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Full Length Research Paper

α-glucosidase inhibitory *in vitro* and antidiabetic activity *in vivo* of *Osmanthus fragrans*

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In this study, EtOAC extract of *Osmanthus fragrans* were evaluated by *a*-glucosidase inhibitory activity in *in-vitro*, and showed stronger inhibiting activity of *a*-glucosidase (IC_{50}=12.5 µg/ml) compared with acarbose as positive control (IC_{50}=1081.27 µg/ml). Based on the result, two active compounds of ursolic acid and oleanolic acid were isolated from EtOAC extract of *O. fragrans* and exhibited great inhibitory activity of *a*-glucosidase (IC_{50}=3.38 and 3.3 µg/ml, respectively). The antidiabetic effect of EtOAC extract, ursolic acid and oleanolic acid were investigated on diabetic mice induced by tail vein injection with alloxan. Compared with diabetic control group, oral administration of ursolic acid (300 mg/kg) and oleanolic acid (120 and 40 mg/kg) could significantly decrease the fasting blood glucose and postprandial blood glucose, oral administration of EtOAC extract (160 mg/kg), ursolic acid (300 mg/kg) and oleanolic acid (120 and 40 mg/kg) could significantly increase liver glycogen content. Each treatment group could significantly decrease serum TG (triglyceride) level and MDA (malondialdehyde) content in diabetic rats. Oral administration of EtOAC extract (160 mg/kg), ursolic acid (100 mg/kg) and oleanolic acid (120) could significantly decrease serum TC (total cholesterol) level, and oral administration of EtOAC extract (500 mg/kg), ursolic acid (300 mg/kg) could significantly increase SOD (superoxide dismutase) level. Results demonstrate the antidiabetic and antioxidant potential of *O. fragrans* and suggest that the plant may have therapeutic value in diabetes and related complications.

Key words: *Osmanthus fragrans*, α-glucosidase, antidiabetic activity.

INTRODUCTION

Diabetes mellitus and its complications is a major health problem, and postprandial hyperglycemic excursion has been implicated as an independent risk factor for cardiovascular disease not only among diabetes patients, but also among nondiabetic subjects in general population with mildly elevated postprandial glucose levels. α-Glucosidase inhibitors as treatment drug, including acarbose and voglibose, can reduce the postprandial glucose levels and insulin responses (Casirola and Ferraris, 2006). Such inhibitors have also reduced sensitivity of postprandial oxidative stress along with reducing the increased risk of cardiovascular diseases (Kawamura et al., 1998).

Plants have been a rich source of α-glucosidase inhibitors and screening of α-glucosidases inhibitors from plants sources is increasing (Kang et al., 2011). *Osmanthus fragrans*, belongs to Oleaceae family, is a flower distributed in the eastern part of the Himalayas in Southwest of China, Japan, India, Nepal and Cambodia. *O. fragrans* as one of the ten famous flowers in China include more than 150 varieties, and can be divided into three groups according to color which are *O. fragrans* luteus (gold-orange), *O. fragrans* albus (silver-white) and *O. fragrans* aurantiacus (reddish). Pharmacological investigations showed that *O. fragrans* have alleviating pain, relieving coughs, neuroprotection, reducing aging, antioxidant and curing aphtha activity (Deng et al., 2003; Lee et al., 2007; Zhang, 2005; Ding et al., 2009; Yuan and Yuan, 2002). The fatty acid components in four differrent varieties of *O. fragrans* were analyzed and the inhibitory activity of α-glucosidase was evaluated (Chen et al., 2009). In this study, EtOAC extract of *O. fragrans* showed α-glucosidase inhibitory activity and two active

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compounds were isolated from this extract and protective effect for alloxan-induced diabetic rats was evaluated using alloxan-induced diabetic rat model *in vivo*.

