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INVESTIGATION OF HYPOGYCEMIC AND HYPOLIPIDEMIC EFFECTS OF AN AQUEOUS EXTRACT OF *LUPINUS ALBUS* LEGUME SEED IN STREPTOZOTOCIN-INDUCED TYPE I DIABETIC RATS.

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Abstract

Background: *Lupinus albus* (LA) seed is a legume food used traditionally for the treatment of diabetes. The aim of this study was to investigate the effects of an aqueous extract of LA on lipid and glucose levels in normal and STZ induced Type 1 diabetic rats.

Methods: Aqueous extract of LA was prepared and used for animal treatments. Diabetes was induced by a single intraperitoneal injection of streptozotocin (60mg/kg body weight). Effects of LA on oral glucose tolerance in normal and diabetic rats were investigated by giving a single dose of distilled water (controls), 200 or 400mg/kg LA extract, metformin 300mg/kg or glibenclamide 500µg/kg after 12 hours of fasting (time 0 glucose). After 15 minutes, a glucose load (3g/kg) was given. Glucose levels were measured at 30, 60, and 120 minutes after glucose loading. To investigate long term effects, animals were given similar treatments daily for 4 weeks. At the end of the study, serum glucose, insulin, total cholesterol (TC), triglycerides (TG), low density lipoprotein (LDL-C), very low density lipoprotein (VLDL-C) and high density lipoprotein (HDL-C) cholesterol levels were measured or calculated.

Results: LA demonstrated significant ($P<0.001$) hypoglycaemic effects in normal rats but not in diabetic rats after acute and long term treatment. Normal LA treated rats showed higher ($P<0.001$) insulin levels compared to normal controls but insulin remained very low in diabetic rats. Diabetic rats showed diabetes associated weight loss in both treated and untreated rats. However, LA was effective in reducing atherogenic lipid levels (TC, TG, LDL-C VLDLC; $P<0.001$) with no change ($P>0.05$) in HDL-C.

Conclusion: We conclude that the use of *Lupinus albus* among various communities may not be effective in treating hyperglycaemia in type 1 diabetes but effective for treating diabetes induced dyslipidemia.

Key words: glucose tolerance, lipids, *Lupinus albus*, streptozotocin, type 1 diabetes.

Introduction

Plants have historically served as a source of food and drugs. The term "nutraceutical" was coined by Stephen De Feliceto blending the two plant functions (Scarafoni et al., 2007). Nutritionally, there is a worldwide shift towards using plant sources as protein in place of animal sources. This is deemed a healthier dietary choice towards the reduction of diet related diseases such as obesity, hypertension, cardiovascular disease and type II diabetes (Arnoldi and Greco, 2011). As such, storage proteins from legumes have been the choice substitute for animal proteins. Soya bean is the most used legume that also possesses cholesterol reducing effects (Anderson et al., 1995; Sirtori et al., 1998; Sirtori et al., 2007).

Lately, there has been a growing interest in other legumes as protein sources for human nutrition. One such legume is white lupin, *Lupinus albus*. This is an annual plant belonging to the class Leguminosae. It is grown in the Middle East and Africa as a food legume. In South Africa it is mostly grown in the Western Cape for animal feed (Brand and Brandt, 2000). In addition to being consumed as food, *L. albus* seed is used medicinally. In traditional medicine the lupin seed is used as an anti-helminthic and anti-parasitic agent as well as an anti-diabetic agent (Eskander and Won Jun, 1995; Mansour et al., 2002). *L. albus* is rich in lupin proteins that belong to the vast families of 11S and 7S globulins of which conglutin-γ makes up 5% of the total globulins. Isolated lupin conglutin-γ has demonstrable hypoglycaemic effects in glucose loaded non diabetic experimental animals (Duranti et al., 1981; Magni et al., 2004; Bertoglio et al., 2011) and show insulin-mimetic effects in cultured cells (Komatsu and Hirano, 1991; Terruzzi et al., 2011). In addition, alkaloids from *L. albus*, is responsible for the bitter taste, also possess hypoglycaemic effects via increased insulin secretion in cultured pancreatic cells (Garzia Lopez et al., 2004; Oraby et al., 2008). Furthermore, crude aqueous extract of *L. albus* stimulates the release of insulin from cultured pancreatic islet cells (Pereira et al., 2001).

