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Antidiabetic activity of *Croton klozchianus* in rats and direct stimulation of insulin secretion in-vitro

R. Govindarajan, M. Vijayakumar, Ch. V. Rao, P. Pushpangadan, Henry Asare-Anane, Shanta Persaud, Peter Jones and Peter J. Houghton

Pharmacognosy and Ethnopharmacology Division, National Botanical Research Institute, Lucknow, India

R. Govindarajan, M. Vijayakumar, Ch. V. Rao

Amity Institute for Herbal and Biotech Products, Trivandrum, India

P. Pushpangadan

Reproductive Health Endocrinology and Development Division, School of Biomedical and Health Sciences, King's College London, UK

Henry Asare-Anane, Shanta Persaud, Peter Jones

Pharmacognosy Research Laboratories, Pharmaceutical Sciences Research Division, School of Biomedical and Health Sciences, King's College London, UK

Peter J. Houghton

Correspondence: P. J. Houghton, Pharmacognosy Research Laboratories, Pharmaceutical Sciences Research Division, School of Biomedical and Health Sciences, King's College London, 150 Stamford Street, London SE1 9NH, UK. E-mail: peter.houghton@kcl.ac.uk

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Abstract

Croton klozchianus is a relatively uninvestigated species with no pharmacological or phytochemical reports available, although it has been used clinically by Ayurvedic physicians to treat diabetes. We have investigated this use by studying the insulin secretion and antidiabetic activity of *C. klozchianus*. Treatment of diabetic rats with aerial parts of *C. klozchianus* extract (CK, 100 and 300 mg kg^{-1} body weight) for three weeks showed significant reduction in blood glucose (45.8% after 14 days for 300mg kg^{-1}). *C. klozchianus* extract caused a significant concentration-dependent increase in insulin secretion (8-fold at 2 mg mL^{-1} for cells challenged with 20 mM glucose) from MIN6 cells grown as monolayers and as pseudoislets, indicating that the antidiabetic activity may have been as a result of increased insulin secretion. It also had a role on the lipid profile of the rats by causing reduction in cholesterol and triglycerides and increasing high density lipoprotein significantly. The results obtained gave some scientific support to the traditional use of the plant as a treatment for diabetes.

Introduction

Croton klozchianus (L.) DC, Family: Euphorbiaceae is a wild herb found in the Western Ghats of India, and has been used successfully by Ayurvedic practitioners of Kerala, India, for the treatment of diabetes and its complications. There are no scientific studies on its activity, but there is an ethnobotanical report on a related species (Pushpangadan & Atal 1984). This study was carried out to investigate the antidiabetic activity of an aqueous alcoholic extract of the aerial parts of *C. klozchianus*. In particular, the effects of *C. klozchianus* on fasting blood glucose levels in streptozotocin diabetic rats were examined, together with its effects on the lipid profile in-vivo. A diabetic state induced by streptozotocin in rats is a common model for testing antidiabetic plant extracts and reduced glucose levels are a measure of restoration of insulin-like activity. It is important to determine the lipid profile since a change in this is an effect of diabetes that leads to several of the serious effects of the disease. The direct effect of *C. klozchianus* on insulin secretion in-vitro from mouse insulinoma MIN6 β-cells was also studied, as an approach to determining at least part of the mechanism by which the extract might exert its hypoglycaemic effect noted in-vivo. Finally, HPLC analysis of the total extract was recorded as a standardization tool and to quantify the amount of quercetin and quinic acid present.

Materials and Methods

Plant material and extraction

Aerial parts of *C. klozchianus* (L.) DC. were collected from Trivandrum, Kerala (India) during March 2005. The plants were authenticated and the voucher specimen (code No.

LWG) was lodged in the herbarium of the National Botanical Research Institute, Lucknow. Dried plant material (1 kg) was exhaustively extracted with 50% aqueous alcohol (1 L × 3 times) by cold percolation. The extract was concentrated under reduced pressure and lyophilized (Labconco, US) to obtain dry residue (12.3%, w/w), defined hereafter as CK.

