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Hypoglycemic and anti-hyperglycemic study of *Gynura procumbens* leaf extracts

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PEER REVIEW

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Comments

The work is of high quality. Sufficient data has been generated on a time line basis which has allowed relevant metabolic changes of the diabetic state to be monitored. Structural activity relationship based on different phytochemical ingredient solubilised by the different ethanol-water solvent system in relation to their anti-diabetic effects is relevant in drug development.
(Details on Page 365)

ABSTRACT

Objective: To study the antidiabetic activity of *Gynura procumbens* (*G. procumbens*) used in the traditional management of diabetes in Southern Asia. **Methods:** *G. procumbens* leaves were extracted sequentially with graded percentage of ethanol in water (95%, 75%, 50%, 25% and 0%), and the extracts were tested for antidiabetic activity using acute (7 h), subcutaneous glucose tolerance test and sub-chronic (14 d) test in non-diabetic and streptozotocin-induced diabetic rats. The extracts were further subjected to phytochemical studies. **Results:** In acute dose (1 g/kg), the extracts significantly lowered fasting blood glucose (FBG) in streptozotocin-induced diabetic rats ($P<0.05$). However, the FBG-lowering effect of the 25% extract compared to the other extracts, was rapid (47% after 2 h) and the highest: 53%, 53% and 60% in the 3rd, 5th, and 7th h, respectively ($P<0.05$), comparable only to the effect of metformin. Furthermore, the extracts suppressed peak FBG in subcutaneous glucose tolerance test, but only the 0% and 25% extracts, and metformin sustained the decrease until the 90th min ($P<0.05$). Moreover, in the 14 days study, the 25% extract exerted the highest FBG-lowering effect, namely 49.38% and 65.43% on days 7 and 14, respectively ($P<0.05$), similar to the effect of metformin (46.26% and 65.42%). Total flavanoid and phenolic contents in the extracts were found to decrease with increase in polarity of extraction solvents. The composition of reference compounds (chlorogenic acid, rutin, astragalin and kaempferol-3-O-rutinoside) followed a similar trend. **Conclusions:** *G. procumbens* contains antidiabetic principles, most extracted in 25% ethanol. Interaction among active components appears to determine the antidiabetic efficacy, achieved likely by a metformin-like mechanism.

KEYWORDS

Antidiabetic, *Gynura procumbens*, Fasting blood glucose, Subcutaneous glucose tolerance test, Streptozotocin-induced diabetes, Flavanoids, Phenolics

1. Introduction

Gynura procumbens (Lour) Merr (*G. procumbens*), a composite known locally in Malaysia as Sambung Nyawa, is an annual evergreen shrub that grows extensively in Southeast Asia, particularly in Indonesia, Malaysia, and Thailand, where it is traditionally used for treatment of eruptive fevers, rash, kidney disease, migraines, constipation, hypertension, diabetes mellitus, and cancer[1]. Some of these traditional claims have been validated in scientific and pharmacological studies, including, anti-herpes simplex virus, anti-inflammatory, and anti-hyperlipidemic and anti-hypertensive activities[2].

G. procumbens has recently received particular attention in the pharmacology of antidiabetic medicinal plants, probably because of its avowed empirical evidence and efficacy in the traditional management of diabetes mellitus. However, the scientific reports on the antidiabetic activity

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of this plant have been conflicting and inconsistent. For instance, Zhang and Tan had reported that 95% ethanol extract improved glucose tolerance in STZ-induced diabetic rats, but not in normal rats[3]. Its aqueous extract was also reported by these authors to exert significant anti hyperglycemic action in STZ-induced diabetic rats. Later on, Akowuah *et al.* on the contrary indicated its glucose lowering effect in normal rats[4]. In the most recent study, the extract of *G. procumbens* was reported to produce significant elevation in the fasting blood glucose (FBG) levels of normal rats, but a decrease in diabetic rats[5]. There is a basic need to stream line these reports, given the widespread traditional use of *G. procumbens*.

Moreover, these study designs are not targeted at natural product discovery or production of standardized herbal forms. Adequate research on medicinal plants beyond screening for biological activity should be conducted with the aim to systematically standardize and develop them into natural products or dosage forms which should effectively complement or supplement existing conventional measures[6].

Consequently, the present investigation using an ethnomedical drug discovery program, evaluated the antidiabetic activity of *G. procumbens* used in the traditional health system of the Southeast Asia, as an effective remedy and management for diabetes mellitus and other ailments. This systematic screening is a fundamental requirement for natural product exploration and development of therapeutic agents from medicinal plants.

2. Materials and methods

2.1. Plant material

Fresh leaves of *G. procumbens* collected from Herbagus Sdn Bhd, Kepala Batas, Penang, 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, then dried in an oven at 45 °C and milled into powder (1 200 g).

2.2. Preparation of plant extracts

The powdered plant material (1 200 g) was first extracted via maceration (45 °C) in 2 L of 95% ethanol, with solvent replenished every 6 h for 3 d. These were pooled together, and then filtered using Whatman No.1 filter paper. The filtrate was concentrated in vacuo in a rotary evaporator (Buchi Labortechnik AG, Switzerland) at 60 °C to about 10% of original volume and thereafter freeze-dried (Lebconco Corporation, Missouri USA) yielding 5 g (0.42%) of dried 95% ethanol extract. The residue of the plant material from the above was dried and re-extracted with 75% ethanol using the same procedure as for 95% (v/v) ethanol, then repeated for 50%, 25% and 0% ethanol (100% water). The respective yields for these subsequent extracts were 8.0 g (0.67%), 17.3 g (1.44%), 25.3 g (2.12%), and 30.1 g (2.51%). The solvents used for the extraction were prepared according to ratios shown in Table 1.