MATERIALS AND METHODS

α-Glucosidase (EC 3.2.1.20), 4-N-trophenyl-α-D-glucopyranoside (PNPG, 026K1516) and acarbose (Lot 16869) from Sigma. The kits of total cholesterol (TC), triglyceride (TG), MDA and SOD purchase from Nanjing Jiancheng Chemical Factory (China). The kits of glucose from Shanghai Rongsheng Biotechnology company (China).

O. fragrans was collected from Guilin, Guangxi province of China in October 2006. It was identified by Prof. Changqin Li (Institute of Chinese Materia Medica, Henan University), and a voucher specimen was deposited in the Institute of Chinese Materia Medica, Henan University (No. 06070103).

Extraction and isolation of bioactive compounds

Dried powder of *O. fragrans* flower (1 000 g) was refluxed three times with EtOAC and MeOH for 2 h respectively. The solution was concentrated under reduced pressure to yield EtOAC (33.4 g) and MeOH extract (100 g).

Based on *a*-glucosidase inhibition assay, EtOAC extract had activity, and was further subjected to CC over silica gel (200-300 mesh) developing with $CHCl_3$-MeOH (100:1 to 8:2) to yield 3 fractions. Fraction 1 was separated on a silica gel H with petroleum ether-EtOAC (10:1), and further chromatographed on Sephadex-LH 20 ($CHCl_3$-MeOH=1:1) to yield ursolic acid and oleanolic acid.

α-Glucosidase inhibitory activity *in vitro*

α-Glucosidase inhibitory activity was determined spectrophotometrically in a 96-well plate based on PNPG as substrate (Kang et al., 2011; Li et al., 2011). The assay mixture (160 µL) contained 8 µl of a sample 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), mixed and incubated at 37°C for 15 min, and then, 20 µL substrate solution (2.5 mM PNPG prepared in the same buffer) added.

The reaction was processed at 37°C for 15 min and stopped by adding 80 µL of 0.2 M Na_2CO_3 solution. Amount of the *p*-nitrophenol released from PNP-glycoside was quantified on a 96 microplate reader at 405 nm. The inhibitory rates (%) were calculated according to the formula: [1-(sample OD_{test}- sample OD_{blank})/ (control OD_{test}- control OD_{blank})] ×100%. Sample OD_{test} stand for solution of sample + enzyme + substrate. Sample OD_{blank} stand for solution of sample + buffer. Control OD_{test} stand for solution of buffer+ enzyme + substrate. Control OD_{blank} stand for solution of buffer.

All reactions were carried out with three replications. Acarbose was used as positive control.

Hypoglycemic effect assay *in vivo*

Experimental animals

Male KM normal rats weighted 20±2 g from the Experimental Animal Center of Henan Province. (Zhengzhou, Henan, China). (12 h light/dark cycle, 25°C and humidity 45-65%) 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 (HNPR-2009-05003). Animals were housed in polycarbonate cages.

Experimental design

A freshly prepared solution of 4% alloxan (80 mg/kg body weight) in normal sodium was injected into the caudal vein of overnight fasted mice. The blood glucose level was determined at the 96 h after injection from 6 h fasted mice. Mice with the blood glucose level more than 11.1 mmol/L were taken as which were successful induction of diabetes for experiments.

Normal and hyperglycemic mice were randomly allocated and similarly grouped into nine groups of 12 animals each. Group 1 normal control (NC). Group 2 diabetic control (DC). Group 3 diabetic rats given EtOAC extract (500 mg/kg). Group 4 diabetic rats given EtOAC extract (160 mg/kg). Group 5 diabetic rats given ursolic acid (300 mg/kg). Group 6 diabetic rats given ursolic acid (100 mg/kg). Group 7 diabetic rats given oleanolic acid (120 mg/kg). Group 7 diabetic rats given oleanolic acid (40 mg/kg). Group 9 diabetic rats given acarbose (75 mg/kg). Normal control and diabetic control were not given the extract but instead received equal volumes of vehicle everyday for the same period.

