HYPOGLYCAEMIC EFFECTS OF AQUEOUS EXTRACT OF GYNURA *PROCUMBENS*

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Summary

The present study investigates the effect of oral administration of the aqueous extract of *Gynura procumbens* on blood glucose levels in normal and streptozotocin (STZ) diabetic rats. However, the mechanism of its action is still not known. Hence, *in vivo* and *in vitro* studies were carried out to study the role of *G. procumbens* on blood glucose lowering effect. In *in vivo* studies, single administrations of aqueous extract 1g/kg b.w. to 16-h fasted streptozotocin (STZ)-induced diabetic rats reduced the mean blood glucose level at hour 2, 5, 6, 7 ($P<0.05$) but not in normal rats when administered by gastric intubation. The plasma insulin of STZ-induced diabetic rats was also measured using rat insulin ELISA kits. The result showed that plasma insulin has not elevated in these rats, which suggested that the hypoglycaemic activity was not due to an insulinotropic effect of *G. procumbens*. In glucose tolerance test where the rats were loaded with glucose (500mg/kg b.w.) intraperitoneally to induce hyperglycaemia, the extract did not reduce the glucose levels ($P>0.05$) in both normal and STZ-induced diabetic rats. The activity of *G. procumbens* was also tested on RIN-5F cell line, clonal pancreatic β-cells. It was found that the extract did not produce a stimulation of insulin secretion. Taken together, these findings indicate that the hypoglycaemic activity of the aqueous extract of *G. procumbens* leaves involves an extra-pancreatic action and not due to its insulinotropic activity.

Keywords: Gynura procumbens; Hypoglycemic activity; Streptozotocin-induced diabetic rats; Insulin; RIN5F cell line

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Introduction

Diabetes mellitus is associated with hyperglycaemia, altered metabolism of lipids, carbohydrates and proteins along with an increased risk of complications from vascular disease [1]. Two types of diabetes are recognized clinically - juvenile-onset or insulin-dependent diabetes (Type I) and maturity-onset or non-insulin-dependent diabetes (Type II). Type I diabetes is associated with low plasma insulin and require exogenous insulin therapy. Type II patients may have normal or elevated levels of insulin but show decreased sensitivity to insulin often correlating with a reduction in insulin receptor concentration [2]. The use of chemical agents such as alloxan [3] and streptozotocin [4] in animal models mimicked type 1 diabetes.

Modern treatment of diabetes based on oral hypoglycaemics (sulphonylureas, biguanides and α-glucosidase inhibitors) and insulin showed an adverse effect with relatively high rates of secondary failure [5]. As an alternative approach, scientific investigation on traditional remedies has led to the discovery of herbal drugs for the treatment of diabetes mellitus [6]. 90% population in rural areas of developing countries still relies on traditional medicines for their primary health care.

Gynura procumbens (Lour.) Merr, family Compositae also known locally as "Sambung Nyawa" is cultivated in South East Asia, especially Indonesia, Malaysia and Thailand for medicinal purposes. It is reported to be useful for hypertension, anti-inflammatory, anti-herpes simplex virus, in eruptive fevers, remedy for kidney troubles, prevention of rheumatism, treatment of colon cancer, haemorrhoids and diabetes [7]. The ethanolic extract of *G. procumbens* possesses anti-hyperglycemic and anti-hyperlipidaemic activities in diabetic rats by improving glucose tolerance, but these were not seen in normal rats [8].

The methanol extract and n-butanol fraction of *G. procumbens* (1g/kg b.w.) also showed significant hypoglycaemic effect in diabetes rats [9].

In the present study, the hypoglycaemic activity of the aqueous extract of *G. procumbens* leaves was studied in normal and STZ-induced diabetic rats. In addition, we also performed experiments using insulin secreting β-cell line, RIN 5F to further evaluate the mechanism of action of aqueous extract of *G. procumbens* leaves.

MATERIAL AND METHODS

In vivo test

Preparation of *G. procumbens* extract

Leaves of *G. procumbens* (L.) Merr. (Compositae) were collected at the Malaysian Research on Agriculture Development Institute (MARDI), Kepala Batas, Penang, Malaysia and identified. A voucher specimen (10117) was deposited in the herbarium of the School of Biological Sciences.

The leaves were dried in oven at 40^0C and milled into powder. The dry powdered leaves weighing 162.5g was extracted with distilled water (3 L) for 24 hours under reflux and heated to 60^0C. The extract was filtered with cotton wool and concentrated at 55^0C by using rotary evaporator (Buchi Labortechnik AG, Switzerland). The extract was then freeze-dried. The final weight of the aqueous extract was 47.5g which was then dissolved in 0.9% NaCl before use.

Animals

Male and female Sprague-Dawley (SD) rats (200-250g) were obtained from the animal house of the School of Pharmaceutical Sciences, Universiti Sains Malaysia, Penang.

They were housed in standard environmental conditions (24 ± 1^0C) with 12 h light: 12 h dark cycles and fed with commercial diet and water *ad libitum.*

Streptozotocin-induced diabetic rats

Diabetes was induced by intraperitoneal injection of streptozotocin (STZ) (Sigma, St. Louis, MO, USA) (65mg/kg body weight in 0.9% NaCl pH 4.5) to 16 hour fasted rats. Their diabetic condition was confirmed by the symptoms of polydipsia, polyuria and by measuring fasting blood glucose concentration 72 hours after injection of STZ. Rats with a blood glucose level above 15.0 mmol/L were considered to be diabetic and used in the experiment.

