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D-*chiro*-Inositol found in *Cucurbita ficifolia* (Cucurbitaceae) fruit extracts plays the hypoglycaemic role in streptozocin-diabetic rats

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Abstract

Cucurbita ficifolia is commonly used as an antihyperglycaemic agent in Asia. However, the mechanism of its action is unknown. Chemically synthesized D-*chiro*-inositol (D-CI), a component of an insulin mediator, has been demonstrated to have antihyperglycaemic effects in rats. In this study, we found that *C. ficifolia* contained fairly high levels of D-CI, thus, *C. ficifolia* may be a natural source of D-CI for reducing blood glucose concentrations in diabetics. We evaluated *C. ficifolia* fruit extract, containing D-CI, for its antihyperglycaemic effect in streptozotocin-induced diabetic rats. Oral administration of *C. ficifolia* fruit extract containing 10 or 20 mg D-CI kg^{-1} body weight for 30 days resulted in significantly lowered levels of blood glucose, and increased levels of hepatic glycogen, total haemoglobin and plasma insulin. An oral glucose tolerance test was performed in fasted diabetic and normal rats, in which there was a significant improvement in blood glucose tolerance in the diabetic rats treated with *C. ficifolia* fruit extract. The effects were compared with 20 mg kg^{-1} body weight chemically synthesized D-CI. Findings from this study demonstrated that *C. ficifolia* fruit extract was an effective source of D-CI for its hypoglycaemic effects in rats, and therefore may be useful in the treatment of diabetes.

Introduction

Medicinal plants are widely used in many countries for the treatment of diabetes mellitus. The antihyperglycaemic effect of several plant extracts and herbal formulations that are used as antidiabetic remedies has been confirmed (Grover et al 2000; Pari & Saravanan 2002). Therefore, the search and study for more effective and safer antihyperglycaemic agents has become an area of active research. Nevertheless, there is no knowledge about the chemical nature of the plant drugs' active components or about the effects caused by their chronic administration that permit the establishment of a basis for their clinical use in the control of diabetes mellitus. Hence, many questions still remain regarding this medicinal resource.

In the last two decades, research has indicated that inositol molecules, particularly D-*chiro*-inositol (D-CI), are important mediators for insulin action (Larner 2001). D-CI is normally present in the urine and blood but is absent or at much reduced levels in urine and blood from animal models and patients with type 2 diabetes. This suggests that type 2 diabetic patients may not produce enough D-CI and therefore exhibit a decrease in insulin sensitivity (Larner 2002). Moreover, the effectiveness of acute administration of chemically-synthesized D-CI on lowering plasma glucose has been evaluated in rats. A single dose of intragastric D-CI (10 mg kg^{-1}) administered to streptozotocin fed rats produced a 30–40% decrease in plasma glucose concentrations (Ortmeyer et al 1993). The acute effects of D-CI on plasma glucose were also demonstrated in streptozotocin diabetic rats, when a single 15 mg kg^{-1} dose attenuated elevated plasma glucose concentrations by 21% in 120 min (Fonteles et al 2000). These studies used chemically-synthesized D-CI. Therefore, it is of considerable interest to find new natural sources of D-CI and extract those molecules as new drug candidates and dietary supplements for type 2 diabetes.

The stereoisomeric family of nine inositols includes myo-, cis-, allo-, epi-, muco-, neo-, scyllo- and the optical isomers D- and L-*chiro*-inositols. *Myo*-inositol is the most

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commonly occurring isomer, whereas D-CI is naturally rare. Free *myo*-inositol and D-CI have been found in plants and seeds, such as pinewood, lupine, pigeon pea, soybean, chickpea, mungbean, and buckwheat (Horbowicz & Obendorf 1994; Horbowicz et al 1998). Besides their existence in plant sources, D-CI and *myo*-inositol have been identified as components of two different inositolphosphoglycan (IPG) molecules in mammalian systems (Larner et al 1988, 1989). The role of IPG molecules as putative insulin secondary messengers has been demonstrated in numerous studies (Varela-Nieto et al 1996; Field 1997; Jones & Varela-Nieto 1998, 1999). IPGs are released from glycosylphosphatidylinositols (GPIs) in cell membranes in response to insulin. Following GPI hydrolysis by phospholipases, IPGs are incorporated into the cell, where they can affect enzymes implicated in insulin action.

