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RESEARCH ARTICLE

Antidiabetic Properties and Mechanism of Action of *Orthosiphon stamineus* Benth Bioactive Sub-fraction in Streptozotocin-induced Diabetic Rats

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Abstract
Orthosiphon stamineus is a popular folk medicine widely used to treat many diseases including diabetes. Previous studies have shown that the sub-fraction of chloroform extract was able to inhibit the rise of blood glucose levels in a glucose tolerance test. This study was carried out to evaluate the chronic effect and possible mechanism of action of the bioactive chloroform sub-fraction of *O. stamineus* using streptozotocin-induced diabetic rats and *in vitro* methods. Administration of the chloroform extract sub-fraction 2 (C*f*2-b) at a dose of 1 g/kg twice daily on diabetic rats for 14 days showed a significant lowering ($p < 0.05$) of the final blood glucose level compared to the pretreatment level. However, there were no significant differences in the plasma insulin levels post-treatment compared to the pretreatment levels for all doses of C*f*2-b. Conversely, C*f*2-b at a concentration of 2 mg/mL significantly increased ($p < 0.001$) the glucose uptake by the rat diaphragm muscle. The increase in glucose uptake was also shown when the muscle was incubated in a solution containing 1 IU/mL of insulin or 1 mg/mL of metformin. Furthermore, the effect of this sub-fraction on glucose absorption in the everted rat jejunum showed that C*f*2-b at concentrations of 0.5 mg/mL, 1 mg/mL and, 2 mg/mL significantly reduced the glucose absorption of the jejunum ($p < 0.05$–0.001). Similarly, the absorption of glucose was also inhibited by 1 mg/mL and 2 mg/mL of metformin ($p < 0.001$). These results suggest that the effect of C*f*2-b may be due to extra-pancreatic mechanisms. There was no evidence that the plant extract stimulated the release of insulin in order to lower the blood glucose level.

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1. Introduction

The pathogenesis of diabetes mellitus and possibility of its management by the oral administration of antidiabetic agents, including those from folk medicines, have stimulated great interest in recent years. *Orthosiphon stamineus* Benth [syn: *Orthosiphon aristatus* (B1) Miq., *Orthosiphon grandiflorus* Bold., *Orthosiphon spicatus* (Thumb.) Bak.; Lamiaceae] [1] is one of the popular traditional folk medicines extensively used in Southeast Asia for the treatment of a wide range of diseases. It is used: (1) in Indonesia for rheumatism, diabetes, hypertension, tonsillitis, epilepsy, menstrual disorder, gonorrhea, syphilis, renal calculus, and gallstones [2], (2) in Vietnam for urinary lithiasis, edema, eruptive fever, influenza, hepatitis, jaundice, and biliary lithiasis [3], and (3) in Myanmar to alleviate diabetes, and urinary tract and renal diseases [4,5].

Due to its popularity and demonstrated effectiveness, phytochemical [6—8] and pharmacological studies [9—11] of this plant have been conducted since 1930. Highly-oxygenated isopimarane-type diterpenes, known as orthosiphols A—E, have been isolated, together with monoterpenes, triterpenes, saponins, flavonoids, hexoses, organic acids, rosmarinic acid, chromene, and myo-inositol. *O. stamineus* has also been reported to possess hypoglycemic and antihyperglycemic activities [12], and an aqueous extract exhibited a hypoglycemic effect in normal and streptozotocin (STZ)-induced diabetic rats, respectively. The present study tried to elucidate the antihyperglycemic or hypoglycemic effects of the plant on normal and diabetic rats when it has been extracted serially with different solvents. This method of extraction helps to streamline the chemical contents of the plant according to the polarity of the solvent. The profile and the dynamics of the blood glucose level of extract-treated rats were then determined using a subcutaneous glucose tolerance test. Theoretically, these tests should be able to screen hypoglycemic and antihyperglycemic agents. A hypoglycemic agent is an agent that is capable of reducing the blood glucose level below fasting levels, whereas antihyperglycemic agents lower the blood glucose level but not beyond the fasting level. Glibenclamide is a hypoglycemic agent, while metformin is antihyperglycemic or euglycemic agent.

The aim of the present study was to evaluate the antihyperglycemic activity of a sub-fraction (C*f*2-b) of chloroform extract from *O. stamineus* in normal and STZ-induced diabetic rats. The effects of this sub-fraction on the uptake of the glucose in the muscle and the inhibition of the glucose absorption in the gastrointestinal tract were also investigated (Fig. 1).

2. Materials and methods

2.1. Chemicals

Metformin (UPHA, Malaysia), glibenclamide (Hovid, Malaysia), STZ (Sigma, USA), and human insulin 100 IU/mL (Novo Nordisk, Denmark) were all used, and analyses were performed using thin layer chromatography (TLC plate Art-5554, Merck, Darmstadt, Germany; Fig. 2) and high-performance liquid chromatography (HPLC). Blood glucose levels were determined using the Accu-chek Advantage II Clinical Glucose meter (Roche Diagnostics Co. USA). The insulin concentration in the plasma samples was assayed by enzyme-linked immunoassay (ELISA) using a Rat Insulin ELISA Kit (Crystal Chem, IL, USA).

2.2. Animals

The normoglycemic female Sprague-Dawley rats weighing 200—250 g used in this study were obtained from the animal house of the School of Pharmaceutical Sciences, Universiti Sains Malaysia, Penang. The animals were kept at 25—30 °C, acclimatized with free access to food (normal laboratory chow, Gold Coin) and water *ad libitum* for 1 week under a 12-hour light, 12-hour dark cycle. All animals were carefully monitored and all of the experimental work with the animals was carried out after obtaining approval from the Universiti Sains Malaysia Animal Ethical Committee. For experimental purposes, animals were fasted overnight but had free access to water.

2.3. Plant material and preparation of the active fraction

O. stamineus Benth was collected from Kepala Batas, Pulau Pinang Malaysia. The plant was identified and a voucher specimen (No: 10810) was deposited at the herbarium at the School of Biological Sciences, Universiti Sains Malaysia. The plant material was prepared according to a previously performed guided fractionation of this plant [13]. It was found that the most active antihyperglycemic principles were in sub-fraction C*f*2-b of the chloroform extract. The C*f*2-b obtained was filtered and the solvent evaporated under vacuum using Rotavapor (Buchi, Switzerland). The extract was then frozen and freeze-dried (Labconco Cooperation, Denmark) at −40 °C for 24 hours to give a dried extract. The dried extract was stored in an airtight container, labeled and stored until use [13].

2.4. Phytochemical investigation of active sub-fraction C*f*2-b

Phytochemical tests were carried out to identify the various constituents of sub-fraction C*f*2-b and were compared to the reference compound (sinensetin) using the following chemical reagents: Dragendorff's reagent was used to determine the presence of alkaloids; a natural product reagent was used for flavonoids; the reagent antimony trichloride was used for terpenoids; and sulfuric acid was used for coumarins [14].