HPLC quantification of quercetin and quinic acid

An HPLC finger print profile for *C. klozchianus* was established using a Waters model (Water Corp, Milford, MA), equipped with a pump (Waters 515) with a Spherisor ODS2 column RP-18 (250 × 4.6 mm, i.d., 5-μm pore size) and a Waters PCM Rheodyne injector with a 25-μL loop. Detection was performed at 254 nm using a 2996 PDA detector. *C. klozchianus* (1 mg mL^{-1}) 20 μL was injected and elution was carried out at a flow rate of 0.5 mL min^{-1} with water:phosphoric acid (99.7:0.3, v/v) as solvent A, and acetonitrile:water:phosphoric acid (79.7:20:0.3 v/v) as solvent B, using a gradient elution (0–5 min with 85–60% A, 5–10 min with 60–40% of A, 10–15 min with 40–25% of A and 15–20 min with 25–0% of A). LC-MS was carried out to identify the peaks using API 2000 triple quadrupole LC-MS/MS mass spectrometer (Applied Biosystems, Ontario, Canada) with a turbo ion spray source.

Animals

Male Sprague–Dawley rats (160–180 g) were purchased from the animal house of the Central Drug Research Institute, Lucknow, India. They were kept in the departmental animal house at 26 ± 2°C and relative humidity of 44–55% with light and dark cycles of 10 and 14 h, respectively, for one week before the experiments. Animals were provided with rodent diet (Amruth, India) and water was freely available. All studies were conducted in accordance with the CPCSEA (Committee for the Purpose of Control and Supervision of Experiment on Animals) India guidelines (Reg. No. 222/2000/CPCSEA).

Oral toxicity test

Groups of six rats were given one of a series of doses of *C. klozchianus* (50, 500, 1000 or 2000 mg kg^{-1}, p.o.) and continuously observed for 2 h to detect changes in gross abnormal behaviour and mortality. Any mortality during the experiment and the following seven days was recorded. A group of animals treated with vehicle (gum acacia 5%) served as control (Turner 1965).

Experimental induction of diabetes

Rats were rendered diabetic by injecting a freshly prepared aqueous solution of streptozotocin (50 mg kg^{-1}, i.p.). Diabetes was confirmed in streptozotocin rats by measuring the fasting blood glucose concentration after 96 h following the administration of streptozotocin. The rats with a blood glucose level above 200 mg dL^{-1} were considered to be diabetic and were used experimentally.

Oral glucose tolerance test

Oral glucose tolerance tests (Bonner-Weir 1988) were performed in overnight-fasted (18 h) normal rats. Rats were divided into two groups of six rats each (n = 6) and these were administered drinking water or *C. klozchianus* 300 mg kg^{-1} in water orally, respectively. Rats were loaded with glucose (2 g kg^{-1}) 30 min after administration of the extracts. Blood was withdrawn from the retro orbital sinus under ether inhalation at 0, 30, 60 and 120 min after glucose administration and serum glucose level was estimated by enzymatic GOD-POD method using a glucose diagnostic kit (Qualigens Diagnostics, Mumbai, India), in which glucose is oxidized by glucose oxidase to gluconic acid and hydrogen peroxide. Hydrogen peroxide in the presence of enzyme peroxidase oxidizes phenol which combines with 4-aminoantipyrine to produce a red-coloured quinoneimine dye. The intensity of the red colour so developed was measured at 505 nm and was directly proportional to the glucose concentration (Trinder 1969).

Experimental design

After induction of diabetes, the rats were divided into five groups. Group 1 control rats, received vehicle solution (2% gum acacia); group 2 diabetic control, received 2% gum acacia; group 3, diabetic rats treated with CK 100 mg kg^{-1} in 2% gum acacia; group 4, diabetic rats treated with CK 300 mg kg^{-1} in 2% gum acacia; group 5, diabetic rats treated with glibenclamide 1 mg kg^{-1} in aqueous solution. The vehicle and drugs were administered orally using an intragastric tube daily for three weeks. After three weeks of treatment, the rats were fasted overnight and blood samples were analysed for serum glucose concentration (Trinder 1969).