Cucurbita ficifolia is a member of the Cucurbitaceae family and is commonly used as a traditional remedy for diabetes in Asia, Africa and South America, where the plant extract has been referred to as vegetable insulin. Previous studies have reported on the hypoglycaemic activity of *C. ficifolia* in animals and man (Roman-Ramos et al 1995; Acosta-Patino et al 2001; Alarcon-Aguilar et al 2002). However, the active components have not been identified. We have investigated the active fractions from *C. ficifolia* to establish the precise mechanism of its hypoglycaemic effects. Analysis of the carbohydrate content of *C. ficifolia* fruit extract revealed that it contained fairly high levels of D-CI. *C. ficifolia* fruit extract was evaluated to see whether it contained sufficient amounts of naturally occurring D-CI towards the effective control of blood glucose, oral glucose tolerance test, liver glycogen, total haemoglobin and glycosylated haemoglobin in streptozotocin-induced diabetic rats.

Materials and Methods

Standards and chemical reagents

The *myo*-inositol standard, D-CI standard, phenyl-α-D-glucoside (internal standard), trimethylsilylimidazole, pyridine, and streptozotocin were purchased from Sigma Chemical Co. (USA).

Preparation of extract

The fruits of *C. ficifolia* were collected from local farmers. *C. ficifolia* fruit extract was prepared by extracting 0.5 kg dried whole fruits without the seeds using 70% methanol in a ratio of 1:10. The extraction was carried out at 50 °C for 1 h with stirring at regular intervals. It was then filtered and evaporated using a rotary evaporator under vacuum until dryness or an approximate 50-fold reduction in solvent volume was achieved. This form was used for administration to rats in this study.

Analysis of inositols and soluble carbohydrates in *C. ficifolia* fruit extract

Samples (100 μL) of the concentrate solution were transferred to silylation vials (Pierce) and evaporated to dryness under nitrogen at 40°C. The dry residues were derivatized with 1.6 mL silylation reagent (trimethylsilylimidazole/pyridine, 1:1, v/v, containing 200 μg phenyl-α-D-glucoside) at 75–80°C for 1 h. Derivatization and analysis by high resolution GC was performed according to Horbowicz & Obendorf (1994). Briefly, derivatized carbohydrates were injected into a Shimadzu gas chromatograph GC-17A (Columbia, MD) equipped with a flame ionization detector and split injector. Carbohydrates were separated on an RTX-5MS capillary column (25 m length, 0.25 mm i.d., and 0.25 μm film thickness; Restek, Bellefonte, PA) programmed for a temperature range from 150 to 200°C at the rate of 3°C min^{-1} and then to 325°C at the rate of 7 °C min^{-1}. Initial and final temperatures were held for 5 and 20 min, respectively. The injector and detector temperatures were held at 270 and 350°C, respectively. The hydrogen was used as the carrier gas at a flow rate of 1.5 mL min^{-1}, whereas the split ratio used was 1:40. Soluble carbohydrates including inositols were quantified using phenyl-α-D-glucoside as the internal standard. Calibration curves of each standard with r^2 in the range 0.989–0.993 were obtained. D-*chiro*-Inositol standard was added to confirm peak identification.

Animals

Male Wistar rats (180–200 g; from the Medical Center of Fudan University) were fed on a standard pellet diet and water was freely available. All the experimental protocols for animal care procedures were approved by the Ethical Committee of Fudan University.

Experimental induction of diabetes

Diabetes mellitus was induced in rats by a single intraperitoneal injection of freshly prepared streptozotocin (45 mg kg^{-1}) in 0.1 M citrate buffer (pH 4.5) in a volume of 1 mL kg^{-1} (Kamalakkannan & Stanely Mainzen Prince 2003). Two days after streptozotocin administration, the blood glucose level of each rat was determined. Rats with a blood glucose range of 250–300 mg dL^{-1} were considered diabetic and included in the study. Blood was collected by sinocular puncture.