Table 1
Graded ratios of ethanol to water used in preparation of the different extracts.

Stock solvents (v/v)	Ethanol 95% (mL)	Water (mL)
95%	1 000	10
75%	789	211
50%	526	474
25%	263	737
0%	0	1 000

2.3. Experimental animal

Sprague Dawley rats (200-250 g) obtained from the Animal Research and Service Centre, Universiti Sains Malaysia (USM) were used in this study. The rats were acclimatized for a period of 7 d 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.) and tap water *ad libitium*. Temperature of facility was (22±3) °C and light/darkness alternated 12 h apart. The experimental procedures were approved by the Animal Ethics Committee, Universiti Sains Malaysia Penang, Malaysia and the National Institutes of Health Principles of Laboratory Animal Care (1985) were observed.

2.4. Experimental protocol

Diabetes were induced in rats by intraperitoneal injection of 55 mg/kg of streptozotocin (STZ, Sigma Aldrich Chemical Co., USA) reconstituted in 0.1 mol/L cold citrate buffer (pH 4.5) after an overnight fast. After 72 h of STZ administration, blood glucose level was measured in blood collected from tail vein puncture using Accu-check Advantage II clinical glucose meter (Roche Diagnostics Co., USA). Rats with FBG≥15 mmol/L (270 mg/dL) were considered diabetic and included in the study.

The percentage change in blood glucose was calculated thus:

Percentage of clycaemic change=(Gx- Gi)/Gx×100

where Gx is the glycaemia at time x and Gi is the glycaemia at the initial time (i). Prior to diabetes induction, an optimum STZ dose selection study was carried out to determine the appropriate dose that will produce the needed chronic hyperglycemia, but with moderate mortality. To 6 groups (n=4) of overnight fasted rats (200-250 g), varying doses of STZ (65, 60, 55, 50, 45 and 40 mg/kg) reconstituted in freshly prepared buffer (0.1 mol/L cold citrate buffer of pH 4.5) were administered intra-peritoneal. These rats were monitored for

12 d for mortality, and the blood glucose level was measured on the first and last days. Clinical features of diabetes including polyurea, polyphagia, polydipsia and glycosuria were also observed.

2.5. Acute/single dose glucose response test in normal rats

In this test, 42 normal rats were randomly categorized into seven groups (n=6). After an over night fast, Group 1 and 2 which consisted the normal and positive controls were respectively treated with 1% carboxymethyl cellulose (vehicle) and metformin (500 mg/kg body weight). Group 3-7 accordingly received single oral doses (1 g/kg) of 95%, 75%, 50%, 25% and 0% ethanol extracts of *G. procumbens* respectively. Blood was collected from tail vein before (0 min) and at 1 h, 2 h, 3 h, 5 h and 7 h post treatment for glucose measurement.

2.6. Acute/single dose glucose response test in STZ-induced diabetic rats

The procedure for this test was same as the above, except that STZ-induced diabetic rats were used in place of normal rats for Group 2-7.

2.7. Subcutaneous glucose tolerance test (SGTT) in normal rats

Forty-two rats were divided into seven groups with six rats per group. After an overnight fast (but with free access to water), five groups were respectively treated with 1 g/kg body weight of 95%, 75%, 50%, 25% and 0% ethanol extracts of *G. procumbens*. Group 1 and 2 were served as the normal and positive controls respectively and received equivalent volume of vehicle (1% carboxymethylcelulose) and metformin (500 mg/kg body weight). Fifteen minutes after oral treatment, 50 mg/kg glucose was administered subcutaneous to all the rats, and glucose was measured in blood samples obtained via tail vein puncture at 15 min (just before the extract was administered) and at 15, 30, 45, 60, 90 and 120 min post glucose load.

2.8. SGTT in STZ-induced diabetic rats

In this test, the procedure including animal grouping, doses of extracts used, duration and glucose measurement and glucose loading was as in the section above, except that animal Group 2-7 rather consisted of STZ-induced diabetic rats.

2.9. Fourteen days consecutive oral administration of extracts of G. procumbens in STZ-induced diabetic rats

Five normal and 35 STZ-induced diabetic rats were assigned to eight groups of five rats each and treated consecutively for 14 d according to the scheme shown below. Group 1 was normal rats, received 1% carboxymethylcelulose (normal control). Group 2 was diabetic rats, received 1% carboxymethylcelulose (diabetic control). Group 3 was diabetic rats, received 500 mg/kg of metformin (positive control). Group 4 was diabetic rats, received 1 g/kg of 95% ethanol extract of *G. procumbens*. Group 5 was diabetic rats, received 1 g/kg of 75% ethanol extract of *G. procumbens*. Group 6 was diabetic rats received 1 g/kg of 50% ethanol extract of *G. procumbens*. Group 7 was diabetic rats received 1 g/kg of 25% ethanol extract of *G. procumbens*. Group 8 was diabetic rats received 1 g/kg of 0% ethanol extract of *G. procumbens*.

FBG and body weight of the rats were measure at outset of the experiment (baseline fasting blood glucose), day 7 and at the end of study (day 14).