Intraperitoneal glucose tolerance test (IPGTT)

Intraperitoneal glucose tolerance test (IPGTT) was conducted on normal and diabetic rats, which had been fasted overnight (at least 16 hours). The rats were divided into six groups, each group comprising 6 animals, as follow:

Group I Control rats given oral saline (10ml/kg b.w.)

Group II Normal rats given aqueous extract (1g/kg b.w.)

Group III Normal rats given glibenclamide (10mg/kg b.w.)

Group IV Diabetic rats given oral saline (10ml/kg b.w.)

Group V Diabetic rats given aqueous extract (1g/kg b.w.)

Group VI Diabetic rats given metformin (500mg/kg b.w.)

Glucose (500mg/kg) was administered intraperitoneally 60 minutes after treatment. Blood samples were collected from the tail veil at –60 minutes (just before the administration of the extract by gastric intubation), time 0 (prior to the glucose load), 15, 30, 45, 60, 90 and 120 minutes after the glucose load. Blood glucose concentration was measured using the Accu-check Advantage II Clinical Glucose meter (Roche diagnostics Co. USA).

Hypoglycaemic test

Normal and diabetic rats weighing 200-250g were fasted overnight. The rats were divided into six groups, each group consisting of six rats, as follows:

Group I Control rats given oral saline (10ml/kg b.w.)

Group II Normal rats given aqueous extract (1g/kg b.w.)

Group III Normal rats given glibenclamide (10mg/kg b.w.)

Group IV Diabetic rats given oral saline (10ml/kg b.w.)

Group V Diabetic rats given aqueous extract (1g/kg b.w.)

Group VI Diabetic rats given glibenclamide (10mg/kg b.w.)

Blood samples were drawn from the tail vein at 0, 1, 2, 3, 4, 5, 6 and 7 hours after the administration of the extract. Blood glucose concentration was measured using the Accu-check Advantage II Clinical Glucose meter (Roche diagnostics Co. USA).

Measurement of plasma insulin concentration

Plasma insulin levels were measured in diabetic rats from hypoglycaemic tests. Blood samples (about 0.5ml) were collected from the tail vein at 0, 1, 2, 3, 5 and 7 hours after administration of the extract. The samples were collected into haematocrit-capillary tubes (Hirschmann Laborgerate GmbH & Co. KG) containing Na-heparin (15 units/ml of blood sample) and centrifuged at 12,000 rpm at 4^0C for 3 minutes. Plasma samples were stored at -20^0C.

The assay was done using kits based on one-step enzyme-linked immunoassay (ELISA) technique purchased from Crystal Chem. (IL, USA).

***In vitro* test**

Culture of RIN5F cells

RIN5F cells which were routinely cultured in RPMI 1640 supplemented with 11mM glucose, antibiotics (50 000 IU/I penicillin-streptomycin) and fetal calf serum (10%, v/v) [10] were passaged 2-4 days before each experiment and plated in 24-well Nunclon multiwell plates or tissue culture inserts at a density of 0.2 x 10^6 cells/well (Falcon, NJ, USA).

Measurement of insulin secretion

Insulin secretion was measured a method described by [11]. Multiwells were seeded with 0.2 x 10^6 cells and insulin release measured after 4-5 days as follows. Cells were washed thrice with Kreb's-Ringer bicarbonate buffer (KRB; 115 mM NaCl, 4.7 mM KCl, 1.28 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 24 mM $NaHCO_3$, 10 mM Hepes-free acid, 1 g/l bovine serum albumin, 1.1 mM glucose; pH 7.4) and preincubated for 40 min at 37^0C. Unless otherwise stated, cells were then incubated for 20 min with 1 ml KRB at 1.1 mM glucose in the absence and presence of the extract and glibenclamide at difference doses. Following incubation, aliquots were removed from each well and stored at -20^0C for insulin assay. Insulin release was measured by rat insulin ELISA kit (Crystal Chem., USA).

Statistical analysis

Results were expressed as mean $\pm$ standard error of mean (S.E.M.). The blood glucose levels of extract-treated animals, the reference drug-treated and vehicle-treated controls were compared by one way ANOVA. Differences with $p<0.05$ were considered to be statistically significant. For the *in vitro* studies, the insulin secretion was expressed as mean $\pm$ standard error of mean (S.E.M.). The significance of the effects of *G. procumbens* extract on RIN5F insulin release was assessed using the Student's t-test. P-values less than 0.05 were considered to be significant.

Results

Effects of aqueous extract of *G. procumbens* on intraperitoneal glucose tolerance test in rats

As shown in Fig.1, the control and extract treated groups demonstrated basal hyperglycaemia subsequent to intraperitoneal glucose load and failed to return to fasting levels after 120 minutes (4.5 $\pm$ 0.2 mmol/L for the extract-treated group as against 4.1 $\pm$ 0.2 mmol/L for the control group), indicating glucose intolerance. Glucose tolerance significantly improved in glibenclamide, reference drug compared to the control. This change shows a significant improvement in 30 to 120 minutes (6.3 $\pm$ 0.3 mmol/L to 2.6 $\pm$ 0.2 mmol/L) after glucose administration ($P<0.001$).

In the STZ-induced diabetic rats, blood glucose levels were 4.0 times higher than those in normal rats. The effect of the aqueous extract was summarised in Figure 2. The oral administration of the aqueous extract of *G. procumbens* to STZ-induced diabetic rats did not cause any significant changes in the fasting blood glucose levels when compared with the control group. The reference drug, metformin (500mg/kg) significantly reduced the fasting blood glucose levels of the diabetic rats at 30, 45 and 120 minutes ($P<0.05$) compared with the control group.