Biochemical parameters

The mice were treated for 8 days. Blood samples were collected from the eyes (venous pool) of 2 heated mice to analyze the level of postprandial blood glucose and 6 h fasted mice to analyze the level of blood glucose on an empty stomach, TC, TG, SOD and MDA. Immediately after the collection of blood samples the mice were sacrificed by single stunning and liver were excised to measure glycogen status. The level of blood glucose, TC, TG, SOD, MDA and the concentration of hepatic glycogen was determined using enzymatic kits according to the manufacturer's instructions.

Statistical analysis

All the grouped data were statistically evaluated with SPSS 17.0 software. Hypothesis testing methods included one-way analysis of variance followed by least significant difference (LSD) test. $P < 0.05$ was considered to indicate statistical significance. All results are expressed as mean ± standard deviation (SD) for eleven mice in each group.

RESULTS

Structure elucidation of compounds

^{1}H-(400 MHz) and ^{13}C-NMR spectra (100 MHz) were recorded on a Bruker Avance DRX-400 spectrometer. Chemical shifts are expressed in ppm referenced to the residual solvent signals. Coupling constants (*J*) are reported in Hz. Two compounds were isolated from the EtOAC extract. By correlating with spectral data (^{1}H and ^{13}C NMR) of literature values, two compounds were identified as ursolic acid and oleanolic acid (Sahu and Mahato, 1994).

Ursolic acid

ESI-MS *m/z*: 455 ([M-H] $^+$). ^{1}H-NMR(400 MHz, C_5D_5N) *δ*:

Table 1. Inhibitory effect of extracts and compounds from *O. fragrans* against α-glucosidase.

Samples	Concentration	α-glucosidase inhibition	
	(μg/ml)	I%	IC_{50}(μg/ml)
EtOAC extract	1500	99.4	12.5
Ursolic acid	50	107.1	3.38
Oleanolic acid	50	106.9	3.3
Acarbose	1500	68.43	1081.27

Table 2. Effect of EtOAC extract and active compounds from *O. fragrans* on blood glucose and liver glycogen level. (*n*=12).

Group	Dose	The level of blood glucose			Liver glycogen
	(mg/kg)	Day 0	Day 8		(mg/g)
			Postprandial	Fasting	
Group 1	0.5%CMC	3.84±1.56***	6.09±1.17***	3.96±0.86***	69.95±0.51***$^{\#}$
Group 2	0.5%CMC	31.37±6.45$^{\triangle\triangle\triangle}$	58.54±7.88$^{\triangle\triangle\triangle}$	28.77±1.96$^{\triangle\triangle\triangle}$	8.87±0.62$^{\triangle\triangle\triangle\#\#\#}$
Group 3	500	30.82±8.07$^{\triangle\triangle\triangle}$	40.07±5.12$^{\triangle\triangle\triangle}$	26.59±1.63$^{\triangle\triangle\triangle}$	18.65±4.91$^{\triangle\triangle\triangle\#\#\#}$
Group 4	160	30.16±5.12$^{\triangle\triangle\triangle}$	45.522±3.46$^{\triangle\triangle\triangle}$	19.63±0.92*$^{\triangle\triangle\triangle}$	25.52±3.30*$^{\triangle\triangle\triangle\#\#\#}$
Group 5	300	31.84±6.21$^{\triangle\triangle\triangle}$	34.39±7.13*$^{\triangle\triangle\triangle}$	18.09±0.52*$^{\triangle\triangle\triangle}$	36.71±2.64**$^{\triangle\triangle\triangle\#}$
Group 6	100	30.35±3.16$^{\triangle\triangle\triangle}$	36.34±4.18*$^{\triangle\triangle\triangle}$	21.84±2.26$^{\triangle\triangle\triangle}$	18.02±2.91$^{\triangle\triangle\triangle\#\#\#}$
Group 7	120	30.65±3.01$^{\triangle\triangle\triangle}$	29.31±1.08*$^{\triangle\triangle\triangle}$	19.93±1.54*$^{\triangle\triangle}$	65.66±2.84***$^{\#}$
Group 8	40	30.11±6.38$^{\triangle\triangle\triangle}$	36.96±2.45*$^{\triangle\triangle\triangle}$	20.94±2.87*$^{\triangle\triangle}$	31.24±5.95*$^{\triangle\triangle\#}$
Group 9	75	31.27±1.43$^{\triangle\triangle\triangle}$	25.89±1.42**$^{\triangle\triangle\triangle}$	16.63±1.05*$^{\triangle\triangle\triangle}$	49.98±3.22*$^{\triangle}$

