Alleviating Effects of Active Fraction of Euonymus alatus Abundant in Flavonoids on Diabetic Mice

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Abstract: Euonymus alatus (E. alatus) has been used as a folk medicine for diabetes in China for more than one thousand years. In order to identify major active components, effects of different fractions of E. alatus on the plasma glucose levels were investigated in normal mice and alloxan-induced diabetic mice. Our results show that ethyl acetate fraction (EtOAc Fr.) displayed significant effects on reducing plasma glucose. In oral glucose tolerance, EtOAc Fr. at 17.2 mg/kg could significantly decrease the blood glucose of both normal mice and diabetic mice. After 4 weeks administration of the EtOAc Fr, when compared with the diabetic control, there were significant difference in biochemical parameters, such as glycosylated serum protein, superoxide dismutase and malondialdehyde, triglyceride, and total cholesterol, between alloxan-induced diabetic mice and the control group. Additional histopathological studies of pancreatic islets also showed EtOAc Fr. has beneficial effects on diabetic mice. Chemical analysis with three-dimensional HPLC demonstrated that the major components from EtOAc Fr. were flavonoids and phenolic acids, which had anti-oxidative effects on scavenging DPPH-radical in vitro. All these experimental results suggest that EtOAc Fr. an active fraction of E. alatus and can prevent the progress of diabetes. The mechanism of E. alatus for glucose control may be by stimulating insulin release, improving glucose uptake and improving oxidative-stress.

Keywords: Euonymus alatus; Diabetes; Flavonoids; Phenolic Acid; Oxidative Stress.

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Introduction

Diabetes mellitus is characterized by abnormal high levels of glucose in the blood and affects about 5% of population. The world wide average growth rate of type 2 diabetes is expected to be 6% per annum, probably reaching a total of about 250 million cases in 2010 (DeFronzo, 1999; Groop and Toumi, 1997; Moller, 2001). The mechanism of current marketed drug for treatment of type 2 diabetes include increasing insulin secretion, delaying glucose absorption and improving insulin sensitivity. Acarbose acts by delaying intestinal absorption of carbohydrate and in particular, attenuating postprandial blood glucose elevations (Lebowitz, 1998). As specific targets of peroxisome proliferator-activated receptor-γ (PPAR-γ), thiazolidinediones (TZDs) not only improve insulin sensitivity and enhance glucose utilization by adipocytes and skeletal muscle but also have beneficial effects on a variety of cardiovascular risk determinants, such as lipids, blood pressure and cytokines and inflammatory markers (Sheehan, 2003). However, these agents also have limitations such as toxicity/side-effects and undesired therapeutic effects.

There are considerable amounts of data which indicate that oxidative stress may be an important underlying pathogenic mechanism of diabetes and its complications (Ceriello and Motz, 2004; Evans et al., 2003). Hyperglycemia and free fatty acid (FFA) results in the generation of free-radical production involving glucose oxidation followed by oxidative degeneration and protein glycation (Maritim et al., 2003). A myriad of reactive oxygen species (ROS) impair the endogenous antioxidant defense system and induces a dysfunction of both β cells and the endothelium in many ways during diabetes (Ceriello, 2000). In peripheral tissues, the skeletal muscle cells and adipocytes produce a resistance to the action of insulin in order to protect themselves from damage by oxidative stress. Both in vitro and in vivo studies supported that antioxidants, such as vitamin E, vitamin C, or glutathione (Ceriello, 2000; Paollisso and Giugliano, 1996) can improve insulin sensitivity and ameliorate diabetic symptoms. It is well known that plants are potential sources of natural antioxidants and bioactive natural products can be drug candidate for further development (Kim et al., 2006; Manonmani et al., 2005).