Human trials using lupin conglutin-γ on healthy non-diabetic individuals confirmed its insulin releasing actions (Bertoglio et al., 2011). All these data were obtained in experimental conditions with intact pancreatic beta cells *in vitro* and *in vivo*. However, no work has been done to investigate the effects of *L. albus* in conditions of depleted pancreatic beta cells mirroring type 1 diabetes. Type 1 diabetes is characterized by impaired insulin secretion leading to hyperglycaemia. Because insulin plays a major role in lipid metabolism, the absence of insulin is associated with dyslipidemia, a risk factor for cardiovascular disease and stroke (Pereira et al., 2001; Carmena, 2009). As such, in current management of diabetes, statins are included to control the dyslipidemia. Indeed, hypolipidemic and anti-atherosclerotic effects of *L. albus* were demonstrated in high-fat diet fed animals (Fontanari et al., 2012; Marchesi et al., 2008).

We therefore set out to investigate the effects of crude aqueous extract of *L. albus* in non diabetic and beta cell depleted streptozotocin (STZ)-induced type 1 diabetic rats. The rationale for using the aqueous extract is because alkaloids and peptides, components already demonstrated to have hypoglycaemic and hypolipidemic effects, are highly soluble in water.

Materials and Methods

Preparation of Crude Aqueous Extract of *Lupinus albus* (LA)

250 grams of dried and crushed LA seeds were soaked in 1000ml of distilled water overnight with continuous agitation. The suspension was vacuum filtered through Whatman No 1 filter paper to remove the residue and the filtrate was freeze-dried (Modulyo Edwards) to give a solid yield of 28%. The powder was stored at -80°C until used for animal treatments.

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Animals

Adult male Wistar rats (250-300 grams weight) were purchased from a local supplier and housed in the Department of Physiology animal holding facility. They were maintained at a temperature averaging 25^{o}C, 12:12 hour light: dark cycle, with water and pellet food (Epol-SA) *ad libitum*. Institutional and national guide for the care and use of laboratory animals was followed at all times. The study was approved by the institutional ethical committee, Walter Sisulu University.

Induction of diabetes

After two weeks of acclimatization in our facility, diabetes was induced in a group of normoglycemic rats as previously described by us (Duze et al., 2012). Briefly, animals were fasted overnight and diabetes was induced by a single intra-peritoneal injection of freshly prepared streptozotocin (STZ; Sigma) at a dose of 60mg/kg body weight in 0.1M citrate buffer (pH 4.5) to induce severe diabetes. Rats were given 5% glucose solution in place of water on the first night post STZ-treatment to counter STZ induced hypoglycaemia due to insulin leakage from damaged β cells (Szudelskit , 2001). After one week, fasting glucose levels were determined from the tail vein using a portable glucometer (Accucheck Active®) which is based on the glucose oxidase method. Severely diabetic animals with blood glucose >300mg/dL were used for this study.

Oral Glucose Tolerance Test

The oral glucose tolerance test (OGTT) was performed in both diabetic and non diabetic rats. The rats were randomly assigned into ten treatment groups of 6 animal groups. Distilled water was used as vehicle for all treatment solutions. Oral treatments were made using LA extract at 200 and 400 mg/kg body weight (b.w.); metformin 300 mg/kg b.w. and glibenclamide 500 µg/kg b.w. (Sigma Aldrich). LA doses chosen are equivalent to 50 (200mg/kg) and 100g (400mg/kg) dry weight of bean serving in a 70kg individual. Treatment groups for the OGTT were as follows:

1. Normal control
2. Diabetic control
3. LA (200mg/kg b.w) normal
4. LA (400mg/kg b.w) normal
5. LA (200mg/kg b.w) diabetic
6. LA (400mg/kg b.w) diabetic
7. Glibenclamide (500µg/kg b.w) normal
8. Glibenclamide (500µg/kg b.w) diabetic
9. Metformin (300mg/kg b.w) normal
10. Metformin (300mg/kg b.w) diabetic