$^{\triangle}$P<0.05, $^{\triangle\triangle}$P<0.01, $^{\triangle\triangle\triangle}$P<0.001 compared with Group 1, *P<0.05, ** P<0.01, ***P<0.001 compared with Group 2, $^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001 compared with Group 9, Group 1=normal control (NC), Group 2=diabetic control (DC), Group 3, 4= EtOAC extract (500 and 160 mg/kg, respectively), Group 5,6=ursolic acid (300 and 100 mg/kg, respectively.

5.49 (1H, brs, H-12), 3.38 (1H, dd, *J*=12.0, 6.0 Hz, H-3), 2.42 (1H, d, *J*=11.2 Hz, H-18), 1.24 (3H, s, H-27), 1.22 (3H, s, H-23), 1.05 (3H, s, H-25), 1.02 (3H, d, *J*=6.0 Hz, H-30), 0.98 (3H, s, H-24), 0.95 (3H, d, *J*=6.2 Hz, H-29), 0.85 (3H, s, H-26). ^{13}C-NMR (100 MHz, C_5D_5N) *δ*: 39.2 (C-1), 29.1 (C-2), 78.1 (C-3), 39.8 (C-4), 55.6 (C-5), 18.6 (C-6), 32.6 (C-7), 37.0 (C-8), 53.3 (C-9), 37.0 (C-10), 23.1 (C-11), 124.7 (C-12), 136.7 (C-13), 41.6 (C-14), 27.9 (C-15), 42.6 (C-16), 47.8 (C-17), 39.3 (C-18), 39.2 (C-19), 33.4 (C-20), 30.9 (C-21), 33.0 (C-22), 24.7 (C-23), 16.3 (C-24), 15.5 (C-25), 17.2 (C-26), 24.0 (C-27), 179.9 (C-28), 28.5 (C-29), 24.7 (C-30).

Oleanolic acid

^{1}H-NMR (400 MHz, C_5D_5N) *δ*: 5.49(1H, brs, H-12), 3.32 (1H, dd, *J*=4.0, 13.0 Hz, H-3), 1.24, 1.22, 1.05, 1.02, 0.98, 0.95, 0.88 (each 3H, s). ^{13}C-NMR (100 MHz, C_5D_5N) *δ*: 38.6(C-1), 28.1(C-2), 78.0(C-3), 39.2(C-4), 55.7(C-5),18.7(C-6), 33.1(C-7), 39.7(C-8), 48.0(C-9), 37.2(C-10), 23.6 (C-11), 122.4(C-12), 144.7(C-13), 42.1(C-14), 28.2(C-15), 23.6(C-16), 46.6(C-17), 41.9(C-18), 46.4(C-19), 30.8(C-20), 34.1(C-21), 33.1(C-22), 28.6(C-23), δ16.3(C-24), 15.4(C-25), 17.3(C-26), 25.9(C-27), 179.9(C-28), 33.1(C-29), 23.7(C-30).

Inhibitory activity against α-glucosidase *in vitro*

The inhibitory effect against α-glucosidase was evaluated by the IC_{50} value in a 96-well plate. EtOAC extract, ursolic acid and oleanolic acid (IC_{50}=12.5, 3.38 and 3.3 μg/mL respectively) showed stronger inhibiting activity compared with acarbose as positive control (IC_{50}=1081.27 μg/mL) in Table 1.

Effects on alloxan-induced diabetic mice *in vivo*

Based on *a*-glucosidase inhibitory activities of *O. fragrans in vitro*, hypoglycemic, antioxidant activities and the effect of blood lipid level of EtOAC, oleanolic acid and ursolic acid from *O. fragrans* were investigated on normal and alloxan-induced diabetic mice *in vivo*.