Unlike the chemical agents, the plants used for medicine usually have several active components and could modulate multiple aspects of pathogenesis of disease. Euonymus alatus (E. alatus), browses and twigged attachments of Euonymus alatus (Thunb.) sieb., is a folk medicine in China. This plant has reliable therapeutic efficacy and a long history of clinical application, which was first recorded in “ShenNongBenCaoJing” in about 2700 BC. E. alatus has been recorded to be used in regulating blood circulation, resolving stagnant blood, relieve pain and so on (Xing et al., 2005; Zhang and Zhao, 2005). Moreover, it has been found recently that E. alatus possesses abilities to reduce plasma glucose and lipid levels by regulating NO production, matrix metalloproteinase-9 activity and intratumoral aromatase activity (Cha et al., 2003; Chung et al., 2002; Lee et al., 2004). Although the anti-hyperglycemic activity of E. alatus has been demonstrated in many clinical cases and researches, the mechanism and its active components for this activity are still unclear. In this study, we screened fractions of E. alatus, primarily clarifying the components of the major active fraction (EtOAc Fr.) through analysis of total phenolic
content and by use of three-dimensional HPLC. We also carried out a systematic evaluation on the glucose- and lipid-reducing effects of the EtOAc Fr., performing anti-oxidation in vitro experiment to further understand its components and possible mechanism.

Materials and Methods

Plant Material and Reagents

*Euonymus alatus* was obtained from Nanjing Medical Co. Ltd. (Nanjing, Jiangsu, China), which were taxonomically authenticated by Dr. Yu in our department. A voucher specimen (No. 20020118) of the plant has been deposited at the Herbarium of China Pharmaceutical University. Alloxan and 1, 1-diphenyl-2-picryl-hydrazil (DPPH) were obtained from Sigma. The bioassay kits for glucose, glycosylated serum protein (GSP), triglyceride (TG), total cholesterol (TC), superoxide dismutase (SOD) and malondialdehyde (MDA) were purchased from Nanjing Jiancheng Bioengineering Institute.

Extractions and Preparations

*E. alatus* (5 kg) were decocted twice with 6 times water for 2 hours (v/w). The aqueous decoction was filtered immediately at hot temperature and concentrated to the desired level, yielding an aqueous extract (yield 500 g; 10%). Subsequently, 250 g aqueous extract was dissolved in distilled water and its suspension was successively extracted with petroleum ether (PE), ethyl acetate (EtOAc) and water-saturated n-butanol (n-BuOH) (3 times each). Organic reagents and water were evaporated and reclaimed to dryness under reduced pressure and at a low temperature (< 60°C) in a rotary evaporator. The yield for each fraction was 0.625 g of PE fraction (0.025%), 10.75 g of EtOAc fraction (0.43%), 24.13 g of n-BuOH fraction (0.97%) and 215 g of residues fraction (8.4%).

Experimental Animals

Male Kunming strain mice with body weight of 18–20 g were obtained from the Central Animal House of Chinese Pharmaceutical University. The animals were housed in standard environmental conditions of temperature (21 ± 2°C), humidity (55 ± 10%) and 12-hour light-dark cycles. Mice were supplied with a standard pellet diet and tap water ad libitum. Before initiation of experiment, the mice were acclimatized for a period about 3 days. Animals were fasted without food overnight (at least 12 hours), but had free assess to water. The animals of all protocols were handled humanely according to current ethical regulations on animal research (National Research Council of USA, 1996).

Design for Doses of Administration

All test samples and positive control drug (metformin) were solved with 0.5% sodium carboxymethylcellulose (CMC-Na). The doses of fractions from *E. alatus* were calculated.
according to their yields in the extraction and preparation, which were all equivalent to 4 g/kg of unprocessed *E. alatus*. They were 400 mg/kg for aqueous extract (Aqu. Ext.), 0.994 mg/kg for petroleum ether fraction (PE Fr.), 17.2 mg/kg for EtOAc fraction (EtOAc Fr.), 38.6 mg/kg for *n*-BuOH fraction (*n*-BuOH Fr.) and 334 mg/kg for residue fraction (Re. Fr.).

**Induction of Alloxan-Diabetic Mice and Diabetic Mice with High-Fat Diet**

**Alloxan-Induced Diabetic Mice**: After fasting for 24 hours, mice were induced diabetic by a quick injection of alloxan (60 mg/kg, freshly dissolved in normal sodium) in the tail vein (Silva *et al.*, 2002). Three days later, the blood samples were collected from retro-orbital plexus of mice, which were fasted overnight. Fasting blood glucose (FBG) levels were measured by glucose-oxidative method. Mice with FBG levels higher than 11.1 mM were considered to be diabetic and used in the experiment.

