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In vivo assessment of antidiabetic and antioxidative activity of natural phytochemical isolated from fruit-pulp of *Eugenia jambolana* in streptozotocin-induced diabetic rats

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ABSTRACT

Objectives: *Eugenia jambolana* (*E. jambolana*) is well known for its antidiabetic potential. The aim of the present study was to investigate the antidiabetic and antioxidative effect of an active compound (FIIc) isolated from fruit-pulp of *E. jambolana* in streptozotocin (45 mg/kg body weight)-induced diabetic rats.

Methods: FIIc was isolated from the crude aqueous extract of fruit-pulp by ion-exchange column chromatography and high-performance column chromatography. Detailed UV, NMR, and IR spectra suggested that FIIc is *α-hydroxy succinamic acid*. FIIc was orally administered to diabetic rats at a dose of 10, 15, and 20 mg/kg body weight (mg/kg bwt.) to determine its effective dose. Thereafter, effective dose was administered to 8 weeks to determine its antidiabetic and antioxidative activity by estimation of glycemic index, lipid profile, key enzymes of carbohydrate metabolism, and oxidative stress parameters.

Results: Administration of 15 mg/kg dose daily for 8 weeks led to significant ($P < 0.001$) fall in fasting blood glucose. Treatment with FIIc (15 mg/kg bwt.) showed significant improvement ($P < 0.001$) in all the biochemical parameters.

Discussion: The results demonstrate that FIIc possesses significant antidiabetic and antioxidative activity.

KEYWORDS

Eugenia jambolana; diabetes mellitus; streptozotocin; antidiabetic activity; antioxidative activity

Introduction

Diabetes mellitus is a syndrome, characterized by hyperglycemia together with impaired metabolism of glucose and other energy-yielding fuels such as lipids and proteins [1]. In spite of the presence of known antidiabetic medicine in the pharmaceutical market, there is growing interest in herbal remedies because synthetic drugs lead to undesirable side effects [2]. The search for efficient and safer antidiabetic plant drug is then of great importance.

Traditional medicinal plants having antidiabetic properties can be used as drugs or simple dietary adjuvant to existing therapies of diabetes. The hypoglycemic activity of many plants/plant products has been evaluated and confirmed in animal models, as well as in human beings [3]. In India, several indigenous plant products have been used by the practitioners of the Ayurvedic system of medicine to treat diabetes [4].

E. jambolana (Family: Myrtaceae) is a large tree found in most of the forests all over India from the Sub-Himalayan tract to the extreme south. Fruits are oval to elliptical 1.5–3.5 cm long, dark purple or nearly black, luscious, fleshy, and edible. The antihyperglycemic activity of the seeds of *E. jambolana* is well documented [5–10]. Recently, we have demonstrated the antidiabetic effect of active principle isolated from the seeds of *E. jambolana* [11]. However, studies on the antihyperglycemic activity of fruit-pulp of *E. jambolana* are lacking. The previous study showed that water extract of fruit-pulp had been found to show hypoglycemic activity immediately or as early as 30 minutes [4], while seeds require 24 hours for the same effect. However, hot water extract of dried fruit-pulp is found to be inactive in alloxan-induced hyperglycemia. In our previous study [12], we have shown that water extract of fruit-pulp is more potent in reducing the fasting blood glucose (FBG) as compared to the ethanolic extract. So far, no study has been reported about purification/isolation of active principle from aqueous fruit-pulp extract of *E. jambolana*. In our lab, we have isolated/purified an active principle from the aqueous extract of fruit-pulp of *E. jambolana* [13,14] and patents for isolation have already been granted (US Patent No. 6,428,825 August 2002; Indian Process Patent No. 188759 May 2003; Indian Product Patent granted No. 230753, February 2009). In this paper, we have investigated the antidiabetic and antioxidative effect of an active compound in streptozotocin (STZ)-induced diabetic rats, which is not reported earlier anywhere in literature.

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Material and methods

Plant material

Fresh fruits of *E. jambolana* were taken from the Azadpur Mandi (Herbal market) at Delhi. The identity was done with the help of a botanist using taxonomic rules (voucher specimen no: P-96/7) and a specimen was deposited for further references in Botanical Garden, Kolkata, India.