2.5. High-performance liquid chromatography study of C*f*2-b

HPLC analysis was performed using a Shimadzu LC system (Shimadzu, Japan) equipped with a CBM-20A controller, dual LC-20AT pumps, a DGU-20A5 degasser, an SIL-20A autosampler, a SPD-20AV detector, and a CTO-10ASvp column oven. Chromatographic separations were achieved using an Agilent Eclipse Plus C18 (250 × 4.6 mm i.d.; 5 μm)

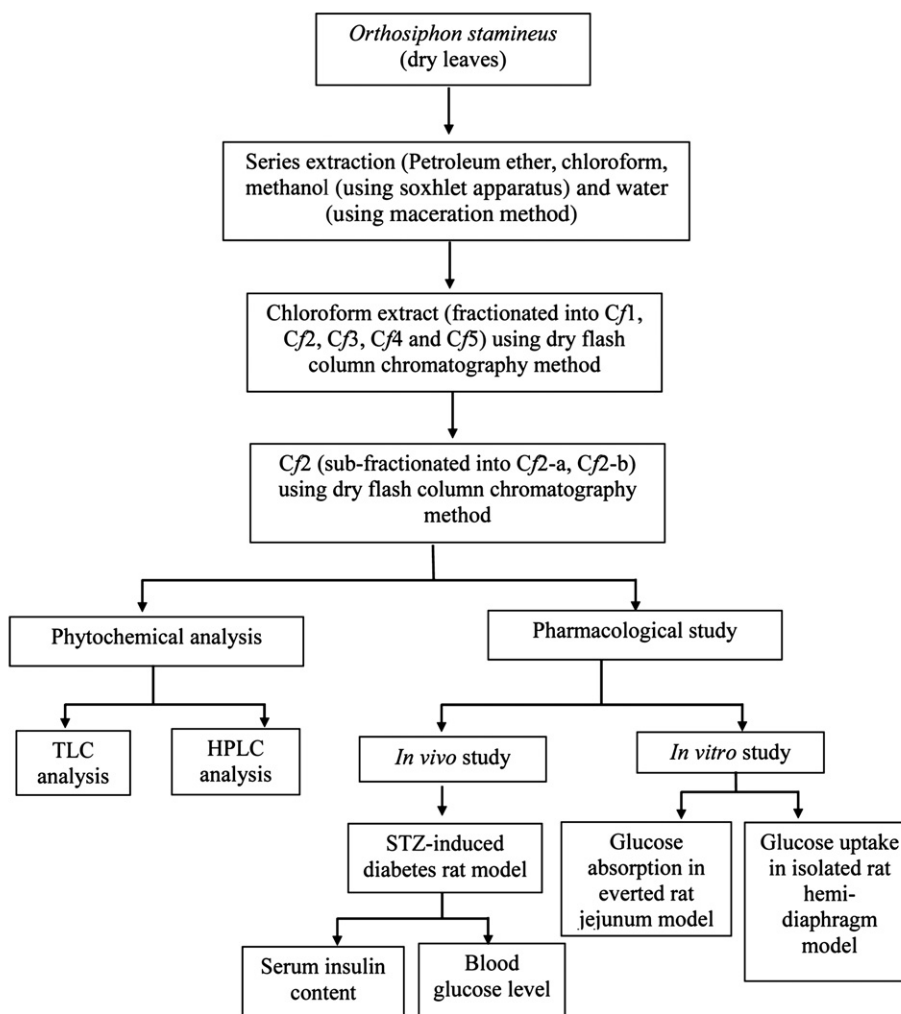

Figure 1 Schematic diagram of the antidiabetic study of *Orthosiphon stamineus*.

(Agilent Technologies, California, USA). A Zorbax guard fitting kit packed with a replaceable Eclipse Plus C18 Guard column (12.5 × 4.6 mm i.d.; 5 μm) (Agilent Technologies, California, USA) was used to protect the analytical column. A reverse-phase HPLC assay was carried out using an isocratic system with a flow rate of 1 mL/min, a column temperature of 25 °C, and a mobile phase of acetonitrile, isopropyl alcohol and 0.02 M phosphate buffer (NaH_2PO_4) (30:15:55, v/v) with pH adjusted to 3.5 using 85% phosphoric acid. The ultraviolet detection was set at 340 nm. The injection volume was 20 μl. The total run time was less than 20 minutes for each injection. Data were acquired and processed using LC-Solution Software. The peaks were detected at 340 nm and identified using standard substances, namely sinensetin, eupatorin, and 3′-hydroxy-5,6,7,4′-tetramethoxyflavone [15].

2.6. *In vivo* studies

2.6.1. Induction of experimental diabetes

Hyperglycemia was induced in rats via a single intraperitoneal injection of STZ (65 mg/kg) [16]. The STZ was freshly dissolved in citrate buffer (0.01 M, pH 4.5) [17] and kept on ice prior to use. The injection volume was 1 mL/kg [18,19]. One week after STZ administration, the blood glucose was measured and the animals were considered to have diabetes if the fasting blood glucose value was over 16.7 mmol/L [19,20].

2.6.2. The repeated administration of C*f*2-b in diabetic rats

Diabetes was successfully induced in 24 Sprague-Dawley rats (200—250 g) and these animals were randomly divided into four groups of six rats ($n = 6$). The groups were treated with either 5 mL/kg of normal saline as a negative control, 500 mg/kg of metformin as a positive control, or a sub-fraction C*f*2-b of *O. stamineus* extract (0.5 g/kg and 1.0 g/kg) orally twice daily for 14 days. The fasting blood glucose levels were measured after confirmation of diabetes and 14 days after the chronic oral treatment for comparison.

2.6.3. Determination of blood glucose and serum insulin levels

Blood samples were obtained from the tail vein of the diabetic rats at the beginning of the experiment and 14 days after the twice-daily treatment. A drop of blood was used each time for the determination of blood glucose levels using the Accu-chek Advantage II Clinical Glucose meter. In order

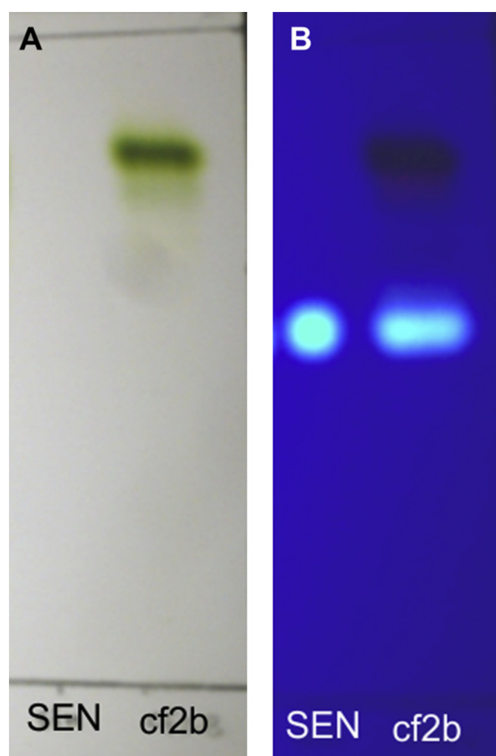

Figure 2 The thin-layer chromatography profile of sub-fraction (C*f*2-b) and sinensetin (SEN) after being developed with ethyl acetate:chloroform (75:25) as a mobile phase and sprayed with a natural product (NP/PEG) reagent under (A) visible light and (B) ultraviolet 365 nm.

to determine the concentration of insulin in the serum, a 75 μL blood sample was collected into hematocrit-capillary tubes (Hirschmann Laborgerate GmbH & Co. KG, Eberstadt, Germany) and centrifuged at 5000 rpm for 3—5 minutes. The plasma samples obtained were stored at −20 °C until they were measured for insulin concentration. The insulin concentration in the plasma samples (5 μL) was assayed by ELISA using a Rat Insulin ELISA Kit.

