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ORIGINAL ARTICLE

Protective Effect of *Schisandra chinensis* Extracts against H_2O_2-induced Oxidative Damage in RINm5F Cells

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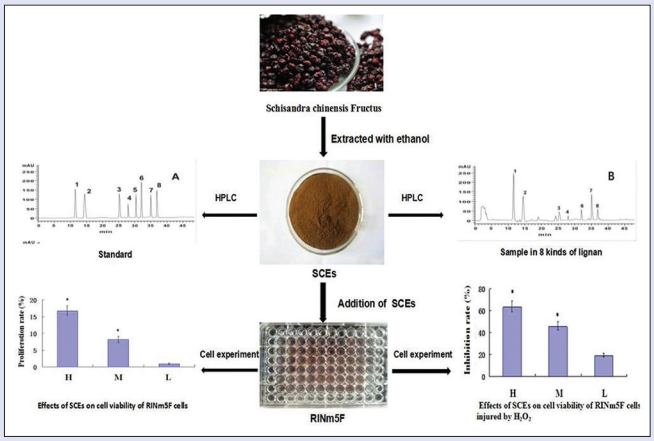
ABSTRACT

Objective: To study the protective effect of *Schisandra chinensis* extracts (SCEs) on the RINm5F cells against H_2O_2-induced oxidative damage and to provide a basis for the study of the role of SCEs in the prevention and treatment of diabetic oxidative stress. **Materials and Methods:** We performed ultrasonic preparation of SCEs and verified the presence of 8 lignans by high-performance liquid chromatography (HPLC). An *in vitro* model of H_2O_2-induced oxidative damage was established using RINm5F cells and treated with various concentrations of SCEs (high dose, medium dose, and low dose). The antioxidative activity of SCEs was observed and its mechanisms were investigated by 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide assay, and measuring malondialdehyde (MDA) and superoxide dismutase assay (SOD) according to the instructions of kits. **Results:** The total lignan content in SCEs was 18.86 mg/mL; the concentration of 8 kinds of lignans were schisandrin, 5.99 mg/mL; schisandrol B, 3.27 mg/mL; schisantherin A, 1.99 mg/mL; schisanhenol, 0.94 mg/mL; anwulignan, below the detection limit; deoxyschisandrin, 1.33 mg/mL; schisandrin B, 3.95 mg/mL; and schisandrin C, 1.39 mg/mL. SCEs promoted proliferation of islet cells and exhibited a protective effect against H_2O_2-induced oxidative damage in islet cells. The cell survival rates in the high-dose and medium-dose SCEs groups were greater than that in the model group by 63.4% and 45.6%, respectively; the protective effect of SCEs against oxidative damage was dose dependent. The intracellular MDA content was significantly decreased ($P < 0.05$), the degree of oxidative damage was reduced, and the activity of superoxide dismutase was significantly increased ($P < 0.05$) in the SCEs groups. **Conclusion:** SCEs exhibited a dose-dependent protective effect against H_2O_2-induced oxidative damage in RINm5F cells. The underlying mechanism was related to the antioxidant activity of the SCEs.

Key words: Antioxidation, diabetes, high-performance liquid chromatography, lignan, *Schisandra chinensis*

SUMMARY

- Experimental study of the protective effect of *Schisandra chinensis* extracts on oxidative damage of RINm5F was induced by H_2O_2. The content of 8 lignans in *Schisandra chinensis* was determined by high-performance liquid chromatography. The total lignan content was 18.86 mg/mL. The content of 8 kinds of lignans were schisandrin, 5.99 mg/mL; schisandrol B, 3.27 mg/mL; schisantherin A, 1.99 mg/mL; schisanhenol, 0.94 mg/mL; anwulignan, below the detection limit; deoxyschisandrin, 1.33 mg/mL; schisandrin B, 3.95 mg/mL; and schisandrin C, 1.39 mg/mL. The efficacy of *Schisandra chinensis* extracts was also studied. The antioxidant activity and the underlying mechanisms of *Schisandra chinensis* extracts in the *in vitro* model were studied by 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide assay, superoxide dismutase activity, and malondialdehyde content in cell and culture media. This study provides a theoretical basis for further study of the role of *Schisandra chinensis* extracts in the prevention and treatment of diabetic oxidative stress.