**Alloxan-Induced Diabetic Mice with High-Fat Diet**: First, the mice were fed with high-fat diet, which nutrient composition expressed as a percentage of weight content was 34% carbohydrate, 19% protein, and 22% lipid with equal quantities of fiber, vitamins, and minerals as in the mouse standard diet. After feeding for 1 week, fasted mice received an intravenous injection of alloxan (60 mg/kg) (Sabu *et al.*, 2002). After 72 hours, the mice with FBG higher than 11.1 mM were considered as diabetic and used for the study. Meanwhile, this FBG was used as starting blood glucose at 0 week. In the entire experimental period, the mice were fed with high-fat diet.

**Screening for Glucose-Lowering Activities of Different Fractions from E. alatus**

In normal mice: Healthy mice were randomly divided by body weight into 7 groups of 10 mice per group. The groups were control, Met (metformin) group (140 mg/kg), Aqu. Ext. group (400 mg/kg), PE Fr. group (0.994 mg/kg), EtOAc Fr. group (17.2 mg/kg), *n*-BuOH Fr. group (38.6 mg/kg) and Re. Fr. group (334 mg/kg). Each mouse received intragastrically 0.2 ml/10 g/day for 7 consecutive days. Mice were fasted for 4 hours before last administration and FBG levels were measured after 2 hours of administration. Blood samples were collected by retro-orbital plexus puncture method with capillary and glucose levels were measured immediately by glucose-oxidative method.

In diabetic mice: The alloxan-induced diabetic mice were used in this experiment. Mice were divided according to FBG into different groups, the same as above. It was noted that the average of blood glucose in each group was not higher than 11.1 mM. After administration for 7 days, blood samples collection and analysis were the same as above.

**Oral Glucose Tolerance Test (OGTT) in Normal Mice and Alloxan-Induced Diabetic Mice**

After 7 consecutive days, the normal and diabetic mice were fasted for 4 hours before last administration. The blood samples from retro-orbital plexus were served as 0 hour
blood glucose level. The mice received various concentrations of samples and glucose (2.5 g/kg) 1 hour later (Park et al., 2005). Subsequently, blood samples were collected and centrifuged at 30, 60 and 120 min after the glucose loading, and glucose level was measured by glucose-oxidative method.

**Analysis of EtOAc Fr. by HPLC and Total Phenolic Content**

It had been reported that the antioxidant activity of plant materials was well correlated with the content of their phenolic components (Sabu et al., 2002). In our preliminary test for chemical components, the results showed that there was great amount of phenolic acid by using 1% (v/v) FeCl₃ reagent. Meanwhile, the isolated compounds were also rich in phenolics. Therefore, it is important to consider the phenolic acid content in EtOAc Fr., which might explain the hypoglycemic activity through antioxidative action.

The total phenolic content was determined by using potassium ferrocyanide-ferric chloride (K₃Fe(CN)₆-FeCl₃) colorimetry (Wang et al., 2001). The EtOAc Fr. (1.08 mg/ml in absolute ethanol) were mixed with 5.0 ml absolute ethanol, 0.3% (w/v) sodium dodecylsulfate (SDS) and 1.0 ml 0.6% K₃Fe(CN)₆-0.9% FeCl₃ (0.9:1, v/v) reagent in order in volumetric flask, which was followed by standing in dark for 5 min. Subsequently, 0.01 M hydrochloric acid (HCl) was added to appointed scale and stored for 20 min at dark. The absorbance of test sample was measured at 720 nm by using developer as a blank. Standard curves were established for the experiment by using various concentrations of protocatechuic acid in absolute ethanol, which was isolated from EtOAc Fr. Absorbance values were converted to mg of protocatechuic acid equivalent per 100 mg sample. The analysis was performed in triplicate.

EtOAc Fr. (30 mg) was solvated completely with 1 ml of methanol under ultrasonication for 10 min. The analysis of the filtrated solution by HPLC equipped with Waters 600 pumps, an Waters 996 photodiode-array detector was performed using a Alltima C18 column (5 µm, 250 x 4.6 mm, Alltech, USA). The solvents were (A) 1% methanoic acid-water, (B) acetonitrile. A linear gradient of 90% A and 10% B changed over 20 min to 80% A and 20% B, which lasted for 20 min. In the next 30 min, it changed to 65% A and 35% B. The flow rate and column temperature were 0.8 ml/min and 30ºC, respectively. The UV data of the effluent from the column ranging from 200 to 400 nm were collected, and the peak analysis and assignment were performed using the system analysis software, Millennium³² (Waters).

