Hypoglycemic Effects of *Gynura procumbens* Fractions on Streptozotocin-induced Diabetic Rats involved Phosphorylation of GSK3β (Ser-9) in Liver

(Changes Hipoglisek Fraksi *Gynura procumbens* di dalam Tikus Diabetes Aruhan- Streptozotocin Melibatkan Pemfosfatan GSK3β (Ser-9) Hepar)

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INTRODUCTION

Diabetes mellitus is a metabolic disease characterised by disordered metabolism and abnormally high levels of blood glucose. It is a major public health problem affecting 285 million or 6.4% of the world population for the year 2010 (International Diabetes Federation 2009). By 2030, this number is estimated to rise to 435 million. Despite considerable progress in the management of diabetes...
mellitus using synthetic drugs, the search for improved and safe natural anti-diabetic agents with minimal side effects is ongoing.

_Gynura procumbens_ Merr. (Compositae), locally known as _Sambung Nyawa or Kecam Akar_ is an evergreen shrub with medicinal properties widely distributed in South East Asia. It has been used by rural communities to treat various ailments including diabetes (Perry 1980). The ethanol and methanol extracts prepared from leaves of the plant were reported to exhibit anti-diabetic effects in streptozotocin (STZ)-induced diabetic rats (Akowuah et al. 2001a,b; Zhang & Tan 2000). In addition, Rasadah et al. (2002) showed that the methanol extract of _G. procumbens_ was able to increase insulin secretion in the insulin-secreting cell line, BRIN-BD11.

More recently, it was reported that the aqueous extract of _G. procumbens_ not only decreased the blood glucose levels but also caused an increase in the glucose uptake by muscle tissues in STZ-induced diabetic rats (Zurina et al. 2010). In contrast to what was reported earlier by Rasadah et al. (2002), there were no significant changes in plasma insulin levels in the diabetic rats following administration with the extract (Zurina et al. 2010). Zurina et al. (2010) also proposed that the hypoglycemic effect observed could be attributed to the phytoconstituents of _G. procumbens_ mimicking insulin action by promoting glucose uptake. However, no experimental evidence was provided to show the involvement of any component of the insulin biosignaling pathway for the effect observed.

In addition to stimulating glucose uptake, insulin promotes phosphorylation and inhibition of glycogen synthase kinase 3 (GSK3), a component of insulin biosignaling (Lee & Kim 2007). GSK3 was first described in glycogen metabolism as an enzyme which phosphorylates and inactivates glycogen synthase, the rate-limiting enzyme in the synthesis of glycogen in response to insulin (Embi et al. 1980). Although GSK3 is constitutively active in cells, it is inactivated in response to insulin stimulation of glycogen synthesis in muscles (Wang et al. 2009). The aim of the present study was to investigate the involvement of GSK3, a component of insulin biosignaling in the hypoglycemic effects of the active fractions from _G. procumbens_ by examining the phosphorylation state of GSK3 in the liver of normal and STZ-induced diabetic rats treated with the fractions.

**MATERIALS AND METHODS**

**PREPARATION AND FRACTIONATION OF _G. PROCUMBENS_ LEAF EXTRACT**

_G. procumbens_ plants were collected from the Green House Facility at the Faculty of Science and Technology, Universiti Kebangsaan Malaysia. Fresh leaves from the plant were washed and air-dried at room temperature. The dried leaves (2 kg) were ground into a powder form, macerated and soaked with 95% ethanol (3 L) for three days at room temperature. This procedure was repeated thrice. The extracts were filtered and evaporated under reduced pressure at a temperature of 40°C using a rotary evaporator to give a residue. This crude extract was partitioned successively between hexane/water, ethyl acetate/water and n-butanol/water (Si et al. 2010). Then, each fraction was concentrated to dryness to obtain three fractions (hexane, ethyl acetate and n-butanol) for biological tests. The yields obtained for each fraction with respect to the initial dry material were hexane 5.4%, ethyl acetate 2.1% and n-butanol 2.9%.