2.10. Phytochemical analysis of the crude G. procumbens extracts

2.10.1. Determination of total phenolics

Total phenolic content of three 95% ethanol extracts prepared from different extraction methods (soxhlet, maceration and ultrasonication) and various ethanol-aqueous (75%, 50%, 25% and 0%) extracts of *G. procumbens* leaves was determined using Folin-Ciocalteu reagent method. Briefly, 0.4 mL (1 mg/mL) of each extract was pipetted into test tubes, and 2 mL (10%, v/v) of Folin-Ciocalteu reagent was added into the extract sample. Five minutes later 1.6 mL (7.5%) of sodium carbonate solution was added into the sample, then the sample mixture was incubated for 1 h at room temperature and the absorbance measured using Perkins Elmer UV-Visible spectrometer (USA) at 760 nm. A series of standard gallic acid solutions (20-200 μg/mL) were prepared and absorbance measured at same wavelength and data used to plot the calibration curve. The total phenolic content was calculated as μg/mL of gallic acid equivalent of extracts[7]. All samples were analyzed in triplicates.

2.10.2. Determination of total flavonoids

Total flavonoids of three 95% ethanol extracts prepared from different extraction methods (soxhlet, maceration and ultrasonication) and various ethanol aqueous extracts (75%, 50%, 25% and 0%) of leaves of *G. procumbens* was determined using aluminium chloride colorimetric method adapted from the procedure reported by Gursoy *et al*[8]. Briefly, 1.5 mL of extract solution in the test tube was mixed with 1.5 mL of 2% aluminium chloride solution prepared in methanol. The absorbance was measured at 415 nm after 10 min of incubation at room temperature using a double beam Perkins Elmer UV-Visible spectrophotometer (USA). The flavonoid content of the extracts was calculated in μg/mL as quercetin equivalent by using the equation obtained from the quercetin calibration curve. The calibration curve was constructed

using six different concentrations (3.125-100.000 μg/mL) of quercetin solution prepared in methanol. All samples were analyzed in triplicates.

2.10.3. TLC profiling of the crude extracts of G. procumbens

TLC analysis was carried out on a 10×20 silica gel F254 TLC plate (Merck, Germany) for qualitative identification of some standard compounds earlier isolated from leaves of *G. procumbens*. Approximately 10 μL of each extract sample (95%, 75%, 50%, 25% and 0% ethanol-water extracts) (0.5 mg/mL) each was applied on the TLC plate along with reference standards, chlorogenic acid, rutin, astragalin and kaempferol-3-O-rutinoside. These were applied as spots on the TLC plate 1 cm from the bottom of the plate and then developed in a 24 cm×24 cm TLC chamber saturated with the developing solvent system namely, ethyl acetate: methanol: water (100:13.5:10). The chromatogram was developed separately, using natural product reagent spray and viewed under UV light at 254 nm and 365 nm.

2.11. Statistical analysis

The results were expressed as the mean±SEM and analyzed using One-way analysis of variance (ANOVA). A difference in the mean at $P<0.05$ was considered statistically significant. The SPSS software version 15.0 subscribed by Universiti Sains Malaysia, was used for the analysis.

3. Results

3.1. Optimum dose of STZ for induction of experimental diabetes

Table 2 shows a 12-day variation in blood glucose of rats administered a single dose each of graded concentrations of STZ intra-peritoneal. It was observed that the number of deaths increased with the concentration of STZ, and this correlated with the extent of increase in blood glucose. The doses 65 and 60 mg/kg respectively increased the blood glucose by 86.25% and 85.52% after 6 d, an elevation higher than the upper maximum ethically recommended (25 mmol/L) for diabetic models at outset of experiment. On other hand, doses 45 and 40 mg/kg failed to sustain the hyperglycemia within the defined minimum cut off of 11.1 mmol/L for at least 12 d. These criteria were met by administration of 50 and 55 mg/kg of STZ, with yet minimal number of deaths. Hence, 55 mg/kg was selected as the optimum dose for preparation of the diabetic models used in this study.

Table 2
Effect of single intra-peritoneal injection of graded doses of STZ on blood glucose level (n=4).

Dose of STZ (mg/kg)	Blood glucose (mmol/L)			Number of deaths
	Day 1	Day 6	Day 12	
65	4.5±1.2	32.1±1.9[a]	–	4
60	4.2±1.8	29.0±1.3[a]	–	4
55	4.7±0.8	21.0±2.1[a]	29.3±2.1[a]	1
50	3.9±0.5	18.3±2.0[a]	29.1±1.3[a]	1
45	4.3±1.1	12.3±0.3[a]	10.3±2.1[a]	0
40	4.7±0.7	11.4±1.1[a]	9.8±1.4[a]	0

a: $P<0.05$ *vs.* Day 1.

3.2. Effect of acute/single oral administration of extracts/metformin on normal

As indicated in Table 3, administration of 1 g/kg each of 95%, 75%, 50%, 25% and 0% extracts of *G. procumbens* caused 30%, 29%, 17%, 23% and 10% reductions in blood glucose level respectively, 7 h after treatment. These decrease, however, are not significant both compared to normal control and metformin treatment (22%). Hence, the treatments in this test did not significantly impact blood glucose in normal rats, at the dose administered and within the observed duration.

In diabetic rats, the effects of the extracts were more striking (Table 4). Compared to the diabetic control, the 95% ethanol extract showed a significant 31% reduction ($P<0.05$) in blood glucose level after 3 h and this decline was sustained until the end of 7 h (51%). The 75% and 50% ethanol extracts also achieved a respective 40% and 34% reductions in blood glucose level, but only after 5 h ($P<0.05$). This decline was also sustained till end of study ($P<0.05$) compared to the diabetic control. The effect of the 25% ethanol extract was very peculiar. Compared to the diabetic control, the extract caused a 47% significant decline in blood glucose, just 2 h after oral administration and sustained this extent of reduction in the 3rd (53%), 5th (53%) and 7th (60%) hours ($P<0.05$). The effect of the 25% extract on blood glucose was also the closest to the effect of the standard drug, metformin, of the five treatments considered in this study. On this basis, the 25% extract was considered the most effective.