Effects of the aqueous extract of *G. procumbens* on hypoglycaemic test in rats

In normal rats, treatment with a single dose of 1g/kg b.w. of the aqueous extract significantly higher the fasting blood glucose levels when compared with the control at 1st, 2nd, 3rd, 5th and 6th hour after oral administration. Glibenclamide (10mg/kg) caused a significant reduction in the fasting blood glucose levels of the normal rats throughout 7-hour study when compared with the control. A maximum decrease of 50.0% was observed after 7 h of treatment (Fig. 3).

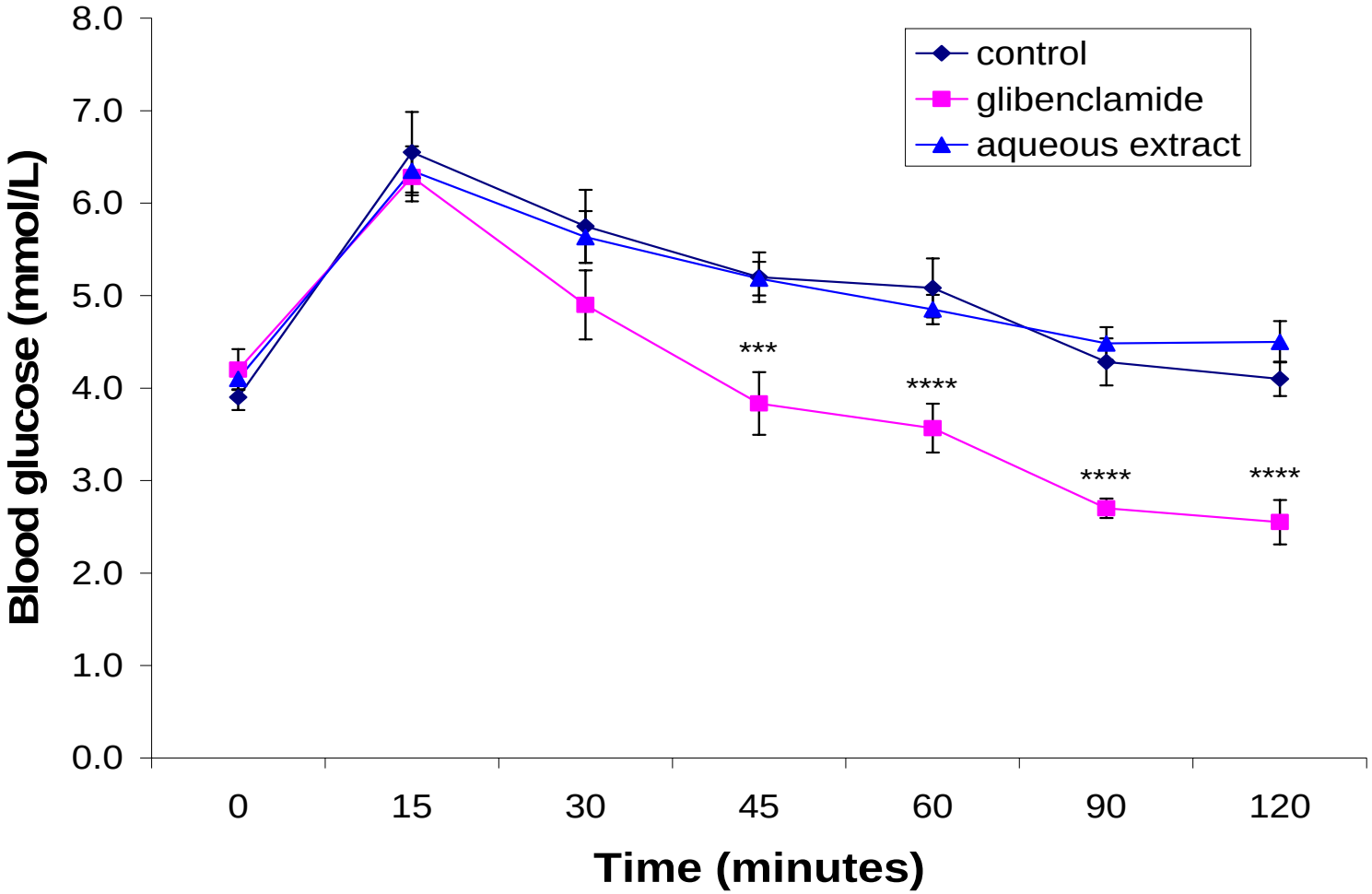
Administration of the aqueous extract at a dose of 1g/kg in STZ-induced diabetic rats brought about fall in the fasting blood glucose levels and showed hypoglycaemic activity (Fig. 4, $P<0.05$). This occurs at the second, fifth, sixth and seventh hour of the experiment during which the fasting blood glucose levels of the STZ-induced diabetic rats were lowered by as much as 30%, 22%, 22% and 23% respectively compared to control groups. Glibenclamide treated diabetic rats showed no significant difference in the fasting blood glucose levels when compared to the control group.

Effects of the aqueous extract of *G. procumbens* on the plasma insulin levels

Plasma insulin concentration was determined in the 0, 1, 2, 3, 5 and 7 hour samples of hypoglycaemic test in diabetic rats (Table I). It seems that the aqueous extract of G. procumbens did not significantly increase ($P>0.05$) the plasma insulin concentration throughout the hours compared to the control group. The same result obtained for the standard drug, glibenclamide, that the plasma insulin concentration did not increased in the diabetic rats.