Plasma lipid profile

The serum cholesterol level was estimated by the Wybenga & Pileggi method (Wybenga et al 1970) using a cholesterol diagnostic reagent kit (Span Diagnostics, Surat, India). Cholesterol reacts with a hot solution of ferric perchlorate, ethyl acetate and sulphuric acid (cholesterol reagent) and gives a lavender-coloured complex which is measured at 560 nm. The total lipid was estimated by the Phosphovanillin method using a Total Lipids diagnostic reagent kit (Merck, Mumbai) (Zollner & Kirsch 1962). Lipids formed a coloured complex when treated with Phosphovanillin in sulphuric acid solution, and the absorbance at 520 nm was proportional to the amount of total lipids present. Triglyceride was estimated by the glycerol phosphate oxidase method using Triglyceride kit (Qualigens Diagnostics, Mumbai) (McGowan et al 1983). Triglycerides in the sample were hydrolysed by microbial lipases to glycerol and free fatty acids (FFA). Glycerol was phosphorylated by adenosine 5-triphosphate (ATP) to glycerol-3-phosphate (G-3-P) in a reaction catalysed by the enzyme glycerol-kinase (GK). G-3-P was oxidized to dihydroxyacetone phosphate (DAP) in a reaction catalysed by the enzyme glycerol phosphate oxidase (GPO). In this reaction H_2O_2 was produced in equimolar concentration to the level of triglycerides present in the sample. H_2O_2 reacted with 4-aminoantipyrine (4-AAP) and 4-chlorphenol in a reaction

catalysed by peroxidase (POD). The result of this oxidative coupling was a chinonimine red-coloured dye. The absorbance of this dye in solution was proportional to the concentration of triglycerides in the sample.

In-vitro studies

Cell culture

MIN6 insulin-secreting cells were obtained from Professor J.-I. Miyazaki (University of Osaka, Japan). The cells were maintained in culture at 37°C under a 5% CO_2 atmosphere in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS; Invitrogen) and 2 mM glutamine, 100 U mL^{-1} penicillin/0.1 mg mL^{-1} streptomycin. All reagents and chemicals were purchased from Sigma Chemical Co (Dorset, UK) unless otherwise stated.

Insulin secretion by MIN6 cells

MIN6 insulin-secreting cells were seeded at a density of 30 000 cells per well in 96-well plates and left to adhere for 1–2 days before use. Cells were pre-incubated in a physiological salt solution (Gey & Gey 1936) supplemented with 2 mM glucose for 2 h then incubated for 30 min in the absence or presence of *C. klozchianus*. In some experiments the effects of *C. klozchianus* on the insulin secretory profile were examined by perifusion of MIN6 cells aggregated as three-dimensional pseudoislet clusters (Hauge-Evans et al 2002). Insulin secretion was measured by radioimmunoassay.

Statistical analysis

The statistical analysis of all the pharmacological studies was carried out using Graph pad prism. The in-vivo data were presented as mean ± s.e.m. for six rats and as described in the figure legends for in-vitro experiments. Differences between treatments were assessed using analysis of variance, followed by Bonferroni's test for multiple comparisons. Differences were considered significant when $P < 0.05$.

Results

Acute in-vivo toxicity studies revealed the non-toxic nature of *C. klozchianus*. There was no mortality or any toxic reactions found at doses selected up to the end of the study period. The oral glucose tolerance test revealed that animals treated with *C. klozchianus* showed significant reductions in plasma glucose levels compared with control rats treated with gum acacia (Figure 1). Streptozotocin has been widely used for inducing type I diabetes in a variety of animals by promoting degeneration and necrosis of pancreatic β-cells (Merzouk et al 2000). Induction of diabetes by streptozotocin in the experimental rats was confirmed by the presence of high fasting plasma glucose levels (Table 1, group 2). *C. klozchianus* induced significant reduction in serum glucose level in streptozotocin diabetic rats ($P < 0.001$) after 14 and 21 days, reducing the glucose concentration by 45.8 and 44.3%, respectively, when administered at 300 mg kg^{-1} (Table 1). The rapid onset of the glucose-lowering effect of *C. klozchianus* in diabetic rats was unlikely to be related to β-cell

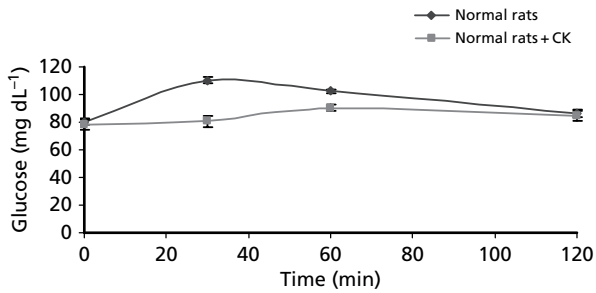


Figure 1 Effect of CK on oral glucose tolerance test in rats. Rats were loaded with glucose (2 g kg^{-1}) 30 min after administration of the extracts. Blood was withdrawn from the retro orbital sinus under ether inhalation at 0, 30, 60 and 120 min after glucose administration and serum glucose level was estimated.

