumulative effects on vital organs and tissues including the liver, kidneys and blood elements. Within the 28 days, no abnormal behaviour, disease or death were observed in both male and female rats that received the plant extract.

Body Weight and Relative Organ Weight

Figure 1 shows the observed changes in the experimental animals' body weight within the 28 days. Although the animals recorded a body weight gain at the end of the 28 days, the gain in weight was not significant in the treatment groups compared to their respective controls. Compared to the controls, the extract had no effect on the ROW of the treated rats (data not shown). Moreover, it was observed that the sex of the animals and the dose of the extract used had no significant effect on both the absolute and relative weights of the organs. Therefore, the extracts have no observable toxic effects on the internal organs of the animals.

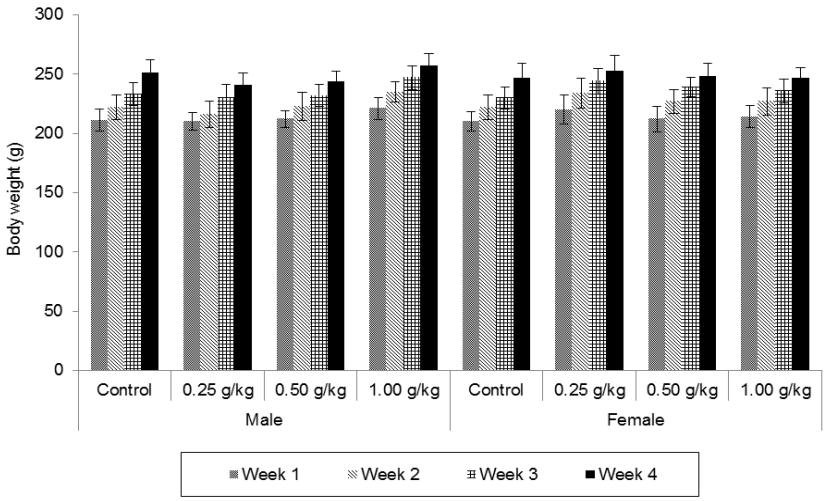

Figure 1: The body weight of control and treated rats measured during and at the end of the 28-day sub-chronic toxicity study. Values are expressed as the mean±SE (n = 5).

Liver and Kidney Profiles

Tables 1 and 2 show liver and kidney profiles of the respective male and female rats administered 250, 500 and 1000 mg/kg extract of GP for 28 days. From the results, the extract did not cause any significant effect on the activities of aspartate transaminase (AST) and alanine transaminase (ALT), cardinal markers of hepatocellular function, when compared to their respective controls. The sub-chronic oral doses of 25% EE did not result in any significant changes in alkaline phosphatase (ALP) activity in any of the treated groups compared to the controls. The measured levels of total proteins, albumin and globulin in the treated groups were not significantly different from those in the control groups in both sexes. Similar to liver profile, the levels of serum creatinine, urea, uric acid and electrolytes of the treated rats were also not significantly altered compared to the control groups after the 28-day treatment. Overall, the biochemical data suggest that the 25% EE of GP may be non-hepatotoxic and non-nephrotoxic at the doses used.

Haematological and Immunological Indices

The effect of the 25% EE on haematological indices after extract administration for 28 days was also evaluated in female and male rats (Table 3). The measured indices, including haemoglobin (HB), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC) and red blood cells (RBC) did not produce any significant effect in the male and female treated groups compared to the controls irrespective of the extract dose used. The differential WBC and platelet counts in female and male treated rats also were not significantly altered compared to the controls at any of

the three doses used, indicating that the 25% EE of GP did not cause any haematological or immunological defects after 28 days of administration.

Acceptable Daily Intake (ADI)

From the acute study data, it was concluded that the LD_{50} of the 25% EE of GP exceeded 2000 mg/kg accordingly, the extract dose of 1000 mg/kg used in the antidiabetic study was within the no-observed-adverse-effect-level (NOAEL), which is defined as the maximum dose of a substance found to have no adverse effects on the test subject. Given that the NOAEL was derived from studies conducted in mammals exposed to the substance via oral routes including gavage, the acceptable daily intake (ADI) equals the product of multiplying 1/100 (safety factor) of the NOAEL given in milligrams of extract per day per kilogram of test species weight (mg/kg-d) by the average weight of an adult human of 70 kilograms (kg): ADI = 1000 × 1/100 = 10 mg/kg; for a 70 kg adult human, ADI = 10 × 70 = 700 mg/kg/day. Since 1 g of dried leaves yielded 252 mg of 25% EE (i.e., 25.2% yields) 700 mg of the extract will be contained in 2.78 g of dried leaves of GP.

