Original Article

Orthosiphon stamineus as a potential antidiabetic drug in maternal hyperglycemia in streptozotocin-induced diabetic rats

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ABSTRACT

Background: Maternal hyperglycemia is associated with increased risk of adverse outcomes for both mother and offspring. Insulin is the standard treatment of hyperglycemia with the aim to reduce risks of complications, however, due to several restrictions, the search for more effective drugs from traditional medicinal plants continues.

Methods: The antidiabetic effects of *Orthosiphon stamineus* (*O.stamineus)* in non-pregnant and pregnant streptozotocin-induced Sprague Dawley rats were identified. The effect of different concentrations of *O. stamineous*on insulin level using isolated pancreatic islets in response to low and high concentrations of glucose was identified. Oral glucose tolerance test was performed in both pregnant and non-pregnant rats prior to and after treatment with *O. stamineus* (0.1 g/100 g of body weight). *O. stamineus* was given orally daily for 2 weeks in non-pregnant and 10 days in pregnant rats.

Results: Oral glucose tolerance test indicated that treatment with *O. stamineus* in non-pregnant and pregnant rats significantly reduced blood glucose level and stimulated glucose-induced insulin secretion. No mortality was recorded throughout the study and no signs of toxicity during the experimental period including in both mother and foetus. For plasma analysis, the interactions of peptides such as GLP-1 and ghrelin level might contribute to the glucose lowering effect by *O. stamineus* via stimulation of insulin. The incubation of islets showed that *O. stamineus*significantly stimulated insulin release in response to high glucose.

Conclusion: O. stamineus could be a potential source of a specific oral hypoglycaemic agent to treat glucose intolerance in pregnancy.

1. Introduction

Gestational diabetes mellitus (GDM) is defined as glucose intolerance in pregnancy which includes women with undiagnosed pre-existing diabetes and with first-onset hyperglycemia without history of pre-existing diabetes mellitus.[1,2] Plasma glucose level for GDM when diagnosed, is either above 5.6 mmol/L (fasting) or 7.8 mmol/L (after 2-h), as defined by National Institute for Health and Care Excellence (NICE) Guidelines.[3] Hyperglycemia in pregnancy leads to increased risk of various adverse maternal and infant outcomes such as caesarean delivery, preeclampsia, shoulder dystocia, macrosomia, neonatal hypoglycemia and perinatal death.[4,5]

Insulin has been used for a long time in diabetes management when dietary and lifestyle modifications inadequately achieve persistent euglycaemia. However, administration of insulin requires multiple daily injections causing discomfort to patients. In addition to insulin, for the past few years, there has been increasing research conducted including the observational, randomized controlled trials studies and various types of diabetic animal models to identify if the use of certain oral hypoglycaemic agents such as metformin and glyburide may be suitable in pregnancy. Oral agents have been increasingly viewed to be a potential substitute to insulin

for their easier administration, lower cost and better acceptance. However, concerns over the efficacy and safety of its use during pregnancy for both mother and foetus are still being studied.[6,7] There are also several issues concerning currently available diabetes therapies including limited actions, secondary failure rates and side effects such as hypoglycaemia, weight gain and gastrointestinal disturbances.[8]

Following the restrictions with currently available therapies, there is a great demand for research using natural products based with antidiabetic properties since plants are considered to be less toxic with less side effects compared to the synthetic ones. To date, numerous research and literature has focused on the discovery of new antidiabetic drug from traditional plants and the mechanisms involved in glucose lowering effects in certain plants have also been explored either by improving insulin secretion or extra pancreatic mechanisms.[9,10]

In this study, we explored the potential effects of medicinal plant, *Orthosiphon stamineus* (*O. stamineus*) which is known as 'Misai kucing' against gestational diabetes. It belongs to the *Lamiaceae* family and is widely grown in many countries particularly Southeast Asia including Malaysia,[11] has gained interests due to the beneficial pharmacological effects such as diabetes, diabetic wound healing,[12] hypolipidemic and antiobesity,[13] bone-protective and antiosteoporotic,[14] prevention of bladder and kidney infection.[15] The biological activities of *O. stamineus* are attributed to the presence of several active compounds including rosmarinic acid, phenolic, flavonoid, amino acids and coumarin.[16,17]

The effects of *O. stamineus* in diabetes have been performed previously in animal and *in vitro* studies, however the mechanisms are not fully elucidated particularly in maternal hyperglycaemia.[18–21] In this current study, *O. stamineus* extract was given orally to streptozotocin-induced Sprague Dawley rat to identify if it has the potential in managing maternal hyperglycemia and to understand the mechanism of actions involved in lowering blood glucose level. This finding is important for the development of an alternative methods rather than insulin or oral hypoglycaemic agents for the treatment of maternal hyperglycemia.