Table 1 Effect of a hydroalcoholic extract of *C. klozchianus* on serum glucose levels in streptozotocin-induced diabetic rats

Groups	Blood glucose (mM)			
	Day 0	Day 7	Day 14	Day 21
Group 1 (normal)	4.36 ± 0.69	4.23 ± 0.65	4.30 ± 0.57	4.38 ± 0.52
Group 2 (diabetic control)	13.79 ± 1.80	12.62 ± 2.00	12.74 ± 1.78	11.79 ± 1.45
Group 3 (CK 100 mg kg^{-1})	13.24 ± 0.14 (ns)	11.63 ± 0.19*	9.35 ± 0.18**	7.34 ± 0.17**
Group 4 (CK 300 mg kg^{-1})	14.25 ± 0.12 (ns)	9.02 ± 0.12**	6.91 ± 0.13***	6.57 ± 0.06***
Group 5 (glibenclamide)	14.31 ± 0.12 (ns)	8.57 ± 0.13**	5.89 ± 0.14**	5.12 ± 0.12***

The values of blood glucose in the table represent the means ± s.e.m. for six rats per group upon treatment with normal saline, CK and glibenclamide. *P* values were calculated based on the paired-*t*-test. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ compared with diabetic control group. Not significant, ns.

neogenesis. However, it was likely that not all β-cells were destroyed by the single streptozotocin dose of 50 mg kg^{-1} used in these experiments, since glibenclamide, a sulphonylurea that stimulates insulin secretion by acting at β-cell ATP-sensitive K^+ channels, restored blood glucose levels to the normal range in streptozotocin-diabetic rats (Table 1). Thus, *C. klozchianus* may also have exerted its glucose-lowering effects by directly stimulating insulin secretion from β-cells that had not been destroyed by streptozotocin treatment.

Measurements of the effects of *C. klozchianus* on insulin secretion from the MIN6 β-cell line indicated that it did exert direct stimulatory effects on the exocytotic release of insulin (Figure 2). Thus, *C. klozchianus* (0.0.06–1 mg mL^{-1}) initiated a concentration-dependent stimulation of insulin secretion from MIN6 cells at 2 mM glucose ($P < 0.01$ by analysis of variance), with a threshold stimulatory concentration of 0.25 mg mL^{-1}. Plants such as *Caesalpinia bonduc* (Chakrabarti et al 2005) and *Scoparia dulcis* (Latha et al 2004) have been shown to possess antidiabetic activity in animal models along with insulin secretion properties, but information is not available on the profile of the insulin secretory responses to those extracts.

In this study in-vitro perifusion experiments were carried out to analyse the time-course and reversibility of insulin secretion in response to *C. klozchianus*. For these experiments MIN6 β-cells were configured as three-dimensional pseudoislets, which act in a manner similar to primary islets (Hauge-Evans et al 2002). It can be seen from Figure 3 (upper panel) that 2 mg mL^{-1} *C. klozchianus* caused a 13-fold increase in insulin secretion at 2 mM glucose. However, the stimulation of secretion was transient, and insulin secretion rapidly returned to basal levels despite the continued presence of *C. klozchianus*. As well as initiating insulin secretion at 2 mM glucose, *C. klozchianus* also potentiated glucose-stimulated insulin secretion. Thus, Figure 3 (lower panel) demonstrates that MIN6 pseudoislets responded to an elevation in glucose from 2 to 20 mM with an 8-fold increase in insulin output, followed by a sustained plateau. Exposure of pseudoislets to 2 mg mL^{-1} *C. klozchianus* resulted in a rapid enhancement of the secretory response to glucose. Similar to the observations made at 2 mM glucose (Figure 3, lower), the effect of *C. klozchianus* at 20 mM glucose was transient, with insulin secretion declining within approximately 10 min of the 20-min exposure period. These in-vitro results display a similar pattern to that observed in the in-vivo experiments using *C. klozchianus*. It is possible that, in the in-vivo experiments, residual β-cells following streptozotocin-induced diabetes might be stimulated to secrete insulin, and so lower the level of fasting blood glucose.