**HPLC ANALYSIS OF _G. PROCUMBENS_ FRACTIONS**

The fractions were dissolved in 45% methanol as 3 mg/mL, filtered through membrane filter and 20 µL injected for high-performance liquid chromatography (HPLC) analysis. HPLC-diode array detector-electrospray ionisation-tandem mass spectrometry (HPLC-DAD-ESI-MS) with Shimadzu liquid chromatography VP ODS column (150 mm × 4.6 mm i.d., 5 µm particle size) was used for identification for the chemical compositions present in the fraction. The mobile phase consisted of water-0.1% formic acid (A) and acetonitrile (B) and a linear gradient program of 10-30% B in 0-30 minutes, 30-100% B in 30-40 minutes was used at a flow rate of 0.8 mL/min and a setting of 347 nm for the DAD detector (Akowuah et al. 2001a, b).

**CELL-BASED ASSAY FOR GSK3 INHIBITOR**

In this assay, a yeast gsk-3 null mutant YTA003W with genotype MATa his3 leu2 ura3 trp1 ade2 mck1::TRP1 mds1::HIS3 mtk1::YTA003W::LEU2 was transformed with mammalian gsk3β. The yeast gsk3 null mutant is temperature sensitive at 37°C and this phenotype is suppressed by the expression of mammalian GSK3β (Andoh et al. 2000). The transformant was grown in 25 mL of SC-Ura medium at 37°C with shaking at 180 rpm in an incubator (Jeio Tech Co., Ltd., Korea) for 2 days. A volume of 400 µL culture was inoculated into 100 mL of screening agar (0.67% yeast nitrogen base without amino acids and ammonium sulphate, 0.5% ammonium sulphate, 2% D(+)-glucose anhydrate, 1 mL each of 0.03 mg/mL of adenine (hemisulphate salt), 0.03 mg/mL of L-tryptophan, 0.03 mg/mL of L-phenylalanine, 0.03 mg/mL of L-histidine and 1.5% bacteriological agar, pH 5.6).

Paper disc diffusion assays were performed in the presence and absence of _G. procumbens_ fractions dissolved in their respective solvent extraction at various concentrations and plates incubated at both permissive temperature (25°C) and high temperature (37°C). H7667, a Streptomyces toxic to yeast was used as the positive control (Cheenpracha et al. 2009) in this assay. Growth was scored after 5 days.

**EXPERIMENTAL ANIMALS**

Male Sprague-Dawley rats (n = 36) aged six weeks and weighing approximately 250 g were obtained from the Animal House Facility at the Faculty of Science
and Technology, Universiti Kebangsaan Malaysia. The study animals were housed in cages and maintained on standard laboratory rat chow with water *ad libitum*. All rats were acclimatised for two weeks before the initiation of the experiment in order to adapt the rats with the environmental conditions. The experiments were performed after the approval of the protocol by Universiti Kebangsaan Malaysia Animal Ethics Committee (UKMAEC) and were carried out in accordance with the current guidelines for the care of laboratory animals (Approval No. FST/SBB/2010/HALIMAH/24-AUGUST/322-SEPTEMBER-2010-NOVEMBER-2011).

INDUCTION OF DIABETES

Diabetes was induced in overnight-fasted rats (8 weeks old) (*n* = 30) each via a single intravenous injection of STZ (Calbiochem, La Jolla, USA) (45 mg/kg body weight; freshly dissolved in ice cold citrate buffer 0.1 M, pH4.5). Normal control animals (*n* = 6) received injection of sodium citrate buffer (pH4.5).

After one week, overnight-fasted rats (*n* = 30) with marked hyperglycemia (blood glucose level exceeding 12.0 mmol/L) were selected and used for the study (Pandit et al. 2010).

EXPERIMENTAL PROTOCOL

The experimental design involved 36 rats which were divided into six groups (*n* = 6 per group).