2. Methods

2.1. Chemicals used for O. stamineus extraction

Formic acid, methanol and LCMS grade acetonitrile were purchased from MERCK (Malaysia). HPLC grade water was prepared from distilled water using a Milli-Q-system (Millipore, MA,USA) and was used during analytical UHPLC analysis. Standards used were rosmarinic acid (Chromadex, CA, USA), caffeic acid, chlorogenic acid, eupatorin and sinensetin (Extrasynthase, Genay, France). All of other solvents and chemicals used in this study were analytical grade. Stock and working standards were prepared by dissolving these analytes in 100% methanol. The standard solutions stored at 4°C were stable for at least 3 months.

2.2. Extract and standard preparation

Fresh *O. stamineus* leaves were purchased from HerBagus (Penang, Malaysia). Samples were cleaned and washed thoroughly prior to drying at 40°C, then grinded to fine powder. Samples were extracted with a boiling water and filtered using Whatman No. 1 filter paper. The filtrate was filtered further using 22 μm membrane filter before subjected to HPLC analysis.

2.3. Ultra high performance liquid chromatography–mass spectrometry electron spray inonization source analysis

The ultra high performance liquid chromatography (UHPLC) was performed on a Dionex 3000 UHPLC system (Thermo Fisher Scientific, MA, USA) that consisted of an autosampler equipped with a column oven, a tray compartment cooler, and a binary pump with built in solvent degasser. The chromatographic separation was performed on a BEH C18 UHPLC column, 100 mm × 2.5 μm, 1.7 μm (Waters, MA, USA) at a flow rate of 0.2 mL/min. The mobile phases used were (A) 0.1% formic acid in water and (B) 0.1% formic acid in acetonitrile. The gradient started with 15% mobile phase B, reaching 50% mobile phase B at 20 min, at isocratic elution of 90% B for 3 min. The gradient reached the initial conditions were held for 2 min as a re-equilibration step. The injection volume was 10 μL and the column temperature was maintained at 40°C.

The UHPLC system was coupled to a linear ion-trap-Orbitrap mass spectrometer Q Exactive (Thermo Fisher Scientific, MA, USA) equipped with an electrospray ionization (ESI) source. The mass detection was performed in a range of 150–1500m/z. The ESI source was operated in negative ion mode under the following specific conditions: source voltage, 3.2 kV; sheath gas, 35 arbitrary units; auxiliary gas, 15 arbitrary unit; sweep gas, 10 arbitrary unit; and capillary temperature, 320°C. Nitrogen (>99.98%) was employed as sheath gas, auxiliary and sweep gas. Instrument control and data acquisition were performed with Chameleon 6.8 software and Xcalibur 2.2 software (Thermo Fisher Scientific, MA, USA).

2.4. Animal

Healthy virgin females and confirmed fertile males (2:1) Sprague Dawley rats with a body weight ranging from 170 to 200 g and 200 to 250 g, respectively were obtained from the Animal Resource Unit, Medical Resource Centre, Institute for Medical Research, Kuala Lumpur. The animals were housed in individual ventilated cages, supplied with osmosis drinking water and diet (Specialty Feeds Pty Ltd., Glenn forrest, Australia), *ad libitum* with controlled temperature of (20 ± 2°C), 40–60% humidity under 12 hours of light and dark cycle. The animals were acclimatized for about a week prior to start of the study. Ethical approval for this study was obtained from the Animal Care and Ethics Committee, Ministry of Health Malaysia (ACUC No: ACUC/KKM/02 (08/2016)).