**Evaluation of Glucose- and Lipid-Lowering Activities of EtOAc Fr. in Diabetic Mice with High-Fat Diet**

Alloxan-induced diabetic mice with high-fat diet were used in this experiment and fed with high-fat diet during the entire experimental period. Normal control and diabetic control were administrated with 0.5% CMC-Na, 0.2 ml/10 g body weight. The positive control was metformin with a dose of 140 mg/kg. The dose for mice with EtOAc Fr. was
17.2 mg/kg. Body weight and food intake were measured every other day. After 4 weeks of administration, the blood samples were collected after picking out eyeball and handled for biochemical analysis.

Biochemical Analysis

FBG at each week (1, 2, 3 and 4) was determined by glucose-oxidative method. At the 4th week, GSP, SOD and MDA, TG, TC of serum were detected using kits.

Histopathological Procedures

The pancreatic tissues were harvested when the mice were sacrificed and immediately fixed in 10% neutral formalin solution. Subsequently, 3–4 mm serial section were prepared and stained with hematoxylin and eosin for light microscope examination when samples had been embedded by paraffin.

DPPH Radical-Scavenging Activity

The antioxidant activities of the EtOAc Fr. and its primary compounds, based on the scavenging activity of the stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical, were determined by modified method (Braca et al., 2001). Test samples were solved in absolute ethanol and 100 µl of sample was added to 100 µl of DPPH-ethanol solution (2 × 10^{-4} M) in 96-well plate. Absorbance at 517 nm was determined after 30 min. The percentage inhibition activity was calculated as \[
\left\{\frac{A_0 - A_1}{A_0}\right\} \times 100
\]
where \(A_0\) was the absorbance without sample and \(A_1\) was the absorbance with sample. Then, the IC\(_{50}\) was calculated according to the concentration and its percentage inhibition activity.

Statistical Analysis

Data are expressed as mean ± SD. The significance of the differences in the value was performed by student’s t-test. Differences were considered statistically significant at p < 0.05.

Results

Effect of Extract and Fractions from E. alatus on Blood Glucose Levels in Normal and Alloxan-Induced Diabetic Mice

Glucose-lowering effects of E. alatus on normal and diabetic mice are shown in Fig. 1. EtOAc Fr. at 17.2 mg/kg exhibited effects of reducing the blood glucose levels both in normal (p < 0.05) and diabetic mice (p < 0.01), in which the effect was similar to metformin (140 mg/kg). Aqu. Ext. (400 mg/kg) and Re. Fr. (334 mg/kg) also had significant glucose
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control effect (p < 0.01) on diabetic mice, but no effects on normal mice. Summarily, the glucose-reducing activities of fractions from *E. alatus* were in the order as EtOAc Fr. (17.2 mg/kg) > Aqu. Ext. (400 mg/kg) > Re. Fr. (334 mg/kg) > PE Fr. (0.994 mg/kg) > n-BuOH Fr. (38.6 mg/kg).

*Effects of EtOAc Fr. on OGTT in Normal and Alloxan-Induced Diabetic Mice*

In an attempt to verify the glucose-lowering effects of EtOAc Fr., OGTT was performed after 7 days of administration. Hyperglycemia in OGTT was induced by oral glucose load (2.5 g/kg). Blood glucose levels were determined before glucose administration (0 min) and 30, 60, 120 min later. The results in Fig. 2 showed the changes in blood glucose levels in normal and diabetic control and experimental groups. EtOAc Fr. (17.2 mg/kg) could lower the baseline glucose levels in normal mice (p < 0.01 and p < 0.05) as compared to the untreated normal control. Glucose tolerance was impaired.
in the alloxan-induced mice compared to their normal mice (Fig. 2C). EtOAc Fr. could significantly improve the glucose tolerance, leading to a 15% correction of blood glucose area under the curve (AUC) (Fig. 2D). Metformin corrected glucose excursion by 10%.