Table 3
Effect of a single/acute dose of extracts/metformin on blood glucose of non diabetic rats (n=6).

Treatment groups	Baseline FBG	1 h	2 h	3 h	5 h	7 h
Control	4.0±0.5	4.2±0.4	4.1±0.6	3.6±0.4	4.1±0.4	4.1±0.5
Extract (95%)	4.7±0.8	4.3±0.9	3.8±0.4	3.9±0.5	3.4±0.6 (28%)	3.3±0.5 (30%)
Extract (75%)	4.7±1.0	4.3±0.4	3.8±0.5	3.9±0.2	3.4±0.3	3.3±0.3 (29%)
Extract (50%)	4.2±0.6	4.5±0.5	4.0±0.4	3.7±0.7	3.7±0.6	3.5±0.5 (17%)
Extract (25%)	4.8±0.9	4.8±0.6	4.2±0.6	4.2±0.7	3.8±0.7	3.7±0.6 (23%)
Extract (0%)	4.0±0.5	4.5±0.6	4.2±0.3	3.6±0.2	3.5±0.2 (13%)	3.6±0.2 (10%)
Metformin	4.5±0.5	3.9±0.7	4.1±0.6	3.6±0.4	3.4±0.5 (24%)	3.5±0.3 (22%)

FBG: values in parentheses represent % change in FBG w.r.t baseline.

3.3. Effect of extracts/metformin on subcutaneous glucose tolerance

About 50 mg/kg glucose was selected from a preliminary dose response study as the optimum dose which produced reversible peak blood glucose 25 min after subcutaneous administration in normal rats (data not shown). Table 5 shows the effect of extracts on blood glucose of non diabetic rats after a subcutaneous glucose load. Compared to the control, metformin and the extracts (except the 95% extract) significantly suppressed the peak blood glucose after 15 min (P<0.05). However, only 0% and 25% extracts along with metformin sustained the significant decrease until the 90th min.

Table 6 shows the effect of extracts of leaves of *G. procumbens* and metformin on SGTT. The 95%, 25% and 0% extracts exerted significant effects on blood glucose starting from the 15th min until 120th min (P<0.05), whereas the 50% ethanol extract did not show any significant effects throughout the study period. Metformin, the positive control, demonstrated a significant effect in all time points, with a higher reduction in glucose level than that of the extracts (P<0.05).

3.4. Effect of extracts/metformin on body weight and blood glucose of STZ-induced diabetic rats

The result of changes in body weight of control and experimental rats treated with extracts and metformin are shown in Table 7. Whereas STZ injection caused significant loss in body weight (16%) after 14 d, compared to normal control, treatment with extracts of *G. procumbens* significantly improved the weight loss within the period (P<0.05). Also, daily administration of the extracts (1 g/kg) for 14 d caused significant reduction (P<0.05) in blood glucose level when compared to diabetic control (Table 8). Although all the test extracts in this study caused significant reductions in blood glucose at days 7 and 14, the extent of reduction by the 25% extract (49.38% and 65.43% for 7 and 14 d, respectively) was highest, and is the most correspondingly to the effect of metformin (46.26% and 65.42%). Hence the 25% extract is

Table 4
Effect of a single/acute dose of extracts/metformin on blood glucose of STZ-induced diabetic rats (n=6).

Treatment groups	Diabetic rats					
	Baseline FBG	1 h	2 h	3 h	5 h	7 h
Control	22.0±0.9	20.3±1.5	23.8±2.7	25.4±3.6	23.7±2.6	22.9±2.9
Extract (95%)	25.7±3.0	18.7±2.8	18.8±2.0	17.8±1.5[a] (31%)	16.3±1.4[a] (36%)	12.6±1.2 (51%)
Extract (75%)	26.4±1.3	21.3±1.3	18.5±1.0[a]	19.2±1.0[a] (27%)	15.7±1.0[a] (40%)	16.7±1.4 (37%)
Extract (50%)	23.2±3.0	18.3±2.1	19.2±2.0	18.3±2.0[a] (22%)	15.3±1.6[a] (34%)	15.7±1.8 (32%)
Extract (25%)	27.3±2.0	18.0±3.9	14.4±2.6[a] (47%)	12.8±2.6[a] (53%)	12.9±3.6[a] (53%)	10.9±2.4 (60%)
Extract (0%)	23.2±2.6	19.9±2.4	18.9±1.0[a]	16.5±1.8[a] (29%)	17.7±1.1[a] (24%)	16.7±0.8 (28%)
Metformin	21.5±1.6	18.8±1.0	13.5±1.2[a] (28%)	11.4±1.5[a] (47%)	9.8±0.4[a] (54%)	7.8±0.1 (64%)

a: P<0.05 *vs.* control; FBG: values in parentheses represent % change in FBG w.r.t baseline.

Table 5
Effect of the extracts/metformin on blood glucose level after subcutaneous loading of 50 mg/kg glucose in non diabetic rats (n=6).