Abbreviation used: *Schisandra chinensis* Extracts (SCEs).

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INTRODUCTION

Diabetes mellitus is a chronic disease that is often accompanied by serious complications. It is one of the most common causes of morbidity and mortality globally. The condition is caused by insufficient secretion of insulin or insulin resistance in the body. Diabetes is of two types: type I and type II. Type II diabetes accounts for a vast majority of cases of diabetes.[1-5] Studies have shown that reactive oxygen species (ROS)-dominant oxidative stress is one of the important

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pathogenetic mechanisms of type II diabetes. Antioxidant enzyme treatment or oxidase scavengers have been shown to interfere with the development and progression of diabetes.[1,6-8]

Several gaps continue to persist in our understanding of the pathogenesis of diabetes. The current management approach is heavily reliant on the use of chemical drugs; however, their use is associated with varying degrees of side effects, aggravation of symptoms of diabetes, and induction of drug dependence on prolonged usage. Therefore, identification of novel antidiabetic drugs with lower toxicity is a key research imperative.

Antidiabetic agents used in Traditional Chinese medicine (TCM) have mild and stable hypoglycemic effects and are associated with low toxicity. These offer a distinct advantage in terms of lowering of blood sugar levels, regulation of lipid metabolism, and amelioration of insulin resistance. The TCM Fructus Schisandrae (*Schisandra chinensis*) acts as an astringent, replenishes qi, nourishes the kidney, and reinforces cardiac function. It contains lignans, polysaccharides, volatile oils, fatty acids, vitamins, and amino acids; among these, lignans are the most important active ingredients.[9,10] In a previous study, schisandra oil was shown to exhibit a protective effect on islet cells in a rat model of type II diabetes established using high-fat diet combined with low-dose streptozotocin. The main component of schisandra oil is lignans. Therefore, in this study, we focused on the 8 kinds of lignans in *S. chinensis* Fructus; the pharmacological activities of lignans have been extensively studied.[11-16] We prepared the *S. chinensis* extracts (SCEs) and verified the lignan content using high-performance liquid chromatography (HPLC). The protective effect of SCEs against H_2O_2-induced oxidative damage in RINm5F cells was studied and the underlying mechanisms were investigated. Our results may provide a basis for further study of the role of SCEs in the treatment of type II diabetes.[17-21]

MATERIALS AND METHODS

Experimental materials

S. chinensis Fructus was obtained from Wangqing County of Jilin and was identified by Wang Weili, the chief pharmacist of the Chinese Medicine Office of the Jilin Food and Drug Testing Institute. The cell strain, rat islet cell tumor (RINm5F), and RPMI 1640 culture medium were purchased from Gibco Company. Fetal bovine serum was purchased from the Tianjin Hao Yang Biological Products Technology Co. Ltd.

Trypsin, ampicillin, and streptomycin were purchased from the Ding Guo Company; hydrogen peroxide was purchased from Sigma Company; 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) was purchased from Anlersco Company; malondialdehyde (MDA) test kit and superoxide dismutase assay (SOD) assay kit were purchased from Nanjing built Biotechnology Co. Ltd.; Methanol is chromatographic purity.

Instruments and equipment

SHIMADZU LC-20A HPLC was purchased from the Shimadzu Corporation; 680 automatic enzyme labeling instrument was purchased from the Bole company of America; TGL-20M variable speed-refrigerated centrifuge was purchased from the Changsha Yida Technology Co. Ltd.; and SYDS-30 liquid nitrogen biological container was purchased from the Chengdu Jinfeng Liquid Nitrogen Container Co. Ltd.

Preparation of *Schisandra chinensis* extracts

The ripe fruit of *S. chinensis* Fructus was crushed to powder form. Forty gram of powder was dissolved in 600 mL of 90% ethanol (powder to liquid ratio 1:15). Three iterations of ultrasonic extraction were performed for 20 min each. The extracted liquid was decompressed to recover ethanol and the remnant was freeze dried to obtain SCE.

