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Full Length Research Paper

Antioxidant, α-glucosidase inhibitory activities *in vitro* and alloxan-induced diabetic rats' protective effect of *Indigofera stachyodes* Lindl. root

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The antioxidant capacity of *Indigofera stachyodes* Lindl. root (ISR) extract had been determined by using 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2-azinobis (3-ethyl-benzothiazoline-6-sulfonic acid) (ABTS and ferric reducing antioxidant power (FRAP) assay, and α-glucosidase inhibitory activity using 96-microplate-based method test *in vitro*. Extracts of EtOAC (ISREA) and *n*-BuOH (ISRBU) were studied on protective effects of alloxan-induced diabetic rats *in vivo*. The results showed that ISREA and ISRBU had good total antioxidant activity *in vitro*, ISREA had the highest antioxidant activity (DPPH: IC_{50} = 7.82 µg/ml, ABTS: IC_{50} = 2.37 µg/ml and FRAP = 2270.80±8.48 µmol TE/g, respectively). Extracts of ISR showed higher α-glucosidase inhibitory activity. ISRBU (IC_{50} = 7.01 µg/ml) had the highest α-glucosidase inhibitory activity. Compared with diabetic control mice, oral administration of ISREA and ISRBU could decrease fasting blood glucose and postprandial blood glucose without statistical significance. Administration of ISRBU (400 mg/kg) could significantly increase liver glycogen content and superoxide dismutase (SOD) levels and significantly decrease serum total cholesterol (TC), triglyceride (TG) and malondialdehyde (MDA) levels in diabetic rats.

Key words: *Indigofera stachyodes* Lindl. root, antioxidant activity; α-glycosidase inhibition activity, diabetes.

INTRODUCTION

Type 2 diabetes mellitus is a complex, multifactorial disease. Oxidative stress has been suggested to be a contributory factor in development and complication of diabetes (Itoi et al., 2007; Pidaran and Leelavinothan, 2006). In recent years, natural antioxidants are used in dietary, pharmaceutical and cosmetic to replace synthetic antioxidants (Riadh et al., 2009). Research founded that some antioxidant compounds isolated and identified from medicinal plants had good effect on antioxidation in *vitro* and in *vivo* (Lee et al., 2011; Sharma et al., 2008). Postprandial hyperglycemia is the most important health issue in the 21st century. α-Glucosidase inhibitor reduce postprandial glucose level. Screening for potent natural glycosidase inhibitors is very important for diabetes (Shibano et al., 2008). *Indigofera stachyodes* Lindl. belongs to Leguminosae family.The roots of *I. stachyodes* (ISR) (Chinese name Xue-Ren-Shen) are used as a traditional Chinese herbal medicine for the treatment of rheumatism, cirrhosis, dysentery and wound (Hu et al.,2005). Phytochemical research showed flavonoids, phenolic acids and sterols were main compounds in the genus *Indigofera* (Thangadurai et al., 2001; Hasan et al., 1996; Su et al., 2004; Walmir et al., 2003; Zhang et al., 2006; Su et al., 2008). In our previous study, volatility of ISR was analyzed by head-space solid micro-extraction for the first time (Tian et al., 2011). Antibacterial, antifungal, insulin sensitizing property, antidyslipidemic activities, Dalton's ascitic lymphoma and nephrotoxic effects on several different species in this genus have been reported (Dahot, 1999; Esimone et al., 1999; Christina et al., 2003; Chakrabarti et al., 2006; Tadigoppula et al., 2006; Palani et al., 2009).

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However, there was no report concerning antioxidant, *a*-glucosidase inhibitory activities *in vitro* and protective effect for alloxan-induced diabetic rats *in vivo*. In this work, we found ISR extracts as effective glycosidase inhibitor and antioxidant. In order to investigate the protective effect of ISR extracts in treating diabetes, antihyperglycemic, lipid modulating, and antioxidant effects of ISR extracts were assayed using the alloxan-induced diabetic rat model.

MATERIALS AND METHODS

Plant material and extract preparation

Air-dried plant of ISR was collected in Guizhou, China, in August 2008. Identified by Professor Fan Liu. The specimen was deposited in Institute of Chinese Materia Medica, Henan University. The air dried ISR were extracted two times with methanol for 3 days at room temperature. After evaporation of solvent *in a vacuum,* the concentrated extract was suspended in water and extracted with petroleum ether, EtOAC and *n*-BuOH, respectively. The solution was concentrated under reduced pressure to yield petroleum ether extract (ISRPE), EtOAC extract (ISREA) and *n*-BuOH extract (ISRBU), respectively.