2.7. *In vitro* studies

2.7.1. Measurement of glucose absorption in the everted rat jejunum

Female Sprague-Dawley rats weighing 150—200 g were fasted for 16 hours before the experiment. They were then killed by a blow to the head, and dissected to remove the small intestine [21]. Segments located between 24.0 cm and 34.5 cm (jejunum) and 84.0—94.5 cm away from pylorus (ileum) [22] were excised, and placed in ice-cold Krebs Ringer solution (154 mM NaCl, 5.6 mM KCl, 5.5 mM glucose, 20.1 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, and 25 mM $NaHCO_3$, adjusted to pH 7.4 with NaOH). Then, the segments were rinsed with the buffer and everted onto a glass rod. They were then cut into 10 cm pieces ($n = 6$), rinsed and placed on filter paper to dry before being weighed. Each piece of the intestine was tied tightly around one end with a thread, while the other end only required a loose knot. Two milliliters of glucose-Ringer solution was injected into each sample using a slipping blunt needle, and the knot was then tightened before any excess tissue was trimmed off at each end. The jejunum samples were incubated in 30 mL of the following Krebs Ringer solutions in 50 mL beakers:

- Krebs Ringer solution (control);
- Krebs Ringer solution + different concentrations of metformin;
- Krebs Ringer solution + different concentrations of glibenclamide; and
- Krebs Ringer solution + different concentrations of C*f*2-b of *O. stamineus*.

The preparations were kept in an atmosphere of 95% oxygen and 5% carbon dioxide at 37 °C throughout the experiment. At the end of the 90-minute incubation period, the glucose concentration of the inside sacs and the incubation media were determined using the Accu-chek Advantage II Clinical Glucose meter. Results are expressed as mg of glucose absorption or amount of glucose transported/mg of tissue weight.

2.7.2. Measurement of glucose uptake in an isolated rat hemi-diaphragm

Glucose absorption by the rat diaphragm was examined according to the method devised by Frayn and Adnitt [23]. Rats weighing 150—200 g were killed by a blow to the head

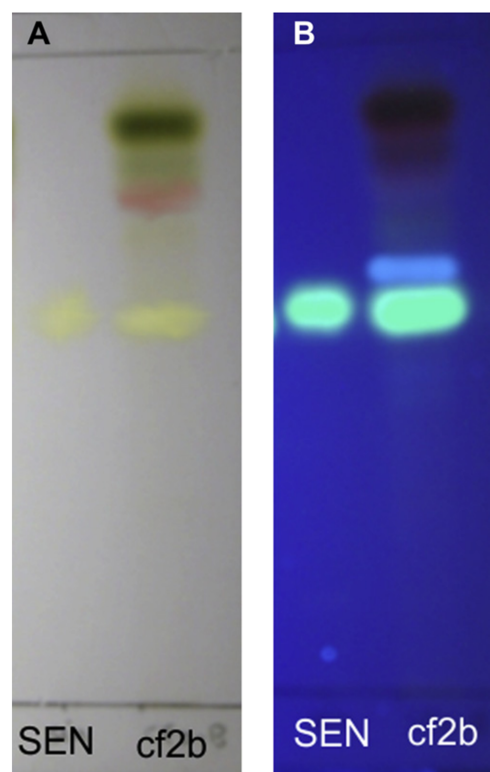

Figure 3 The thin-layer chromatography profile of sub-fraction (C*f*2-b) and sinensetin (SEN) after being developed with ethyl acetate:chloroform (75:25) as a mobile phase and sprayed with an antimony trichloride reagent under (A) visible light (B) ultraviolet 365 nm.

and dissected to remove the diaphragm. Each diaphragm was rinsed in ice-cold Krebs Ringer solution and then cut into two equal hemi-diaphragms, gently blotted and weighed. One hemi-diaphragm was placed in a small conical flask containing 2 mL of Krebs Ringer solution in an atmosphere of 95% O_2 and 5% CO_2. The other hemi-diaphragm from the same animal was placed into Krebs Ringer solution containing the test substance. The tissues were constantly shaken at 37 °C and incubated for 90 minutes. Duplicate samples of each medium were taken for glucose estimation before the placement of the tissue and at the end of the experiment. One hemi-diaphragm was used as a control and the other hemi-diaphragm from the same animal was used for the test experiment (using C*f*2-b of the *O. stamineus* extract). Equal numbers of left and right hemi-diaphragms were used for controls and test samples, respectively. The results obtained were expressed as the amount of glucose utilized by the hemi-diaphragms in 1 μg of glucose per gram of tissue during the 90-minute incubation period. The glucose concentration was measured using the Accu-chek Advantage II Clinical Glucose meter.

2.8. Statistical analysis

Statistical analysis of data was performed using the one-way analysis of variance, independent-sample *t* test and paired-sample *t* tests. The differences between the means were considered significant at the probability level $p < 0.05$. The statistical analysis was performed using the computer program SPSS (Release 11.5, SPSS Inc., 2001).

3. Results

3.1. Phytochemical investigation of C*f*2-b

Phytochemical screening of C*f*2-b in the present study indicated the presence of terpenoids and flavonoids, with