Animals in Group 1 were normal rats and served as the non-diabetic control group. Animals in Groups 2 through 6 were STZ-induced diabetic rats. Group 2 served as diabetic-untreated control. Animals in Group 3 were administered orally with the reference drug, Glibenclamide (Sigma Chemical Co., St. Louis, USA) (5 mg/kg b.w./day) which served as the diabetic positive control group. Rats in Groups 4, 5 and 6 were force-fed orally with *G. procumbens* fractions (hexane, ethyl acetate and n-butanol) respectively at 250 mg/kg b.w./day. Animals were given assigned treatments for two weeks (1 mL/rat). *G. procumbens* fractions and Glibenclamide were suspended in 0.2% carboxymethyl cellulose (CMC) prepared in distilled water prior to oral administration to experimental animals. Control Groups 1 and 2 were only administered the vehicle (0.2% CMC).

Fasting blood glucose levels were determined on day 0 (start of treatment) and day 14 (end of treatment) for determination of blood glucose levels using AccuCheck® Active Glucometer (Roche Diagnostics, Laval, Canada)

DETERMINATION OF GLYCOGEN CONTENT

Liver glycogen content was determined according to the method described by (Ong & Khoo 2000). Weighed amounts of liver tissues were homogenised in 30% potassium hydroxide (KOH) and boiled at 100°C for 30 min. Glycogen was precipitated with 95% ethanol, pelleted, washed and resolubilised in distilled water. Glycogen content was determined by a color reaction with anthrone reagent and measured spectrophotometrically at a wavelength of 590 nm.

WESTERN BLOTTING

Proteins from liver tissues were extracted using protein extraction buffer (Lipina et al. 2005) and the amount of protein measured using the Bradford Reagent following manufacturer instructions, with bovine serum albumin (BSA) as a standard. A total of 40 µg protein from each sample was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli 1970), transferred onto polyvinylidene fluoride (PVDF) (Towbin et al. 1979) membranes and then blocked in 2.5% BSA in tris buffered saline-Tween 20 (TBST) (0.1% (w/v) Tween-20 in TBS). The membranes were then probed using a rabbit monoclonal antibody to rat GSK3β (Cell Signaling Technology, Inc., Danvers, USA) or rat monoclonal antibody to rat phospho-Ser GSK3β (Cell Signaling Technology, Inc., Danvers, USA) or mouse monoclonal anti-β-actin (Santa Cruz Biotechnology, Inc., Santa Cruz, USA). After incubation with secondary horseradish peroxidase-conjugated antibody, immunoreactive bands were developed using Enhanced Chemiluminescence (ECL) Plus western blotting detection system (Thermo Scientific, Rockford, USA). Band area intensity was quantified using a densitometer (Vilbert Lourmat 302, France).

STATISTICAL ANALYSIS

All data were expressed as mean ± Standard Error Mean (SEM). Statistical significance between groups was determined using one-way ANOVA and using Student’s *t*-test unpaired between two groups. *p* values < 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

The present study was undertaken to re-evaluate the reported anti-diabetic activities of *G. procumbens* (Zhang & Tan 2000; Akowuah et al. 2001a,b; Rasadah et al. 2002; Zurina et al. 2010) by using three different fractions prepared from the ethanolic extract of the plant (hexane, ethyl acetate and n-butanol fractions) in an experimentally-induced animal diabetes model. Western analyses of liver samples from plant fraction-treated diabetic rats...
were performed to determine whether GSK3 is involved in the anti-hypoglycemic effects of *G. procumbens*. In order to examine the potential of *G. procumbens* fractions in reducing blood glucose levels, a commercial anti-diabetic drug, Glibenclamide was used as a positive control. Glibenclamide, an anti-diabetic drug in a class of medication known as sulfonylureas, is established to act by stimulating release of insulin from the pancreatic beta (β) cells (Luzi & Pozza 1997).