Materials in experiments *in vitro*

α-Glucosidase (EC 3.2.1.20), 4-Nitrophenyl-*α*-D-glucopyranoside (PNPG, 026K1516), acarbose, dimethyl sulfoxide (DMSO), gallic acid propyl (PG), butyl-p-hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) were obtained from Sigma. 1,1-Diphenyl-2-picrylhydrazyl (DPPH) from Tokyo Chemical Industry Co. 2,4,6-Tripyridyl-S-triazine (TPTZ) from Acros organics. 6-Hydroloxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid (Trolox) from Aldrich; 2,2′-Azino-bis(3-ethylbenzo-thiazoline-6-sulfonicacid) (ABTS) were obtained from Fluka.

Antioxidant activity using DPPH assay

DPPH radical scavenging activity was assayed according to the method of Kang and wang (2010). 0.1 ml different extracts of ISR in methanol had been mixed with 3.5 ml DPPH methanol solution (0.06 mmol/L). The solution was measured at 515 nm after 30 min at room temperature with PG, BHA and BHT as positive control. The antioxidant activity was expressed as an IC_{50} value, that is, the concentration in µg/ml that inhibits DPPH absorption by 50%, and was calculated from the concentration-effect linear regression curve.

Antioxidant activity using ABTS assay

Scavenging activity on ABTS radical of different extract from ISR was evaluated in accordance with the literature (Kang and Wang, 2010). The different extracts of IRS (0.15 ml) were mixed with ABTS radical stock solution (2.85 ml) and incubated at 37°C. The absorbance was observed at 734 nm after 10 min with PG, BHA and BHT as positive control. The percentage inhibition of $ABTS^{+}$ was calculated using the formula: % Inhibition = $[(A_0-A_1)/A_0] \times 100$, where A0 was the absorbance of the control and A1 was the absorbance of the sample and the standard compound. FRAP reducing activity assay. According to the literature, Thaipong et al. (2006), the ISR (0.2 ml) and fresh prepared TPTZ stock solution (3.8 ml) were mixed and incubated at 37°C for 30 min. The absorbance was measured at 593 nm. Trolox was used as a reference standard. The standard curve was linear between 25 and 400 µmol/L Trolox. Results were expressed in µmol Trolox equivalents (TEAC) (TE)/g sample. In this study, $RACT_{50}$ was used to express Trolox equivalent ($RACT_{50}$ = the concentration of Trolox cleared 50% free radical/ the concentration of compound or condensate cleared 50% free radical).

α-glucosidase inhibition assay

The *α*-glucosidase-inhibitory activity was screened by the method of the microplate-based method based on PNPG as substrate according to the (Kang et al., 2009). The assay mixture (160 µl) contained 8 µl of IRS extracts in DMSO (or DMSO itself as control), 112 µl phosphate buffer (pH 6.8) and 20 µl enzyme solution (0.2 U/ml *α*-glucosidase in phosphate buffer) was mixed and incubated at 37°C for 15 min.

Then, substrate solution (20 µl, 2.5 mM PNPG prepared in the same buffer) was added. The reaction was terminated by adding 80 µl of 0.2 M Na_2CO_3 solution after incubating at 37°C for 15 min. Enzymatic activity was quantified by measuring the *p*-nitrophenol released from PNP-glycoside at 405 nm wavelength in a 96 microplate reader. Enzymatic inhibition data were expressed as IC_{50} values (concentration of inhibitor required for 50% inhibition against *α*-glucosidase). The inhibitory rates (%) were calculated according to the formula: $[1-(OD_{test}-OD_{blank})/(\text{control } OD_{test}-\text{control } OD_{blank})] \times 100\%$. All reactions were carried out with three replications. Acarbose was used as positive control.

Materials and animals in experiments *in vivo*

Male KM normal rats weighted 20 ± 2 g were obtained from the Experimental Animal Center of Henan Province (Zhengzhou, Hennan, China) (12 h light/dark cycle, 25°C and humidity 45 to 65%) and were fed with standard rodent diet and water *ad libitum*. All animal procedures were approved by the ethical committee in accordance with the 'Institute ethical committee guidelines' for Animal Experimentation and Care. Animals were housed in polycarbonate cages.