2.5. In vitro experiments

2.5.1. Isolation of islets with collagenase digestion

Non-diabetic Sprague Dawley female rats were selected to study the effects of *O.stamineus* on healthy rat islets. The rats ($n = 6$) (mean body weight: 219.4 ± 21.3 g) were euthanized in a tank supplied with CO_2. Isolation of islets using collagenase digestion and batch incubation of islets were performed using methods as previously described.[22] The bile duct was injected with collagenase solution. The pancreas collected was then incubated at 37°C in a digital block heater (Select Bioproducts, NJ, USA), resuspended with a syringe and washed with Hanks' Balanced Salt Solution (HBBS) buffer (Sigma Aldrich, MO, USA), Histopaque 1119 (Sigma Aldrich) and Histopaque 1077 (Sigma Aldrich) for several times. The islets found between the Histopaque 1077 (Sigma Aldrich) and Hanks buffer layer were then collected, picked and transferred into filtered RPMI-1640 medium (Sigma Aldrich) supplied with L-glutamine, 11 mM glucose and PESTcontaining Fetal Bovine Serum (FBS) (10%). The islets were then incubated in the CO_2 incubator overnight at 37°C.

2.5.2. Insulin release with batch incubation methods

D(+)-Glucose (HmBG, Malaysia) was prepared at low (3.3 mM) and high (16.7 mM) concentration in KRB solution (Krebs-Ringer bicarbonate buffer) pH 7.4 containing stockchlorin, KH_2PO_4, $\cdot CaCl_2$ $2H_2O$, $NaHCO_3$, hepes and albumin. The overnight cultured islets were pre-incubated in low glucose for approximately 30–45 min. For batch incubation of islets, 3 islets were incubated in O. stamineus extract at different concentrations; 0.1, 1.0 and 10.0 µg/mL. To assess the insulin secretory response to glucose, the islets were incubated in low and high glucose. To assess the amount of insulin secreted during the islet incubation, ELISA kit (Cat. No. EZRMI-13K) (Merck, Darmstadt, Germany) was used and then further analysed using Multiscan Go. Rat/Mouse Insulin ELISA kit (Cat No EZRMI-13K) (Merck, Darmstadt, Germany) was used to measure the insulin content.

2.6. In vivo experiments

2.6.1. Diabetic induction, mating and treatment

For diabetic induction, the rats were administered with a single-dose intra peritoneal injection with Streptozotocin at 30 mg/kg (Sigma Aldrich) to induce mild hyperglycaemia by partially destroying the β-cells of the islets of Langerhans. The rats with fasting blood glucose level more than 7 mmol/L were selected in this study. For mating of the animals, diabetic female rats were mated overnight with non-diabetic male rats (2:1). The day of sperm detected in vaginal smear was considered as Day 0 of pregnancy and pregnant female rats were housed in the individually ventilated cage. For mortality and maternal clinical observations, rats were monitored closely and once a day after treatment to observe for any clinical signs of maternal toxicity such as piloerection, vaginal bleeding, diarrhoea, alteration in locomotion, dull fur, emaciation, sedation, soft stool, urination or maternal deaths.

For both non-pregnant and GDM groups, the rats were randomly assigned to four treatment groups, caged individually. *Group 1*: control non-diabetic (vehicle); *Group 2*: control diabetic group (vehicle); *Group 3*: positive control (Glibenclamide) (0.6 mg/kg); *Group 4*: treatment (0.1 g/100 g of body weight of O. stamineus extract). For non-pregnant rats, O.stamineus was given daily for 14 days whereas in pregnant rats, for 10 days after organogenesis period. Several parameters such general behaviour, body weight, feed and water intake were recorded throughout the study.

2.6.2. Oral glucose tolerance test

Glucose tolerance test was performed to measure the body's ability to utilize glucose. Rats were left fasted overnight prior to glucose tolerance test. Fasting blood glucose level for all rats were measured prior to glucose load by tail prick using a glucometer, Accucheck Aviva Plus (Roche, Mannheim, Germany), followed by glucose (0.2 g/kg of body weight) administration to all rats. Blood glucose level was measured at 0, 30, 60 and 120 min using a glucometer, Accucheck Aviva Plus (Roche, Mannheim, Germany). Blood was also collected at 0, 30, 120 min in heparin tubes for insulin content from plasma measurement. Blood was then centrifuged at 2500 × g for 10 min and plasma was transferred into 1.5 mL Eppendorf tubes and kept at −20°C.