Out of a total of 44 experimental animals injected with a single intravenous injection of STZ (45 mg/kg body weight), diabetes was successfully induced in 31 (70%) of the rats with blood glucose levels ranging from 15.8 to 21.3 mmol/L (Table 1) i.e. exceeding the limit of 12.0 mmol/L recognised as the accepted minimum blood glucose level for experimental hyperglycemia (Murphy & Anderson 1974). One of the major symptoms in diabetes is the impairment of glucose storage capacity attributed to the lack of activity of glycogen synthase, the rate-limiting enzyme in glycogenesis, in addition to defective glucose uptake activity of the cells (Cline et al. 2002).

In the present study, the impaired capacity of the liver to synthesise glycogen in diabetes is reflected by the reduced glycogen content in liver of diabetic rats (Figure 1).

Repeated oral administration of hexane, ethyl acetate and n-butanol fractions of *G. procumbens* into diabetic rats for 14 days caused significant \( p < 0.05 \) reduction in blood glucose levels of 29.7%, 60.1% and 33.5%, respectively (Table 1). At the dose tested (250 mg/kg b.w/day), the ethyl acetate *G. procumbens* fraction exhibited glucose-reducing activity that is doubled the activities detected in the other

### TABLE 1. Effects of *G. procumbens* leaf fractions on blood glucose levels in STZ-induced diabetic rats

<table>
<thead>
<tr>
<th>Test model</th>
<th>Groups</th>
<th>Dose (mg/kg)</th>
<th>Blood glucose levels (mmol/L) (Inhibition %)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Day 0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(Start of treatment)</td>
</tr>
<tr>
<td>Normal</td>
<td>Control (Vehicle)</td>
<td>-</td>
<td>5.4 ± 0.21</td>
</tr>
<tr>
<td>Diabetic</td>
<td>Control (Vehicle)</td>
<td>-</td>
<td>21.1 ± 1.01</td>
</tr>
<tr>
<td></td>
<td>Glibenclamide</td>
<td>5</td>
<td>20.1 ± 1.33</td>
</tr>
<tr>
<td></td>
<td>Hexane fraction</td>
<td>250</td>
<td>19.5 ± 1.06</td>
</tr>
<tr>
<td></td>
<td>Ethyl acetate fraction</td>
<td>250</td>
<td>21.3 ± 1.62</td>
</tr>
<tr>
<td></td>
<td>n-butanol fraction</td>
<td>250</td>
<td>20.0 ± 1.13</td>
</tr>
</tbody>
</table>

Values are given as mean ± S.E.M. Diabetic controls were compared with normal controls and treated groups at the corresponding time. Values are statistically significant at *\( p < 0.05 \) vs diabetic control.

![Figure 1](image-url)  
**FIGURE 1.** Effects of *G. procumbens* fractions on glycogen content of liver tissue in STZ-induced diabetic rats. Values are given as mean ± S.E.M. Diabetic controls were compared with normal controls and treated groups at the corresponding time-interval. Values are statistically significant at *\( p < 0.05 \) vs diabetic control.
two fractions; and was as pronounced as the activity observed with the standard oral hypoglycemic drug, Glibenclamide which reduced blood glucose by 61.7% \((p < 0.0001)\). The differential blood glucose-reducing activities of the \(G. procumbens\) fractions examined in the present study may be accounted for by the differences in phytochemical constituents and composition among the three fractions. In the present investigation, phytochemical analysis of the plant fractions using HPLC revealed that the hexane and n-butanol fractions contain the flavonoid, kaempferol whilst the ethyl acetate fraction contained kaempferol-3,7-di-O-\(\beta\)-D-glucoside. This latter compound has been shown to be responsible for anti-hyperglycemic activity in an ethanol extract of \(Gynura medica\) (Liu et al. 2010).

Oral administration of the fractions or Glibenclamide into diabetic rats also increased liver glycogen content significantly \((p < 0.05)\) (Figure 1). The increase in liver glycogen level was higher in rats administered with hexane and n-butanol fractions of the plant compared with rats given the ethyl acetate fraction. Treatment with the ethyl acetate fraction however, increased hepatic glycogen content to a similar level as seen in Glibenclamide-treated rats. The results obtained with \(G. procumbens\) fractions as well as Glibenclamide indicated that the impaired glycogen storage of the diabetic state was partially corrected by the fractions of the reference drug.