In Table 2, compared with Group 1, blood glucose in the serum from Group 2 induced by alloxan showed a significant increase (P<0.001) and it indicated the model

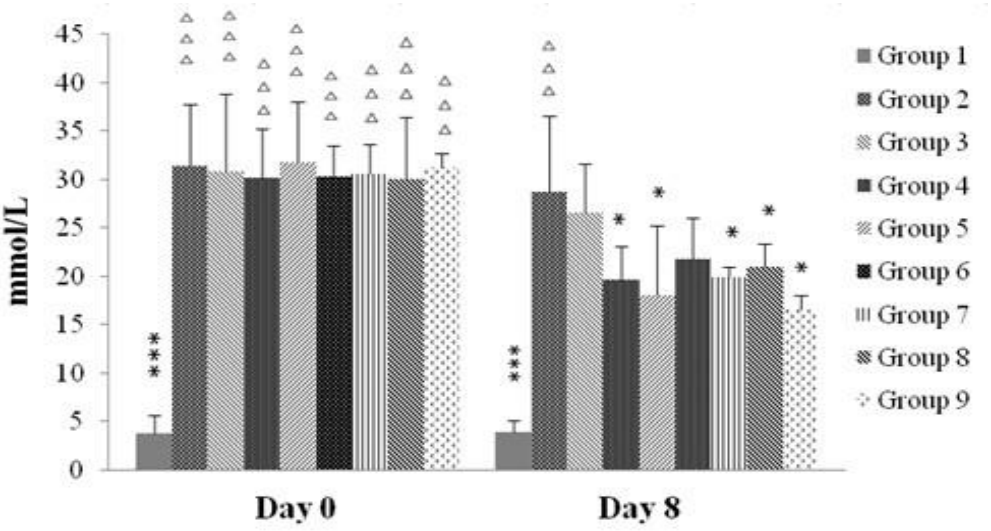

Figure 1. Effect of EtOAC extracts and compounds from *O. fragrans* on blood glucose level in the serum.

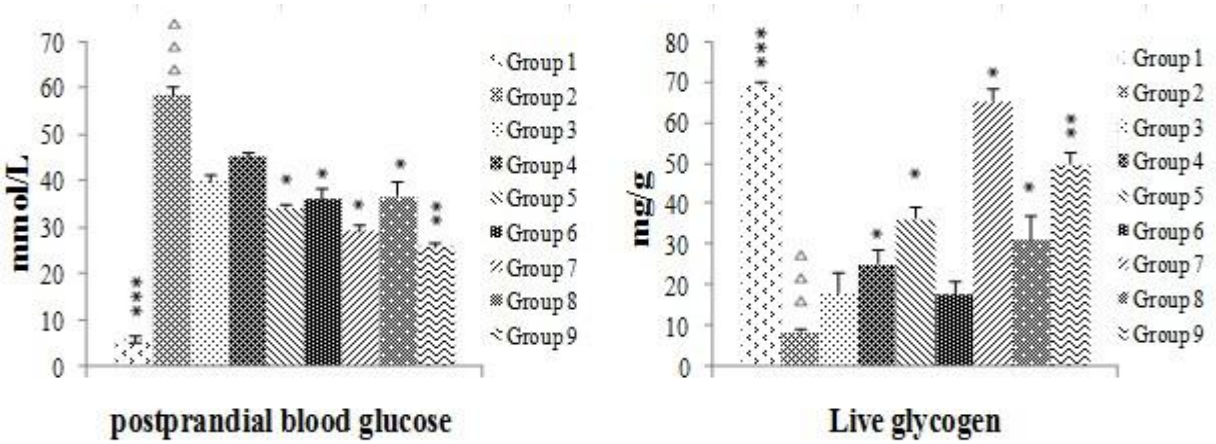

Figure 2. Effect of EtOAC extracts and compounds from *O. fragrans* on postprandial blood glucose level in the serum and live glycogen.

of alloxan-induced diabetic mice was established.