Effects of the aqueous extract of *G. procumbens* on insulin release by RIN5F cells

Figure 5 shows the effect of aqueous extract of *G. procumbens* leaves on glucose stimulated insulin release in a clonal ß-cell, RIN 5F. The aqueous extract (0-10mg/ml) had no dose dependent stimulatory effect on insulin secretion by clonal ß-cell at 1.1 mM glucose. On the other hand, glibenclamide, a reference drug (0-10mg/ml) stimulated basal RIN 5F insulin release in a dose dependent manner (Fig. 6). Glibenclamide at a dose 10mg/ml induced a stimulation of insulin secretion to 1.0 ± 0.03 ng/million cells per 20 minutes.


8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
Blood glucose (mmol/L)
0
15
30
45
60
90
120
Time (minutes)
control
glibenclamide
aqueous extract
***
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****
****

Figure 1

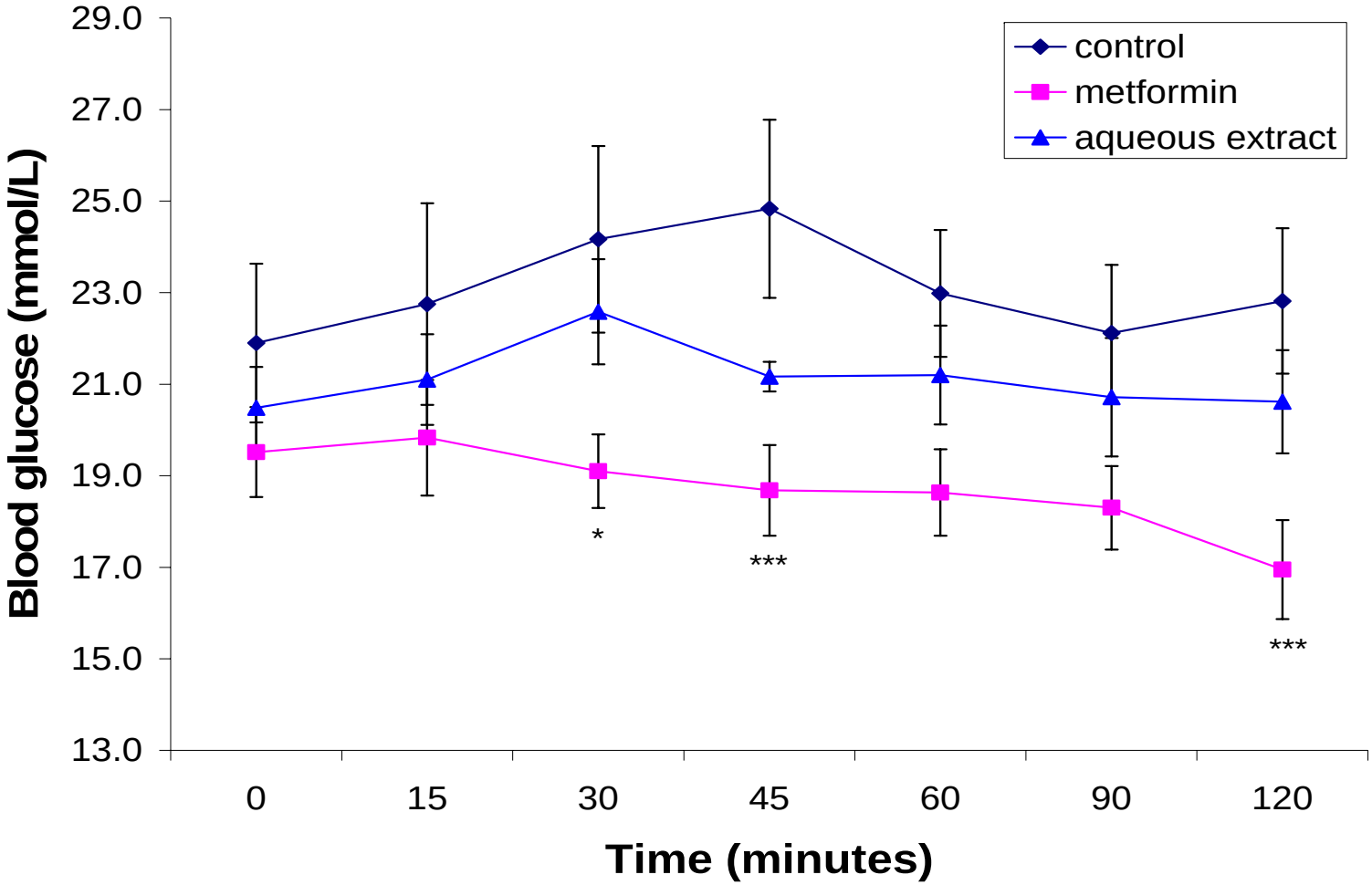
29.0
27.0
25.0
23.0
21.0
19.0
17.0
15.0
13.0
Blood glucose (mmol/L)
0
15
30
45
60
90
120
Time (minutes)
control
metformin
aqueous extract
*
***
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Figure 2