Table 1: Serum biochemical indices of male rats treated with 25% extract of GP recorded at the end of the 28-day sub-chronic toxicity study.

Parameters measured		Study groups			
		Control	250 mg/kg	500 mg/kg	1000 mg/kg
Liver profile	AST (U/l)	168.0±21.0	166.0±19.0	170.8±17.3	165.4±15.3
	ALT (U/l)	73.5±11.2	75.5±10.3	72.0±11.3	73.4±10.3
	ALP (U/l)	210.0±23.4	213.0±18.3	210.4±15.3	217.0±17.3
	GGT (U/l)	<4.0	<4.0	<4.0	<4.0
	Total protein (g/l)	66.0±8.2	66.0±10.3	69.5±8.3	68.0±7.3
	Albumin (g/l)	11.3±1.8	11.3±2.4	11.6±3.3	12.0±2.9
	Globulin (g/l)	54.7±8.2	54.7±7.2	57.6±9.3	56.0±8.9
Kidney profile	Ca (mmol/l)	2.7±0.4	2.4±0.3	2.6±0.3	2.7±0.1
	Na (mmol/l)	141.5±13.2	141.5±11.4	143.8±17.2	144.0±18.3
	K (mmol/l)	5.8±1.4	5.8±1.7	6.5±2.2	6.9±1.5
	Cl (mmol/l)	103.0±10.3	113.0±9.4	108.3±10.9	112.0±11.8
	Phosphorus (mmol/l)	2.3±0.3	2.2±0.5	2.9±0.4	2.5±0.8
	Creatinine (µmol/l)	45.6±4.3	45.6±6.3	48.3±4.3	45.6±9.4
	Urea (mmol/l)	9.3±2.3	9.3±3.0	8.3±2.9	8.4±2.4
	Uric acid (mmol/l)	164.0±19.2	161.0±17.3	169.9±18.3	166.6±18.9

Note: Values expressed as the mean±SE, n = 5; GGT – γ-glutamyl transferase

Table 2: Serum biochemical indices of female rats treated with 25% extract of GP recorded at the end of the 28-day sub-acute toxicity study.

Parameters measured		Study groups			
		Control	250 mg/kg	500 mg/kg	1000 mg/kg
Liver profile	AST (U/l)	171.5±16.3	173.5±15.3	175.5±19.3	178.3±16.3
	ALT (U/l)	55.3±6.4	53.8±4.6	55.0±7.3	54.3±7.3
	ALP (U/l)	213.5±20.3	211.2±18.3	213.0±18.3	212.7±17.9
	GGT (U/l)	<4.0	<4.0	<4.0	<4.0
	Total protein (g/l)	72.0±8.3	74.3±8.2	76.3±7.3	77.0±7.3
	Albumin (g/l)	18.3±3.2	17.3±2.3	17.0±3.7	17.6±2.9
	Globulin (g/l)	53.8±7.3	57.0±8.9	59.3±8.2	59.4±7.9
Kidney profile	Ca (mmol/l)	2.6±0.3	2.6±0.9	2.6±0.7	2.7±0.7
	Na (mmol/l)	148.8±16.9	146.8±19.3	147.7±18.3	149.0±17.3
	K (mmol/l)	4.9±0.5	5.1±0.6	4.9±0.8	4.5±0.3
	Phosphorus (mmol/l)	1.7±0.4	1.8±0.3	1.8±0.7	1.7±0.4
	Cl (mmol/l)	103.8±11.3	105.5±9.5	108.0±8.9	105.3±9.3
	Creatinine (μmol/l)	53.8±6.4	55.1±5.5	54.1±7.6	53.8±8.4
	Urea (mmol/l)	7.8±2.5	7.1±1.9	7.2±2.5	7.6±1.3
	Uric acid (mmol/l)	173.9±18.3	172.7±16.3	173.5±16.4	174.7±21.3