2.6.3. Caesarean hysterectomy and assessment of maternal visceral organs, reproductive performance and foetal parameters

After treatment, on day 21 of pregnancy, the rats were euthanized using isofluorene supplied with O_2 and maternal blood was collected by cardiac puncture. Plasma was collected from blood after being centrifuged at 3000 × g for 15 min to measure blood glucose and several analytes including insulin, cholesterol, glucagon, glucagon like peptide 1 (GLP-1) and ghrelin level. The rats were then necropsied and examined by a veterinarian. The number of live and dead foetuses was recorded. The foetuses were then removed and dried from amniotic fluid. Total implantation sites, plus early and late resorptions were counted and recorded.

2.6.4. Analytes analysis

Glucose and cholesterol level were measured using PrimePlex Hematology Kit using BioLis 24i Premium Chemistry Analyzer machine. Insulin, ghrelin and GLP-1 were processed using Elisa Kit (Elabscience, Wuhan, China).

2.7. Statistical analysis

Results were presented as mean ± standard deviation (SD). The results were evaluated using non-parametric, Kruskal–Wallis test, followed by Dunn–Bonferroni *post hoc* test for multiple comparison between experimental groups. Bonferroni adjustment correction values were used and *p*-values < 0.05 were considered to be statistically significant. All statistical analysis was performed using SPSS 21.0.

3. Results

3.1. Identification of phenolic compounds in O. stamineus

O. stamineus was analysed based on the accurate mass data of the molecular ions, in which ions detected were tentatively identified by their generated molecular formula, through the software Data analysis (Xcalibur) which provided list of possible elemental formulas, together with the use of standard when available and after thorough survey of the literature. The widely accepted accuracy threshold for confirmation of elemental compositions was established at 5 ppm. The UHPLC-ESI analysis of *O. stamineus* extract revealed the presence of 5 polyphenols compounds such as rosmarinic acid, chlorogenic acid, caffeic acid, sinensetin and eupatorin. The peak number, retention time, compound name, observed *m/z*, and the generated molecular formula are presented in Fig. 1. The compounds were identified based on their retention time with the reference standards implied which is similar to the previous study.[23] Sinesetin and eupotorin found in this plant were reported to have diuretic activity in rats.[24]

3.2. In vitro study: effects of different concentrations of O. stamineus on isolated diabetic pancreatic islets

Different concentrations of *O. stamineus* were added to pancreatic islets obtained from diabetic Sprague Dawley rats to identify the effects of the extract on the stimulation of insulin release. At low glucose, no stimulation of insulin was observed at baseline level and even in the presence of *O. stamineus* at different concentrations. At high glucose, in the presence of *O. stamineus*, insulin release was increased by 7.7, 30.3 and 102.4 times than control islets at 0.1 (0.6 ± 0.2), 1.0 (2.3 ± 0.6; *p* < 0.05) and 10.0 (7.9 ± 0.4; *p* < 0.001) ng/mL respectively.

3.3. Morbidity, mortality, abnormalities and body weight changes, feed and water intake

All control and both treated pregnant and non-pregnant rats survived to scheduled sacrifice. No behavioural alterations and other clinical signs of toxicity were observed among animals treated with *O. stamineus*. The average number of lives fetuses was comparable between control, positive and treated groups. No significant external abnormalities on fetuses were found in all groups.

No significant differences were observed in body weight changes between different groups in both non-pregnant and pregnant rats as shown in Table 1. In non-pregnant rats, feed intake
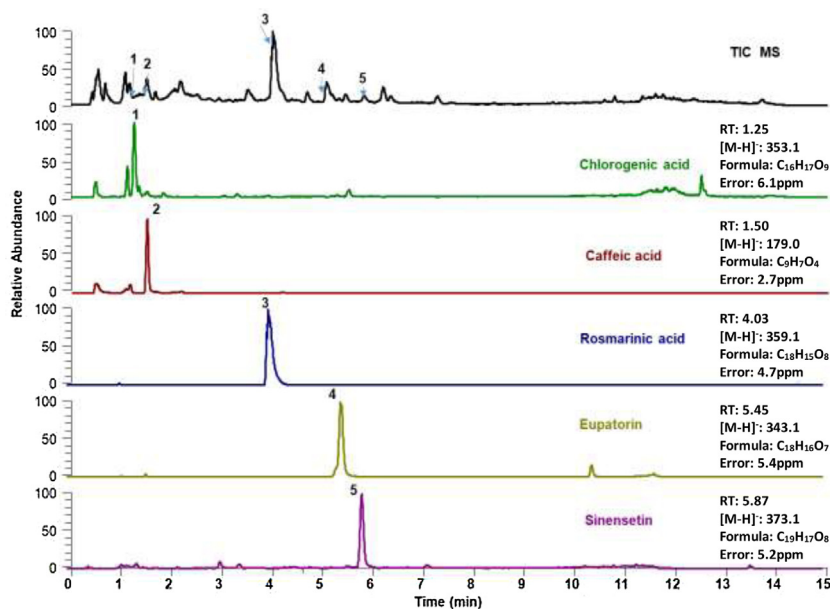
Fig. 1. Representative UHPLC-MS traces of *O. stamineus* in negative ion mode. Assigned peaks number labelled the chemical marker found in this plant. RT: retention time.