Determination of the content of *Schisandra chinensis* extracts

The reference substances – schisandrin, schisantherin A, and anwulignan were purchased from the China National Institute of Pharmaceutical and Biological Products. Schisandrol B, deoxyschisandrin, schisandrin B, and schisandrin C were prepared in this laboratory with a purity of >98%. Schisanhenol (purity: 98.5%) was provided by the Schisandra Institute, Beihua University. Ten milligrams each of the reference substances – schisandrin, deoxyschisandrin, schisandrin B, and schisandrol B were added to methanol to prepare a control solution with a concentration of 1 mg/mL. Ten milligrams each of the reference substances – schisandrin C, schisantherin A, schisanhenol, and anwulignan were added to methanol to prepare a control solution with a concentration of 0.8 mg/mL. One milliliter of each reference substance was added to 10 mL methanol in a volumetric flask, shaken, and filtered with a 0.45 μm membrane to obtain the reference solution.

SCEs (10 g) extract was placed in a 10 mL volumetric flask; methanol was added to constant volume to scale, shaken well, and filtered with a 0.45 μm membrane to obtain the sample solution.

Agilent ZORBAX 300SB-C18 column (4.6 mm × 250 mm, 5 μm) was selected for the column; Shimadzu GVP (4.6 mm) was selected for the protection column and the mobile phase was methanol (A) and water (B). The gradient elution is shown in Table 1. The column temperature was 27° C; the flow rate was 0.8 mL/min; the detection wavelength was 230 nm; and the injection volume was 10 μL.

Cell experiment

Sample grouping

SCEs were prepared into high-dose (100 μg/mL), medium-dose (50 μg/mL), and low-dose (l. 25 μg/mL) group, respectively.

3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide assay for cell viability

A cell suspension of the logarithmic growth phase of the islet cell strain was prepared at a concentration of 5×10^4 cells/mL. The suspension was seeded in a 96-well plate (100 μL per well). After inoculation, the cells were incubated in a 37° C, 5% CO_2 incubator. After 24 h, the cells were completely adherent, and the culture fluid was discarded. Control group, high-dose group, medium-dose group, and low-dose group of SCEs were prepared with 6 parallel holes in each group. 100 μL of the sample was added into each of the SCEs groups while 100 μL of the culture fluid was added in the control group. Both SCEs and control groups were incubated for 48 h. Subsequently, the culture broth was discarded and 100 μL of the culture solution was added. 10 μL of 5 mg/mL MTT solution was added to each well followed by incubation for 3 h. After discarding the supernatant, 100 μL of DMSO was added and shaken for 15 min. OD was measured at 570 nm using an automated enzyme-linked detector and repeated three times.

Table 1: The gradient condition of high-performance liquid chromatography

Time/(min)	A/(%)	B/(%)
0-15	60-75	40-25
16-20	75	25
21-30	75-90	25-10
31-40	90-100	10-0
41-45	100	0
46-55	100-60	0-40

Protective effect of *Schisandra chinensis* extracts against H_2O_2-induced oxidative damage in islet cells

The logarithmic growth phase of the islet cell strain was made into a cell suspension, and 1×10^5 cells/mL was seeded in 96-well plates (100 μL per well). After inoculation, the cells were incubated in 37°C, 5% CO_2 incubator. After 24 h, the cells were completely adherent and the medium was discarded. The experiments were divided into control group, model group, and high-dose, medium-dose, and low-dose SCEs groups, with 6 parallel holes in each group. 100 μL SCEs solution was added into the wells in the high-dose, medium-dose, and low-dose SCEs group, respectively, and 100 μL culture medium was added into the wells in the control and model groups, which were then incubated for 12 h. In the model group and SCEs groups, 10 μL H_2O_2 was added to achieve H_2O_2 final concentration of 0.8 mmol/L; in the control group, 10 μL phosphate-buffered saline (PBS) was added and continued to culture for 4 h. The medium in the well was aspirated and 100 μL of culture medium was added; 10 μL of 5 mg/mL MTT solution was added to each well and incubated for 3 h. Subsequently, the supernatant was discarded. 100 μL of dimethyl sulfoxide was added and shaken for 15 min. The OD value was measured at 570 nm with an automated enzyme-linked detector and repeated three times.

Determination of superoxide dismutase activity and malondialdehyde content in islet cell culture fluid

The culture liquid of the control group, model group, and the high-dose, medium-dose, and low-dose SCEs groups were taken. In accordance with the instructions of SOD and MDA kits, the OD value of each cell culture liquid was measured by automatic enzyme-linked detector and the MDA content of SOD activity in the culture medium was calculated.