Metformin (a biguanide) and glibenclamide (a sulphonylurea) are oral hypoglycaemic drugs prescribed for the treatment of diabetes. They were used as positive controls to establish whether LA acts to release insulin from beta cells (glibenclamide) or by increasing uptake by peripheral tissues (metformin). Glucose levels were measured after a 12 hour fast (time 0). Animals were then given the respective treatments (in 1ml volume) followed 15 minutes later by a glucose load (3 g/kg p.o. in 1ml volume). Control animals received 1ml distilled water p.o. Blood glucose levels were measured at 30, 60 and 120 minutes after glucose load using an Accucheck Active ® glucometer.

Long term treatment protocol

Eight animal groups from the OGTT study were continued on respective treatments for 28 days with weekly body weight and fasting blood glucose measurements. However, glibenclamide and metformin were not used for normal rats to avoid drug-induced hypoglycaemia. On day 28, fasting glucose levels were recorded from the tail vein using an Accucheck Active ® glucometer. Animals were euthanized by an overdose of phenobarbitone (65 mg/kg; Sigma). Blood was collected by cardiac puncture into plain dry tubes. After clotting, the blood was centrifuged at 10 000 rpm (Sigma) for 10 minutes and serum collected and stored at -70^{o}C for insulin and lipid measurements.

Assay for Insulin

A high range rat insulin ELISA (Mercodia, AB, Uppsala, Sweden) was used for the analysis of insulin levels in serum samples as per manufacturer's instructions. This was a colorimetric assay employing a double antibody method. Absorbance was read at 450nm and sample insulin concentrations were calculated from the standard curve and expressed as µU/mL.

Lipid profiles

Total cholesterol (TC), HDL-C and triglyceride (TG) concentrations were determined using an enzymatic direct colorimetric ELISA method as per manufacturer's instructions (Labtest Diagnostics, Brazil). Values were reported in mg/dL. For the determination of very low density lipoprotein cholesterol (VLDL-C) and low density lipoprotein cholesterol (LDL-C), Friedwald formulae were used as: VLDL-C=TG/5 and LDL-C = TC – (VLDL-C+ HDL-C) (Friedewald et al., 1972).

Acute toxicity studies

Acute toxicity was determined in healthy adult Wistar rats of either sex as previously described (OECD, 2001; Asare et al., 2011). Two groups of 5 rats/group received single oral dose of LA extract at 2500 and 5000 mg/kg body weight. The animals were observed continuously for 1 hour, then hourly for the next 4 hours, intermittently over the next 48 hours and at least once a day for two weeks. Physical manifestations of toxicity such as writhing, gasping, salivation, hyperactivity, drowsiness and death were looked for during these animal observations.

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Statistical analysis

All data were presented as mean ± standard error of the mean (SEM). Statistical analysis between groups was performed using two-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. GraphPad Prism statistical package version 6.0 was used for statistical analyses. A difference in the means with P<0.05 was considered statistically significant.

Results

OGTT

Initial mean fasting glucose levels were five-fold higher in diabetic compared to normal rats (Fig. 1A & B). As expected, there was increase in blood glucose levels after giving an oral glucose load for both normal and diabetic rats. Blood glucose concentrations decreased to preloading or lower (metformin and glibenclamide) levels in normal rats but remained high in diabetic rats. The rise in glucose concentration after the glucose load in LA treated normal rats was significantly lower (P<0.001) compared to normal controls. This trend was similar to that obtained for reference drugs. In LA (200 and 400mg/kg) and reference hypoglycaemic drug (metformin and glibenclamide) treated rats $AUC_{glucose}$ was lower (P<0.001) compared to normal control rats (Fig. 1C). In contrast, LA extract showed no observable effect on glucose levels in STZ-induced diabetic rats showing a high $AUC_{glucose}$. However, metformin showed hypoglycemic effects while glibenclamide, an insulin secretagogue did not (Fig. 1B & C).