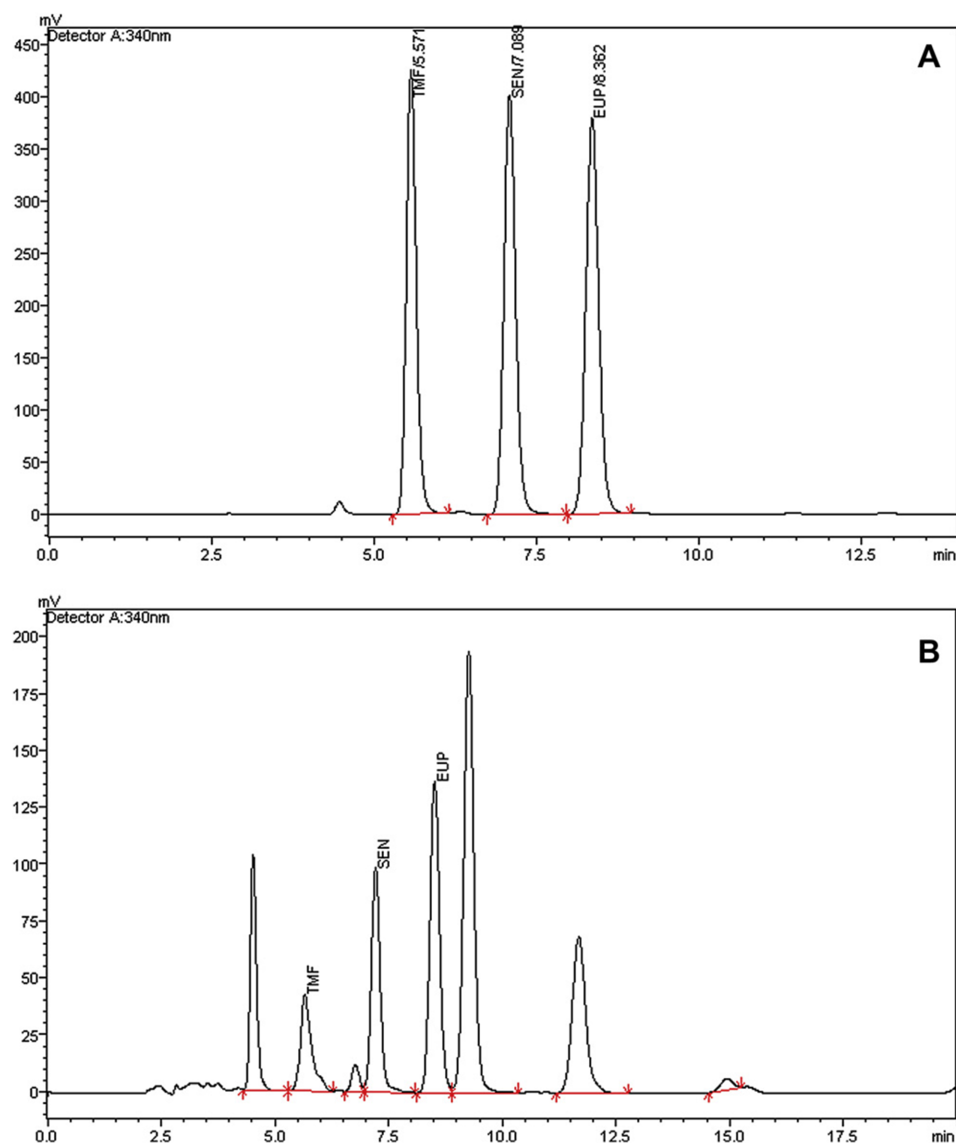

Figure 4 (A) High-performance liquid chromatogram of standard markers. Peaks for 3′-hydroxy-5,6,7,4′-tetramethoxyflavone (TMF), sinensetin (SEN), and eupatorin (EUP) are indicated. (B) High-performance liquid chromatogram of C*f*2-b. Peaks for TMF, SEN, and EUP are indicated.

one of the flavonoids being identified as sinensetin. However, alkaloids and coumarins were absent (Figs. 1 and 3).

3.2. High-performance liquid chromatography analysis

The percentages of sinensetin, eupatorin, and 3′-hydroxy-5,6,7,4′-tetramethoxyflavone in C*f*2-b were 1.48%, 2.26%, and 0.58%, respectively (Fig. 4).

3.3. *In vivo* studies

3.3.1. The effect of repeated administration of C*f*2-b on the blood glucose and insulin levels of diabetic rats

The twice-daily oral treatment with C*f*2-b at 1.0 g/kg for 14 days caused a significant decrease ($p < 0.05$) in blood glucose levels in the post-treatment samples, compared to the pretreatment levels of diabetic rats (Fig. 5). Similarly, metformin (500 mg/kg) caused a significant lowering ($p < 0.001$) of blood glucose levels at the post-treatment compared to the pretreatment level (Fig. 6). Fig. 6, however, shows that no significant increase was found in the post-treatment plasma insulin levels compared to the pretreatment levels of the metformin 500 mg/kg- and C*f*2-b 1.0 g/kg-treated groups.

3.4. *In vitro* studies

3.4.1. The effects of sub-fraction C*f*2-b on glucose absorption in the everted rat jejunum

Fig. 7A shows that the everted small intestine absorbed glucose during incubation in Krebs solution over a 90-minute period. The addition of glibenclamide at concentrations of 0.01 mg/mL, 0.1 mg/mL, and 1 mg/mL did not significantly change the amount of glucose absorbed. By contrast, incubation in metformin 0.5 mg/mL began to reduce the amount of glucose absorbed, although this was not statistically significant (Fig. 7B). Moreover, the

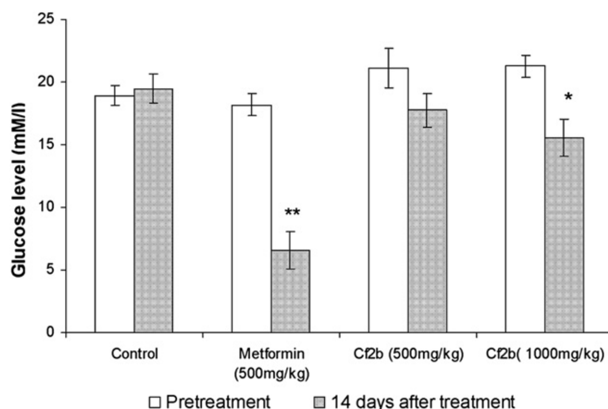

Figure 5 The effects of the oral administration of sub-fraction C*f*2-b (0.5 g/kg and 1 g/kg) on the blood glucose levels of streptozotocin-induced diabetic rats, twice daily for 14 days. The values are presented as mean ± S.E.M. ($n = 6$). * Significant difference ($p < 0.05$); ** significant difference ($p < 0.01$) between pretreatment and post-treatment.

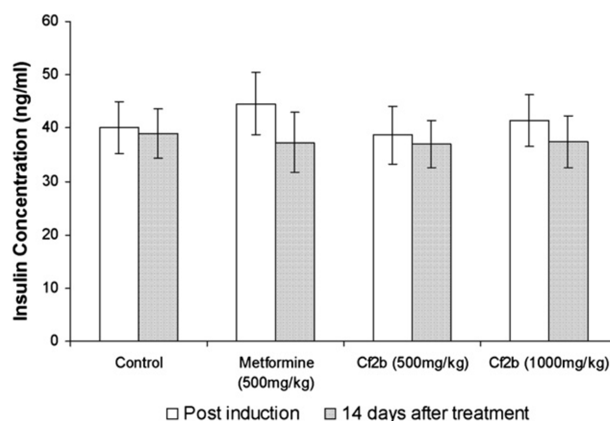

Figure 6 The effects of the oral administration of sub-fraction C*f*2-b (0.5 g/kg and 1 g/kg) twice daily for 14 days on insulin levels of streptozotocin-induced diabetic rats. The values are presented as mean ± S.E.M. ($n = 6$).

increased concentration of metformin of 1 mg/mL significantly ($p < 0.05$) inhibited the absorption of glucose in the everted small intestine. Further doubling of the concentration of metformin to 2 mg/mL reduced the extent of the inhibition of glucose absorption, although it was still statistically significant ($p < 0.05$) when compared to Krebs Ringer solution alone. The amount of glucose absorption of the everted small intestine was also significantly ($p < 0.05$) inhibited by sub-fraction C*f*2-b at concentrations of 0.5 mg/mL, 1.0 mg/mL, and 2 mg/mL (Fig. 7C). Similar to Fig. 7B, the addition of metformin 1 mg/mL significantly inhibited ($p < 0.05$) glucose absorption by the intestine in the Krebs Ringer solution (Fig. 7D). However, the further addition of sub-fraction 2 mg/mL of C*f*2-b in the Krebs Ringer solution containing metformin 1 mg/mL did not inhibit the absorption further (Fig. 7D).