Table 1
Effects of Treatment With *O.stamineus* (0.1 g/100 g of Body Weight) on Body Weight Change in Non-pregnant (2 Weeks' Treatment) and Pregnant Rats (10 Days' Treatment)

Parameter	Treatment groups							
	Non-pregnant				Pregnant			
	Control non-diabetic	Control diabetic	Glibenclamide (0.6 mg/kg)	OS (0.1 g/100 g)	Control non-diabetic	Control diabetic	Glibenclamide (0.6 mg/kg)	OS (0.1 g/100 g)
Initial weight (g)	167.9 ± 3.7	192.4 ± 16.8	184 ± 16.0	190.5 ± 13.3	207.8 ± 18.5	210.5 ± 13.1	214.9 ± 2.0	217.7 ± 20.3
Final weight (g)	197.5 ± 7.0	222.6 ± 22.9	218 ± 12.7	222.8 ± 17.2	291.4 ± 14.0	286.7 ± 26.6	288.7 ± 30.3	282.7 ± 42.6
Body weight change (g)	29.6 ± 7.0	30.2 ± 6.5	34 ± 6.6	32.3 ± 8.7	83.6 ± 6.4	76.1 ± 16.8	73.8 ± 14.2	65 ± 28.3
Percentage body weight change (%)	17.6 ± 4.3	15.6 ± 2.1	18.7 ± 4.8	16.9 ± 4.4	40.7 ± 6.6	36.0 ± 6.9	34.4 ± 6.2	29.5 ± 11.4

Values are presented as mean ± SD ($n = 6$ in each group). OS: *O. stamineus*.

was the highest in the *O. stamineus* group at 358 ± 20.7 g, followed by diabetic 357 ± 42.6 g, glybenclamide 348.1 ± 16.3 g and control non-diabetic 328.5 ± 21.6 g, with no significant differences between different groups. In pregnant rats, rats treated with glybenclamide group consumed feed most at 267.7 ± 3 g, followed by control diabetic group at 254 ± 25.5 g, *O. stamineus* at 252.6 ± 27.9 g and control non-diabetic at 235.8 ± 14.6 g, with no significant different observed in different groups. In non-pregnant rats, the mean water intake was the highest in the *O. stamineus* group followed by control diabetic, glybenclamide and control diabetic at 721.2 ± 51.3, 721 ± 27.8, 713 ± 75.8 and 711.7 ± 64.1 mL respectively with no significant difference observed in different groups. The mean water intake was the highest in the glybenclamide group followed by control diabetic, control non-diabetic and *O. stamineus* at 546.8 ± 43.4, 535.7 ± 54.7, 534.3 ± 32.0 and 508.8 ± 36.8 mL respectively in pregnant rats with no significant difference observed in different groups.

3.4. Oral glucose tolerance test in non-pregnant rats

O. stamineus was administered in rats to identify the effects on blood glucose and insulin levels. After 2 weeks' treatment, significant differences were observed between different groups (Fig. 2a). At 120 min, mean blood glucose level for control non-diabetic, control diabetic, glybenclamide and *O. stamineus* was at 4.5 ± 0.6, 8.2 ± 1.2, 4.9 ± 1.1, 4.8 ± 0.5 mmol/L respectively. For area under the glucose curve (AUC), significant differences were observed in the

different groups (Fig. 2b). For insulin level, significant differences were observed at different time points between different groups (Fig. 2c).

3.5. Oral glucose tolerance test in pregnant rats

As shown in Fig. 2d, after 10 days' treatment, significant differences were observed between different groups. At 120 min, mean blood glucose level for control non-diabetic, control diabetic, glybenclamide and *O. stamineus* was at 5.2 ± 1.1, 9.8 ± 1.3, 5.1 ± 0.4 and 4.7 ± 0.2 mmol/L respectively. For AUC, after treatment, significant differences were observed in the different groups (Fig. 2e). For insulin level, significant differences were observed at each time point between different groups (Fig. 2f).