**Total Phenolic Content of EtOAc Fr. and Three-Dimensional HPLC Profile**

In order to comprehend the components of active fraction (EtOAc Fr.), total phenolic content was analyzed and the components were presented by three-dimensional HPLC profile. The total phenolic content was 16.6 mg protocatechuic acid equivalent per 100 mg EtOAc Fr. By using potassium ferrocyanide-ferric chloride ($K_3Fe(CN)_6\cdot FeCl_3$) colorimetry. The three-dimensional HPLC profile of methanol solution of EtOAc Fr. is shown in Fig. 3. The analysis based on UV-absorption clearly showed the presence of the following constituents in EtOAc Fr.: protocatechuic acid, catechin, $p$-hydroxybenzoic acid, vanillic
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Figure 3. Three-dimensional HPLC profile of the methanol solution of EtOAc Fr.

Acid, naringenin, quercetin-7-O-β-D-glucoside, quercetin, kaempferol, 5, 7-dihydroxy-4H-benzopyran-4-one, kaempferol-7-O-β-D-glucoside, aromadendrin, 3, 5-dimethoxy-4-hydroxybenzoic acid. We have isolated these compounds from EtOAc fraction.

Effects of EtOAc Fr. on Body Weight, Food Intake and Plasma Glucose in Diabetic Mice with High-Fat Diet

To further evaluate the glucose- and lipid-lowering effects of the EtOAc Fr., we prepared alloxan-induced mice with high-fat diet. The mice were fed with high-fat diet for 1 week and then obtained an intravenous injection alloxan in the tail, the purpose of which was to form hyperglycemia and hyperlipidemia. In the next 4 weeks, the mice received a administration per day and high-fat diet. During the 4-week period, diabetic mice treated with EtOAc Fr. could delay body weight gain compared to the diabetic control (Fig. 4A). The changes in body weight were parallel to the changes in food intake (Fig. 4B), but there was no statistical significance. The FBG was collected each week and analyzed as shown in Table 1. The results showed that a dose (17.2 mg/kg) of EtOAc Fr. significantly lowered FBG from second week, which was similar to the effect of metformin (140 mg/kg).

Effects of EtOAc Fr. on GSP, SOD, MDA, TG and TC in Diabetic Mice with High-Fat Diet

Glycosylated serum protein, an interim product of AGE (advanced glycated end products), expresses direct ratio with blood glucose level. Figure 4 shows the results of the effects of
Figure 4. Effects of EtOAc Fr. on body weight gain and food intake in diabetic mice with high-fat diet. With high-fat diet for 1 week, mice were given an intravenous injection of alloxan (60 mg/kg) in tails. After 3 days, the mice with FBG more than 11.1 mM were used in the study. Meanwhile, the diabetic mice were fed high-fat diet throughout the experiment. Body weight (A) and food intake (B) were recorded in the morning postprandial state every the other day. Data are reported as mean ± SD for body weight and mean for food intake.

Table 1. Effects of Oral Administration of EtOAc Fr. on FBG of Alloxan-Induced Mice with High-Fat Diet at Different Weeks

<table>
<thead>
<tr>
<th>Group</th>
<th>Dose (mg/kg)</th>
<th>0 Week</th>
<th>1st Week</th>
<th>2nd Week</th>
<th>3rd Week</th>
<th>4th Week</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>—</td>
<td>5.63 ± 1.04</td>
<td>5.96 ± 1.04</td>
<td>5.26 ± 1.05</td>
<td>5.21 ± 0.81</td>
<td>5.18 ± 1.36</td>
</tr>
<tr>
<td>Dia-C</td>
<td>—</td>
<td>32.40 ± 2.37**</td>
<td>32.14 ± 5.47**</td>
<td>30.18 ± 8.57**</td>
<td>29.47 ± 3.69**</td>
<td>28.15 ± 1.74**</td>
</tr>
<tr>
<td>Met</td>
<td>140</td>
<td>31.29 ± 2.15</td>
<td>19.17 ± 6.75**</td>
<td>18.29 ± 5.88**</td>
<td>15.56 ± 4.96**</td>
<td>14.91 ± 4.72**</td>
</tr>
<tr>
<td>EtOAc Fr.</td>
<td>17.2</td>
<td>31.24 ± 4.84</td>
<td>23.99 ± 7.43**</td>
<td>20.67 ± 6.95**</td>
<td>18.95 ± 5.93**</td>
<td>7.87 ± 2.67**</td>
</tr>
</tbody>
</table>