Superoxide dismutase activity and malondialdehyde content in islet cells

The cells in the control group, the model group, and the high-dose, medium-dose, and low-dose SCE groups were suspended in PBS at 0°C, and the cells were disrupted by an ultrasonic cell disrupter under an ice bath; the absence of cells was observed under the microscope. According to the instructions for the SOD and MDA kits, the OD value of each cell culture fluid was measured with an automatic enzyme-linked enzyme detector, and the MDA content of the SOD activity in the culture fluid was calculated.

Statistical analysis

All data analyses were performed using SPSS statistical software (IBM, Armonk, New York, USA). The results are expressed as mean ± standard deviation. One-way analysis of variance and Q-test were performed for each experiment. $P < 0.05$ was considered indicative of statistically significant between-group difference; $P < 0.01$ was significant difference.

RESULTS AND ANALYSIS

Determination of the content of *Schisandra chinensis* extracts

The mixed control solution and the sample solution were injected into the HPLC and measured. The chromatograms of the mixed control solution and the sample solution are shown in Figure 1. The regression equation for the 8 kinds of reference products was obtained with the concentration of reference substance as the abscissa and the peak area as the ordinate [Table 2]. The contents of 8 lignans in the sample solution are shown in Table 3.

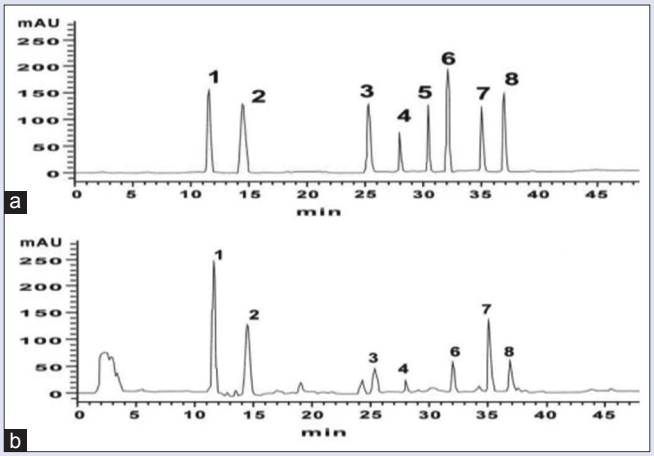

Figure 1: Standard (a) and the sample (b) in 8 kinds of lignan [Peak identification: 1, schisandrin; 2, schisandrol B; 3, schisantherin A; 4, schisanhenol; 5, anwulignan; 6, deoxyschisandrin; 7, schisandrin B; 8, schisandrin C. Other high-performance liquid chromatography conditions are described in Table 1]

Table 2: The regression equation of 8 kinds of schisandra lignans in standard

Analysis compound	Regression equation	Linear range (μg/ml)	R^2
Schisandrin	y=2192.5x+586.4	25-500	0.9997
Schisandrol B	y=2288.5x+300.3	25-500	0.9997
Schisantherin A	y=2642.2x+336.8	20-400	0.9996
Schisanhenol	y=1142.9x+108.5	20-400	0.9994
Anwulignan	y=2032.8x+85.29	20-400	0.9995
Deoxyschisandrin	y=2473.6x+35.86	25-500	0.9995
Schisandrin B	y=1278.9x+123.3	25-500	0.9996
Schisandrin C	y=2298.5x+578.5	20-400	0.9997

The effect of *Schisandra chinensis* extracts on islet cell proliferation

Compared with that in the control group, the number of islet cells in the high-dose, medium-dose, and low-dose SCEs groups increased in varying degrees in a dose-dependent manner, and that in the high-dose group increased significantly [Table 4 and Figure 2].

Protective effect of *Schisandra chinensis* extracts on islet cells induced by H_2O_2

The cell survival rate in the model group was significantly lower than that in the other groups ($P < 0.01$). Compared with the model group ($P < 0.05$), the cell survival rate increased with increase in the concentration of SCEs [Table 5 and Figure 3].