3.6. Plasma analysis in non-pregnant and pregnant rats

Plasma from both non-pregnant and pregnant rats was also collected during necropsy for measurement of analytes such as blood glucose, insulin, cholesterol, glucagon-like peptide (GLP-1) and ghrelin level during fasting state and significant differences were observed in different groups (Table 2). Ghrelin which is produced in several tissues including pancreas and liver influences glucose homeostasis through the modulation of insulin secretion and insulin receptor signalling.[25] GLP-1 is released from intestinal influences blood glucose homeostasis by delaying gastric emptying,
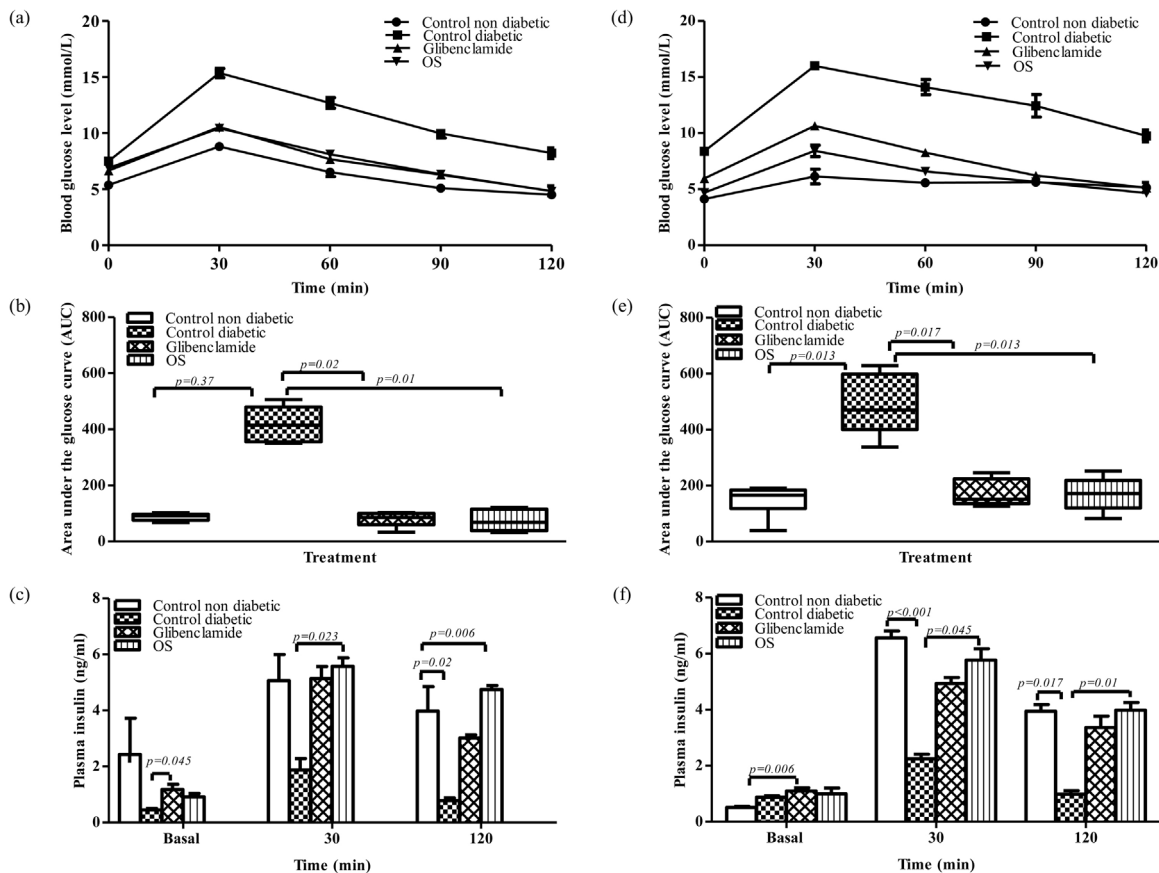
Fig. 2. (a) Blood glucose level; (b) Area under the glucose curve; (c) Insulin content after 2 weeks' treatment in non-pregnant rats. (d) Blood glucose level; (e) Area under the glucose curve; (f) Insulin content after 10 days' treatment in pregnant rats. All data were expressed as mean ± SD (n = 6 in each group).OS:O. stamineus.