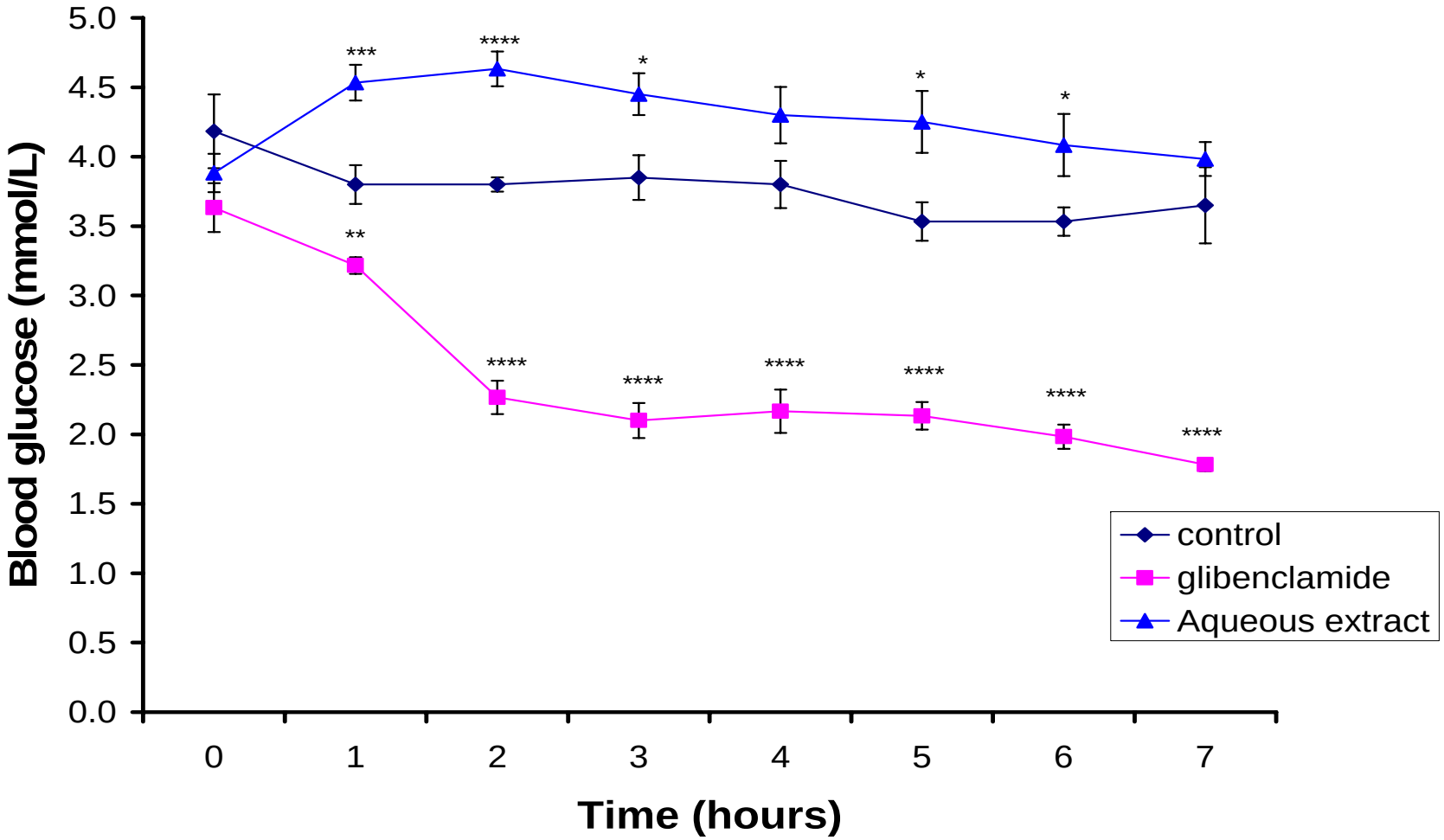
Blood glucose (mmol/L)
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
1
2
3
4
5
6
7
Time (hours)
***
****
*
*
*
**
****
****
****
****
****
****
control
glibenclamide
Aqueous extract