The materials includes alloxan (Alfa Aesar A Johnson Matthey, USA); maleicdialdehyde (MDA), superoxide dismutase (SOD) and glycogen from the Nanjing Jianchen Bioengineering Institute (Jiangsu, China); total cholesterol (TC) and triglyceride (TG) from Shanghai Beihai Biotechnology Engineering Co., Ltd. (Shanghai, China), and blood glucose test kit from Shanghai Rongsheng Biotech Co., Ltd. (Shanghai, China).

Experimental design and treatment schedule

Mice were made diabetic by a single tail vein injection of alloxan monohydrate (80 mg/kg b.w.) after overnight fasting for 12 h. Alloxan was weighed individually in Eppendorf's tube for each animal according to the weight and then solubilized with 0.2 ml saline (154 mmol/l NaCl) just prior to injection. Fasting for 96 h after injection of alloxan, mice with marked hyperglycemia (fasting blood glucose＞11.0mmol/L) were included in the study. Experimental mice were randomly divided into nine groups of ten animals each; Group 1 (normal mice treated with distilled water), Group 2 (diabetic mice treated with distilled water), Group 3 to 5 received 250, 500 and 1000 mg/kg of ISREA, respectively. Group 6 to 8 received 200, 400 and 800 mg/kg of ISRBU, respectively. Group 9 was vehicle control (diabetic mice treated with 75 mg/kg Acarbose) and then treated with drugs at 24 h later. The duration of treatment

Table 1. α-Glucosidase inhibitory activity of extracts of *I. stachyodes* Lindl.

Sample	Concentration	α-glucosidase inhibition	
	(μg/ml)	I (%)	IC_{50} (μg/ml)
ISRPE	1500	96.69	68.44±0.55
ISREA	1500	100.04	14.31±0.12
ISRBU	1500	98.99	7.01±0.02
Acarbose*	1500	57.26	1103.01±12.15

*Acarbose was used as positive control.

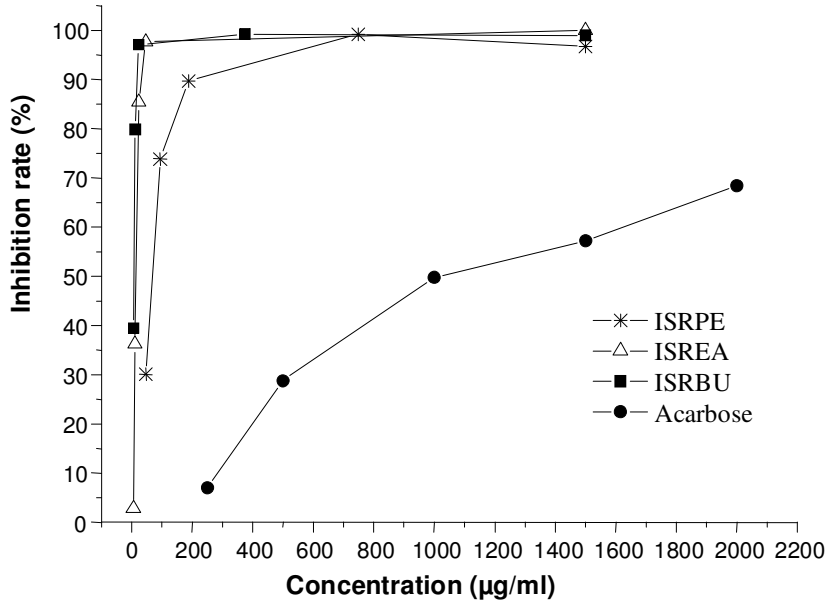

Figure 1. The mass concentration of extracts of *I. stachyodes* Lindl..effect on α-glucosidase inhibitory activity.

was 7 days for diabetic by intragastric administration. Blood was collected from the eyes after 2 h by intragastric administration on day 7. Blood was collected from the eyes after fasting 12 h on the day 8; animals were then sacrificed by cervical dislocation, the liver, kidney and spleen were removed promptly, and weighed. Blood samples were centrifuged (3000 rpm for 15 min at 4℃) for separating the serum. After that, the serum was stored at -20℃ for the following biochemical analysis. The tissues were also stored at -20℃ until required.