Preparation of crude aqueous extract

The crude aqueous extract was prepared from fresh fruits of *E. jambolana* by separating seeds from fruit-pulp. Approximately, 100 g of fruit-pulp was mixed with 200 ml of double distilled water and ground in electric grinder which was allowed to stand overnight. The pulp was then filtered through 5–6 layers of muslin cloth. The filtrate was spun in a refrigerated centrifuge for 15 minutes at 10,000 rpm and then the supernatant was lyophilized. The whole procedure was carried out at 4°C in a cold room. The yield of lyophilized water extract was about 10 g from 650 g of fruit-pulp, obtained from 1 kg fruits of *E. jambolana*.

Isolation and purification of antihyperglycemic compound (FIIc)

The lyophilized aqueous extract of the pulp was dissolved in distilled water (10%) and then applied on to the bed of the pre-packed column without disturbing it. Fractions were then eluted with 0.1 M phosphate buffer (pH 6.0) at a flow rate of 5–6 drops/minute. Out of the four fractions, fraction two (FII) showed potent antihyperglycemic activity. The separation of fraction two from other fractions was clear without any overlapping. Fraction two was further subjected to purification by re-chromatography resulted in a fraction FIIc, i.e., purified active principle. The yield of active compound was about 100 mg from 10 g of lyophilized water extract. Detailed UV, NMR, and IR spectrum suggested that FIIc is a α-hydroxy succinamic acid which is a small aliphatic organic compound having molecular formula $C_4H_7O_4N$.

Experimental animals

Male Wistar albino rats (weighing 160–200 g) were taken from Central Animal House of University College of Medical Sciences (UCMS), Delhi, India. The animals were housed under standard conditions of temperature (22 ± 2°C) and at 12 hours light–dark cycle. The rats were fed with a commercial diet (Hindustan Lever Ltd., Mumbai) and water *ad libitum*. The experimental protocol was approved by the Institutional Animal Ethical Committee (IAEC) of UCMS, Delhi, India. All experimental procedures were conducted in accordance with the ethical guidelines of International Association for the Study of Pain [15].

Induction of experimental diabetes

STZ was purchased by M/S Sigma Aldrich, USA and at a dose of 45 mg/kg in 0.1 M citrate buffer, pH 4.5 was injected intraperitoneally to overnight fasted rats. STZ injected animals exhibited hyperglycemia within 48 hours. FBG levels were measured after 48 hours and again repeated twice at an interval of three days. The rats with stabilized diabetes having FBG values of 300 mg/dl or above were considered severely diabetic and included in the study.

Experimental procedure

The rats were randomized into the following groups of five rats each. Group 1, control animals; Group 2, diabetic animals; Group 3, diabetic treated with FIIc (10 mg/kg); Group 4, diabetic treated with FIIc (15 mg/kg); Group 5, diabetic-treated with FIIc (20 mg/kg); Group 6, diabetic treated with glibenclamide (600 µg/kg), a standard antidiabetic drug.

Glibenclamide and FIIc were dissolved in 1 ml of distilled water and administered orally via a standard orogastric cannula. Antihyperglycemic activity was assessed by fall in FBG. Blood samples were collected from overnight fasted rats before administration of the drug and simultaneously 90 minutes, 3 hours, and 5 hours after giving the drugs to determine the blood glucose levels. The antihyperglycemic effect of FIIc at a dose of 15 mg/kg bwt. was also studied at 24 hours, 48 hours, and 72 hours after drug administration to know how long it is effective in maintaining the blood glucose levels.

After assessment of antihyperglycemic activity with different doses of FIIc, the effective dose was found to be 15 mg/kg bwt. Therefore, subsequent studies were carried out with 15 mg/kg bwt. for 8 weeks in diabetic rats. After 8 weeks of treatment, blood samples were taken from overnight fasted rats and then all the rats were sacrificed for tissue collection.