Figure 3

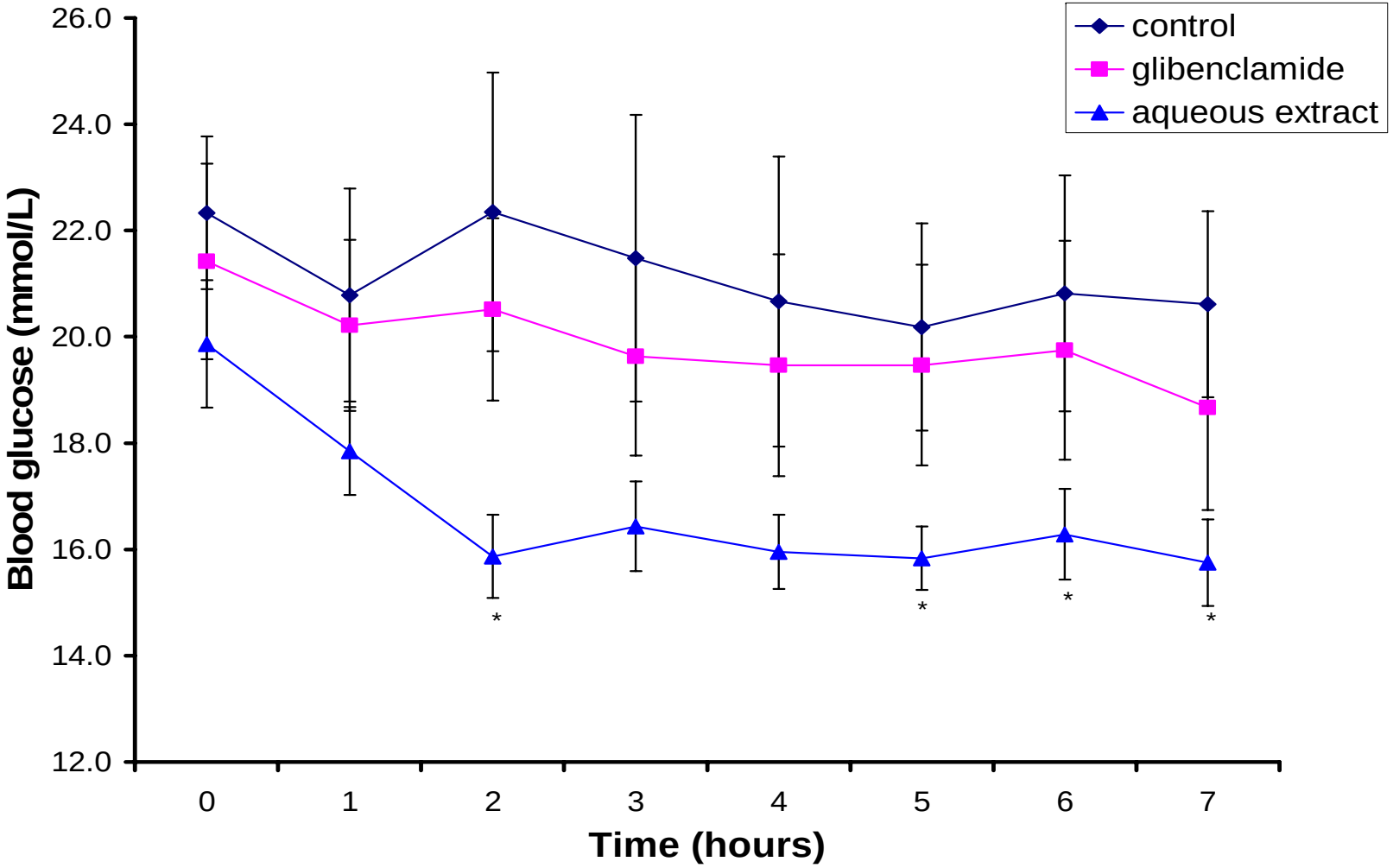
control
glibenclamide
aqueous extract
Blood glucose (mmol/L)
26.0
24.0
22.0
20.0
18.0
16.0
14.0
12.0
0
1
2
3
4
5
6
7
Time (hours)
*
*
*
*

Figure 4

Table I

Glucose induced insulin response before and after oral administration of the aqueous extract of *G. procumbens* leaves

Treatment	N	Plasma insulin (ng/ml)					
		Fasting	**1 h**	**2 h**	**3 h**	**5 h**	**7 h**
Control	6	2.00 ± 0.07	2.06 ± 0.08	2.11 ± 0.04	2.18 ± 0.10	2.08 ± 0.06	2.29 ± 0.07
Glibenclamide	6	2.02 ± 0.03	2.02 ± 0.04	2.07 ± 0.04	2.05 ± 0.03	2.12 ± 0.04	2.36 ± 0.08
G. procumbens	6	2.05 ± 0.03	2.07 ± 0.02	2.11 ± 0.03	2.07 ± 0.01	2.09 ± 0.02	2.07 ± 0.04

Data are expressed as means ± standard error of mean, N = 6 rats in each group.

Result shows not significant between extract-treated and glibenclamide-treated compared to control-treated group.

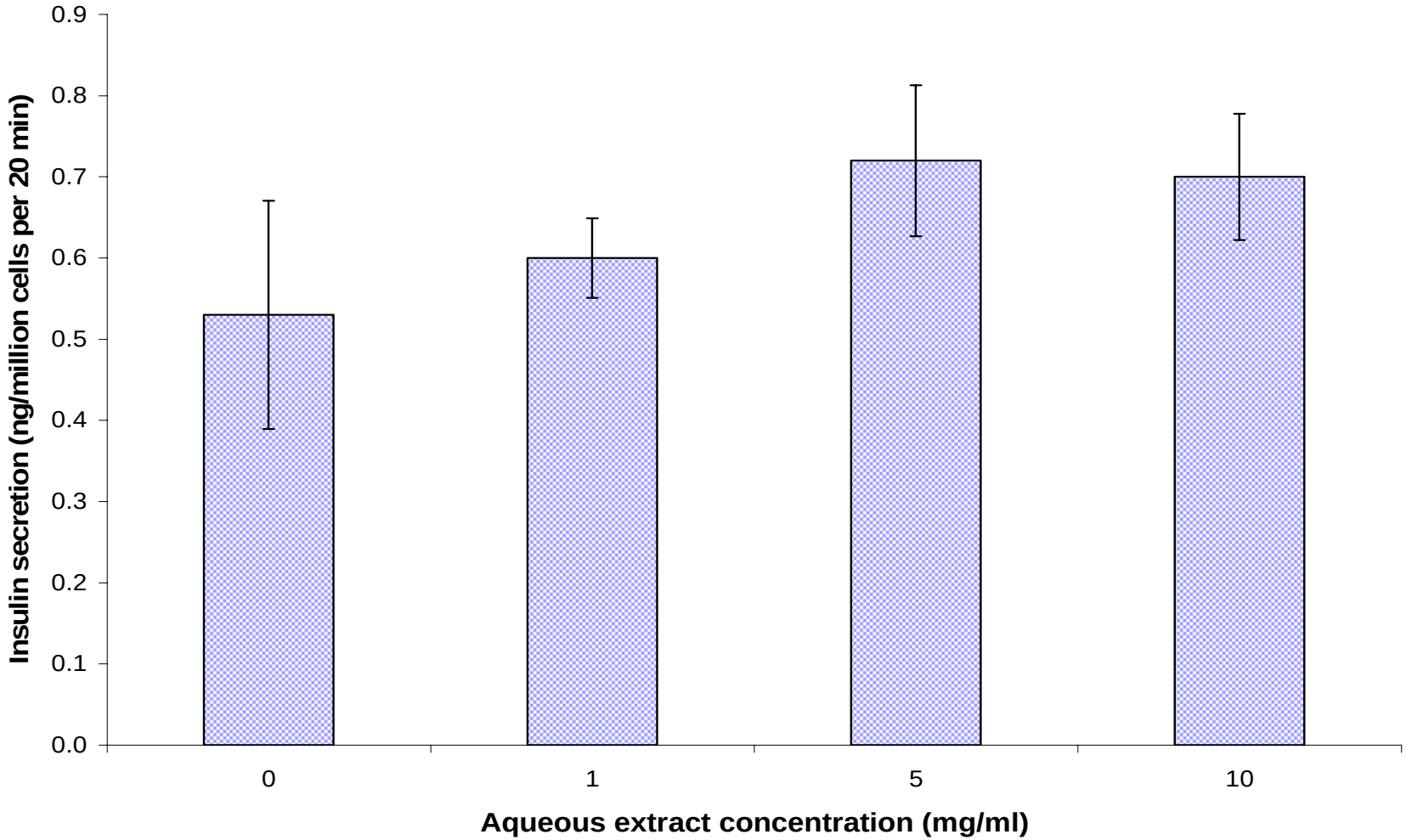
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Insulin secretion (ng/million cells per 20 min)
0
1
5
10
Aqueous extract concentration (mg/ml)