After treatment of eight days, the level of postprandial blood glucose was determined. In Table 2 and Figure 2, compared with Group 2, the level of postprandial blood glucose in group 5, 6 (ursolic acid 300 and 100 mg/kg, respectively) and Groups 7, 8 (oleanolic acid 120 and 40 mg/kg, respectively) decreased ($P<0.05$), and Group 9 significantly decreased ($P<0.01$).

After 8 h fasted, the blood glucose level of mice was determined again. In Table 2 and Figure 1, the level of blood glucose in Groups 4, 5 ,7 ,8 and 9 decreased ($P<0.05$) compared with Group 2, and the low dose of EtOAC extracts showed greater activity than that of high dose. The result showed that intragastric administration of EtOAC extract, oleanolic acid and ursolic acid to alloxan-induced diabetic mice could decrease the level of postprandial and fasting blood glucose (Figures 1 and 2), and the hypoglycemic activities also exhibited strong activity in dose dependent manner.

In order to evaluate glycogen status of EtOAC extract, oleanolic acid and ursolic acid from *O. fragrans* in *vivo*, animals were sacrificed at the end of experiments and liver were removed as described in Table 2 and Figure 2. Compared with Group 2, EtOAC extract (160 mg/kg) and oleanolic acid (40 mg/kg) increased the concentration of liver glycogen in tissue ($P<0.05$), and ursolic acid (300 mg/kg) could significantly increased ($P<0.01$). Oleanolic acid (120 mg/kg) reversed the concentration of liver

Table 3. Effect of EtOAC extract and active compounds from *O. fragrans* on the levels of MDA, SOD, TG and TC in serum.

Group	TG (mmol/L)	TC (mmol/L)	SOD (U/ml)	MDA (nmol/ml)
Group 1	0.63±0.14*	2.56±0.24*	167.41±57.15*	10.25±1.15***
Group 2	1.30±0.50$^{\Delta\Delta\Delta}$	4.10±1.53$^{\Delta\Delta\Delta}$	98.38±3.86$^{\Delta\Delta\Delta}$	25.38±4.93$^{\Delta\Delta\Delta}$
Group 3	0.58±0.25**	2.99±0.78	175.30±4.24*	10.45±1.15***
Group 4	0.67±0.21***	2.87±0.57*	118.21±49.57	11.47±2.68***
Group 5	0.61±0.19**	3.43±0.43	180.31±44.56*	10.61±0.96***
Group6	0.73±0.15*	2.71±0.49*	147.96±11.41	17.12±2.08***
Group7	0.64±0.31**	2.41±0.12*	144.72±24.31	10.64±2.56***
Group8	0.56±0.30***	2.86±0.7	146.92±17.07	11.3±3.77***
Group9	0.65±0.31**	2.72±1.19*	200.48±74.04***	10.25±1.07***

$^{\Delta}$P<0.05, $^{\Delta\Delta}$ P<0.01, $^{\Delta\Delta\Delta}$ P<0.001 group1, *P<0.05, **P<0.01, *** P<0.001 compared with Group 2, $^{\#}$P<0.05, $^{\#\#}$ P<0.01, $^{\#\#\#}$P<0.001 compared with Group 9