Note: Values expressed as the mean±SE, n = 5; GGT – γ-glutamyl transferase

Acute Antihyperglycaemic Effect

The 7-hour effect of a single dose (500 mg/kg, 1000 mg/kg or 2000 mg/kg) of 25% EE of GP and fractions prepared from the 25% EE (EAF, n-BF and AF) on blood glucose in SDRs were independently evaluated. Figure 2 shows the effect of the 500 mg/kg dose on measured blood glucose concentration. The 25% EE significantly reduced blood glucose level by 31%, while the EAF, n-BF and AF lowered blood glucose by 41%, 35% and 30%, respectively, after 7 hours (p<0.05) compared to concentrations at outset of administration. Only the EAF could significantly lower blood glucose comparably to metformin treatment after 3 (27%) and 5 (37%) hours (p<0.05).

The 1000 mg/kg dose of EE, EAF, n-BF and AF exerted a maximum reduction of 44%, 27%, 50% and 30%, respectively, in glucose levels at 7 hours after treatment (p<0.05), relative to values obtained at 0 hour (Fig. 3). Conversely, a significant lowering in glucose levels in the EAF-treated group were observed only at the last hour (7^{th} hour), while the other treatment groups showed a significant reduction beginning at the 2^{nd} or 3^{rd} hours following oral treatment. It was also found that the n-BF and AF closely mimic the effect of metformin.

Table 3: Haematological indices of male and female rats treated with 25% extract of GP recorded at the end of the 28-day sub-chronic toxicity study.

Sex	Parameters measured	Study groups			
		Control	250 mg/kg	500 mg/kg	1000 mg/kg
Male	WBC (10^3/µl)	9.5±2.3	10.4±1.5	11.4±1.9	12.1±2.1
	Neutrophil (%)	21.1±4.5	26.6±4.3	25.2±4.3	23.5±5.3
	Lymphocyte (%)	76.2±12.4	68.5±10.3	67.5±9.4	66.9±9.8
	Monocyte (%)	0.9±0.2	0.9±0.2	0.6±0.1	0.8±0.2
	Eosinophil (%)	1.2±0.9	1.2±0.8	1.4±0.8	2.5±0.9
	Basophil (%)	1.3±0.8	1.9±0.5	1.3±0.4	1.4±0.5
	RBC (10^6/µl)	8.9±0.9	8.8±1.2	9.0±0.8	9.0±0.8
	HB (g/dl)	16.6±1.3	16.6±2.1	16.9±1.9	17.1±1.8
	HCT (%)	0.7±0.1	0.8±0.1	0.8±0.1	0.8±0.1
	MCV (fl)	87.2±7.4	86.8±8.3	88.2±7.5	87.5±5.3
	MCH (pg)	18.8±1.4	18.9±0.9	18.9±0.8	19.2±1.1
	MCHC (g/dl)	21.3±1.6	21.8±2.1	20.9±1.9	21.3±1.8
	PLT (10^3/µl)	870.6±190.0	897.0±201.0	990.2±180.0	931.2±250.0
	MPV (f)	6.7±1.4	6.9±1.6	7.7±1.7	7.4±1.3
Female	WBC (10^3/µl)	10.9±2.9	10.2±1.1	11.4±2.1	10.8±1.8
	Neutrophil (%)	24.9±1.8	26.3±2.1	25.9±1.9	25.5±2.2
	Lymphocyte (%)	70.9±13.4	68.8±12.6	65.6±11.8	69.9±11.3
	Monocyte (%)	0.7±0.3	0.7±0.2	0.8±0.4	0.7±0.2
	Eosinophil (%)	0.9±0.1	0.8±0.1	0.9±0.1	0.9±0.1
	Basophil (%)	2.3±0.5	1.4±0.9	1.4±0.8	1.4±0.7
	RBC (10^6/µl)	9.2±2.3	8.8±1.3	8.6±1.5	8.5±2.1
	HGB (g/dl)	18.1±2.1	17.2±1.8	16.9±1.1	17.0±1.3
	HCT (%)	0.8±0.1	0.7±0.1	0.7±0.1	0.7±0.1
	MCV (fl)	89.5±8.9	87.2±7.1	87.3±5.3	87.8±6.4
	MCH (pg)	18.6±2.7	17.4±2.9	17.2±2.5	17.8±2.4
	MCHC (g/dl)	19.2±2.1	18.2±3.1	18.4±2.8	18.9±2.7
	PLT (10^3/µl)	1123.0±230.0	1066.0±232.0	978.0±288.0	946.40±244.0
	MPV (fl)	7.3±2.1	7.2±2.2	7.2±1.9	7.1±1.5