3.4.2. The effects of sub-fraction C*f*2-b on glucose uptake in the isolated rat hemi-diaphragm

The uptake of glucose by the hemi-diaphragm in the Krebs solution was not significantly increased by metformin 0.01 mg/mL or 0.1 mg/mL. The higher concentration of 1 mg/mL of metformin, however, significantly increased ($p < 0.05$) the uptake of glucose by the hemi-diaphragms during the 90-minute incubation period (Fig. 8A). Fig. 8B shows that the uptake of glucose from the incubation media (Krebs Ringer solution) by hemi-diaphragms in the presence of insulin 1 IU/mL was significantly higher ($p < 0.05$) than in the Krebs Ringer solution alone. The addition of metformin 1 mg/mL in the Krebs solution containing insulin 1 IU/mL significantly ($p < 0.05$) increased the uptake of glucose compared to the Krebs Ringer solution containing same amount of insulin (Fig. 8C). By contrast, the uptake of glucose by the hemi-diaphragm during the 90-minute incubation period was not significantly increased by sub-fraction C*f*2-b 0.5 mg/mL and 1 mg/mL. However, when incubated in the presence of a higher concentration of C*f*2-b (2 mg/mL), the glucose utilized by the hemi-diaphragm was significantly higher ($p < 0.05$) than in the Krebs Ringer solution alone (Fig. 8D). Although the utilization of glucose by the hemi-diaphragm was increased in the

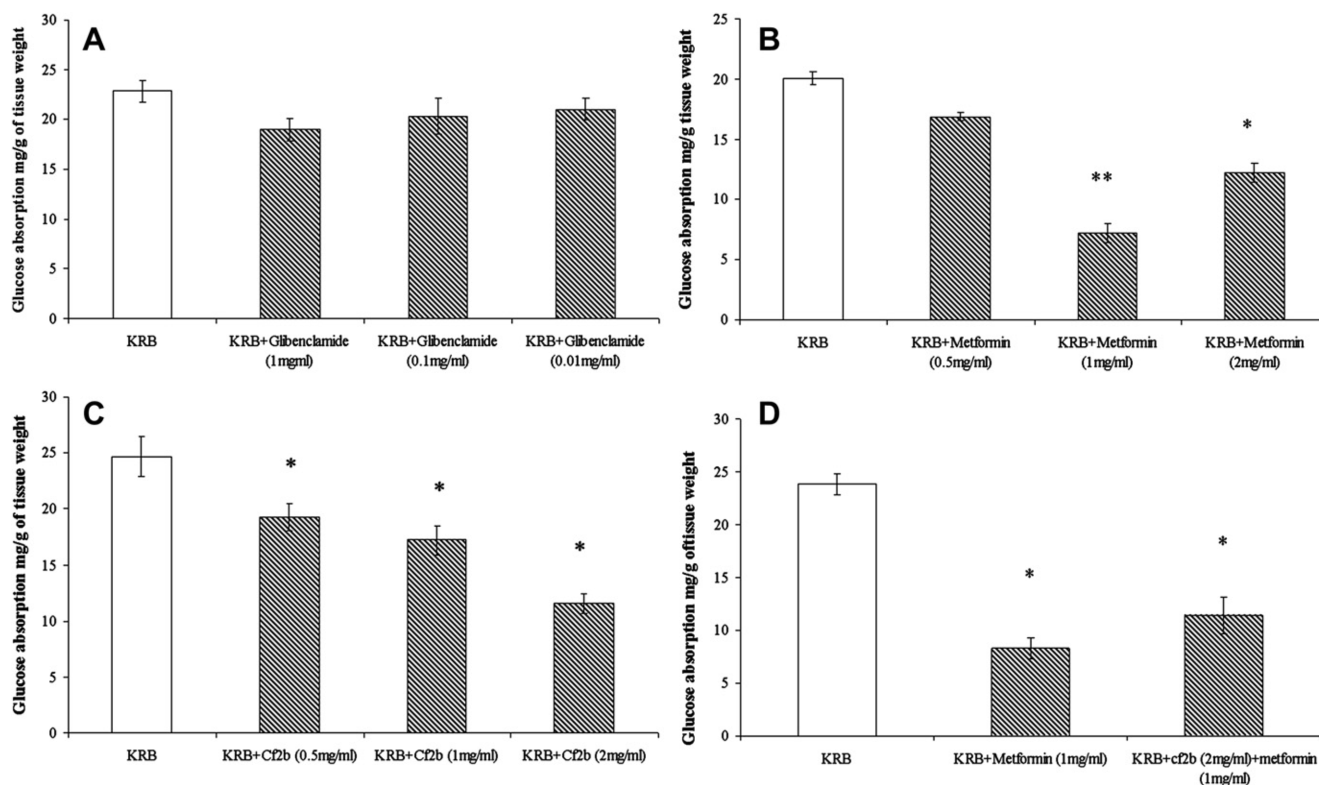

Figure 7 The effect of different concentrations of (A) glibenclamide, (B) metformin, (C) sub-fraction (C*f*2-b), and (D) sub-fraction (C*f*2-b) 2 mg/mL and metformin 1 mg/mL on the glucose absorption of isolated everted rat small intestine incubated for 90 minutes in Krebs solution. The values are presented as mean $\pm$ S.E.M. ($n = 6$). * Significant difference ($p < 0.05$); ** significant difference ($p < 0.01$) between the control group and glibenclamide, metformin, and C*f*2-b treated groups.

presence of insulin 1 IU/mL (Fig. 8B), the further addition of sub-fraction C*f*2-b 2 mg/mL in the presence of insulin 1 IU/mL did not significantly change the amount of glucose uptake as compared to the Krebs Ringer solution containing same amount of insulin (Fig. 8E).

4. Discussion

The use of STZ to induce diabetes in rats was widely utilized due to its specificity in destroying only the β-cells of the islets of Langerhans. The intermediate dose of 65 mg/kg intraperitoneally manages to induce the diabetic state, and the rats were able to survive without insulin supplementation up to the end of the 14-day study period. This finding corresponded with those of Junod et al and Ar'Rajab and Ahren [24,25]. A novel result reported in the present study indicates that the twice daily treatment with a sub-fraction of chloroform extract from *O. stamineus* (C*f*2-b) at a dose of 1.0 g/kg for 2 weeks results in the reduction of fasting blood glucose level in STZ-induced diabetic rats. This finding is in line with that of Mariam et al [12], who reported that the aqueous extract of the *O. stamineus* plant reduced the blood glucose level of STZ-induced diabetic rats.