Biochemical analyses

Blood glucose was estimated by commercially available glucose kit based on glucose oxidase method (Jun et al., 2008). Glycogen content in the liver, TC, TG, superoxide dismutase (SOD) and malonaldehyde (MDA) levels in serum were measured following the commercial kit's instructions.

Statistical analysis

All the grouped data were statistically evaluated with SPSS 17.0 software. Statistical comparisons were compared by one-way analysis of variance (ANOVA). The results were considered statistically significant if the *p* values were 0.05 or less. All results are expressed as mean ± standard deviation (SD) for ten mice in each group.

RESULTS

a-Glucosidase inhibitory activity *in vitro*

In the Table 1, ISRPE, ISREA and ISRBU showed stronger activity against a-glucosidase (IC_{50} = 68.44±0.55, 14.31±0.12, 7.01±0.02 μg/ml, respectively) than that of acarbose (IC_{50} = 1103.01±12.15 μg/ml) as positive control. The result in Figure 1 showed that the α-glucosidase inhibitory activities of ISRPE, ISREA and ISRBU exhibited strong activity in mass concentration dependent. The a-glucosidase inhibitory activity of different extracts from ISR was reported for the first time.

Table 2 Antioxidant activity of extracts of *I. stachyodes* Lindl.

Sample	DPPH radical scavenging capacity IC_{50} (μg/ml)	ABTS radical scavenging capacity IC_{50} (μg/ml)	ferric reducing antioxidant power $RACT_{50}$ (μmol/g)
ISRPE	NT	NT	167.93±6.54
ISREA	7.94±0.09	2.73±0.08	2270.80±8.48
ISRBU	14.32±1.36	5.12±0.15	1375.40±11.31
BHA[b]	3.20±0.04	1.88±0.01	6633.04 ±114.04
BHT[b]	18.72±0.50	7.77±0.01	1581.68 ± 97.41
PG[b]	0.89±0.01	0.76±0.01	10675.79± 89.32

NT, not available because of low activity, BHA, BHT and PG were used as positive control.

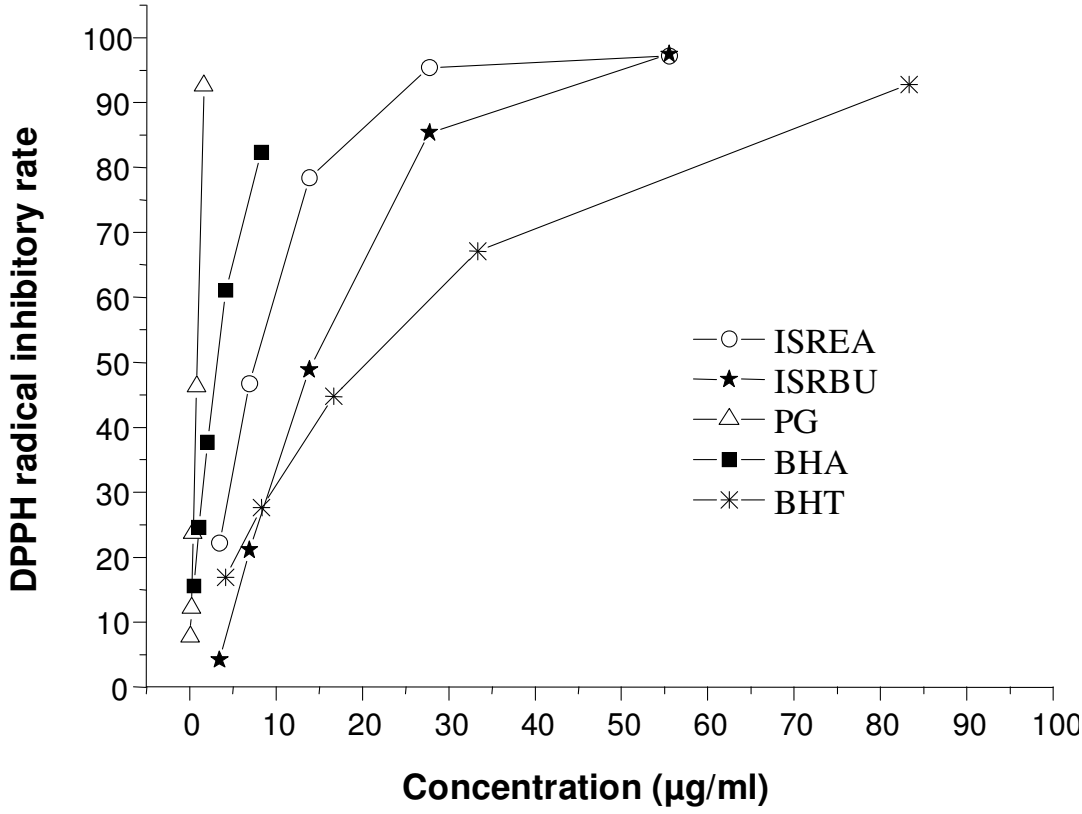

Figure 2. DPPH radical scavenging activity of extracts of *I. stachyodes* Lindl.