Note: Values are expressed as the mean±SE, n = 5; HCT – haematocrit, PLT – platelets; MPV – mean platelet volume

The acute effect of 2000 mg/kg on blood glucose is shown in Figure 4. At this concentration, the EAF and AF were found to exert non-significant lowering of blood glucose levels (10.13% and 8.65%, respectively) when compared to the diabetic control. However, the 25% EE and n-BF significantly reduced blood glucose levels by 18.44% and 13.46%, respectively, after 7 hours compared to the diabetic control. The effect of metformin was 33.01% at the same conditions. Overall, it was found that the 25% EE of GP and the fractions displayed

antihyperglycaemic effects under acute conditions. However, this action appears to not depend on the dose administered. Moreover, the effect of the n-BF was more consistent at the three dose levels tested and was therefore considered the fraction with the highest composition of effective antihyperglycaemic bioactives.

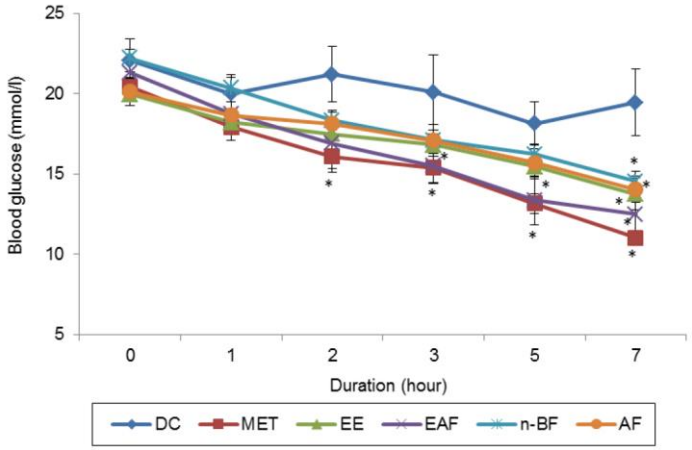

Figure 2: The effect of a single oral dose (500 mg/kg) of 25% EE and fractions of GP on a 7 hour blood glucose level measured in SDRs. Data are expressed as the mean±SE (n = 6); *p<0.05 vs. diabetic control.
Note: DC – diabetic control; MET – metformin

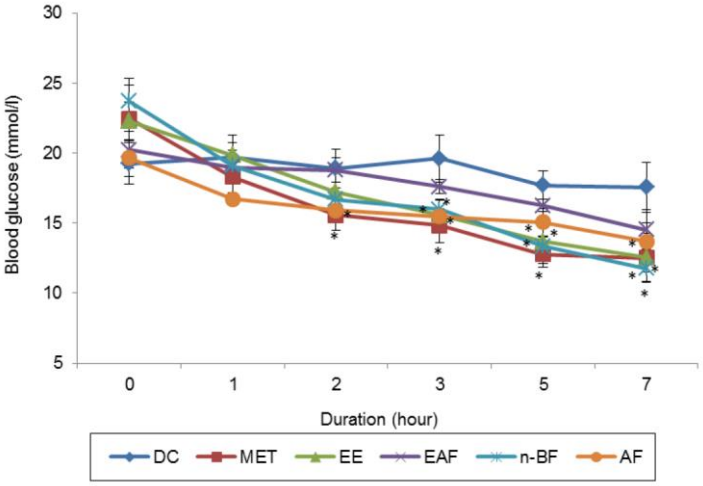

Figure 3: The effect of a single oral dose (1000 mg/kg) of 25% EE and fractions of GP on 7 hour blood glucose level measured in SDRs. Data are expressed as the mean±SE (n = 6); *p<0.05 vs. diabetic control.
Note: DC – diabetic control; MET – metformin