Figure 5

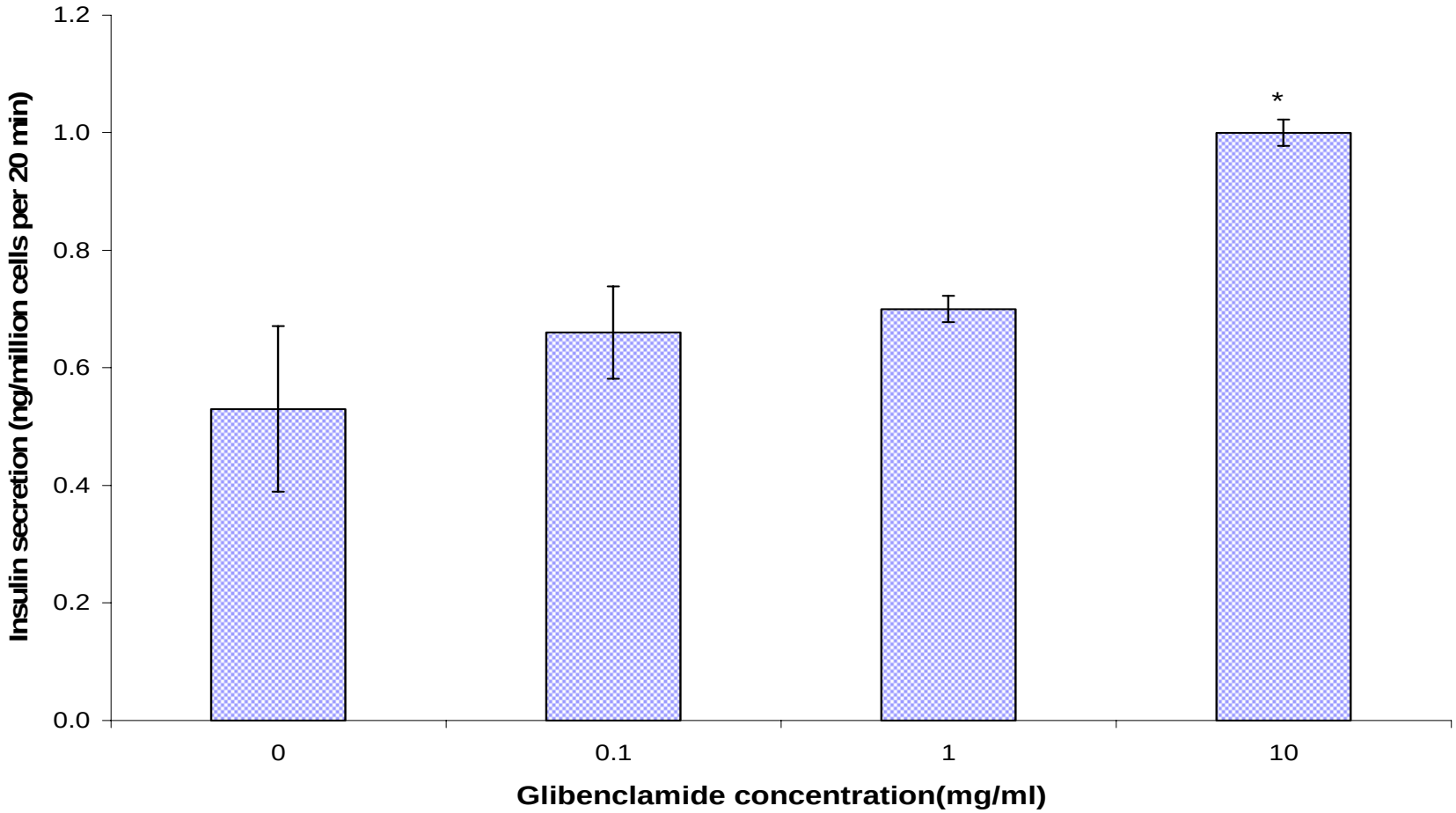
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Insulin secretion (ng/million cells per 20 min)
*
0
0.1
1
10
Glibenclamide concentration(mg/ml)