Superoxide dismutase activity and malondialdehyde content in islet cell culture medium

Compared with the model group, the SOD activity in the medium-dose and high-dose SCEs groups was significantly enhanced ($P < 0.05$), which indicated enhanced antioxidant capacity of the cells. The MDA content decreased significantly ($P < 0.05$), which indicated reduced degree of cell peroxidation injury [Table 6].

Table 3: The contents of 8 kinds of lignans in *Schisandra chinensis* (mg/g, *n*=3)

Schisandrin	Schisandrol B	Schisantherin A	Schisanhenol	Anwulignan	Deoxyschisandrin	Schisandrin B	Schisandrin C	The total lignan
5.99	3.27	1.99	0.94	- b	1.33	3.95	1.39	18.86

-b: Below LOD. LOD: Detection limit

Table 4: Effects of *Schisandra chinensis* extracts on the viability of RINm5F cells

Groups	OD value
Control	0.833±0.425
SCEs (H)	0.972±0.336*
SCEs (M)	0.901±0.426*
SCEs (L)	0.841±0.325

*P<0.05 versus control group. SCEs: *Schisandra chinensis* extracts; OD: Optical density value

Table 5: Effects of *Schisandra chinensis* extracts on the viability of RINm5F cells injured by H_2O_2

Groups	OD value
Control	0.751±0.365
Model	0.423±0.534*
SCEs (H)	0.691±0.445#
SCEs (M)	0.616±0.631#
SCEs (L)	0.505±0.623

*P<0.05 versus control group; #P<0.05 versus model group. SCEs: *Schisandra chinensis* extracts; OD: Optical density value

Table 6: Effects of *Schisandra chinensis* extracts on activity of superoxide dismutase and content of malondialdehyde in RINm5F cells' culture fluid injured by H_2O_2

Groups	MDA contents/(CB/[μmol/L])	SOD activity/(λB/[U/mL])
Control	16.389±3.012	25.301±4.65
Model	23.328±4.981*	17.328±6.321*
SCEs (H)	17.894±3.784#	24.521±5.024#
SCEs (M)	19.781±4.653#	22.801±3.981#
SCEs (L)	20.976±3.674	20.652±4.971

*P<0.05 versus control group; #P<0.05 versus model group. SCEs: *Schisandra chinensis* extracts; SOD: Superoxide dismutase; MDA: Malondialdehyde

Table 7: Effects of *Schisandra chinensis* extracts on activity of superoxide dismutase and content of malondialdehyde in RINm5F cells injured by H_2O_2

Groups	MDA/(CB/[μmol/L])	SOD/(λB/[U/mL])
Control	1.256±4.453	32.234±5.01
Model	2.245±5.643*	23.14±4.452*
SCEs (H)	1.453±4.054#	30.325±5.432#
SCEs (M)	1.726±4.402#	28.235±4.675#
SCEs (L)	2.065±5.078	24.432±5.457

*P<0.05 versus control group; #P<0.05 versus model group. SOD: Superoxide dismutase; MDA: Malondialdehyde; SCEs: *Schisandra chinensis* extracts

Superoxide dismutase activity and malondialdehyde content in islet cells

Compared with the model group, the activity of SOD in the high-dose and medium-dose SCEs groups was significantly enhanced ($P < 0.05$), which indicated a significant increase in the antioxidant capacity of the cells and decrease in the MDA content ($P < 0.05$). These findings indicated that the degree of cell peroxidation injury was reduced; the results were consistent with the changes in SOD activity and MDA content in the cell culture media [Table 7].

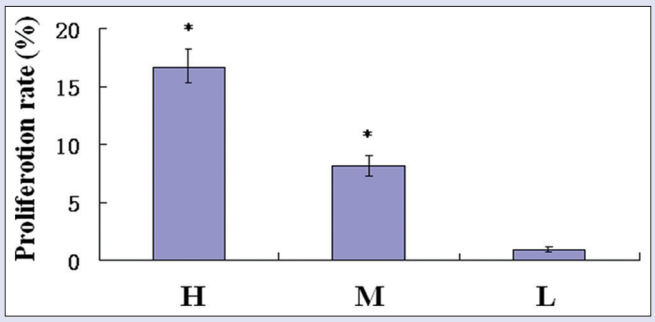

Figure 2: Effects of SCEs on the viability of RINm5F cells. *P < 0.05 versus control group