The blood glucose level of the post-treatment animals after a repetitive dose regimen with metformin was significantly lower compared to the pre-treatment level. This result is similar to that found by Zhang and Tan [26]. The lowering of blood glucose levels after repeated doses of metformin in diabetic rats is not expected, as the antidiabetic drug metformin, a biguanide, is not known to stimulate the release of insulin from pancreatic β-cells. The lowering of blood glucose levels by metformin was presumably a result of the physiological response of several tissues, including increases of glucose uptake by skeletal muscle and adipose tissue [27,28]. In a previous work [13], it was reported that C*f*2-b produced an antihyperglycemic effect in a subcutaneous glucose tolerance test in normal rats. The effect of C*f*2-b mimicked the effect of metformin; it has no hypoglycemic effect in normal rats but an antihyperglycemic effect in the glucose tolerance test. This suggests that the extract may have no direct stimulatory effect on insulin secretion, similar to metformin.

The possible mechanism of action of C*f*2-b was studied using *in vitro* model techniques. In order to study glucose transport in peripheral tissues *in vitro*, the commonly used techniques involved cultures of skeletal muscle strips or cells, and the isolated diaphragm from rat [23] or from humans obtained by surgical excision [29]. The effects of natural products on glucose uptake and metabolism in peripheral tissues have also been studied using fragments or a homogenate of the rat small intestine [30]. Among these methods, the isolated rat diaphragm and everted rat jejunum methods were chosen in the present study to evaluate the glucose uptake and glucose absorption, respectively, in an attempt to elucidate the mechanisms of action of the antihyperglycemic agents of C*f*2-b. The previous study

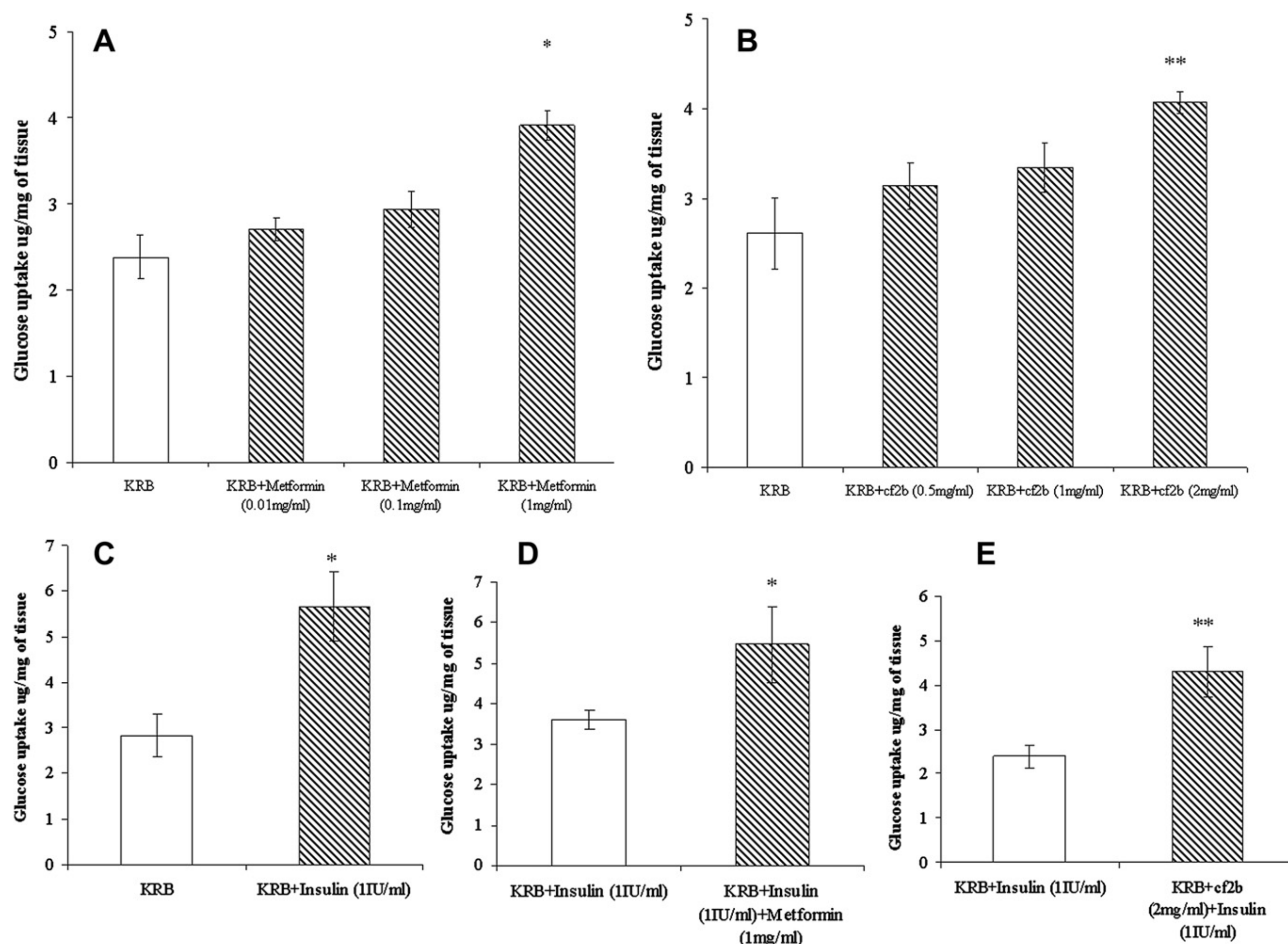

Figure 8 The effect of (A) metformin, (B) insulin, (C) metformin (Met + Ins), (D) sub-fraction (C*f*2-b), and (E) sub-fraction (C*f*2-b) on the glucose uptake of isolated rat hemi-diaphragm incubated at 37 °C for 90 minutes in Krebs solution. The values are presented as mean ± S.E.M. ($n = 6$). * Significant difference ($p < 0.05$); ** significant difference ($p < 0.01$) between the control group and metformin, insulin and C*f*2-b treated groups.

carried out by Gray and Flatt has indicated the usefulness of this preparation for comparative studies of insulin and other drug actions, as well as the insulin-like effects of other natural products [31]. The effect of C*f*2-b was examined at 0.5 mg/mL, 1 mg/mL, and 2 mg/mL in order to study its effect on glucose uptake by the isolated skeletal muscle of the rat diaphragm in the absence and presence of insulin. In the absence of insulin, sub-fraction C*f*2-b induced a dose-dependent increase in glucose uptake by the diaphragm, which means that C*f*2-b may act like insulin regarding the uptake of glucose, by facilitating glucose transport across plasma membranes into cells or by increasing glucose transport into the cells, which increases the metabolism of carbohydrates. In the presence of both insulin and C*f*2-b, the utilization of glucose was not further increased, which means that C*f*2-b might not improve insulin action in the muscle. Instead, it suggests that C*f*2-b has an antihyperglycemic effect due to its extra-pancreatic action. One of the extra-pancreatic actions occurs as a result of stimulating glucose utilization in peripheral tissues, such as the diaphragm [32,33]. The above findings indicate that the effects of C*f*2-b on glucose uptake are probably the result of a direct insulin-like effect on the utilization of glucose, which occurs by increasing the membrane transport of glucose in peripheral tissues. Similarly, metformin at a concentration of 1 mg/mL increased glucose uptake by the diaphragm in the absence of insulin. This metformin-induced improvement in glucose disposal has been attributed predominantly to the action of metformin, which facilitates the translocation of glucose transporters from intracellular sites to the plasma membrane of rat skeletal muscle [34] and increases their intrinsic activity [35]. This finding is in accordance with previous studies in which various concentrations of metformin were used [23]. In the present study, however, metformin at a concentration of 1 mg/mL in the presence of insulin at 1 IU/mL further increased the glucose utilization. These results are in agreement with the previous study by Frayn and Adnitt [23], which showed that metformin not only enhanced the peripheral utilization of glucose, but also improved the insulin action in muscle and fat tissues. This was attributed, in part, to the increased movement of insulin-sensitive glucose transporters into the cell membrane. Another action of metformin involves the insulin-independent suppression of fatty acid oxidation and a reduction in hypertriglyceridemia. The possible

mechanism of action of C*f*2-b in this case is probably its immediate membrane effect, which results in increasing glucose transport as well glucose utilization, especially by muscle. One possible action of C*f*2-b is the direct insulin-like effect on the utilization of glucose, resulting in an increase in the uptake of glucose into muscle and adipose tissue. It is a crucial component of the physiological response to insulin.