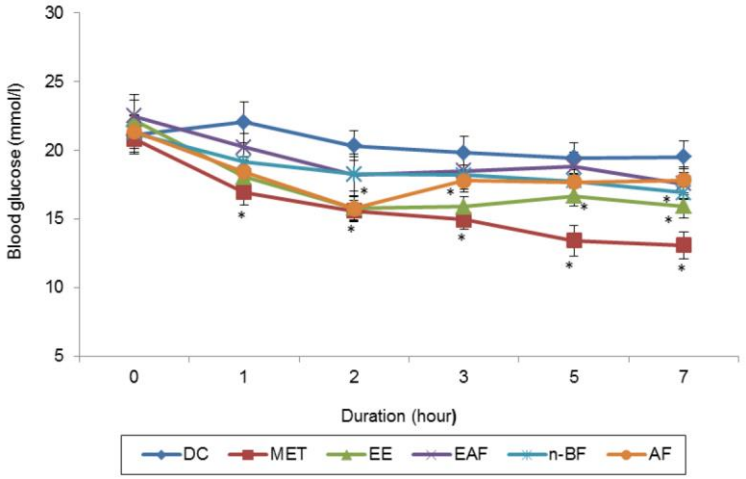

Figure 4: The effect of a single oral dose (2000 mg/kg) of 25% EE and fractions of GP on 7 hour blood glucose level measured in SDRs. Data are expressed as the mean±SE (n = 6); *p<0.05 vs. diabetic control.
Note: DC – diabetic control; MET – metformin

Fourteen-Day Antihyperglycaemic Activity

The effect on body weight and blood glucose was also studied following 14 days of repeated oral administration of the 25% EE and fractions at two dose levels (500 mg/kg and 1000 mg/kg) (Figs. 5 and 6, respectively). The EAF was administered at 1000 mg/kg only due to limited yield from fractionation. The body weights of untreated diabetic rats successively decreased over the study duration compared to normal control rats (p<0.05). However, in the treatment groups, after an initial decrease in weight up to day 7, the EE, n-BF and AF treated groups began to gain weight. At the end of the study (day 14), EE recorded 8.3% and 11.3% increases in weight for the 500 mg/kg and 1000 mg/kg doses, respectively, compared to the diabetic control. The corresponding increases in weight were 12.5% and 12.5% for n-BF and 8.7% and 9.6% for AF placing the effect of n-BF comparable to metformin, which exerted about a 13.3% gain (p<0.05). The blood glucose levels measured before, during and after a 14-day repeated oral administration is shown in Figure 6. Blood glucose levels in the untreated diabetic rats successively increased from the outset of experiment (day 0) up to 17.2% and 34.1% on days 7 and 14, respectively (p<0.05), which was also significant compared to the normal control. The intervention groups had varying degrees of successive decreases in weight. Although all treatment groups recorded significant reductions in weight (p<0.05) at the end of study, the effect of the n-BF was the most remarkable. Not only did the n-BF show the highest ability to lower glucose levels, which was dose-dependent (51.2% and 62.0% at 500 mg/kg and 1000 mg/kg, respectively), the effect of this fraction was the closest to that of metformin (63.6%) after 14 days of treatment (p<0.05). Therefore, after 14 days of repeated oral treatment, the n-BF was shown to be the probable fraction with the highest composition of active glucose lowering compounds as in acute antihyperglycaemic experiments.