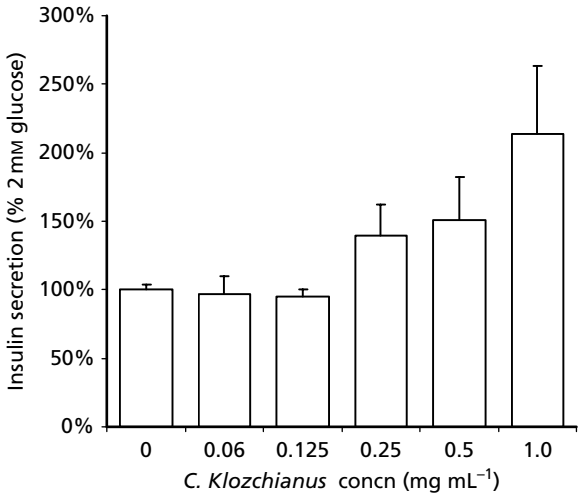


Figure 2 Effect of *C. klozchianus* on insulin secretion from MIN6 cells. MIN6 cells seeded into 96-well plates were incubated for 30 min in the presence of increasing concentrations (0.06–1 mg mL^{-1}) of *C. klozchianus* extract at 2 mM glucose. Bars show mean ± s.e.m. n = 5.

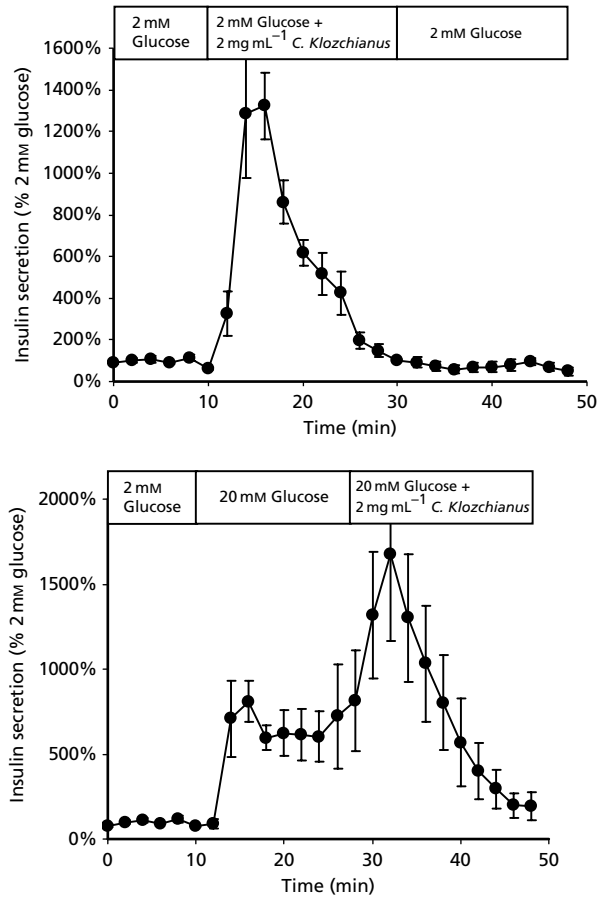


Figure 3 Time-course and reversibility of the effect of *C. klozchianus* on insulin secretion from MIN6 pseudoislets. MIN6 cells configured as islet-like structures (pseudoislets) were perifused (0.5 mL min^{-1}) with buffers containing 2 or 20 mM glucose in the absence or presence of *C. klozchianus* (2 mg mL^{-1}), as shown by the bars. Fractions were collected every 2 min and assayed for insulin. Points show means ± s.e.m., n = 3. Upper panel: *C. klozchianus* induced a rapid, but transient (~10 min) and reversible, stimulation of insulin secretion at 2 mM glucose. Lower panel: a stimulatory concentration of glucose (20 mM) induced a biphasic and maintained insulin secretory response, and *C. klozchianus* caused a transient potentiation of 20 mM glucose-stimulated insulin secretion.