Western analysis showed the presence of phosphorylated GSK3, pGSK3(Ser-9) in liver samples obtained from both groups of rats administered with plant fractions or Glibenclamide (Figure 2). Hepatic GSK3β in non-treated diabetic rats remained in the non-phosphorylated state. This kinase enzyme remains active in the diabetic state to phosphorylate glycogen synthase thus explaining the lowered levels of liver glycogen in the animals with diabetes as described earlier.

Despite the differences in chemical composition between the hexane, ethyl acetate and n-butanol plant fractions, there were no significant differences \((p > 0.05)\) between the hepatic pGSK3(Ser-9) levels in rats administered with these fractions as estimated from the immunoreactive band intensities (Figure 2). It was also interesting to note that the band intensity of the hepatic pGSK3 obtained from Glibenclamide-treated rats was similar to that in plant fractions-administered animals.

Since phosphorylation of Ser-9 in GSK3β leads to inhibition of the kinase enzyme activity (Yin et al. 2009), data obtained in the present study from western analysis using pGSK3β (Ser-9) antibody suggest that \(G. procumbens\) fractions or Glibenclamide inhibited GSK3β. How GSK3β is inactivated by \(G. procumbens\) was next addressed.

In insulin signaling, GSK3β activity is regulated by upstream components of the pathway including the level of insulin interacting at the receptor level. Work in our laboratory using crude ethanol \(G. procumbens\) extract showed that in extract-treated rats, the plasma insulin remained low as in non-treated diabetic control (Table 2). It was unlikely that the action of \(G. procumbens\) involved stimulation of \(\beta\) cells of the pancreas. That \(G. procumbens\) action mimics insulin effects as previously suggested by Zurina et al. (2010) for the aqueous extract of \(G. procumbens\) thus was reiterated by the plasma insulin data (Table 2) obtained in the present study. Therefore, the GSK3β inhibition observed in liver of \(G. procumbens\)-administered diabetic rats was not likely to be caused by potentiation of insulin production in the pancreas. The ethanol and methanol \(G. procumbens\) extracts exerted significant hypoglycemic effects \((p < 0.05)\) in STZ-induced diabetic rats after administration of the extracts (Akowuah et al. 2001a,b; Zhang & Tan 2000). Contrary to the plasma insulin data obtained in the present study for the ethanol \(G. procumbens\) extract, and that obtained by Zurina et al. (2010) using an aqueous extract of the same plant, Rasadah et al. (2002) reported that the methanol extract of \(G. procumbens\) increased insulin secretion significantly \((p < 0.05)\) in BRIN-BD11 cells.

A yeast-based GSK3 assay was further employed to test if the \(G. procumbens\) fraction-mediated phosphorylation of

**FIGURE 2.** GSK3β expression in liver of normal and STZ-induced diabetic rats. Liver protein extracts from glibenclamide-treated and \(G. procumbens\) fractions-treated animals or control animals were subjected to immunoblot analyses with the indicated antibodies. Lanes represent livers from: 1: Normal control rats; 2: Diabetic control rats; 3: Glibenclamide (5 mg/kg)-treated diabetic rats; 4: \(G. procumbens\) hexane fraction (250 mg/kg)-treated diabetic rats; 5: \(G. procumbens\) ethyl acetate fraction (250 mg/kg)-treated diabetic rats; and 6: \(G. procumbens\) butanol fraction (250 mg/kg)-treated diabetic rats.
GSK3β in diabetic rats was directly due to GSK3 inhibitory activities present in the plant fractions. All the three plant fractions tested showed no significant GSK3 inhibitory activities even at concentrations of up to 10 mg of the fractions tested (Table 3). Growth inhibition of the yeast transformant was not observed at 37°C indicating these fractions was not targeting GSK3β. The hypoglycemic action of *G. procumbens* fractions could therefore be affecting component(s) upstream of the pathway that regulates GSK3 activity. In this aspect, possible candidates as targets for anti-diabetic action in the insulin signaling pathway are PI3-kinase and PKB/Akt (Rayasam et al. 2009).