The result showed that ISR might be a good source of natural *α*-glucosidase inhibitor using the therapy of hyperglycemic and its complication.

In *vitro* assay for free radical scavenging activity

The antioxidant activity of ISR with half inhibitory concentration (IC_{50}) and Trolox equivalent ($RACT_{50}$) is shown in Table 2 and Figures 2 and 3). In DPPH assay, the antioxidant activity of ISREA (IC_{50} = 7.94±0.09 μg/ml) was higher than that of BHT (IC_{50} = 18.72±0.50 μg/ml) and higher than that of ISRBU (IC_{50} = 14.32±1.36 μg/ml) and ISRPE. In ABTS assay, the antioxidant activity of ISRBU (IC_{50} = 5.12±0.15 μg/ml) was higher than that of BHT. The ISREA (IC_{50} = 2.73±0.08 μg/ml) was far higher than that of BHT and below BHA (1.88±0.01 μg/ml) slightly. In FRAP assay, the antioxidant activity of ISREA ($RACT_{50}$ = 2270.80±8.48 μmol/g) was higher than that of BHT (1581.68 ± 97.41 μmol/g) and far lower than that of BHA and PG. The results of three methods were summarized that the antioxidant activity of ISRE A was

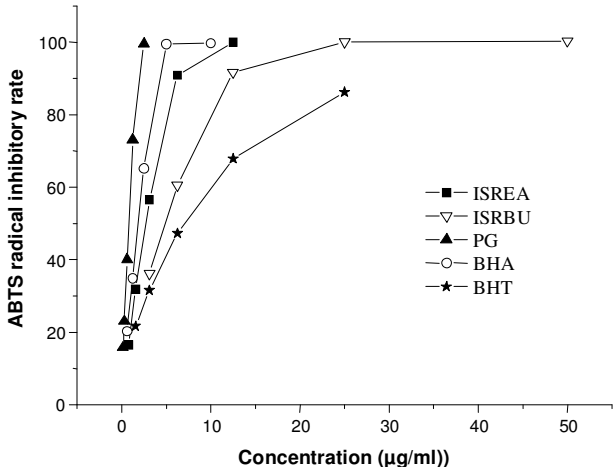

Figure 3. ABTS radical scavenging activity of extracts from *I. stachyodes* Lindl.

Table 3. Effect of ISREA, and ISRBU treatment on fasting blood glucose.

Group	Dose (mg/kg b.w)	Before treatment (mmol/L)	After treatment (mmol/L)
Normal control		3.33±0.30***	5.50±0.41***
Diabetic control		25.98±11.57△△△	30.22±1.48
ISREA	1000	25.22±9.62△△△	28.41±6.33
ISREA	500	24.31±3.97△△△	21.68±12.93
ISREA	250	21.01±7.42△△△	21.69±8.86
ISRBU	800	22.44±7.48△△△	22.31±11.70
ISRBU	400	23.09±7.05△△△	23.06±8.26
ISRBU	200	21.21±7.88△△△	23.92±11.31
Acarbose	75	21.88±4.90△△△	22.41±8.11

Data expressed as means ± s.d (n =10). Acarbose was as the positive control drug. △p < 0.05, △△p<0.01, △△△p<0.001 Alloxan-induced diabetic group compared to vehicle control group. *P＜0.05, **P＜0.01, ***P＜0.001 treated group compared to alloxan-induced group.

higher than that of ISRPE and ISREA had the highest antioxidant activity *in vitro*.