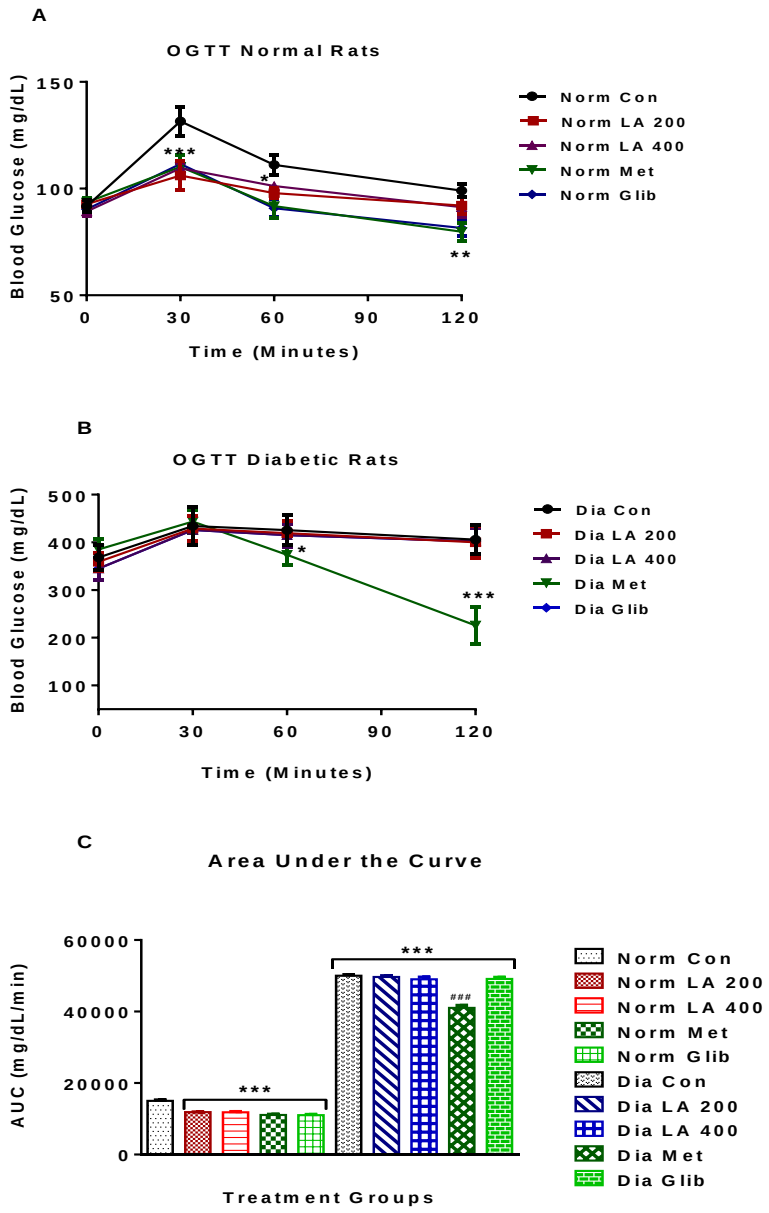

Figure 1: Oral glucose tolerance in *Lupinus albus* treated rats for normal (A) and diabetic rats (B) calculated to show $AUC_{glucose}$ (C). Data are presented as mean ±SEM. LA= *Lupinus albus*; Norm = normal; Dia = diabetic; Met = metformin; Glib = glibenclamide. For OGTT *P<0.05; **P<0.01 ***P<0.001 compared to controls; For $AUC_{glucose}$ ***P<0.001 (compared to normal control); $^{###}$P<0.001 (compared to diabetic control).

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Body weights

Mean body weight gain of rats after 4 weeks of treatment are shown in Table 1. Body weights decreased (P<0.001) in STZ-induced diabetics; controls, LA and glibenclamide treated rats, compared to normal controls. However, metformin partially protected diabetic rats from diabetes associated weight loss (P<0.05 compared to normal controls). Body weights for normal LA treated rats were similar to normal controls (P>0.05; Table 1).