In the insulin signaling pathway, Akt-mediated phosphorylation and inhibition of GSK3β in response to insulin resulted in the activation of glycogen synthase (Lizcano & Aleesi 2002). The activation of glycogen synthase was then promoting glycogen synthesis, which constituted an important mechanism of glycemic control. In diabetes, GSK3β remains non-phosphorylated and activated resulting in lowered glycogen content in liver; this was due to the lack of stimulation of the insulin pathway. In the present study, the hexane, ethyl acetate and n-butanol fractions of *G. procumbens* were able to reduce blood glucose levels, partially restore hepatic glycogen-storage capacity and inhibit liver GSK3β in STZ-induced diabetic rats. In addition to the blood glucose reducing activities, the plant fractions did not exhibit significant GSK3-inhibitory activity as assessed by an *in vitro* assay; and did not affect plasma insulin levels in the diabetic animals.

**CONCLUSION**

A majority of the glucose-reducing agents available for the treatment of diabetes mellitus consists of agents which improve insulin production by β cells. Here it was shown that *G. procumbens* was able to lower blood glucose without affecting plasma insulin levels. Therefore, it was likely that the hypoglycemic action of the fractions observed could be due to direct or indirect effects on the activities of one or more of the upstream components of the insulin biosignaling pathway. Thus, isolation and purification efforts towards identification of the active principle in the *G. procumbens* fractions responsible for these activities as well as determination of upstream kinases involved are pertinent.

**ACKNOWLEDGEMENT**

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**TABLE 2. Effect of *G. procumbens* crude ethanolic extract on plasma insulin in STZ-induced diabetic rats**

<table>
<thead>
<tr>
<th>Test model</th>
<th>Groups</th>
<th>Dose (mg/kg)</th>
<th>Plasma insulin (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Day 42 (End of treatment)</td>
</tr>
<tr>
<td>Normal</td>
<td>Control (Vehicle)</td>
<td>-</td>
<td>1.459 ± 0.113</td>
</tr>
<tr>
<td>Diabetic</td>
<td>Control (Vehicle)</td>
<td>-</td>
<td>0.145 ± 0.0161**</td>
</tr>
<tr>
<td></td>
<td>Glibenclamide</td>
<td>5</td>
<td>0.189 ± 0.0076*</td>
</tr>
<tr>
<td></td>
<td><em>G. procumbens</em></td>
<td>50</td>
<td>0.144 ± 0.0252</td>
</tr>
<tr>
<td></td>
<td><em>G. procumbens</em></td>
<td>100</td>
<td>0.159 ± 0.0169</td>
</tr>
<tr>
<td></td>
<td><em>G. procumbens</em></td>
<td>150</td>
<td>0.156 ± 0.0158</td>
</tr>
</tbody>
</table>

Values are given as mean ± S.E.M. Diabetic controls were compared with normal controls and treated groups at the corresponding time-interval. Values are statistically significant at **p < 0.001 and *p < 0.05**

**TABLE 3. No inhibitory activity of *G. procumbens* against GSK-3 using yeast based GSK-3 assay**

<table>
<thead>
<tr>
<th>Fraction (Solvent)</th>
<th>Concentration (mg/ml)</th>
<th>mg of sample per disk</th>
<th>Inhibition zone (mm)</th>
<th>Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hexane</td>
<td>125 (80 μL)</td>
<td>10</td>
<td>37°C 25°C</td>
<td>No activity</td>
</tr>
<tr>
<td>BuOH</td>
<td>125 (80 μL)</td>
<td>10</td>
<td>0</td>
<td>No activity</td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>125 (80 μL)</td>
<td>10</td>
<td>0</td>
<td>No activity</td>
</tr>
<tr>
<td>H7667 (Positive control)</td>
<td>-</td>
<td>4</td>
<td>15 0</td>
<td>Positive</td>
</tr>
</tbody>
</table>
REFERENCES


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