Experimental design

We hypothesized that a *C. ficifolia* fruit extract equivalent to 10 or 20 mg D-CI kg^{-1} of body weight would improve glucose tolerance and decrease hyperglycaemia in streptozotocin-induced diabetic rats. Treatments of rats included; a sucrose/distilled water solution (2 mL, 8.8% sucrose) that was administered at a dose level equivalent to the amount of sucrose provided in the *C. ficifolia* fruit extract; a low-dose *C. ficifolia* fruit extract (10 mg D-CI kg^{-1}) dissolved in 2 mL 4.4% sucrose/distilled water solution; a high-dose *C. ficifolia* fruit extract (20 mg D-CI kg^{-1}) dissolved in 2 mL distilled water; or chemically synthesized D-CI (20 mg kg^{-1}) dissolved in 2 mL 8.8% sucrose/distilled water. In the experiment, a total of 30 rats (6 normal; 24 streptozotocin-diabetic surviving rats) were used. The rats were divided into five groups of six rats each: groups 1 and 2, normal and streptozotocin-induced diabetic rats given intragastrically solutions of the sucrose/distilled water daily for 30 days, respectively; groups 3 and 4,

streptozotocin-induced diabetic rats administered intragastrically the low- and high-dose *C. ficifolia* fruit extract daily for 30 days, respectively; group 5, streptozotocin-diabetic rats treated intragastrically with chemically synthesized D-CI daily for 30 days.

All the rats were bled at intervals of 10 days for 30 days to measure blood glucose. After 30 days of treatment, the rats were decapitated after an overnight fast. Blood was collected in heparinized tubes and plasma was separated after centrifugation. Liver tissues were excised immediately and stored in ice-cold containers.

Estimation of blood glucose, hepatic glycogen and plasma insulin

Fasting blood glucose was estimated by the o-toluidine method (Sasaki et al 1972). Hepatic glycogen was estimated by the method of Morales et al (1973). Plasma insulin was performed by the ELISA method using a Boehringer Mannheim kit (Boehringer analyzer ES 300), Mannheim, Germany.

Determination of total haemoglobin and glycosylated haemoglobin

Total haemoglobin was estimated by the cyanomethaemoglobin method (Drabkin & Austin 1932) and glycosylated haemoglobin was estimated by the method of Sudhakar Nayak & Pattabiraman (1981).

Oral glucose tolerance test in fasted diabetic and normal rats

Treatments were administered to fasted diabetic and normal rats before an oral glucose tolerance test (OGTT). After 4 h of fasting, diabetic or normal rats were given intragastrically either a sucrose/distilled water solution (8.8% sucrose), a high-dose *C. ficifolia* fruit extract (20 mg D-CI kg^{-1}) or chemically synthesized D-CI (20 mg kg^{-1}). One hour following treatment, blood was collected via the saphenous vein for the 0 time point. Immediately following, rats received 1 g glucose kg^{-1} body weight (70% glucose solution) intragastrically. Blood was collected at 30, 60, 90, and 120 min after administration of glucose. Blood samples were held on ice until centrifuged to obtain serum. Serum samples were stored at –20°C until analysis.

Statistical analysis

All data were expressed as mean ± s.e. for six rats in each experimental group. Data were compared by analysis of variance and only values with $P < 0.01$ were considered as significant.

Results

Carbohydrate content of *C. ficifolia* fruit extract

As shown in Table 1, soluble carbohydrates of the *C. ficifolia* fruit extract included D-CI (2.9 mg g^{-1}), *myo*-inositol (7.8 mg g^{-1}), fagopyritols (33.6 mg g^{-1}) and sucrose (126.8 mg g^{-1}). Although the majority of carbohydrates found in the extract were from sucrose and fagopyritols, there were fairly high levels of D-CI in *C. ficifolia* fruit extract. Galactopyranosyl D-*chiro*-inositols are relatively rare and have been isolated recently in seeds of certain plants: as a minor component of the sucrose fraction of *Glycine max* (Fabaceae) and lupins, and as a major component of *Fagopyrum esculentum* (Polygonaceae) (Horbowicz et al 1998).

Effect of *C. ficifolia* fruit extract on blood glucose levels in diabetic rats

Glucose levels measured in blood of normal and experimental rats are given in Table 2. Streptozotocin-induced diabetic rats showed significantly increased levels of blood glucose as compared with normal rats. *C. ficifolia* fruit extract and chemically-synthesized D-CI significantly ($P < 0.01$) decreased blood glucose in diabetic rats.