DISCUSSION

Islet is one of the tissues with the lowest expression of antioxidant enzymes and the lowest activity in human body.[22] The increase in islet beta cell apoptosis and decrease in their number are important causes of the progressive functional decline of islet beta cells in type 2 diabetes.[23] Excessive oxygen-free radicals in the human body may lead to the apoptosis and dysfunction of islet beta cells.[24] Currently, ROS-dominant oxidative stress is believed to be one of the main mechanisms of the onset of type 2 diabetes, and oxidative stress is mainly caused by ROS.[25,26]

In this experiment, 90% ethanol was used as the extraction solvent with a solid to liquid ratio of 1:15. Ultrasonic extraction was carried out for three times (20 min each), and the content of 8 major lignans in *S. chinensis* Fructus was established by HPLC. Hydrogen peroxide was used to induce oxidative stress in pancreatic islet RINm5F cells, which simulated the level of oxidative stress *in vivo*. The antioxidant activity of SCEs was investigated and its underlying mechanisms were examined by MTT assay and by detection of SOD activity and MDA content in cells and the culture media. The results showed a dose-dependent proliferation effect of SCEs on islet cells; compared with the model group, the cell survival rate in the high-dose and medium-dose SCE groups was significantly increased by 63.4% and 45.6%, respectively ($P < 0.05$). These findings indicated a dose-dependent protective effect of SCE against H_2O_2-induced oxidative damage in islet cells; the greatest effect was observed in the high-dose group.

Our experimental results showed that SCEs could promote the proliferation of islet cells–RINm5F and exert a protective effect against the damage of cells induced by H_2O_2. The effect of SCEs on the decrease in the number of islet beta cells *in vitro* may be inhibited by reducing the effect of oxidative stress injury. Oxidative stress is characterized by the decrease in cell antioxidants and antioxidant enzymes or the increase in cell oxidants, or both. SOD is the main antioxidant enzyme in the body, which can effectively neutralize the superoxide ions and their derivatives of ROS precursors, thus inhibiting oxidative stress-induced damage. The changes in activity of SOD reflect the antioxidative capacity of the body; MDA is a toxic by-product of lipid peroxidation *in vivo*. It has relatively good stability and can be easily measured. It represents the degree of damage caused by ROS over the long term, especially the degree of lipid oxidation.[26] In this experiment, we observed a decrease in the SOD activity and increase in the MDA content in islet cells and

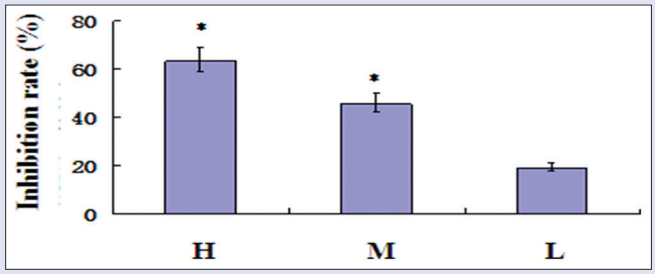

Figure 3: Effects of SCEs on the viability of RINm5F cells injured by H_2O_2. *$P < 0.05$ versus model group

in the culture media, which indicated that the islet cells – RINm5F were in a state of oxidative stress injury. Increase in ROS in the cells and the high concentration of ROS may lead to large-scale, nonspecific oxidative damage which directly impairs the cell membrane integrity and induces cell apoptosis. In the high-dose and low-dose groups pretreated with SCEs, the activity of SOD increased and the content of MDA decreased. This means that the extract of *S. chinensis* increased the activity of antioxidant enzymes in the cultured islet beta cells, reduced the toxic by-products of lipid peroxidation, reduced the rate of ROS production, and attenuated oxidative stress. Our study provides a robust theoretical basis for further study of the effects of SCEs on the prevention and control of oxidative stress in diabetes.

CONCLUSION

In this *in vitro* study, SCEs promoted proliferation of islet cells and exhibited a potent protective effect against H_2O_2-induced oxidative stress in islet cells. The results showed that underlying mechanism of the protective effect of SCEs was related to its antioxidant activity. This study laid a theoretical foundation for further study of the mechanism of therapeutic effects of *S. chinensis* in type II diabetes mellitus.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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