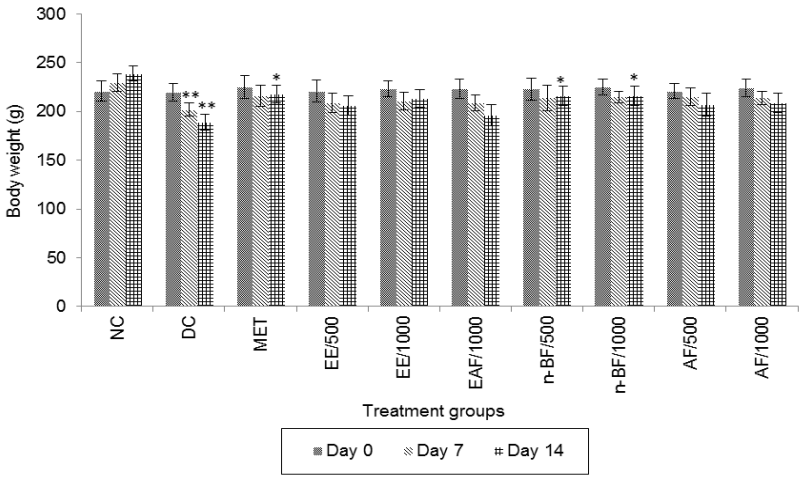

Figure 5: The effect of 14-day oral administration of 25% EE of GP and its fractions on body weight in SDRs. Values are expressed as the mean±SE (n = 6); **p*<0.05 vs. diabetic control; ***p*<0.05 vs. normal control.
Note: NC – normal control; DC – diabetic control; MET – metformin

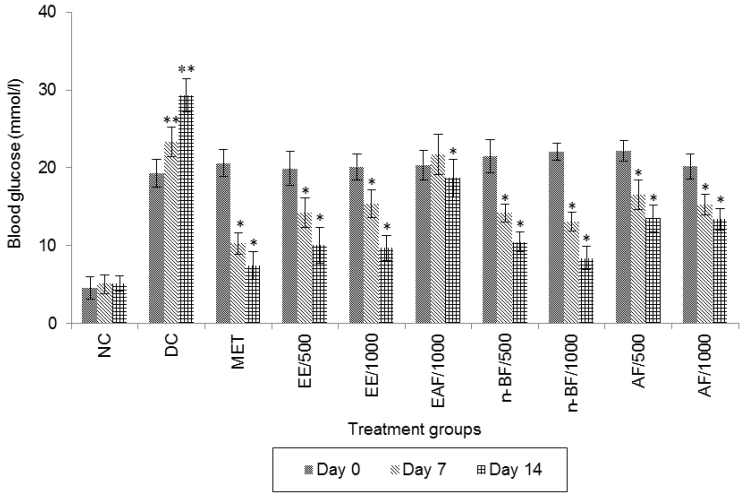

Figure 6: The effect of 14-day oral administration of 25% EE of GP and its fractions on blood glucose level in SDRs. Data are expressed as the mean±SE (n = 6); **p*<0.05 vs. diabetic control; ***p*<0.05 vs. normal control.
Note: NC – normal control; DC – diabetic control; MET – metformin

Total Flavonoid Content

Figure 7 shows the total flavonoid content of the fractions obtained from the 25% EE of GP. Flavonoids were present in all fractions studied. However, the total

flavonoid content of the n-BF was found to be 62.90% and 79.68% higher than that of the EAF and AF, respectively.

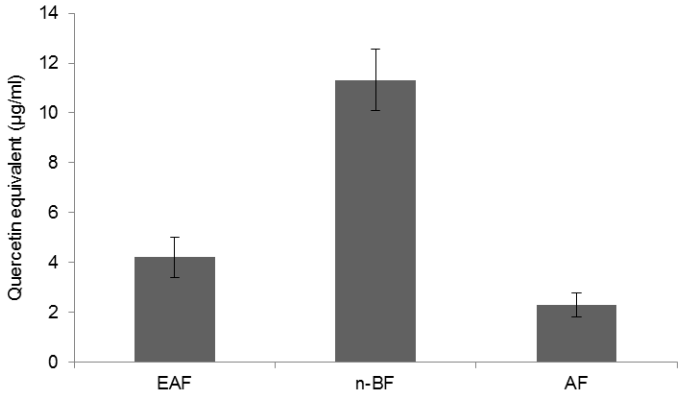

Figure 7: Total flavonoids, measured as quercetin equivalents (µg/ml), in fractions prepared from 25% EE fractions of GP. Values are presented as the mean±SE; n = 3 replicates.

Total Phenolic Content

The composition of phenolics determined in the fractions obtained from the 25% EE of GP is shown in Figure 8. The results indicate that phenolics are present in all three fractions. As in the case with flavonoids, it was also found that the phenolic content of the n-butanol fraction was 61.22% and 49.33% higher than that of the EAF and AF, respectively.