Effect of *C. ficifolia* fruit extract on body weight, glycogen, haemoglobin, glycosylated haemoglobin and plasma insulin in diabetic rats

Table 3 shows the changes in body weight, liver glycogen, total haemoglobin, glycosylated haemoglobin and plasma insulin of normal and experimental rats. In diabetic rats, the body weight, liver glycogen, total haemoglobin and plasma insulin were decreased, but glycosylated haemoglobin was increased as compared with normal rats ($P < 0.01$). *C. ficifolia* fruit extract and chemically-synthesized D-CI-treated diabetic groups showed significant ($P < 0.01$) increases in body weight, liver glycogen, total haemoglobin and plasma insulin, and a decrease in glycosylated haemoglobin compared with untreated diabetic rats.

Effect of *C. ficifolia* fruit extract on oral glucose tolerance test in fasted diabetic rats

The oral glucose tolerance level measured in the blood of diabetic and normal rats after 4 h fasting is shown in Figure 1. In streptozotocin-induced diabetic rats, blood glucose concentrations of both *C. ficifolia* fruit extract- and chemically-synthesized D-CI-treated rats were significantly lower than those of the sucrose/distilled water solution-treated rats. In *C. ficifolia* fruit extract- and chemically synthesized D-CI-treated diabetic rats, a decrease in blood glucose concentration was observed after 60, 90 and 120 min. Meanwhile, administration of *C. ficifolia* fruit

Table 1 Carbohydrate content of *C. ficifolia* fruit extract

Soluble carbohydrate	Amount (mg g^{-1})
D-*chiro*-Inositol	2.9 ± 0.2
Myo-inositol	7.8 ± 0.5
Fagopyritols	33.6 ± 3.9
Sucrose	126.8 ± 9.1

Values are mean ± s.e. of four determinations.

Table 2 Effect of *C. ficifolia* fruit extract on blood glucose levels in diabetic rats

Groups	Blood glucose levels (mg/100 mL)			
	0 Day	10 Days	20 Days	30 Days
Normal + 8.8% sucrose/distilled water solution (group 1)	96.06 ± 8.19	98.06 ± 6.22	92.99 ± 6.98	98.34 ± 5.67
Diabetic + 8.8% sucrose/distilled water solution (group 2)	306.08 ± 6.31[a]	318.66 ± 9.22[a]	308.98 ± 9.32[a]	316.33 ± 7.99[a]
Diabetic + extract containing 10 mg D-CI kg^{-1} (group 3)	309.87 ± 8.13	230.99 ± 6.05[b]	159.65 ± 8.53[b]	100.88 ± 5.03[b]
Diabetic + extract containing 20 mg D-CI kg^{-1} (group 4)	298.99 ± 6.90	213.66 ± 4.77[b]	138.99 ± 7.63[b]	99.22 ± 8.44[b]
Diabetic + synthesized D-CI 20 mg D-CI kg^{-1} (group 5)	308.99 ± 7.70	216.66 ± 5.07[b]	146.99 ± 7.91[b]	99.81 ± 6.33[b]

Each value is mean ± s.e. for six rats in each group. [a]$P < 0.01$, significant difference between diabetic control and normal control. [b]$P < 0.01$, significant difference between experimental groups and diabetic control.

Table 3 Effect of *C. ficifolia* fruit extract on body weight, glycogen, haemoglobin, glycosylated haemoglobin and plasma insulin in diabetic rats