Table 2
Effects of Treatment With *O. stamineus* (0.1 g/100 g of Body Weight) on Analytes in Nonpregnant (After 2 Weeks) and Pregnant Rats (After 10 Days)

Analytes	Treatment groups							
	Non-pregnant				Pregnant			
	Control non-diabetic	Control diabetic	Glibenclamide (0.6 mg/kg)	OS (0.1 g/100 g)	Control non-diabetic	Control diabetic	Glibenclamide (0.6 mg/kg)	OS (0.1 g/100 g)
FBG (mmol/L)	7.1 ± 0.6	10.7 ± 0.5**	9 ± 0.5†	8.2 ± 0.1§	4.6 ± 0.5	8.9 ± 1.2**	6.8 ± 0.4†	5.9 ± 0.2§
Cholesterol (mmol/L)	1.9 ± 0.2	2.7 ± 0.2**	2.4 ± 0.2†	2.2 ± 0.8§	1.4 ± 0.2	2.9 ± 0.5**	2.2 ± 0.1†	1.8 ± 0.2§
Insulin (pg/mL)	94.6 ± 9.2	34.7 ± 14.8**	67.7 ± 12.6	131.2 ± 13.5§§,#	134.7 ± 3	39.4 ± 6**	60 ± 8.1†	94 ± 11.1§
Ghrelin (pg/mL)	427.1 ± 334.1	133.5 ± 10.5*	216.2 ± 85.5	418.2 ± 125.1§§	301.4 ± 28.9	170.9 ± 35.1**	215.2 ± 13†	256 ± 14.1§
GLP-1 (pg/mL)	5905.6 ± 1423.1	2583.8 ± 192.6**	3617 ± 289.8†	4626.2 ± 235.3§	1087 ± 405	184.8 ± 90.2**	359.8 ± 38.4†	559.2 ± 138.8§

Values are presented as mean ± SD (n = 6). FBG: fasting blood glucose; GLP-1: glucagon-like peptide 1; Con = control; OS: *O. stamineus*.

* $p < 0.05$;
** $p < 0.01$, Con non-diabetic vs. Con diabetic;
† $p < 0.05$, Con non-diabetic vs. Gli.
§ $p < 0.05$.
§§ $p < 0.01$, Con diabetic vs. OS.
# $p < 0.05$, Gli vs. OS.

enhancing pancreatic insulin secretion and suppressing pancreatic glucagon secretion.[26]

4. Discussion

Type 2 diabetes is characterized by pancreatic islets dysfunction and insulin resistance which leads to hyperglycemia.[10] Medicinal plants with antidiabetic potential have been explored since ancient times for diabetes management. Antidiabetic effects of medicinal plants are mediated via several mechanisms including enhanced in insulin secretion, glucose utilization by adipose and muscle tissues or inhibition of glucose absorption from intestine and glucose output from liver.[9] To identify the antidiabetic effects by *O. stamineus*, we first confirmed the insulin stimulatory effects with *in vitro* study using isolated pancreatic islets from Sprague Dawley rats. Principally, the mechanisms of insulin secretion from pancreatic beta cells involves glucose uptake, closure of kalium channel, cell depolarization, calcium entry and eventually leads to insulin release.[27] The incubation of islets with different concentrations of *O. stamineus* resulted in a significant stimulation of insulin release in response to high glucose, whereas no stimulatory effect was detected at low glucose concentration. The exact target of OS by which it promotes insulin release in the pancreatic islets however has yet to be identified.