Blood samples were drawn from overnight fasted rats by retro-orbital venepuncture technique [16]. FBG was measured using the glucose oxidase-peroxidase method [17]. Blood samples, collected in EDTA vials, were used for the estimation of glycosylated hemoglobin (GHb) [18]. Plasma insulin and C-peptide levels were estimated by enzyme-linked immunosorbent assay using commercially available kits from Mercodia (Uppsala, Sweden) and DRG (Marburg, Germany), respectively. Hepatic hexokinase [19], phosphofructokinase [20], glucose-6-phosphatase [21], and fructose-1,6-bisphosphatase [22] were assayed. Lipid profile were estimated in serum. Serum triglycerides (TGs)

were determined by the method of Fossati and Prencipe [23]. Total serum cholesterol (TC) [24], high-density lipoprotein-cholesterol (HDL-C) [25], serum malondialdehyde (MDA) [26] as an index of lipid peroxidation and reduced glutathione (GSH) [27] in erythrocytes were estimated by using standard techniques. The activity of superoxide dismutase (SOD) [28,29] and catalase [30] was assayed in erythrocytes.

Toxicity study

The healthy rats were given FIIc at doses of 10 times, 15 times of effective dose daily for 4 weeks (five rats were taken for each dose). The animals were observed for 1 hour continuously and then hourly for 4 hours and finally after every 24 hours up to 4 weeks for any signs of toxicity (such as writhing, gasping, palpitation, and decreased respiratory rate).

Statistical analysis

Values were expressed as the mean ± SEM for five animals in each group. The data were analyzed by using repeated measure analysis of variance (ANOVA) followed by Dunnett's test and repeated measure ANOVA, followed by Tukey's test. The results were considered significant at $P < 0.01$.

Results

Acute treatment

Table 1 shows antihyperglycemic effect of FIIc in diabetic rats at different intervals of time. No statistically significant reduction in FBG was observed from 90 minutes to 5 hours after oral administration of the FIIc at a dose of 10 mg/kg bwt. While 15 mg/kg bwt. and 20 mg/kg bwt., doses showed statistically significant antihyperglycemic activity ($P < 0.001$) from 3 hours onwards. Effect of both the doses, i.e., 15 and 20 mg/kg bwt. was comparable. Hence, 15 mg/kg bwt. dose was considered as an effective dose.

Long- term treatment (8 weeks)

Glycemic control

Treatment with FIIc showed a significant decrease ($P < 0.001$) in the levels of FBG and GHb (Table 2). A significant reduction in the levels of insulin and C-peptide was observed in diabetic animals compared to control animals. Administration of the FIIc and glibenclamide to diabetic animals significantly improved serum insulin and C-peptide levels.

Effect on the key enzymes of carbohydrate metabolism

Table 3 demonstrates the effect of FIIc and glibenclamide on key enzymes of glycolysis (hexokinase and phosphofructokinase) and gluconeogenesis (glucose-6-phosphatase and fructose-1,6-bisphosphatase) in diabetic animals. The activities of hexokinase and phosphofructokinase were found to be significantly suppressed, whereas the activities of glucose-6-phosphatase and fructose-1,6-bisphosphatase significantly increased in diabetic animals compared to controls. Supplementation of FIIc produced significant improvement in the activity of these enzymes. However, effect of glibenclamide on both glycolytic and gluconeogenic enzymes was less than compared to FIIc in diabetic rats.

Serum lipid profile

The effect of FIIc on serum lipid profile in diabetic rats is summarized in Table 4. The levels of TG and TC were significantly ($P < 0.001$) reduced, whereas high-density lipoprotein-cholesterol was significantly ($P < 0.001$) increased in FIIc-treated diabetic animals. The hypolipidemic effect of FIIc was comparable to that of glibenclamide.

Effect on oxidative stress parameters

As shown in Table 5, diabetic animals showed significantly ($P < 0.001$) low levels of erythrocyte's GSH (1.89 ± 0.15 mg/g Hb) when compared with controls (2.89 ± 0.05 mg/g Hb). The levels of GSH were significantly (2.65 ± 0.16 mg/g Hb) improved following supplementation of FIIc. A significant elevation ($P < 0.001$) in the levels of serum MDA (4.21 ± 0.137 nmol/l) was observed in diabetic animals when compared to controls (2.06 ± 0.11 nmol/l). However, oral administration of FIIc significantly decreased ($P < 0.001$) the serum MDA levels (2.23 ± 0.206 nmol/l) in diabetic animals.

The activity of antioxidant enzyme SOD and catalase in erythrocytes of diabetic animals was found to be significantly decreased ($P < 0.001$) when compared to control animals. Following treatment with FIIc, the activity of SOD & catalase was significantly improved (1841.60 ± 129 U/GHb and 3.25 ± 0.113 U/GHb, respectively) in diabetic animals.