Groups	Body weight (g)		Glycogen (g/100 g tissue)	Haemoglobin (g/100 mL)	Glycosylated haemoglobin (mg/100 mL)	Plasma insulin (μU mL^{-1})
	Initial	Final				
Normal + 8.8% sucrose/distilled water solution (group 1)	186.06 ± 6.19	208.06 ± 7.22	3.99 ± 0.28	12.34 ± 0.67	0.45 ± 0.02	17.9 ± 0.91
Diabetic + 8.8% sucrose/distilled water solution (group 2)	201.08 ± 6.31	168.66 ± 9.12[a]	1.78 ± 0.32[a]	6.33 ± 0.49[a]	0.96 ± 0.06[a]	8.3 ± 1.20[a]
Diabetic + extract containing 10 mg D-CI kg^{-1} (group 3)	189.87 ± 8.13	199.99 ± 6.65[b]	2.39 ± 0.33[b]	8.88 ± 0.43[b]	0.76 ± 0.04[b]	13.1 ± 1.03[b]
Diabetic + extract containing 20 mg D-CI kg^{-1} (group 4)	179.89 ± 9.01	196.33 ± 7.35[b]	3.19 ± 0.25[b]	10.98 ± 0.62[b]	0.51 ± 0.05[b]	16.9 ± 0.99[b]
Diabetic + synthesized D-CI 20 mg D-CI kg^{-1} (group 5)	186.12 ± 7.93	198.68 ± 5.95[b]	2.88 ± 0.26[b]	9.18 ± 0.71[b]	0.62 ± 0.05[b]	15.7 ± 0.98[b]

Each value is mean ± s.e. for six rats in each group. [a]$P < 0.01$, significant difference between diabetic control and normal control. [b]$P < 0.01$, significant difference between experimental groups and diabetic control.

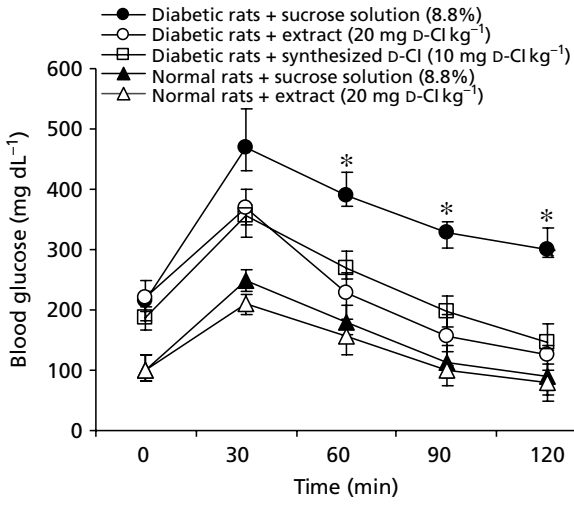

Figure 1 Blood glucose concentrations during an oral glucose tolerance test given 1 h following administration of a sucrose/distilled water solution (8.8%), a high doses *C. ficifolia* fruit extract (D-CI 20 mg kg^{-1}) or chemically synthesized D-CI (20 mg kg^{-1}) in fasted streptozotocin-induced diabetic and normal rats, respectively. Each value is mean ± s.e. for six rats in each group. *$P < 0.01$, significant difference between *C. ficifolia* fruit extract and sucrose/distilled water solution treatment in diabetic rats.

extract to normal rats before an oral glucose tolerance test did not affect blood glucose concentrations.

For all the parameters studied, *C. ficifolia* fruit extract-treated groups at doses of 10 and 20 mg D-CI kg^{-1} showed significant effects. The 20 mg D-CI kg^{-1} dose had a highly significant effect and restored all the parameters to near normal levels when compared with chemically-synthesized D-CI.

Discussion

The major finding of this study was that *C. ficifolia* contained fairly high levels of D-CI (2.9 mg g^{-1} in *C. ficifolia* fruit extract). The *C. ficifolia* fruit extract-treated groups at doses of 10 and 20 mg D-CI kg^{-1} showed that there was a significant reduction in blood glucose and glycosylated haemoglobin, and an increase in body weight, hepatic glycogen, total haemoglobin and plasma insulin when compared with the diabetic control. Oral administration of *C. ficifolia* fruit extract containing 20 mg D-CI kg^{-1} showed a significant effect on orally administered glucose load in diabetic rats without inducing the hypoglycaemic state. It was also found that the antihyperglycaemic effects of *C. ficifolia* fruit extract at a dose of 20 mg (D-CI kg^{-1}) were slightly more significant than chemically-synthesized D-CI at the same dose (20 mg kg^{-1}).