Treatment groups	0 min	15 min	30 min	45 min	60 min	90 min	120 min
Control	5.26±0.10	6.08±0.21	5.92±0.16	5.82±0.14	5.36±0.16	4.76±0.12	4.90±0.02
Metformin	4.26±0.12	4.40±0.19[a]	4.94±0.39[a]	4.66±0.40[a]	3.96±0.32[a]	4.00±0.17[a]	4.14±0.22[a]
Extract (0%)	4.20±0.12	4.40±0.35[a]	4.60±0.59	4.80±0.39[a]	5.12±0.36	4.10±0.25[a]	3.70±0.25[a]
Extract (25%)	3.78±0.13	4.88±0.12[a]	4.77±0.39[a]	4.47±0.36[a]	4.87±0.36[a]	3.48±0.33[a]	4.63±0.40
Extract (50%)	4.30±0.38	5.20±0.21[a]	5.85±0.19	5.28±0.23	4.98±0.19	4.38±0.09	4.54±0.08
Extract (75%)	4.33±0.18	4.60±0.31[a]	4.97±0.35[a]	4.84±0.29	4.84±0.41[a]	4.17±0.13	4.31±0.21
Extract (95%)	4.36±0.13	5.30±0.15	6.34±0.36	5.24±0.14	4.94±0.29	4.50±0.22	4.38±0.24

a: P<0.05 *vs.* control.

Table 6
Effect of the extracts/metformin on blood glucose level after subcutaneous glucose load in STZ-induced diabetic rats (n=6).

Treatment groups	0 min	15 min	30 min	45 min	60 min	90 min	120 min
Control	21.43±1.60	23.62±1.70	23.63±1.10	22.75±0.71	23.07±0.84	22.57±0.86	21.37±0.45
Metformin	20.77±1.77	18.50±1.10[a]	17.78±1.10[a]	18.33±1.40[a]	17.40±1.30[a]	16.98±1.80[a]	16.97±2.30[a]
Extract (0%)	18.56±0.45	20.04±0.40[a]	18.98±0.55[a]	19.24±0.92[a]	20.22±1.00[a]	19.16±1.60[a]	18.30±1.30[a]
Extract (25%)	20.57±0.86	19.67±0.76[a]	20.63±0.54[a]	20.85±0.72	22.62±1.10	19.32±0.84[a]	19.30±0.72
Extract (50%)	19.68±1.30	20.43±1.20	20.27±0.70	19.35±0.94	20.62±1.30	20.23±1.20	19.87±1.70
Extract (75%)	20.23±1.70	21.03±1.70	21.35±1.60	21.80±1.40	22.32±1.70	19.80±0.88[a]	20.08±1.20
Extract (95%)	18.46±1.57	20.06±0.70[a]	19.94±1.10[a]	18.50±1.50[a]	19.50±1.30[a]	19.44±1.20[a]	19.58±1.20[a]

a: P<0.05 *vs.* control.

also considered the most effective in anti-hyperglycemic effect.

Table 7
Effect of oral administration of extracts (1 g/kg) or metformin (500 mg/kg) on body weight (g) of STZ-induced diabetic rats (n=6).

Treatment groups	Day 0	Day 7	Day 14
Normal control	210±9	221±8	231±10 (+10%)
Diabetic control	220±7	205±9	182±8[b] (−16%)
Extract (95%)	219±12	209±8	203±6[a] (−7%)
Extract (75%)	220±10	210±10	203±10[a] (−7%)
Extract (50%)	218±12	207±9	210±8[a] (−7%)
Extract (25%)	20±14	210±7	213±6[a] (−5%)
Extract (0%)	223±14	212±11	205±9[a] (−8%)
Metformin	223±11	215±8	216±8[a] (−4%)

a: P<0.05 *vs.* diabetic control, b: P<0.05 *vs.* normal control; values in parentheses represent % change in blood glucose w.r.t day 0; weight gain (+), weight decrease (−).

Table 8
Effect of daily oral administration of extracts (1 g/kg) or metformin (500 mg/kg) on blood glucose level of STZ-induced diabetic rats (n=6).

Treatment groups	Day 0	Day 7	Day 14
Normal control	4.6±0.3	5.2±0.3 (+11.54%)	5.1±0.7 (+9.80%)
Diabetic control	20.4±2.1	25.3±1.6 (+19.37%)	28.4±2.1 (+28.17%)
Extract (95%)	23.9±1.3	16.9±1.4[a] (−29.27%)	13.1±2.1[a] (−45.17%)
Extract (75%)	22.5±1.4	17.5±2.1[a] (−22.22%)	13.2±1.5[a] (−41.33%)
Extract (50%)	22.9±2.3	13.2±2.1[a] (−42.36%)	10.5±1.2[a] (−54.15%)
Extract (25%)	24.3±1.8	12.3±1.4[a] (−49.38%)	8.4±1.5[a] (−65.43%)
Extract (0%)	23.3±1.3	14.5±2.4[a] (−37.77%)	12.7±1.2[a] (−45.49%)
Metformin	21.4±2.1	11.5±1.9[a] (−46.26%)	7.4±1.1[a] (−65.42%)

a: P<0.05; values in parentheses represent % change in blood glucose w.r.t day 0; +: increase; −: decrease.