Toxicity study

The animals treated with 10 times, 15 times of effective dose (15 mg/kg) of FIIc did not show any drug-induced clinical signs of toxicity (such as writhing, gasping, palpitation, and decreased respiratory rate) during the whole experimental period. All the animals were appeared healthy and active. No death was registered.

Table 1. Acute antihyperglycemic effect of FIIc at various time intervals in diabetic rats.

		Blood glucose (mg/dl)						
		Fasting			After treatment			
Groups	Dose (per kg bwt.)	0 hour	90 minutes	%Fall	3 hours	%Fall	5 hours	%Fall
Control animals	Vehicle	79 ± 3.2	82 ± 2.9	–	80.5 ± 3.4	–	79.2 ± 2.8	–
Diabetic animals	Vehicle	303 ± 8.2	302 ± 6.9	–	306 ± 3.4	–	308 ± 7.1	–
Diabetic + gliben	600 µg	300 ± 7.2	278 ± 4.9[a]	7.2	269.5 ± 5.2[a]	10.2	258.5 ± 4.8[a]	13.8
Diabetic + FIIc	10 mg	305 ± 6.9	292 ± 3.1	4	275 ± 6.2	9.8	265 ± 4.5[a]	13.1
Diabetic + FIIc	15 mg	304 ± 7.1	283 ± 4.2	6.9	249 ± 3.9[a]	18.1	239.5 ± 3.1[a]	21.2
Diabetic + FIIc	20 mg	303 ± 6.6	281.3 ± 3.7[a]	7.16	246.5 ± 3.6[a]	18.6	238 ± 2.9[a]	21.4

Notes: Values are mean ± SEM for five animals in each group. FIIc: purified active compound; gliben: glibenclamide. %Fall was calculated as the difference between the two values/starting value × 100.
[a]$P < 0.001$ vs. compared to 0 hour.

Table 2. Glycemic control by FIIc (15 mg/kg bwt.) after 8 weeks treatment in diabetic rats.

Groups	FBG (mg/dl)	GHb (%)	Insulin (µU/ml)	C-peptide (pmol/ml)
Control animals	93.20 ± 9.6	2.85 ± 0.16	12.09 ± 0.84	0.198 ± 0.005
Diabetic animals	371.20 ± 31.18[a]	15.60 ± 0.57[a]	2.24 ± 0.89[a]	0.028 ± 0.003[a]
Diabetic + FIIc	120.4 ± 12.6[b]	5.84 ± 0.94[b]	10.98 ± 1.01[b]	0.178 ± 0.011[b]
Diabetic + gliben	153.4 ± 30.1[b]	6.6 ± 1.19[a,b]	8.28 ± 0.98[b]	0.159 ± 0.009[b]

Note: Values are mean ± SEM for five animals in each group.
[a]$P < 0.001$ vs. healthy control.
[b]$P < 0.001$ vs. diabetic control.

Discussion

The present study is a preliminary assessment of the antidiabetic and antioxidant activities of active compound purified from fruit-pulp of *E. jambolana*. It was observed that the antihyperglycemic effect of FIIc started from 90 minutes and gradually increases up to 5 hours (the effect was not seen for more than 5 hours due to the restriction of fasting period). However, FIIc at a single dose of 15 mg/kg bwt. decreases FBG steadily up to 48 hours in diabetic animals. This shows that the drug takes the initial time to reach the target tissues in the body, or it gets metabolized and the metabolite (s) is/are active. This also showed that the antihyperglycemic effect of FIIc persist till 48 hours with its single dose (15 mg/kg bwt.) in diabetic animals, which was not observed in glibenclamide-treated animals.

As the effective dose was found to be 15 mg/kg bwt., further studies were carried out with this dose only. The FIIc produced significant ($P < 0.001$) reduction in blood glucose level in diabetic rats after 8 weeks treatment (as shown in Table 2). During diabetes, the excess glucose present in the circulation reacts with hemoglobin to form GHb. The estimation of GHb is a well-accepted parameter used in the prognosis of the diabetic state [31]. In this study, administration of FIIc to diabetic animals exhibited significant reduction in the levels of GHb in the FIIc-treated animals, which reflects improved glycemic control by the FIIc.