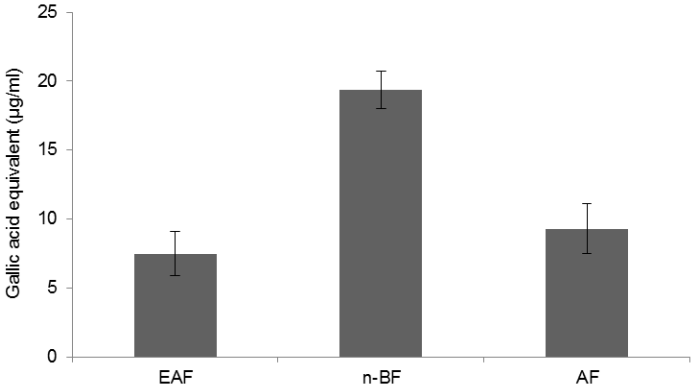

Figure 8: Total phenolics measured as gallic acid equivalents (µg/ml) in fractions prepared from 25% EE and fractions of GP. Values are presented as the mean±SE; n = 3 replicates.

DISCUSSION

Until a radical cure is found for diabetes mellitus, exploring botanical flora to identify potentially new and active agents to treat diabetes is necessary. From empirical data, it was found that a plant in the Malaysian ecosystem, GP, is likely to contain active antidiabetic agents. Therefore, in a preliminary study, we conducted a hypoglycaemic and antihyperglycaemic activity screening of sequential ethanol-water ratio extracts from this plant (Algariri *et al*. 2013). It was found that the 1:3 ethanol-water (25% ethanol) extract recorded the highest effect on glucose reduction both in a tolerance test and in diabetic animal models.

Although there are previous reports on the safety of the methanol (Rosidah *et al*. 2009) and the 95% EEs (Hayouni *et al*. 2007) from GP, it is possible that the present active extract is not safe. Assuming all extracts are non-toxic can be fatal because the variability in solvents largely determines the chemical composition (Falleh *et al*. 2012; Sultana *et al*. 2009) which in turn affects the biological activity and toxicity (Trabelsi *et al*. 2010), of extracts from medicinal plants.

Female rats were chosen for this study because female animals are generally more sensitive than males (OECD 2008a). In an acute toxicity test, the maximum dose of the extract (2000 mg/kg) did not cause any treatment-related mortality throughout the 14 days of observation. Animal growth rate, hepatic, nephritic and haematopoietic function indices were also not adversely affected, suggesting that the extract is safe with no observed acute toxicity because the LD_{50} for the female rats is greater than 2000 mg/kg. This observation is consistent with the reports by Mahmood *et al.* (2010) and Rosidah *et al*. (2009) although both studies used different extraction solvents and a limit dose of 5000 mg/kg.

To assess the possible cumulative effects of repeated exposure to GP, the extract was administered in three dose levels for 28 days (sub-chronic) and similar parameters as the acute study were analysed as recommended by the revised OECD 407 guidelines. In the current study, all of the rats were alive until the end of the experiment and did not show any symptoms of sickness compared to the normal control rats. The weights of both treated and control rats gradually increased with no significant difference between the groups, suggesting that the extract did not impair growth. Changes in body weight and internal organs are, in general, sensitive and indicative markers of toxicity following exposure to potentially toxic substances (Babayi *et al.* 2007).

Plant extracts are typical examples of xenobiotics that are biotransformed and prepared for excretion by the liver organ. Therefore, it is important to evaluate biomarkers of liver function to assess the toxicity (Burke 2002) of medicinal plant extracts. The activities of the transaminases, particularly the ALT that is a specific marker for liver injury (Thapa & Walia 2007), and ALP were similar to the control rats following a 28-day administration of the 25% EE of GP. Other liver function and reaction indices analysed were equally not affected by the extract, implying that the extract was non-toxic to the liver and did not cause any necrosis, cirrhosis or cholestasis.

The primary function of the kidneys is the elimination of metabolic waste products from endogenous and exogenous substances (xenobiotics), including plant extracts. In the present study, 28-day administration of GP extract did not cause any observable toxic changes in the measured kidney profile indices, including electrolytes, creatinine, uric acid and urea. On the basis of our observation, GP extract is considered non-nephrotoxic.