3.7. Total phenolics in extracts of G. procumbens

The result of total phenolic contents of 95% extracts prepared by different methods and extracts prepared in graded ethanol-water ratio by maceration is shown in Figure 1. The 95% soxhlet extract contained lesser phenolic compounds (35.11±0.77) μg/mL gallic acid equivalent compared to extracts prepared by maceration and ultrasonication (50.58±0.32) and (49.52±0.31) μg/mL gallic acid equivalent, respectively. The phenolic content in graded ethanol-aqueous extracts was also found to vary. The 50% ethanol extract contained the highest total phenolic compounds (76.14±0.61) μg/mL followed by 75%, 95% and 25% extracts. Phenolic compounds were found to lowest in water (0% ethanol) extract (39.28±0.18) μg/mL gallic acid equivalent.

3.8. Total flavonoids in extracts of G. procumbens

From the study, 95% ethanol extracts of *G. procumbens* obtained by the three different extraction procedures contained similar amount of flavonoids (Figure 2), indicating probably that the three extraction methods were similarly efficient in extracting flavonoids. The total flavonoid contents were however, varied among the graded ethanol-aqueous extracts. The 75% extract contained the highest amount of phenolics (26.16±0.60) μg/mL quercetin equivalent followed by 50% (16.79±0.28) μg/mL, 25% (11.57 ±0.08) μg/mL and 0% ethanol (2.53±0.25) μg/mL extracts (Figure 2). The flavonoid concentration appears to decrease with increase in water content of the extraction solvent.

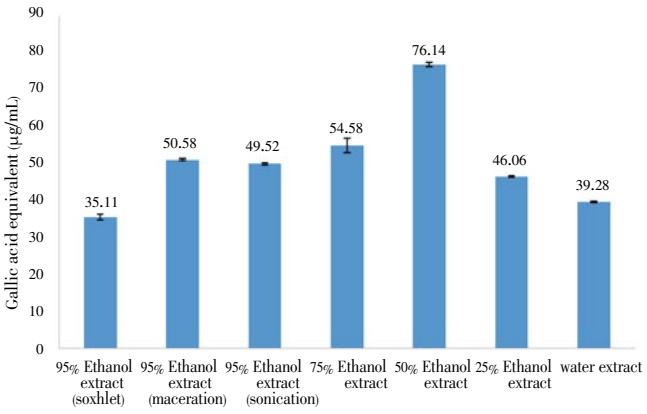

Figure 1. Total phenolic content of extracts of *G. procumbens* (n=3).

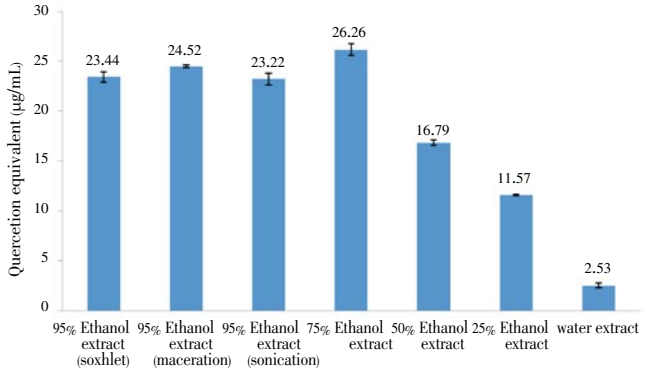

Figure 2. Total flavonoid content of extracts of *G. procumbens* (n=3).

3.9. TLC profiles of G. procumbens extracts

The TLC chromatograms of the extracts compared with marker compounds, namely, chlorogenic acid, rutin, astragalin and kaempferol-3-O-rutinoside are shown in Figure 3a and 3b. TLC profiles of the 95% extracts obtained via soxhlet, maceration and ultrasonication were similar. However, the profiles of the graded ethanol-aqueous extracts showed a gradual decrease in the concentration of marker compounds from 75% to 0% ethanol extract. The intensity of the major compounds present in the extracts also differs significantly. The intensities of astragalin and chlorogenic acid spots in particular, were more intense in 75% ethanol extract compared to 0% ethanol extract, affirming strongly, the role solvent polarity plays in determining the nature and proportion of active compounds extracted from their natural sources.

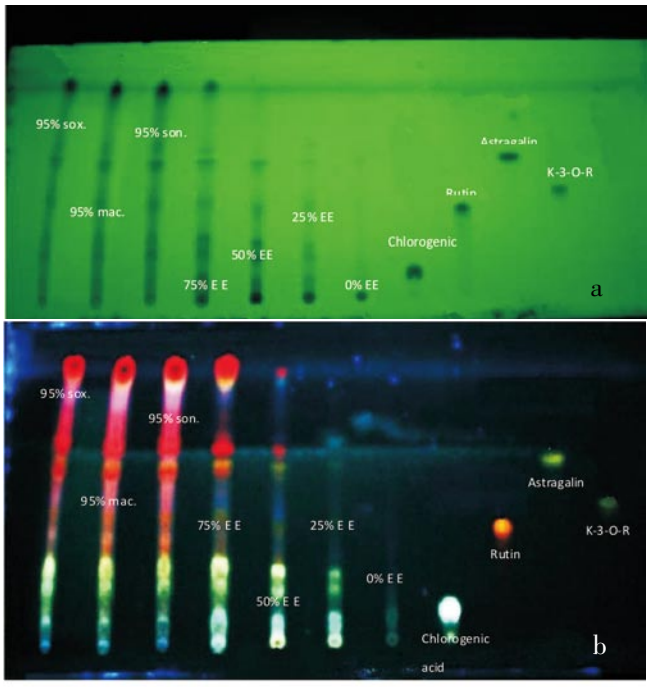

Figure 3. TLC profiles of 95% ethanol extracts of *G. procumbens* obtained via soxhlet (sox.), maceration (mac.) and sonication (son.), and graded ethanol-aqueous extracts (EE) and reference/standards [chlorogenic acid, rutin, astragalin and kaempferol-3-O-rutinoside (K-O-3-R)].
a: viewed under UV light (254 nm), b: viewed under UV light at 365 nm after spraying with natural product (NP) reagent.