In the everted rat jejunum, the results clearly show that C*f*2-b at concentrations of 0.5 mg/mL, 1 mg/mL, and 2 mg/mL inhibited intestinal glucose absorption. Similarly, it was found that the absorption of glucose was also inhibited by metformin. The finding that metformin inhibited glucose absorption is in agreement with the findings of the previous studies by Klip and Leiter [35], Caspary and Creutzfeldt [36], Lorch [37], and Bailey [38]. In addition, Wilcock and Bailey [39] demonstrated that metformin decreases glucose transport in the inverted sacs of the intestine from hamsters, rats, and mice. The present results are consistent with their studies. Furthermore, the lowest dose of metformin (0.5 mg/mL) failed to inhibit glucose absorption while the highest dose (2 mg/mL) inhibited the glucose absorption by less than the 1 mg/mL dose. The extent of the inhibition by metformin may therefore depend upon the concentration of metformin. Love reported that metformin inhibits glucose absorption in the everted rat jejunum at concentrations greater than 1.5 mg/mL or 2 mg/mL, but causes increased absorption of glucose when present at lower concentrations [40]. Lenzen et al reported that metformin significantly increased the energy-dependent sodium-hexose cotransporter (*SGLT1*) gene expression and facilitative hexose transporter *GLUT5* gene expression in the jejunum [41]. This offers the potential for an increase in hexose uptake at the brush border membrane. Increased intestinal glucose utilization by metformin would be expected to consume a greater proportion of the intracellular glucose taken up from the jejunum, which accounts, at least in part, for the apparent reduction in glucose transport [42]. Since glucose absorption from the intestine was decreased significantly by metformin as well as C*f*2-b, the efficacy of C*f*2-b may also be due to the increase in the gene expression of SGLT1 and GLUT5 in the rat jejunum enterocytes. This offers the potential for an increase in hexose uptake at the brush border membrane, and may compensate for other effects of the C*f*2-b that suggest a decrease in glucose absorption by SGLT1. Thus, the inhibition of glucose absorption when C*f*2-b is in direct contact with the brush border membrane of the rat small intestine might be one of the possible mechanisms responsible for its antihyperglycemic activities. In this case, one possible mechanism of the antihyperglycemic effect of C*f*2-b is related to the inhibition of the active transport of intestinal glucose absorption, which is similar to metformin.

Thin layer chromatography screening of C*f*2-b indicated the presence of terpenoids and flavonoids. HPLC analysis also indicated that flavonoids, namely sinensetin, eupatorin, and 3′-hydroxy-5,6,7,4′-tetramethoxyflavone, are present in C*f*2-b. Since previous studies have indicated that flavonoids and terpenoids produce antidiabetic activity [43–45], these compounds could be responsible for the antihyperglycemic effect of C*f*2-b, whether acting separately or synergistically. Although C*f*2-b may have no direct stimulatory action on insulin secretion, it might produce the antihyperglycemic effect through an extra-pancreatic mechanism. Another possible mechanism observed in the present study by which C*f*2-b exerts an antihyperglycemic effect is by inhibition of the active transport of intestinal glucose absorption. The findings of the present study suggest that the extract may be effective in non-insulin dependent diabetes mellitus.

References

1. Suresh A, Yasuhiro T, Banskota AH, Kadota H, Siphonols A-E. Novel nitric oxide inhibitors from *Orthosiphon stamineus* of Indonesia. *Bioorg Med Chem Lett*. 2003;13:31–35.
2. Bwin DM. Plants with reputed hypoglycaemic action. In: *Burmese Indigenous Medicinal plant: 1*. Burma: Burma Medical Research Institute; 1967:126.
3. Eisai PT. *Medicinal Herb Index in Indonesia*. 2nd ed. Indonesia: PT Eisai; 1995:263.
4. WHO. Regional office for the Western Pacific Manila and Institute of Material Medica Hanoi. *Medicinal Plants in Vietnam*. Hanoi: Science and Technology Publishing House; 1970.
5. Ameer OZ, Salman IM, Asmawi MZ, Ibraheem ZO, Yam MF. *Orthosiphon stamineus*: traditional uses, phytochemistry, pharmacology, and toxicology. *J Med Food*. 2012;15:678–690.
6. Takeda Y, Matsumoto T, Terao H, Shingu TF, Nohara T, Kajimoto T. Orthosiphol D and E, minor diterpenes from *Orthosiphon stamineus*. *Phytochemistry*. 1993;33:411–415.
7. Masuda T, Masuda K, Shiragami S, Jitoe A, Nakatani N. Orthosiphols A and B, novel diterpenoid inhibitors of TPA (12-O-tetradecanoylphorbol-13-acetate)-induced inflammation, from *Orthosiphon stamineus*. *Tetrahedron*. 1992;48:6787–6792.
8. Sumaryono W, Proksch P, Wray V, Witte L, Hartmann T. Qualitative and quantitative analysis of the phenolic constituents from *Orthosiphon aristatus*. *Planta Medica*. 1991;57:176–180.
9. Beaux D, Fleurentin J, Mortier F. Effect of extracts of *Orthosiphon stamineus* Benth, *Hieracium pilosella* L., *Sambucus nigra* L. and *Arctostaphylos uva-ursi* (L.) spreng. in rats. *J Phytotherapy*. 1999;13:222–225.
10. Doan DD, Nguyen NH, Doan HK, Nguyen TL, Phan TS, Van DN, et al. Studies on the individual and combined diuretix effects of four Vietnamese traditional herbal remedies (*Zea mays*, *Imperata cylindrical*, *Plantago major* and *orthosiphon stamineus*). *J Ethnopharmacol*. 1992;36:225–231.
11. Nirdnoy M, Muangman V. Effects of folia orthosiphon on urinary stone promoters and inhibitors. *J Med Assoc Thail*. 1991;74:318–321.
12. Mariam A, Asmawi MZ, Sadikun A. Hypoglycaemic activity of the aqueous extract of *Orthosiphon stamineus*. *Fitoterapia*. 1996;67:465–468.
13. Mohamed EAH, Mohamed AJ, Asmawi MZ, Sadikun A, Ebrika OS, Yam MF. Antihyperglycemic effect of *Orthosiphon stamineus* Benth leaves extract and its bioassay-guided fractions. *Molecules*. 2011;16:3787–3801.
14. Wagner H, Bladt S, Zgainski EM. *Plant Drug Analysis: A Thin Layer Chromatography Atlas*. USA: Springer-Verlag; 1984.
15. Yam MF, Mohamed EA, Ang LF, Pei L, Darwis Y, Mahmud R, et al. A Simple Isocratic HPLC method for the simultaneous determination of sinensetin, eupatorin, and 3′-hydroxy-5,6,7,4′-tetramethoxyflavone in *Orthosiphon stamineus* extracts. *J Acupunct Meridian Stud*. 2012;5:176–182.
16. Perez C, Dominguez E, Canal JR, Campillo JE, Torres MD. Hypoglycaemic activity of an aqueous extract from *Ficus carica* (fig tree) leaves in streptozotocin diabetic rats. *Pharm Biol*. 2000;38:181–186.