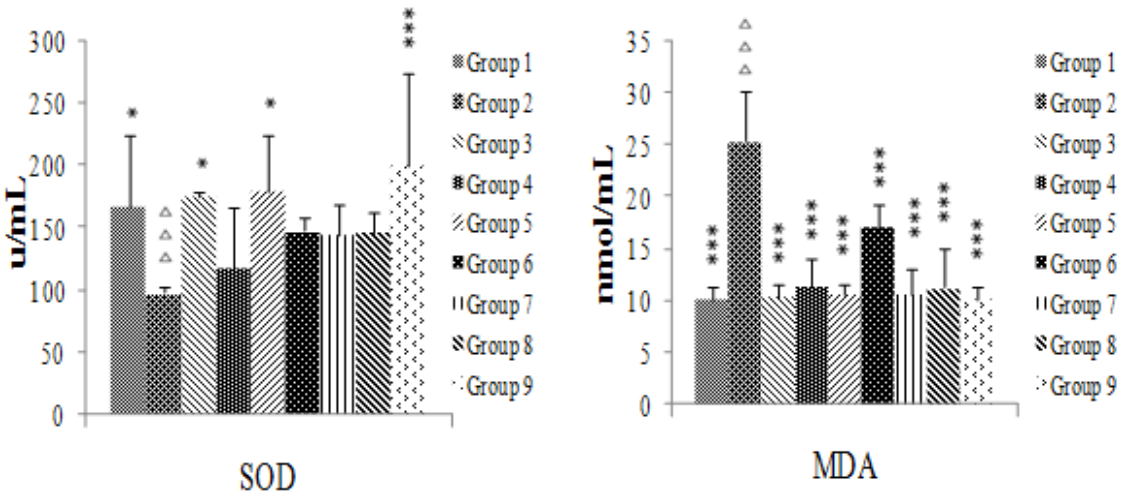

Figure 3. Effect of EtOAC extract and compounds from *O. fragrans* on SOD and MDA in the serum.

glycogen to near normal status (P<0.001), and the activity of increasing the concentration of lliver glycogen better than the positive control of acarbose.

Effect of EtOAC extract, oleanolic acid and ursolic acid from *O. fragrans* on the antioxidant and lowering blood lipid in normal and diabetic mice

In Table 3 and Figure 4, compared with the Group 1, the level of TC, TG and MDA in Group 2 showed a significant increase (*P*<0.001), and the level of SOD in Group 2 decreased significantly (*P*<0.001). After intragastric administration of EtOAC extract and compounds, the level of TC, TG and MDA in therapeutic group decreased, and the level of SOD increased. Compared with Group 2 and the positive control of acarbose, the result showed that intragastric administration of EtOAC extract, oleanolic acid and ursolic acid to alloxan-induced diabetic mice could lower the blood lipid level of TC and TG, and inhibit the oxidant stress and oxidative damage to tissues by decreasing the level MDA and increasing the level of SOD (Figure 3).

DISCUSSION

Glucose metabolic disorder is the most important and fundamental pathological changes in diabetes, so the blood glucose level is the key indicator to evaluate the success of models and the effectiveness of drugs. Experimental results showed that the drugs can significantly reduce high blood sugar, regulate the glycogen synthesis, which was very significant to maintain normal blood sugar and improve glucose tolerance. Lipid metabolism disorders followed the glucose metabolism disorder, the high blood sugar is the direct cause of high hypertriglyceridemia and hypercholesterolemia, the presence of hyperlipidemia is