This result was consistent with previous studies using chemically-synthesized D-CI in streptozotocin rats. Fonteles et al (2000) reported that a single dose of D-CI ($15\,mg\,kg^{-1}$) injected into the jugular vein promoted a 21% decrease in plasma glucose of streptozotocin rats, which was different from the control rats at 80, 100, and 120 min after administration. The glucose lowering effect of the *C. ficifolia* fruit extract demonstrated in this study was of a similar magnitude to that of synthesized D-CI, suggesting that D-CI in the *C. ficifolia* extract was primarily responsible for the observed effects.

D-CI, originally discovered as a component of a putative mediator of intracellular insulin action, is thought to accelerate the dephosphorylation of glycogen synthase and pyruvate dehydrogenase which are the rate limiting enzymes of non-oxidative and oxidative glucose disposal (Ostlund et al 1993; Larner 2001). The mechanism by which administration of D-CI acts to lower blood glucose is not well understood. Fonteles et al (2000) and Ortmeyer et al (1993) suggested that acute administration of D-CI might act to lower plasma glucose by being incorporated into a mediator precursor. Sanchez-Arias et al (1992) demonstrated that streptozotocin rats had impaired GPI-dependent insulin signalling. Isolated hepatocytes from streptozotocin rats had lower amounts of GPI compared with control rats. Streptozotocin-induced diabetes also blocked the hydrolysis of GPI in response to insulin and markedly reduced the uptake of IPG. Larner (2002) reported that urinary *chiro*-inositol excretion was much lower in diabetic rats compared with normal rats. This pattern of inositol excretion may be related to an altered GPI–IPG signalling system. It was possible that administration of D-CI corrected the GPI-dependent signalling defect of streptozotocin rats. Insulin deficiency in streptozotocin-induced diabetes leads to a decrease in glucose utilization by the liver, muscle, and adipose tissue and an increase in hepatic glucose production (Alemzadeh et al 2002). The antihyperglycaemic effect of D-CI may result from inhibition of hepatic glucose output or enhanced glucose transport, glucose utilization, glucose disposal, or glycogen synthesis. Therefore, the antihyperglycaemic effect of *C. ficifolia* fruit extract might result from its containing a significant amount of D-CI, and the potential role of D-CI in regulation of the GPI–IPG insulin signalling system.

In addition to D-CI, the *C. ficifolia* fruit extract contained a large amount of *myo*-inositol ($7.8\,mg\,L^{-1}$), also identified as a component of an IPG with insulin mimetic effects. However, some previous work has suggested that administration of *myo*-inositol has no effects on plasma glucose concentrations. For example, dietary supplementation with 1.0% *myo*-inositol for 14 days had no effects on hyperglycaemia of streptozotocin rats (Greene et al 1975). However, Ortmeyer (1996) was able to observe the improvement of glucose tolerance by *myo*-inositol in rhesus monkeys. Diabetic conditions and/or animal species may produce these differences. A possible explanation raised by these latter authors was that the effectiveness of *myo*-inositol may have resulted from its conversion to *chiro*-inositol in-vivo. *C. ficifolia* contains relatively high amounts of free *myo*-inositol. Its role is not known. We presumed that *myo*-inositol might have contributed to the glucose-lowering effects of the *C. ficifolia* fruit extract by its conversion to *chiro*-inositol in-vivo. Future research may elucidate a beneficial role for such a large amount *myo*-inositol present in the *C. ficifolia* fruit extract in other aspects of health and disease. The *C. ficifolia* fruit extract contained free D-CI ($2.9\,mg\,L^{-1}$), but besides this, the extract contained $33.6\,mg\,g^{-1}$ fagopyritols, an amount more than tenfold the quantity of D-CI. The role of these D-CI derivatives is unknown. Since the fagopyritols are small oligosaccharides containing D-CI as one of the monomer units, they could be hydrolysed in-vivo to provide more D-CI. Also, the fagopyritols themselves structurally resemble rather closely the known insulin-mimetic IPG molecules. Therefore, the fagopyritols might have contributed to the glucose-lowering effects of the *C. ficifolia* fruit extract. Further investigation is needed to elucidate these effects.

Findings from this study have suggested that *C. ficifolia* fruit extract was an effective source of D-CI for hypoglycaemic effects in rats. *C. ficifolia* can provide a concentrated natural source of D-CI in the food supply and therefore may be useful in the treatment of diabetes.

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