To further confirm if *O. stamineus* has an effect on blood glucose and insulin levels, the extract was given orally daily to the non-pregnant diabetic rats. After observing the potent glucose lowering effects by *O. stamineus*, we further treated it in diabetic pregnant rats. The results showed that treatment with *O. stamineus* reduced blood glucose level in both non-pregnant and pregnant rats. No significant changes on the body weight of foetuses and the absence of toxicity signs of non-pregnant and pregnant rats, suggesting that *O. stamineus* did not induce systematic toxicity. This is further supported by our study where no significant changes were found in maternal body weight between all groups in both non-pregnant and pregnant rats with low percentage in body weight changes, indicating no harmful and detrimental effects with *O. stamineus*. Similar findings were reported previously on prenatal developmental studies where no maternal toxicity effects on pregnant rats as well as foetuses after 20 days treatment at 2000 mg/kg body weight/day.[28] In the non-pregnant group, blood glucose, area under the glucose curves and insulin level showed significant differences in response to 2 weeks' treatment with *O. stamineus* as compared to control diabetic rats. The experiment was further carried out in the pregnant diabetic rats with a daily intake of *O. stamineus* for 10 days which also resulted in significant differences in the blood glucose, area under the glucose curves and insulin level. The rats gradually developed diabetes after being injected with streptozotocin one week prior to conception phase. The treatment only started after the completion of embryonic organogenesis stage which takes about 7 days from the gestational day 0. It has also been shown in the previous studies that *O. stamineus* treated up to fourteen days in rats up to 5 g/kg of body weight would not cause any harm, severe toxic effects and organ damages in rats.[29–31]

We also evaluated the insulin, cholesterol, ghrelin and GLP-1 levels from fasting plasma samples to further explore the interactions of these peptides with glucose regulation. In both non-pregnant and pregnant rats, our finding showed that in diabetic animals treated with *O. stamineus*, both ghrelin and GLP-1 level were significantly increased as compared to diabetic group suggesting that the insulin and low glucose lowering effects might be associated with the interactions of proteins. In addition, animals treated with *O. stamineus* showed significantly lower cholesterol level as compared to control diabetic group. Ghrelin which is present in both human and rats is known to be involved in some metabolic functions including control of appetite, energy balance,[32] glucose homeostasis[33] and insulin regulations.[34] Studies have discovered the association of ghrelin with obesity, type 2 diabetes and metabolic syndrome.[26] Our results showed that despite elevated ghrelin level detected in non-pregnant and pregnant rats in the *O. stamineus*-treated group, the amount of feed and water consumed was not affected, which might explain why no significant differences observed in body weight changes.

Previous studies showed that lower concentration of ghrelin was detected in type 2 diabetes mellitus as compared to normal subjects.[27,28] Another peptide, incretin hormone known as GLP-1 which is produced from the intestine, also acts at the islets of Langerhans in the endocrine pancreas to stimulate insulin secretion in response to glucose and suppress glucagon release.[26] Several studies on GLP-1 secretagogues activity of medicinal plants have been reported previously.[35–37] We speculate that the increase of GLP-1 level in the animal treated with *O. stamineus* showed that it contributes, at least in part, to the antidiabetic activity of blood glucose via incretin effect, which is enhanced of insulin secretion.

In addition, based on the *in vitro* results and insulin content from oral glucose tolerance test results, we speculate that the decrease of blood glucose level in the diabetic rats might be associated mainly with the insulin stimulatory activity by *O. stamineus*. This is further supported by a previous study using perfused rat pancreas, indicating potentiated glucose-induced insulin secretion at 100 μg/mL of *O. stamineus* Benth aqueous extract. The presence of phenolic and/or flavonoid compounds in the extract may be associated with antidiabetic activity.[18] In addition, several other classes of chemicals was also found in *O. stamineus* such as terpenoids, caffeic acid derivatives and chromene.[16,17]

The effect of *O. stamineus* previously was found to be associated with increased insulin sensitivity. The OS chloroform extract subfraction 2 (C*f*2-b) showed no direct stimulatory effects on insulin secretion or blood glucose levels in streptozotocin-induced diabetic rats but however exhibited an antihyperglycemic effect in normoglycemic rats.[20] It was shown that C*f*2-B fraction exerts its effect via increased glucose uptake by the rat diaphragm muscle and reduced glucose absorption.[19,21] Our findings suggested that *O. stamineus* extract maybe effective in lowering the blood glucose level in both non-pregnant and pregnant rats partly via stimulation of insulin release which could be probably triggered by several peptides interactions. The presence of active compound present in *O. stamineus* might contribute to its antidiabetic properties. The mechanisms by which *O. stamineus* enhanced insulin secretion should be further investigated. The findings may be important and useful for clinical trial and drug development with the aim to improve health outcomes for patients.

Conflict of interest

The authors declare no conflict of interest.

Funding

Ethical statement

Ethical approval for this study was obtained from the Animal Care and Ethics Committee, Ministry of Health Malaysia (ACUC No: ACUC/KKM/02 (08/2016)).

Data availability

The data will be made available upon request.

Acknowledgements

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