Protective effect of extracts from *I. stachyodes* Lindl. on alloxan-induced diabetic rat

Serum blood glucose

The effect of different doses of ISREA and ISRBU on fasting blood glucose levels of diabetic rats was given in Table 3. The intragastric administration of ISREA and ISRBU to diabetic rats resulted in different degree decreased but no significantly decrease in level of fasting blood glucose (p>0.05). Fasting blood glucose levels of diabetic rats were higher than that of normal rats throughout the experimental period. Table 4 showed the level of post-prandial blood glucose of diabetic control was significantly increased. Administration of ISREA and ISRBU to diabetic rats resulted in no significantly decrease in level of post-prandial blood glucose level of rats (p>0.05).

Table 5 shows the content of glycogen in liver tissues of normal and diabetic animals. There was significantly decrease in content of glycogen in diabetes control when compared with the normal control group (p<0.001). Administration of ISREA (500 mg/kg), ISRBU (800, 400, 200 mg/kg) and acarbose (75 mg/kg) resulted in a significant increase in the level of liver glycogen (p<0.05, p<0.001). The administration of ISRBU at the dose of 200 mg/kg b.w. and acarbose at the dose of 75 mg/kg b.w. showed a highly significant effect and tend to bring the level to near normal (p<0.001). Table 6 showed the MDA, SOD, TC and TG level of normal and diabetic rats. The level of MDA was significantly increased whereas the level of SOD was significantly decreased in diabetic

Table 4. Effect of ISREA, and ISRBU treatment on post-prandial blood glucose.

Group	Dose (mg/kg b.w)	Post-prandial blood glucose (mmol/L)
Normal control		3.85±0.68***
Diabetic control		38.43±5.66△△△
ISREA	1000	32.70±9.35△△△
ISREA	500	34.90±9.57△△△
ISREA	250	32.45±4.35△△△
ISRBU	800	33.74±6.52△△△
ISRBU	400	28.35±8.63△△△
ISRBU	200	36.29±5.89△△△
Acarbose	75	32.65±7.12△△△

Data expressed as means ± s.d (n = 10). Acarbose was as the positive control drug. △p < 0.05, △△p<0.01, △△△p<0.001 alloxan-induced diabetic group compared to vehicle control group. *P＜0.05, **P＜0.01, ***P＜0.001 treated group compared to alloxan-induced group

Table 5. Effect of ISREA, and ISRBU on liver glycogen level.

Group	Dose (mg/kg b.w)	Liver glycogen (mg/g)
Normal control		30.21±1.44***
Diabetic control		4.03±0.71△△△###
ISREA	1000	8.06±3.08△△△###
ISREA	500	12.77±5.68△△△*###
ISREA	250	5.59±3.35△△△###
ISRBU	800	19.70±9.01△△***
ISRBU	400	22.01±6.68△***
ISRBU	200	23.26±6.72***
Acarbose	75	24.35±6.67***

Data expressed as means ± s.d (n = 10). Acarbose was as the positive control drug. △p < 0.05, △△p<0.01, △△△p<0.001 alloxan-induced diabetic group compared to vehicle control group. *P＜0.05, **P＜0.01, ***P＜0.001 treated group compared to alloxan-induced group. #P＜0.05, ##P＜0.01, ###P＜0.001 compared with positive control group.

Table 6. Effect of ISREA and ISRBU treatment on the level of MDA, SOD, TG and TC in serum.

Group	Dose (mg/kg b.w)	MDA (nmol/ml)	SOD (U/ml)	TG (mmol/L)	TC (mmol/L)
Normal control		4.40±0.44***	171.92±10.18***##	0.94±0.18**#	2.67±0.38*
Diabetic control		11.08±2.39△△△##	107.66±16.80△△△	1.40±0.24△△###	3.87±0.53△
ISREA	1000	7.34±1.29△***	127.62±12.12△△	1.21±0.28###	3.90±0.84△
ISREA	500	6.72±0.90***	145.69±20.77*	0.97±0.23**##	4.01±1.21△△#
ISREA	250	6.67±1.63***	142.18±28.78	0.62±0.20△***	3.58±0.69
ISRBU	800	6.61±1.24***	143.89±16.70*	1.03±0.24*##	2.73±0.74**
ISRBU	400	6.83±1.21***	147.82±30.01*	0.67±0.34***	2.20±0.35***
ISRBU	200	5.40±1.49***	134.22±21.14△	1.11±0.30*###	2.58±0.61***
Acarbose	75	7.82±1.41△△**	123.76±13.78△△	0.53±0.10△***	3.07±0.69