4. Discussion

Streptozotocin, 2-deoxy-2-(3-(methyl-3-nitrosoureido)-D-glucopyranose, is by far the most frequently used agent (69%) in preparation of diabetic animal models for the study of multiple aspects of diabetes, and the dose required for inducing diabetes depends on the animal species, route of administration and nutritional status[9]. Consequently, in the present study a preliminary dose response effect of STZ on blood glucose (standardization) was carried out to determine the optimum dose needed to produce stable hyperglycemia in Sprague Dawley rats, as data in the literature could not be relied upon due to broad variability[10,11]. The results indicated that a single intra-peritoneal injection of 40-45, 50-55 and 60-65 mg/kg body weight of STZ could induce hyperglycemia in SD rats after 6 d, but of varying intensities. Whereas the doses 65 and 60 mg/kg increased the blood glucose by 86.25% and 85.52% after 6 d, an elevation higher than the upper maximum ethically recommended (25 mmol/L) for diabetic models at outset of experiment[12], doses 45 and 40 mg/kg failed to sustain the hyperglycemia within the defined minimum cut off of 11.1 mmol/L for 12 d[13]. These criteria were however met by administration of 50 and 55 mg/kg of STZ, with yet minimal number of deaths. Hence, 55 mg/kg was selected as the optimum dose for preparation of the diabetic models used in this study.

Using this diabetic model, serial ethanol extracts of *G. procumbens* were screened for hypoglycemic and anti-hyperglycemic effect. Whereas, the glucose lowering test in normal rats aims to evaluate tendency of an extract/drug to produce hypoglycemia (side effect of some antidiabetic drugs), the glucose lowering test in diabetic rats evaluates its antidiabetic property. In the present study, it was found that at acute dose (1 g/kg), the extracts of *G. procumbens* did not produce any significant effect on FBG, even after 7 h. This feature is desirable, hence hypoglycemia, a side effect of most oral hypoglycemic agents can cause seizures, coma accidents, and death or may even induce permanent brain damage. Hypoglycemia is indeed, more lethal than hyperglycemia.

The result of acute dose glucose response in diabetic rats indicates that all five extracts of *G. procumbens* lower FBG at least at some points within the 7 h. The 25% extract which exerted prompt reduction of FBG (only 2 h after administration) and sustained the reduction till end of study comparable to the effect of metformin, was considered the choice extract on this basis. This implies that 25% ethanol-water solvent combination may be most suitable for extraction of the active antidiabetic compounds in *G. procumbens*. It is plausible that both short- and long-acting anti-hyperglycemic compounds are present in the 25% ethanol extract, hence a good candidate for further studies aimed at novel antidiabetic natural products or standardized herbal formulation.

The extracts of *G. procumbens* were also tested for their ability to impact on the tolerance level of glucose administered parenteral in normal and diabetic rats. In the model, glucose loading was administered subcutaneous, rather than oral, to circumvent the possibility of a false positive response which could result from delayed glucose absorption due to its interaction with the sticky/waxy and viscous extracts, especially the 95% and 75% ethanol extracts. Although, the extracts (except the 95% extract) significantly suppressed the peak blood glucose in normal rats, after 15 min of glucose load, only the 0% and 25% extracts along with metformin sustained this decrease until the 90th min. Again, affirming the relative effectiveness of the 25% ethanol extract. This effect in SGTT test was also replicated in STZ-induced diabetic rats.

In a short term study, 1 g/kg of extracts of *G. procumbens* was administered daily to STZ-induced diabetic rats for 14 d. Body weight and blood glucose was monitored within the period. There was a measured decline in body weight of untreated diabetic rats compared to normal control rats. Body weight gain is an indicator of efficient glucose homeostasis; but in diabetics, the body cells scarcely access glucose, and on the alternative fats and tissue proteins are breakdown for energy supply (muscle wasting) accounting for the loss in body weight[14]. However, of the five treatments, the 25% and 50% extracts showed significant recovery in body weight gain after 14 d administration, compared to the diabetic control, implying that these fractions may possess some protective effect in controlling muscle wasting, probably by reversal of gluconeogenensis, improvement in insulin secretion and/or glycemic control. Similar effects of body weight recovery following treatment with other antidiabetic medicinal plant extracts have also been reported[15,16]. Similarly, all of the five extracts significantly reduced blood glucose in the diabetic rats 14 d after treatment. However, the effect of the 25% extract was more interesting. It exerted the highest reductions

on days 7 (49%) and 14 (65%), an effect, only comparable to metformin. The polarity of ethanol and water are 5.2 and 9, respectively. It is likely from this study that the higher the polarity of ethanol-water solvent combination, the more the concentration of active compounds or their interactions, hence the anti-hyperglycemic effect of the extract.