Table 1: Body weights gained by rats at the end of the 28 day treatment period. Reference hypoglycaemic drugs were only used in treating diabetic rats.

Treatment	Normal Weight gain (g)	Diabetic Rats Weight gain (g)
Control	22.0 ± 1	-24.0 ± 2***
LA 200 mg/kg b.w.	21.0 ± 2	-18.0 ± 2***
LA 400 mg kg b.w.	22.0 ± 2	-18.0 ± 4***
Metformin		13.0 ± 1*
Glibenclamide		-24.0 ± 2***

Data are presented as mean ±SEM (n=6/group). LA= *Lupinus albus*. *P<0.05; ***P<0.001 compared to normal control rats.

Hypoglycaemic effects after long term treatment

Results from daily administration of 200 and 400 mg/kg doses LA for 28 days showed significant (P>0.05, P<0.001) glucose lowering activity in normal rats showing a 34% decrease in fasting blood glucose levels compared to untreated controls on day 28 (Fig. 2A). However, LA extract had no effect on blood glucose levels in STZ- induced diabetic rats at both doses, similar to the insulin secretagogue glibenclamide. However, metformin at 300mg/kg effectively reduced glucose levels (P>0.001) by 52% in STZ induced diabetic rats compared to the diabetic controls (Fig. 2B).

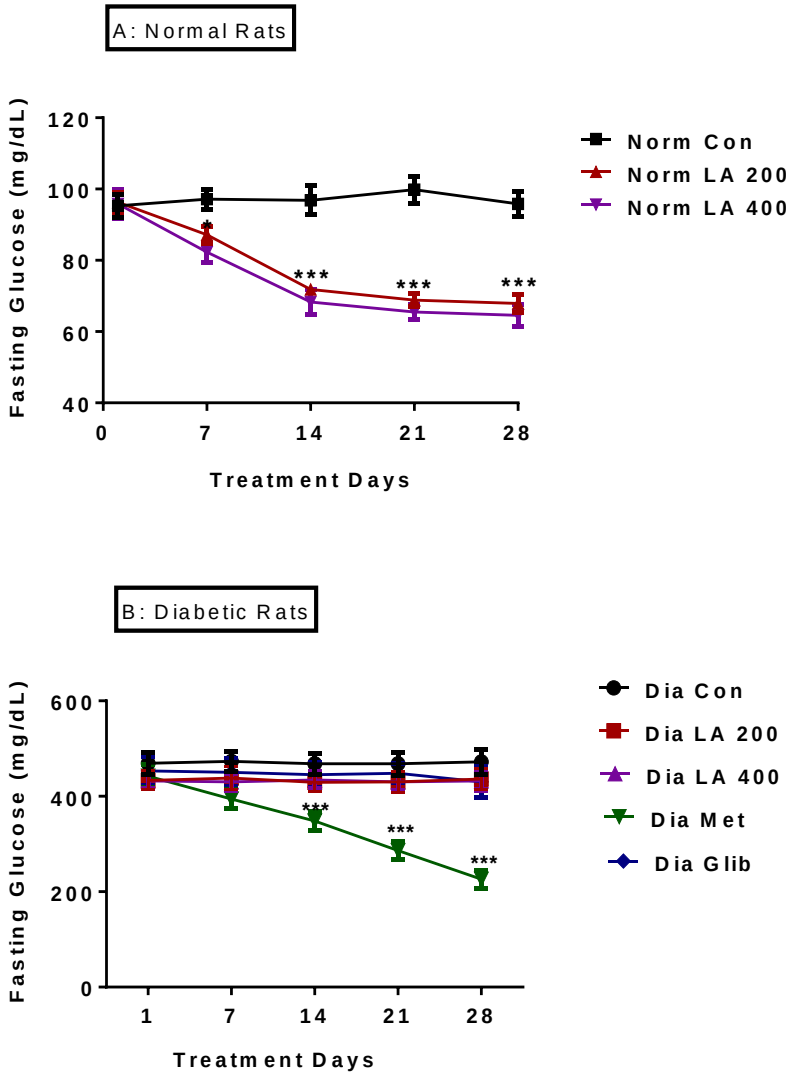

Figure 2: Blood glucose levels for normal (A) and STZ-induced diabetic rats (B) measured every seven days for 28 days. Data are presented as mean ±SEM. LA= *Lupinus albus*; Norm = normal; Dia = diabetic; Met = metformin; Glib = glibenclamide. ***P<0.001 compared to controls.