The haematopoietic and/or immunological system is also one of the most sensitive target tissues for toxic compounds and is therefore an important index of physiological and pathological status in both humans and animals (Almanca *et al*. 2011). In the haematological analysis, there were no differences between the extract treated and control rats indicating that a sub-chronic administration of GP extract was unable to elicit any toxic effects on the haematopoietic system. Although the presence of saponins, erythrocyte lysing detergents (Chunlaratthanaphorn *et al*. 2007), was found in the leaves of GP, erythrocyte counts were not adversely affected possibly due to very low concentration of the saponins or masking of the deleterious action by other bioactive compounds. It is evident from both acute and sub-chronic experiments that the active antihyperglycaemic extract of GP (25% EEs) was found to be safe when administered orally.

Taken together, the toxicity results are consistent with those of a previous study by Rosidah *et al.* (2009), which reported that administration of the methanol extract from GP leaves at 125, 250 and 500 mg\kg\day for 13 weeks did not produce any toxic changes in general conditions of growth, organ weights, haematological and biochemical parameters. This implies that the differences in extraction solvent did not significantly impact the safety profile of GP leaves.

To further characterise the active antidiabetic compounds in GP, the 25% active extract was further fractionated via liquid-liquid solvent extraction, and the fractions (ethyl acetate, n-butanol and aqueous) were subsequently tested for antihyperglycaemic activity in acute and sub-chronic conditions. The 7 hour effect of a single dose (500 mg/kg or 1000 mg/kg or 2000 mg/kg) of these fractions on blood glucose in SDRs was evaluated. The results indicated that all fractions tested displayed antihyperglycaemic effects under acute conditions. However, the effect of the n-BF was more consistent at the three doses tested, and thus was considered the fraction with the highest composition of effective antihyperglycaemic bioactives. The profound action of the n-BF was also observed in the 14-day antihyperglycaemic study. Not only did the n-BF show highest glucose-lowering action that was dose-dependent in the sub-chronic experiment, the effect of the fraction was the closest to that of metformin. Therefore, the data suggests the n-BF as the fraction with the highest composition of active glucose-lowering compounds. However, our observation slightly disagrees with a previous study by Chong *et al*. (2012), which indicated that the EAF was the most effective in lowering glucose (60.1%) compared to the n-hexane (29.7%) and n-butanol (33.5%) fractions. This difference could largely be a function of the variation in extraction procedures employed in the two studies. The present study used 25% ethanol for the crude extract preparation and employed heat (45°C) in the maceration process compared to the 95%

ethanol used at room temperature by Chong *et al*. (2012). Moreover, the order of the fractionation was also inconsistent with the present study.

Selective chemical evaluation of the three fractions was then performed to identify the predominant bioactive compounds, and this evaluation found the presence of phenolic compounds and flavonoids in all three fractions. This finding is in agreement with our previous reports that showed that the flavonoids and phenolic compounds were the predominant compounds identified in ethanol-water extracts of GP (Algariri *et al*. 2013). However, in the present study, the total phenolic and flavonoid composition of the n-BF was found to exceed that of the EAF and AF, which was consistent with the observed antihyperglycaemic activity. The antidiabetic action of several medicinal plants has been attributed to the phenolic and flavonoids contents (Ali *et al*. 2012). The high composition of phenols and flavonoids in the n-BF of GP may also be responsible for it having the highest glucose responsive action.

CONCLUSION

In summary, the 25% EEs of GP were found to be safe in oral acute and sub-chronic toxicity evaluations performed in male and female Sprague Dawley rats, with the LD_{50} exceeding higher than 2000 mg/kg. GP is therefore considered a NOAEL drug with an ADI of 700 mg/kg/day and a safety factor of 100. The n-BF of GP exerted the highest antihyperglycaemic activity, in proportion to the highest phenol and flavonoid compositions. Therefore, the n-BF of GP is a promising potential source for antidiabetic natural products or standardised dosage forms.

ACKNOWLEDGEMENT

The authors sincerely thank the Academy of Science for the Developing World (TWAS) and USM for the joint TWAS-USM fellowship offer to Dr. Item J. Atangwho, which contributed immensely to the completion of this research work and its eventual publication. The research work was funded by a grant from the Ministry of Agriculture and Agro-based Industries, Malaysia; grant number: 304/PFARMASI/650581/K123.

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