Flavonoids and phenolic compounds are the most widely distributed compounds in food plants that account for majority of the observed pharmacological actions, particularly via their well known antioxidant activities. The extracts in this study were therefore evaluated for the flavonoid and phenolic compounds. Results indicated that phenolics and flavonoids were respectively highest in the 50% and 75% extracts. Overall, the phenolic and flavonoid contents appear to decrease with increase in water content of the extraction solvent, contrary to the observed anti-hyperglycaemic activity of the extracts. It was noted earlier that the presence of a certain amount of water in an extraction solvent is necessary to enhance interaction of hydroxyl and/or carboxylic groups with water, to promote the dissolution of phenolics in the solvent[17], but that as the amount of water is increased, the interaction is decreased by the benzene ring present in the structure hence restricting the solubility of phenolic compounds. Whole plant extracts are known to contain numerous compounds whose net observed pharmacological actions or inactions largely depend on synergistic or antagonistic interactions among these compounds[18]. These selective and non predictive interactions of compounds in the 25% extract of *G. procumbens* may have accounted for its potent anti-hyperglycemic effect, rather than the actual amount of phenolics or flavonoids.

In this current study, there is an observed similarity in the hypoglycemic and anti-hyperglycemic activities of 25% ethanol extract and metformin, which may give some insight into the antidiabetic mechanism of the extract. Metformin exerts its anti-hyperglycemic effect primarily through inhibition of increased rates of hepatic gluconeogenesis, as well as improvement of insulin sensitivity via stimulation of peripheral glucose uptake in skeletal muscles and adipose tissues[19]. It is likely that the 25% extract may achieve its effect by similar mechanisms. However, the rapid normalization of blood glucose level in rats treated with the 25% ethanol extract in comparison to the control rats suggests the existence of some residual β-cells which must have been sensitized by compounds such as flavonoids and phenolics in extract. Islam *et al.* had suggested a similar mechanism for leaf extract of *Catharanthus roseus* (a biguanide-like action)[20].

The concentrations of marker compounds including chlorogenic acid, rutin, astragalin and kaempferol-3-O-rutinoside determined in the extracts were found to be similar irrespective of the extraction methods, but gradually decreased with increased proportion of water in the extraction solvent. For instance, the intensity of astragalin and chlorogenic acid spots was higher in 75% ethanol extract than that in 0% extract. The amount of compounds present in the extracts depended on the solubility of compound in the extraction solvent, and since flavonoids have low solubility in water[21], the increase in the water portion of the extraction solvents would reduce the amount of flavonoids extracted. Although chlorogenic acid was determined as a major chemical constituent in *G. procumbens* whose concentration depended on the polarity of extracting solvent system and extraction method, it was found that the total phenolic content was the highest in 50% ethanol extract, while chlorogenic acid content was the highest in 75% ethanol extract. This suggests that besides chlorogenic acid, there are other phenolic compounds in the extract, hence corroborating that interaction of constituents rather than the actual content is responsible for the observed antidiabetic activity.

It is clear from this study, that extracts of *G. procumbens* contain active principles that possess anti-hyperglycemic, but no hypoglycemic effect. This activity is most potent when extracted in 25% ethanol-water solvent combination. The interaction among active components appears to determine the antidiabetic efficacy much more than actual amount of the components present in the extracts. The extract may achieve its antidiabetic action via a mechanism similar to metformin.

Conflict of interest statement

We declare that we have no conflict of interest.

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Comments

Background

The full length paper has evaluated hypoglycemic and anti-hyperglycemic properties of *G. procumbens* extract as well as its phytochemistry. The literature cited is current and the study sought to resolve the conflicting reports on pharmacodynamic interaction and the expressed antihyperglycemic and hypoglycemic effects of *G. procumbens* in normal and diabetic rat models. The background has clear cut aims and objectives that may interest those in the field of pharmacognosy and drug development from medicinal products.

Research frontiers

Research in ethnomedicine has been fraught with problems of which solvent fractions contains phytochemical ingredients for expression of maximum bioactivity. Different solvent system such as ethanol, ethyl acetate, chloroform *etc.* have been in use. Graded ethanol water solvent ratios has not been fully exploited. The use of graded ratio of ethanol-water

solvents for extraction and identification of the 25% ethanol to water solvent extract demonstrating the most potent and effective anti-hyperglycemic effect of *G. procumbens*, with similar anti-diabetic mechanism to metformin is a cutting edge in the field of the research in this paper.

Related reports

Other authors such as Rodisah *et al.* (2009), Zhang *et al.* (2000), and Hassan *et al.* (2010) have used methanol, ethanol, water singly for extraction. Islam also assessed the anti-diabetic and hypolipidemic effects of different solvent fraction in *C. roseus*. The methods used by the authors are outstanding. This is the first comprehensive acute-single dose as well as sub-chronic long term (14 d) studies carried out on the pharmacology of this medicinal plant. The methods covered a wide array of indices such as fasting blood glucose, glucose tolerance test evaluating clinical indices characteristic of diabetes (polyurea, polyphagia, polydipsia and glucosuria).

Innovations and breakthroughs

This is the first study using graded ratios of ethanol to water solvents for extraction of phytochemical ingredients of this medicinal plant and has identified 25% ethanol to water extract as most effective anti-hyperglycemic components to others. In a single study, both acute and sub-chronic assays in diabetic and non-diabetic models which has allowed measurement of fasting blood glucose levels, sub-glucose tolerance test and monitoring of clinical features of diabetes and its possible mechanism makes this model new and innovative.

Applications

This model (graded ratio of ethanol to water) can be used for solvent for evaluation of antidiabetic efficacy of medicinal plants. It is a good model of structural activity relationship relevant in drug development by pharmaceutical concerns.

Peer review

The work is of high quality. Sufficient data has been generated on a time line basis which has allowed relevant metabolic changes of the diabetic state to be monitored. Structural activity relationship based on different phytochemical ingredient solubilised by the different ethanol-water solvent system in relation to their anti-diabetic effects is relevant in drug development.

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