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Insulin levels

Fasting insulin levels were significantly increased (P<0.001) in LA treated normal rats in a non-dose dependant manner. There was a 60% increase in fasting insulin levels in LA treated normal rats compared to controls. Insulin remained low in all treatment groups of STZ-induced diabetic rats (Table 2).

Table 2: Comparison of the effects of LA on serum insulin concentrations in normal and STZ-induced diabetic rats. Reference hypoglycaemic drugs were only used in treating diabetic rats.

Treatment	Normal Insulin (µU/ml)	Diabetic Rats Insulin (µU/ml)
Control	11.25 ± 1.48	2.46 ± 0.07***
LA 200 mg/kg b.w.	17.52 ± 0.32***	2.72 ± 0.05***
LA 400 mg kg b.w.	18.11 ± 0.51***	2.83 ± 0.07***
Metformin		2.57 ± 0.05***
Glibenclamide		2.80 ± 0.04***

Blood samples were collected after 28 days of daily treatment. Data are presented as mean ±SEM (n=6/group). LA= *Lupinus albus*. ***P<0.001 compared to normal control rats. Diabetic rat

Lipid levels

Despite the lack of hypoglycaemic effect in STZ-induced diabetic rats, LA exhibited significant hypolipidemic effects. LA treatment of STZ-induced diabetic rats at both 200 and 400 mg/kg b.w. resulted in a dose dependent decrease in TC (P<0.001; 18% and 27% respectively) and LDL-C (P<0.001; 26% and 37% respectively) compared to diabetic controls (Fig. 3A and C). LA treatment at both 200 and 400mg/kg b.w. resulted in a non-dose dependent decrease in TG (P<0.001; 21%) and VLDL-C (P<0.001; 24%; Figure 3 B and D). LA and glibenclamide treatment had no effect on HDL-C levels but metformin treatment increased HDL-C compared to diabetic controls (Fig 3E). In normal rats, LA treatment reduced TC and LDL-C (P<0.001) but not TG, VLDL-C and HDL-C concentration compared to normal controls (Fig. 3). Overall, diabetic rats demonstrated diabetes induced dyslipidemia.