Figure 6

DISCUSSION

The experiments described here clearly demonstrated that aqueous extract of *G. procumbens* leaves possesses a hypoglycaemic activity. The aqueous extract showed significant hypoglycaemic activity in the fasting blood glucose levels of the STZ-induced diabetic rats at 1g/kg b.w. but not in normal rats. The continued fall in the fasting blood glucose observed even 7 hours after the administration of the extract shows that the hypoglycaemic effect is persistent. In normal rats, however, the extract (1g/kg b.w.) did not produce a hypoglycaemic effect. Instead, the extract produced significant elevation in the fasting blood glucose levels. A study on the chemical constituents of *G. procumbens* has reported the presence of glucosides in the *G. procumbens* extract [9]. It is possible that the increase levels of glucose in normal rats could be due to these sugar-containing molecules.

Our results are in accordance with reported effects of other forms of the plant extract. In 2001, Akowuah et al. [9] reported that a methanol extract and the n-butanol fraction (of the methanol extract) of *G. procumbens* produced a hypoglycaemic effect in STZ-induced diabetic rats and not in normal rats. Zhang and Tan (2000) [8] reported that the ethanolic extract of *G. procumbens* leaves show anti-hyperglycaemic and anti-hyperlipidaemic activities in diabetic rats. The authors also claimed that the effects were similar to that of metformin in improving glucose tolerance in STZ-induced diabetic rats, but the effects were not seen in normal rats. In the present study, we did not find any improvement in glucose tolerance in STZ-induced diabetic rats. These could be due to reduced or no effect of the components present in the aqueous extract at higher doses [12] and the presence of other antagonistic components in the extract.

Our finding that glibenclamide reduced the fasting glucose levels of normal rats but not of diabetic rats is in accordance with a previous report [13]. The use of chemical agents like streptozotocin (STZ) in animals model mimicked Type 1 diabetes [4]. Severe cytotoxicity of STZ on the ß cells of the pancreas resulted in severe damage of islet cells [14, 15]. In diabetic rats, the ß cells of the pancreas are unable to produce insulin to cause hypoglycaemia. Hence, glibenclamide, a sulphonylurea derivative is ineffective in the streptozotocin-induced diabetic rats as shown in the Figure 4. However, biguanides such as metformin ameliorates hyperglycaemia by improving peripheral sensitivity to insulin and reducing hepatic glucose production [16]. Therefore, metformin as shown in Figure 2 is effective in the STZ-induced diabetic rats since it does not depend on the ß cells for its activity.

In vivo results discussed above indicate that the mechanism by which this plant decreased blood glucose levels in diabetic rats is independent of insulin secretion. We tested this contention further by examining the insulin secreting properties of the extract, if any, in *in vitro* experiments. Concomitant exposure of clonal pancreatic ß-cells, RIN5F cell line to the aqueous extract showed no stimulation in insulin secretion. The results, thus, support our *in vivo* experiments in providing evidence that the hypoglycaemic action of the extract does not rely on insulin secretion. Glibenclamide, a sulphonylurea derivative was shown to evoke a dosage-dependent stimulation of basal insulin release from the RIN5F cell line. At the dose 10 mg/ml, glibenclamide was found to stimulate insulin secretion. Glibenclamide is the most well known oral hypoglycaemic agent derived from sulfonic acid. It binds to receptors on the surface of pancreatic β-cells. As a result, the cell membrane creates an influx of calcium ions and a subsequent release of insulin [16].

The results obtained favor that the antihyperglycaemic effects of the aqueous extract of *G. procumbens* could be due to its ability to mimic or improve insulin action at cellular level. It could also be due to the insulin like effect of the active principle(s) present in the extract. A preliminary phytochemical analysis of the *G. procumbens* extract led to isolation of flavonol, flavonol glycoside, including rutin, quercetin and kaempferol [9] from the n-butanol fraction. In addition, qualitative analysis of the aqueous extract of *G. procumbens* by High Performance Thin Layer Chromatography (HPTLC) revealed the presence of astragalin (kaempferol-3-o-glycoside)(data not shown). These flavonoids and their glycosides found to be responsible for blood glucose lowering activity [17].

An extra-pancreatic actions influence the glucose metabolism [18]. The extra-pancreatic actions may be involved in this pharmacological effect include stimulating glucose utilization in peripheral tissue [19], increase in glycolytic [20] and/or glycogenic enzymes activity in peripheral tissues [19], decrease the secretion of the counter-regulatory hormones (glucagons, cortisol and growth hormones) [21] and inhibition of glucose absorption from the intestine [22].

In conclusion, our *in vivo* studies indicate that the aqueous extract of *G. procumbens* leaves possess a significant hypoglycaemic effect in STZ-induced diabetic rats. It is evidence from the *in vitro* studies that the aqueous extract does not possesses insulinotropic properties. The mechanism of this pharmacological effect may be an extra-pancreatic action. However, we cannot exclude the possibility that the other mechanisms may be responsible for lowering the blood glucose level. Further pharmacological and phytochemical studies are currently in progress in order to investigate the mode of action of this plant.

ACKNOWLEDGEMENTS

This research was supported financially by the R & D research grant from the Malaysian Government. The authors wish to thank Abdul Razak Kasmuri for his technical assistance.

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