Data expressed as means ± s.d (n = 10). Acarbose was as the positive control drug. △p < 0.05, △△p<0.01, △△△p<0.001 Alloxan-induced diabetic group compared to vehicle control group. *P＜0.05, **P＜0.01, ***P＜0.001 treated group compared to Alloxan-induced group. #P＜0.05, ##P＜0.01, ###P＜0.001 compared with positive control group.

control mice compared with normal mice (p<0.001). The ISREA and ISRBU treatment group were significantly decreased in the level of MDA (p<0.001) and groups 4 to 8 tend to bring the level to near normal. The ISREA (500 mg/kg) and ISRBU (800, 400 mg/kg) treatment group was significantly increased in the level of SOD compared

with diabetic control mice ($p<0.05$). There was significantly increased in the level of TC and TG in diabetic control compared with normal control groups ($p<0.05$, $P<0.01$, respectively). Administration of groups 4 to 8 and acarbose at the dose of 75 mg/kg b.w. were significantly decreased in the level of TG and tend to bring the level to near normal ($p<0.05$, $p<0.01$, $p<0.001$, respectively). Group 6, 7 and 8 were significantly decreased in the level of TC ($p<0.01$, $p<0.001$, respectively).

DISCUSSION

Diabetes mellitus (DM) is associated with absolute or relative deficiencies in insulin secretion and/or insulin action. It is a metabolic disorder syndrome characterized by hyperglycemia and alterations in electrolyte, fat and protein metabolism. At the same time, it may result in the chronic damage of the eyes, kidneys, neural, cardiovascular, *etc* (Fu et al., 2010). Normally, body organisms are able to control and counter the free radical mediated oxidative damage. However, in diabetes mellitus, oxidative stress could play an important role in the onset and progression of the disease (Sun et al., 2007; Ani and Naidu, 2008). In recent years, studies have shown that there was exists phenomenon that MDA was significantly increased and SOD was significantly decreased in animal models of diabetes mellitus and clinic diabetic sufferer. Endogenous antioxidant enzyme (SOD) is responsible for the detoxification of deleterious oxygen radicals. The amount of MDA to measure the degree of lipid peroxidation; it reflects the reactive oxygen species levels produce in lipid oxidation (Ni et al., 2003; Ramesh and Pugalendi, 2006). In this experiment, the antioxidant activity of ISR was analyzed by three methods *in vitro*. The results of three methods were summarized that the antioxidant activity of ISREA was higher than that of ISRPE, and ISREA had the highest antioxidant activity in *vitro*. The level of SOD was decreased and level of MDA in serum was increased in diabetic rats. Treatment with ISREA (400 mg/kg), ISRBU (800 mg/kg) and ISRBU (400 mg/kg) had reversed the level of SOD and MDA. By reducing the level of MDA and increasing the level of SOD to decreased lipid peroxidation and/or decreased utilization (Sandesh et al., 2010) and enhance antioxidant capability and protect the body to further oxidative damage from free radicals in diabetic mice, which play the role of hypoglycemic effect. α-Glycosidases are hydrolytic enzymes that play a vital glycoproteins (Chao et al., 2010). α-Glucosidase inhibitors increase gastro-intestinal motility, which could competitively inhibite α -glycosidase activity and delay or inhibit the absorption of glucose; the postprandial hyperglycemia reduced effectively (Ortiz et al., 2007; Seifath et al., 1998). α-Glucosidase inhibitory activity from natural products were considered generally safe, effective, well tolerated, low prices and used increasingly in the treatment of NIDDM (Dai et al., 2010; Yeh et al., 2003). A variety of plants extract are known to have α-glucosidase inhibitory activity (Si et al., 2010; Kang and Wang,2010). Thus, we determined α-glucosidase inhibitory activity of extracts of ISR. In the paper, ISRPE, ISREA and ISRBU had good α-glucosidase inhibitory activity *in vitro*; ISRBU had the strongest α-glucosidase inhibition. Oral administration of ISRBU (800, 400, 200 mg/kg b.w.) can decrease postprandial blood glucose but no significant difference, significantly increase liver glycogen and significantly decrease the level of TG, TC in serum to ameliorate concurrent hyperlipidemia.

Conclusion

It could be concluded that the extract of ISR had effective prevention and treatment on the development for diabetes and its complications. Thus, studies should be done on purification and identification of the anti-diabetic chemical compound of ISREA and ISRBU.

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