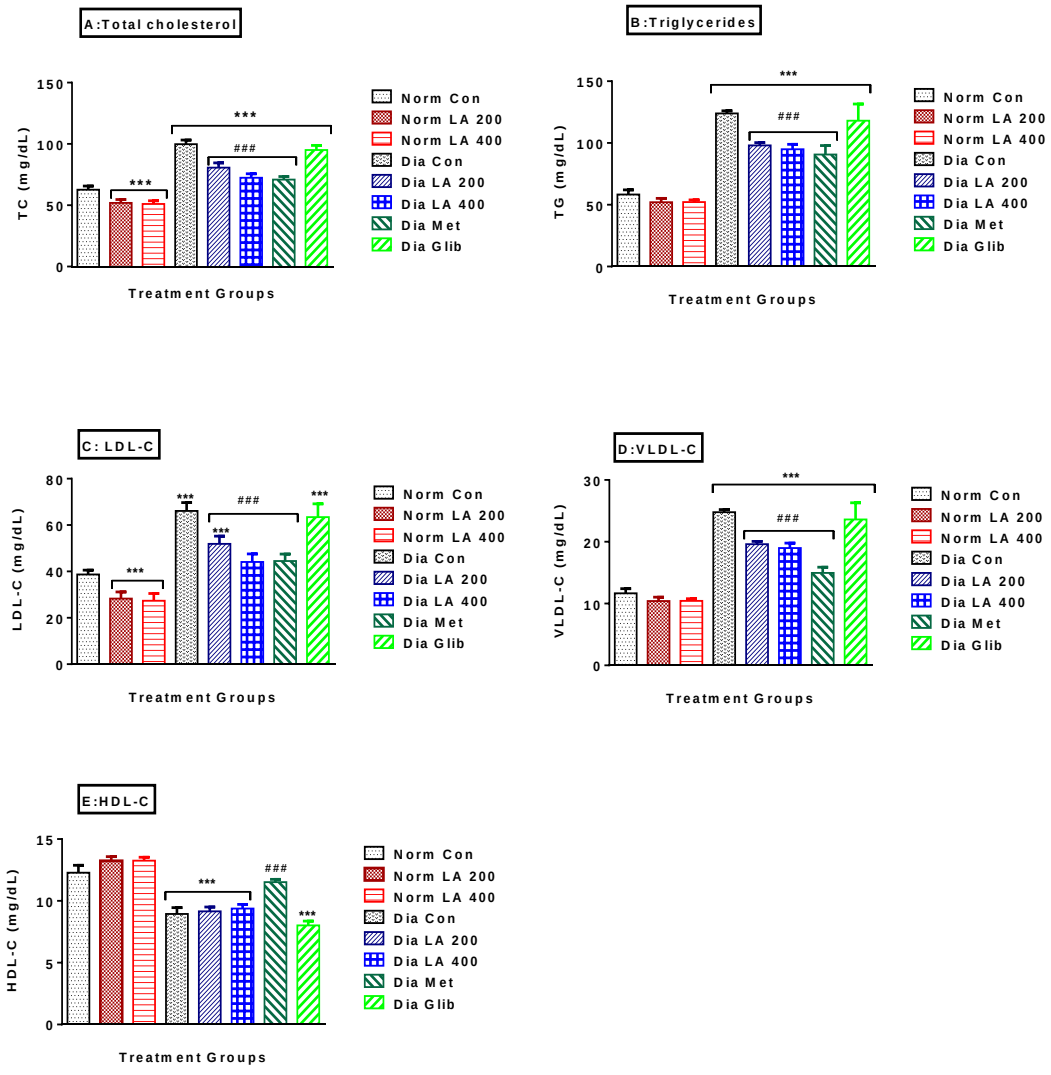

Figure 3: Comparison of the effects of LA on serum lipids in normal and STZ-induced diabetic rats after 28 days of daily treatment. TC (A); TG (B); LDL-C (C); VLDL-C (D); HDLC (E). Reference hypoglycaemic drugs were only used in treating diabetic rats. Data are presented as mean ±SEM. LA= *Lupinus albus*; Norm = normal; Dia = diabetic; Met = metformin; Glib = glibenclamide. ***P<0.001 compared to normal controls; ###P<0.001 (compared to diabetic control).

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Acute toxicity studies

Toxicity studies carried out using oral doses of 2500 and 5000 mg/kg LA aqueous extract did not cause any obvious signs of toxicity as assessed by behavioral changes by the experimental rats. Rats remained active and healthy after 2 weeks of observation.

Discussion

Diabetes mellitus is associated with dyslipidemia in both clinical and experimental diabetes (Rader et al., 2009; Istvan, 2002). Many studies have demonstrated hypoglycaemic and hypolipidemic effects of medicinal plants in streptozotocin induced diabetic animal models (Kim et al., 2006; Aguilar-Santamaria et al., 2009; Gupta et al., 2009; Zhou et al., 2012; Patel et al., 2012). The STZ-induced diabetic rats in our study exhibited both hyperglycaemia and dyslipidemia. Destruction of pancreatic beta cells was confirmed by failure of STZ-induced diabetic rats to respond to the reference drug glibenclamide, an insulin secretagogue. In the present study, we sought to assess anti-hyperglycaemic and anti-lipidemic effects of an aqueous extract of *Lupinus albus* seeds on beta cell depleted STZ-induced diabetic rats and in normal rats with intact pancreatic beta cells. Results showed that LA did not exhibit glucose-lowering effects in STZ induced diabetic rats after acute or chronic treatment. However, the extract acted to reduce fasting glucose levels in normal non-diabetic rats. Despite the absence of hypoglycaemic effects of LA in STZ induced diabetic rats, the extract was effective in lowering atherogenic cholesterol in both STZ-induced diabetic and non diabetic rats.