STZ a highly cytotoxic agent of pancreatic β-cells induces diabetes by damaging the cells that causes the reduction in insulin release. It is reported that treatment of diabetic animals with medicinal plants resulted in insulinogenic effect. The possible mechanism through which FIIc exerts its antihyperglycemic effect might have been due to the increased release of insulin from remnant β-cells or regenerated β-cells. Treatment with the FIIc produced a significant elevation in plasma levels of insulin in diabetic rats. This suggests that FIIc potentiate the release of insulin from pancreatic islets. The estimation of C-peptide further confirms the insulinogenic action of FIIc as increased amounts of C-peptide were observed in FIIc-treated diabetic animals. C-peptide was measured as it permits better quantization of endogenous insulin secretion rather than peripheral insulin levels. The half-life of C-peptide is much longer than that of insulin and due to metabolic inertness of

Table 3. Effect of FIIc (15 mg/kg bwt.) on key hepatic carbohydrate-related enzyme activity in diabetic rats after 8 weeks treatment.

Groups	Hexokinase (U/mg of protein)	Phosphofructokinase (U/mg of protein)	Fructose-1,6-bisphosphatase (U/g of protein)	Glucose-6-pase (U/mg of protein)
Control animals	0.219 ± .021	12.85 ± 0.35	58.01 ± 3.2	17.36 ± 0.48
Diabetic animals	0.082 ± 0.007[a]	4.19 ± 0.31[a]	195.8 ± 6.5[a]	45.17 ± 1.32[a]
Diabetic + FIIc	0.192 ± 0.012[b]	11.21 ± 0.24[b]	75.6 ± 4.9[b]	19.82 ± 0.99[b]
Diabetic + glibe.	0.189 ± 0.016[b]	10.49 ± 0.20[b]	99.8 ± 05.2[b]	23.31 ± 1.12[b]

Note: Values are mean ± SEM for five animals in each group.
[a]$P < 0.001$ vs. healthy control.
[b]$P < 0.001$ vs. diabetic control.

Table 4. Effect of FIIc (15 mg/kg bwt.) on serum lipid profile of diabetic rats after 8 weeks treatment.

Groups	Serum lipid profile (mg/dl)		
	TG	TC	HDL
Control animals	74.60 ± 3.4	67.8 ± 3.0	37 ± 2.1
Diabetic animals	224.2 ± 25.0[a]	164.0 ± 36[a]	19 ± 4.2[a]
Diabetic + FIIc	78.8 ± 19.7[b]	69.0 ± 8.8[b]	32 ± 1.8[b]
Diabetic + glibenclamide	88.4 ± 19.6[b]	85.6 ± 9.8[b]	31 ± 0.9[b]

Note: Values are mean ± SEM for five animals in each group.
[a]$P < 0.001$ vs. healthy control.
[b]$P < 0.001$ vs. diabetic control.

C-peptide, its levels in peripheral venous blood are about five to six times greater than those of insulin [32].

STZ-induced diabetic animals also showed suppression in the activity of glycolytic enzymes (hexokinase and phosphofructokinase) with a concomitant elevation in the activity of gluconeogenic enzymes (glucose-6-phosphatase and fructose-1,6-bisphosphatase). These results are consistent with earlier findings in experimental diabetes [11]. Following supplementation with FIIc, the activity of both glycolytic and gluconeogenic enzymes were significantly improved. Therefore, it seems to increase the flux of glucose into the glycolytic pathway in an attempt to reduce high-blood glucose concentration. Furthermore, suppression of gluconeogenic enzymes following treatment with FIIc shows a decreased release of glucose via gluconeogenesis.