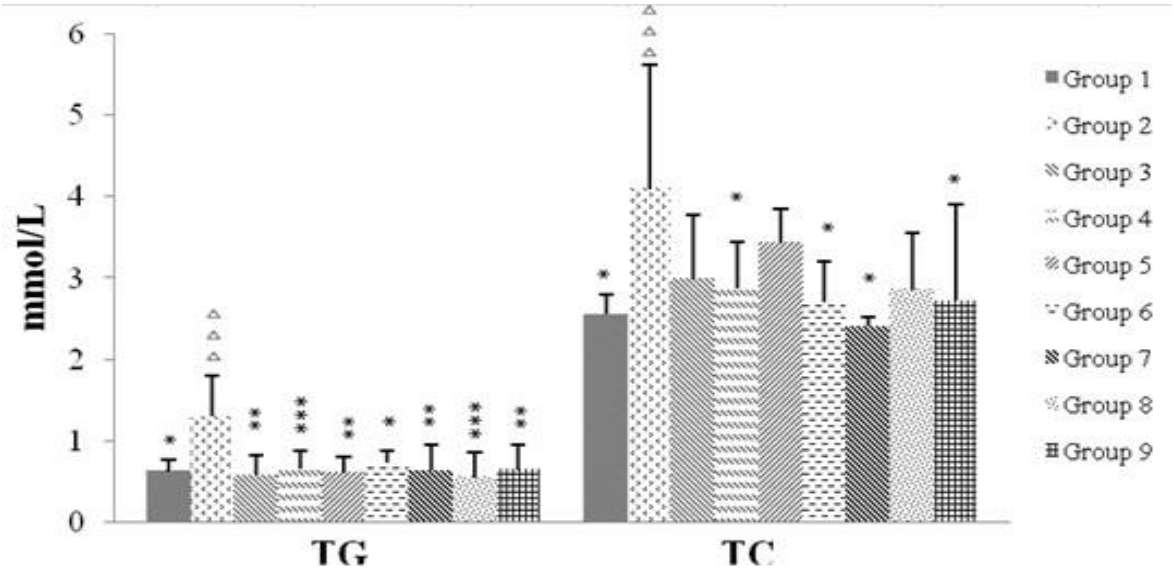

Figure 4. Effect of EtOAC extract and compounds from *O. fragrans* on blood lipid in the serum.

also the main factor to accelerate atherosclerosis and vascular complications in diabetes.

Therefore, reducing the blood lipid level is a goal of delaying the complications of diabetes (Tang et al., 2006). Glycosylation reaction can cause oxygen free radicals increase, while long-term state high blood sugar can enhance glycosylation reactive, the concentration of major blood oxygen metabolite MDA significantly increased. MDA can accelerate the occurrence and development of diabetic vascular disease, and can lead to diabetic retinopathy, kidney disease. So antioxidant is another way to prevent and treatment the diabetic complications. α-Glucosidase inhibitory activity from natural products were considered generally safe, effective, well tolerated, low prices and used in the treatment of diabetes.

In this experiment, the *a*-glucosidase inhibitory activity of EtOAC extract of *O. fragrans* was analyzed in *vitro*. The results were summarized EtOAC extract of *O. fragrans* had good α-glucosidase inhibitory activity in vitro, ursolic acid and oleanolic acid had the strongest α-glucosidase inhibition. Treatment with EtOAC extract, ursolic acid and oleanolic acid, intragastric administration of ursolic acid (300 mg/kg) and oleanolic acid (120 and 40 mg/kg) can significantly decrease the fasting blood glucose and postprandial blood glucose. Treatment with of EtOAC extract (500 mg/kg), ursolic acid (300 mg/kg) could significantly increase SOD (superoxide dismutase) level and could significantly decrease serum MDA (malondialdehyde) content in diabetic rats. Oral administration of EtOAC extract (160 mg/kg), ursolic acid (100 mg/kg) and oleanolic acid (120) could significantly decrease serum TC (total cholesterol) level and could significantly decrease serum TG (triglyceride) level in diabetic rats. Oral administration of EtOAC extract (160 mg/kg), ursolic acid (300 mg/kg) and oleanolic acid (120 and 40 mg/kg) could significantly increase liver glycogen content. The effects of EtOAC extract, oleanolic acid and ursolic acid from *O. fragrans* on lipid metabolism disorders were confirmed. Experimental results also showed the sample drugs had antioxidant activity and possibly play a role in hypoglycemic through oxidative mechanisms.

Conclusion

The present study was undertaken to evaluate *a*-glucosidase inhibitory activity in *in-vitr*o, and hypoglycemic, antioxidant activities and the effect of blood lipid level of EtOAC extract, oleanolic acid and ursolic acid from *O. fragrans in vivo*. To the best of our knowledge, this is the first report of antidiabetic activity of this plant. In this study, EtOAC extract, ursolic acid and oleanolic acid from *O. fragrans* showed stronger inhibiting activity of α-glucosidase compared with acarbose as positive control. The extract of *O. fragrans* had effective prevention and treatment on the development for diabetes and its complications. Thus, studies show that *O. fragrans* may be good sources of natural antioxidants and α-glucosidase inhibitor used for the therapy of diabetes and its complication.

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