We selected hypoglycaemic reference drugs based on their mechanism of action to assist in identifying possible mechanism of action of LA. Metformin exerts hypoglycaemic effects via increased peripheral glucose uptake while glibenclamide is an insulin secretagogue acting on pancreatic beta cells to release insulin. Our results imply that the mode of action of LA as a hypoglycaemic agent is insulin dependent thus ineffective in lowering glucose in type 1 diabetes, as mirrored by the STZ-induced diabetic rats used in this study. The absence of functional beta cells in our rat model was confirmed by the low insulin levels evident in the diabetic rats even after treatment with glibenclamide. LA was effective in lowering fasting glucose in normal rats with intact beta cells, in a similar manner to glibenclamide.

Our most significant finding is the hypolipidemic effect of LA in STZ-induced diabetic rats. It is well established that elevated levels of TC, TG and LDL-C result in an increased risk of cardiovascular disease (Rader et al., 2009; Istvan, 2002). Insulin promotes lipid synthesis and suppresses lipid degradation by stimulation of transcription factors such as steroid regulatory element-binding protein (SREBP)-1c in the liver and in adipose tissue (Ferre, and Foufelle, 2007). The reduced insulin levels in STZ-induced diabetic rats in this study imply a non-insulin dependent mechanism in LA hypolipidemic effects. In a study in which rabbits were fed a high cholesterol diet, LA proteins were shown to be hypolipidemic and anti- atherosclerotic (Marchesi et al., 2008). Additionally, in a study in which subjects with moderate hypercholesterolemia were given daily LA protein as a beverage, reduction in cholesterol levels and blood pressure were observed (Naruszewicz et al., 2006). In an in vitro study, treatment of liver hepatocellular (HepG2) cells with LA proteins resulted in increased activity of LDL receptors (Sirtori et al., 2004). Taken together these studies support the findings in our previous study and may point towards increased LDL receptor activity as the possible mechanism of action of LA in STZ-induced diabetic rats. Inhibition of HMG-CoA reductase (or 3-hydroxy-3-methyl-glutaryl-CoA reductase) the rate-controlling enzyme responsible for cholesterol synthesis via the mavelonate pathway is another potential target for LA action in lowering cholesterol levels. Cholesteryl ester transfer protein (CETP) transfers cholesteryl ester from HDL to apolipoprotein B-containing lipoproteins and plays an important role in regulating the concentration and composition of HDL (Arai et al., 2011; Redondo et al., 2011). Thus, LA treatment may not affect CETP activity hence the unchanged HDL levels observed after 4 weeks of treatment in our study.

Insulin is an anabolic hormone promoting protein synthesized in the tissues. In its absence there is muscle wasting and loss of tissue proteins resulting in weight loss (Marchesini et al., 1982; Basil and Gougeon, 2013). This was evident in the loss of weight observed in the present study. However LAwas not toxic at doses up to 5000 mg/kg. Thus, it has low toxicity. A previous study also reported a low toxicity with an LD50 of >4000 mg/kg in mice (Stobiecki et al, 1993).

This study demonstrates that *L. albus* has potential to treat the complications of Type 1 diabetes owing to its hypolipidemic effects. However, it may not possess hypoglycemic effects in Type 1 diabetic patients. Our findings imply that *L. albus* acts in a manner similar to glibenclamide, by increasing insulin secretion. The traditional use of *L. albus* in the treatment of diabetes among various communities may be associated with type 2 diabetes, the more common form of diabetes worldwide (Herman and Zimmet, 2012). Indeed this plant may be effective for type 2 diabetes in which pancreatic beta cells are still functional. Further studies to investigate effects of *L. albus* in a Type 2 diabetes model are essential.

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