Since lipid abnormalities and increased oxidative stress accompanied by atherosclerosis are the major causes of cardiovascular disease in diabetic patients, the ideal treatment for diabetes should have a favorable effect on the lipid profile and antioxidant system in addition to glycemic control. Therefore, the effect of FIIc on serum lipids and oxidant/antioxidant system was studied. Diabetic hyperglycemia is always accompanied with dyslipidemia. That is characterized by increase in TC, TG and fall in high-density lipoprotein-cholesterol. This altered serum lipid profile was significantly ($P < 0.001$) reversed after treatment with the FIIc for 8 weeks in the diabetic animals. Insulin insufficiency is responsible for the derangement of lipid and lipoprotein metabolism [33]. Insulin decreases TG levels and increases HDL through activation of lipoprotein lipase, an enzyme responsible for the catabolism of TG-rich lipoproteins, which provide a significant portion of HDL. Insulin also increases receptor-mediated removal of LDL-C. Hence, decreased activity of insulin during diabetes leads to increased levels of serum LDL-C and consequently hypercholesterolemia [34]. This suggested that the significant control in the level of serum lipids in FIIc-treated diabetic animals might be due to the improvement in the plasma insulin level on administration of the FIIc. On the other hand, decreased glucose disposal during diabetes leads to increased utilization of fatty acids for energy production, which consequently results in increased formation of acetyl coenzyme A and thus of lipids. Hence, the increased insulin levels brought about by FIIc indicates the possible FIIc effect on sensitizing tissues like skeletal muscles and adipose tissue for uptake of glucose and thus preventing hyperlipidemia a potential factor for coronary heart disease. Thus, prevent the progression of cardiovascular diseases.

During diabetes, an imbalance between enzymatic and non-enzymatic antioxidant defense and generation of free radicals leads to enhanced oxidative stress, as evidenced by a fall in GSH content and activity of antioxidant enzymes, along with a concomitant increment in lipid peroxidation. GSH, a natural antioxidant, is a potent scavenger of reactive oxygen species (ROS) and helps to maintain the structural and functional integrity of erythrocytes. Treatment with the FIIc improves the GSH level in erythrocytes of diabetic animals. SOD and CAT are two major antioxidant enzymes and are involved in the direct elimination of reactive oxygen species [35]. Reduced activity of these free-radical scavenging enzymes may result in many deleterious effects due to the accumulation of superoxide radicals and H_2O_2. However, the activity of SOD and CAT was found to be partially restored following supplementation with the FIIc. Elevation in the GSH level and the activity of SOD and CAT supports the notion that the FIIc may act as a free-radical scavenging agent. Furthermore, the protective effect of FIIc on lipid peroxidation was demonstrated by the significant reduction in the levels of serum MDA in FIIc-treated rats. The restoration of altered lipid peroxidation and antioxidant defense system following treatment with FIIc could be primarily due to the subsequent lowering of blood glucose levels. It has been hypothesized that free circulating glucose is the proximal source of increased oxidative stress in the hyperglycemic condition [36]. Thus, the lowering of blood glucose levels would prevent the formation of reactive oxygen species and oxidative stress.

Table 5. Effect of FIIc (15 mg/kg bwt.) on oxidative stress parameters in diabetic rats after 8 weeks of treatment.

Groups	Serum MDA (nmol/L)	GSH (mg/g of Hb)	SOD (U/g of Hb)	Catalase (U/g of Hb)
Control animals	2.06 ± 0.11	2.89 ± 0.05	1980.20 ± 96	3.39 ± 0.06
Diabetic animals	4.21 ± 0.14[a]	1.89 ± 0.15[a]	1040.60 ± 127[a]	1.63 ± 0.30[a]
Diabetic + FIIc	2.23 ± 0.21[b]	2.65 ± 0.16[b]	1841.60 ± 129[b]	3.25 ± 0.11[b]
Diabetic + glibe	2.75 ± 0.25[b]	2.56 ± 0.12[b]	1842.60 ± 53[b]	3.06 ± 0.18[b]

Note: Values are mean ± SEM for five animals in each group.
[a]$P < 0.001$ vs. healthy control.
[b]$P < 0.001$ vs. diabetic control.

Conclusion

The antihyperglycemic effect of FIIc was comparable with that of glibenclamide. However, the hypolipidemic and antioxidant effect of FIIc was found to be more potent than that of glibenclamide. Since lethal dose of FIIc is very high and it did not reveal any physical signs of toxicity or mortality even after four weeks of treatment, it can be considered relatively safe.

Thus, it is concluded that FIIc possesses potent antidiabetic, hypolipidemic, and antioxidative activity, and it may prove to be effective for the treatment of diabetes and its associated complications. The results of the present study provide an impetus for further molecular and mechanistic studies on the therapeutic action of FIIc before it can be administered as possible insulin replacement or adjuvant in the management of diabetes mellitus.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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