Discussion

Insulin resistance in obesity is evident before the development of chronic hyperglycaemia. Therefore it is unlikely that insulin resistance, at the prediabetic stage, results from oxidative stress triggered by hyperglycaemia itself. However, the strong association of obesity and insulin resistance suggests that a major mediator of oxidative stress mediated at the prediabetic stage might be a circulating factor secreted by adipocytes. There are reports that hypercholesterolaemia in streptozotocin-induced diabetes in rats results from increased intestinal absorption and synthesis of cholesterol. Lipoproteins from diabetic rats are oxidized and demonstrate cytotoxicity, a feature which can be prevented by insulin or antioxidant treatment (Mathe 1995). In patients with severe hypertriglyceridaemia, especially where diabetes is accompanied by genetic hyperlipidaemia, therapy with lipid lowering drug is required. When administered to streptozotocin-diabetic rats at 300 mg kg^{-1} *C. klozchianus* had potent effects on their lipid profile by significantly ($P < 0.001$) decreasing total lipid, triglyceride and cholesterol (Table 2).

HPLC and LC-MS analysis of the extract was carried out to standardize the extract (Figure 4), using quercetin (**1**) and quinic acid (**2**) as marker components (Figure 5). The concentrations of quercetin and quinic acid in the plant were found to be 0.31 and 0.12% (w/w), respectively. Quercetin has been reported to prevent and protect against streptozotocin-induced oxidative stress and β-cell damage in rat pancreas (Coskun et al 2005). Thus, the significant antidiabetic and lipid lowering activity of *C. klozchianus* may be attributed partially to the presence of quercetin, although other compounds, as yet unidentified, probably also contribute.

Conclusion

This is the first report on the pharmacological activity or phytochemical evaluation of *C. klozchianus* and it has confirmed that *C. klozchianus* confers moderate protection against diabetes in-vivo. Our in-vitro data indicated that these protective effects may occur, at least in part, by direct stimulation of β-cells

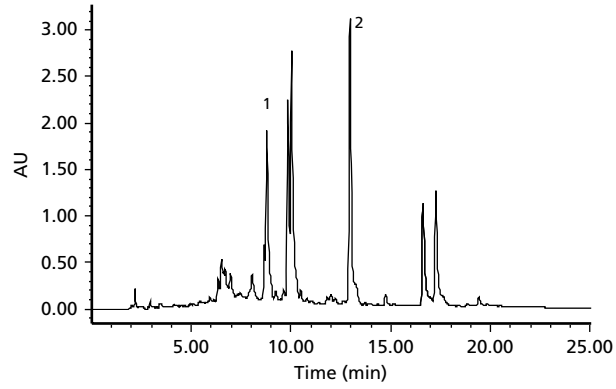
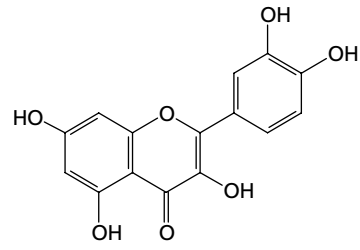
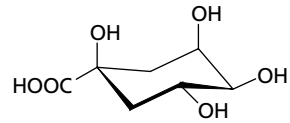


Figure 4 HPLC fingerprint profile of CK.



1 Quercetin



2 Quinic acid

Figure 5 Structure of quercetin and quinic acid.

Table 2 Effect of *C. klozchianus* on the level of serum total lipids, triglycerides and cholesterol in streptozotocin-induced diabetic rats

Group	Total lipids (mg dL^{-1})	Triglycerides (mg L^{-1})	Cholesterol (mg dL^{-1})
Group 1 (normal)	85.14 ± 1.63	68.41 ± 1.54	73.45 ± 2.63
Group 2 (diabetic control)	142.56 ± 2.10	116.42 ± 1.24	159.42 ± 2.86
Group 3 (CK 100 mg kg^{-1})	108.12 ± 2.65***	92.26 ± 2.47***	98.12 ± 2.33***
Group 4 (CK 300 mg kg^{-1})	88.19 ± 1.26***	67.43 ± 1.25***	74.35 ± 2.64***
Group 5 (glibenclamide)	92.15 ± 1.54***	77.35 ± 1.46***	81.32 ± 1.38***

The values of lipid profile in the table represent the means ± s. e. m. for six rats per group upon treatment with normal saline, CK and glibenclamide. *P* values were calculated based on the paired-*t*-test. ***$P < 0.001$ compared with diabetic control group.

to secrete insulin, although in-vivo experiments would have to be carried out to verify this hypothesis. In addition, the capacity of *C. klozchianus* to reduce triglyceride and total cholesterol levels while increasing high density lipoprotein may contribute to its beneficial effects in diabetic rats in-vivo.

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