17. Ozsoy-Sacan O. Effects of chard (*Beta vulgaris* L. varcicla) extract on pancreatic h-cells in STZ-diabetic rats: a morphological and biochemical study. *J Ethnopharmacol*. 2000;73: 251—259.
18. Hamilton K, Eaton EJ, Garland HO, Old S. Effects of experimental diabetes mellitus on gentamicin-induced acute renal functional changes in the anesthetized rats. *Clin Exp Pharmacol Physiol*. 1998;25:231—235.
19. Murali B, Upadhyaya UM, Goyal RK. Effect of chronic treatment with Enicostemma littorale in non-insulin-dependent diabetic (NIDDM) rats. *J Ethnopharmacol*. 2002;81:199—204.
20. Cetto AA, Wiedenfeld H, Revilla MC, Sergio I. A hypoglycemic effect of Equisetum myriochaetum aerial parts on streptozotocin diabetic rats. *J Ethnopharmacol*. 2000;72:129—133.
21. Yongyot M, Thirayudth G. Inhibitory effect of capsaicin on intestinal glucose absorption *in vitro*. *Food Cosmet Toxicol*. 1978;16:469—473.
22. Andres CA. Simple *in vitro* perfusion system to measure intestinal nutrient uptake. *J Nutr Biochem*. 1998;9:52—57.
23. Frayn KN, Adnitt PI. Effect of metformin on glucose uptake by isolated diaphragm from normal and diabetic rats. *Biochem Pharmacol*. 1972;21:3153—3162.
24. Junod A, Lambert AE, Orci L, Pictet R, Gonet AE, Renold AE. Studies of the diabetogenic action of streptozotocin. *Proc Soc Exp Biol Med*. 1967;126:201—205.
25. Ar'Rajab A, Ahren B. Prevention of hyperglyceamia improves the long-term result of islet transplantation in streptozotocin-diabetic rats. *Pancreas*. 1992;7:435—442.
26. Zhang XF, Tan BK. Antihyperglycaemic and anti-oxidant properties of *Andrographis paniculata* in normal and diabetic rats. *Clin Exp Pharmacol Physiol*. 2000;27:358—368.
27. Defronzo RA, Barzilai N, Simonson DC. Mechanism of metformin action in obese and lean non-insulin-dependent diabetic subjects. *J Clin Endocrinol Metab*. 1991;73:1294—1301.
28. Johnson AB, Webster JM, Sum CF, Heseltine L, Argyraki M, Cooper BG. The impact of metformin therapy on hepatic glucose production and skeletal muscle glycogen synthase activity in overweight type II diabetic patients. *Metabolism*. 1993;42: 1217—1222.
29. Kashwagi A, Verso MA, Andrews J, Vasquez B, Reaven G, Foley JE. *In vitro* insulin resistance of human adipocytes isolated from subjects with noninsulin-dependent diabetes mellitus. *J Clin Investig*. 1983;72:1246—1254.
30. Hikino H, Ishiyama M, Suzuki Y, Konno C. Mechanisms of hypoglycemic activity of ganoderan B: glycan of Ganoderma lucidum fruit bodies. *Planta Med*. 1989;55:423—428.
31. Gray AM, Flatt PR. Pancreatic and extrapancreatic effects of the traditional antidiabetic plant *Medicago sativa* (Lucerne). *Br J Nutr*. 1997;78:325—334.
32. Naik SR, Filho JMB, Dhuley JN, Deshmukh A. Probable mechanism of hypoglycaemic activity of basic acid, a natural product isolated from *Bumelia sartorum*. *J Ethnopharmacol*. 1991;33:37—44.
33. Obatomi DK, Bikomo EO, Temple VJ. Anti-diabetic properties of the African mistletoe in streptozotocin-induced diabetic rats. *J Ethnopharmacol*. 1994;43:13—17.
34. Hundal HS, Ramial T, Reyes R, Leiter LA, Klip A. Cellular mechanism of metformin action involves glucose transporter translocation from an intracellular pool to the plasma membrane in L6 muscle cells. *Endocrinology*. 1992;131:1165—1173.
35. Klip A, Leiter LA. Cellular mechanism of action of metformin. *Diabetes Care*. 1990;13:696—704.
36. Caspary WF, Creutzfeldt W. Analysis of the inhibitory effect of biguanides on glucose absorption: inhibition of sugar transport. *Diabetologia*. 1971;7:379—385.
37. Lorch E. Inhibition of intestinal absorption and improvement of oral glucose tolerance by biguanides in the normal and streptozotocin-diabetic rat. *Diabetologia*. 1971;7:195—203.
38. Bailey CJ. Biguanides and NIDDM. *Diabetes Care*. 1992;15: 755—772.
39. Wilcock C, Bailey CJ. Reconsideration of inhibitory effect of metformin on intestinal glucose absorption. *J Pharm Pharmacol*. 1990;43:120—121.
40. Love AHG. The effect of biguanides on intestinal absorption. *Diabetologia*. 1969;5:422.
41. Lenzen S, Tiedge M, Jorns A, Munday R. Alloxan derivatives as a tool for the elucidation of the mechanism of the diabetogenic action of alloxan. In: Shafrir E, ed. *Lessons from Animal Diabetes*. Boston: Springer; 1996:113—122.
42. Ikeda T, Iwata K, Murakami H. Inhibitory effect of metformin on intestinal glucose absorption in the perfused rat intestine. *Biochem Pharmacol*. 2000;59:887—890.
43. Lu YX, Zhang Q, Li J, Sun YX, Wang LY, Cheng WM, et al. Antidiabetic effects of total flavonoids from *Litsea coreana* leave on fat-fed, streptozotocin-induced type 2 diabetic rats. *Am J Chin Med*. 2010;38:713—725.
44. Jung M, Park M, Lee HC, Kang YH, Kang ES, Kim SK. Antidiabetic agents from medicinal plants. *Curr Med Chem*. 2006;13: 1203—1218.
45. Tan MJ, Ye JM, Turner N, Hohnen-Behrens C, Ke CQ, Tang CP, et al. Antidiabetic activities of triterpenoids isolated from bitter melon associated with activation of the AMPK pathway. *Chem Biol*. 2008;15:263—273.