Data were expressed as mean ± SD (n = 10). **p < 0.01 compared to the normal control; *p < 0.05 and **p < 0.01 compared to the diabetic control.
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EtOAc Fr. on these biochemical parameters in alloxan-induced diabetic mice with high-fat diet. Diabetic mice had a significant increase in GSP (p < 0.01) versus normal mice. Metformin and EtOAc Fr. (17.2 mg/kg) revealed obvious reduction in GSP (p < 0.01) compared to the diabetic control at fourth week.

SOD and MDA are antioxidative parameters. There is a widespread understanding that hyperglycemia and hyperlipidemia create excess reactive oxygen species, which are probably involved in some of the complications of diabetes. Table 2 shows that there were significant decrease and increase in SOD (155.31 U/ml, p < 0.01) and MDA (11.45 nM, p < 0.01), respectively, in diabetic mice compared to the normal control, respectively. After the treatment of metformin and EtOAc Fr., the levels of SOD and MDA in the diabetic mice changed significantly (p < 0.01).

The effects of EtOAc Fr. on TG and TC levels are shown in Table 3. The diabetic control showed high levels of TG (2.54 mM, p < 0.01) and TC (13.36 mM, p < 0.01) compared to the normal control. However, levels of TG (1.00 mM) and TC (9.03 mM) had significant reduction (p < 0.01) after administration of EtOAc Fr.

**Histopathological Investigation of Pancreatic Islets**

As shown in Fig. 6, decrease of pancreatic islets number and size, ambiguity of their verges, karyopyknosis and degranulation of cells, vacuolation and invasion of connective tissues were detected in the diabetic mice. Simultaneously, there was fat degeneration in part of islet cells. After administration of metformin (140 mg/kg) and EtOAc Fr. (17.2 mg/kg), there was obvious amelioration in histological signs.
Table 3. Effects of Oral Administration of EtOAc Fr. on TG and TC of Alloxan-Induced Mice with High-Fat Diet

<table>
<thead>
<tr>
<th>Group</th>
<th>Dose (mg/kg)</th>
<th>TG (mM)</th>
<th>TC (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>—</td>
<td>1.43 ± 0.42</td>
<td>2.91 ± 1.13</td>
</tr>
<tr>
<td>Dia-C</td>
<td>—</td>
<td>2.54 ± 0.85</td>
<td>11.45 ± 2.23</td>
</tr>
<tr>
<td>Met</td>
<td>140</td>
<td>184.55 ± 15.79</td>
<td>8.22 ± 0.91</td>
</tr>
<tr>
<td>EtOAc Fr.</td>
<td>17.2</td>
<td>194.16 ± 25.55</td>
<td>6.16 ± 1.08</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SD (n = 10). **p < 0.01 compared with normal control; *p < 0.05 and **p < 0.01 compared with diabetic control.

Table 2. Effects of Oral Administration of EtOAc Fr. on SOD and MDA of Alloxan-Induced Mice with High-Fat Diet

<table>
<thead>
<tr>
<th>Group</th>
<th>Dose (mg/kg)</th>
<th>SOD (U/ml)</th>
<th>MDA (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>—</td>
<td>176.63 ± 21.10</td>
<td>4.97 ± 1.13</td>
</tr>
<tr>
<td>Dia-C</td>
<td>—</td>
<td>155.31 ± 13.18</td>
<td>11.45 ± 2.23</td>
</tr>
<tr>
<td>Met</td>
<td>140</td>
<td>184.55 ± 15.79</td>
<td>8.22 ± 0.91</td>
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</tbody>
</table>

Data were expressed as mean ± SD (n = 10). ##p < 0.01 compared to the normal control; *p < 0.05 and **p < 0.01 compared to the diabetic control.

Figure 6. Histological profiles (× 200) of pancreatic islets in (A) normal control, (B) diabetic control, (C) Metformin (140 mg/kg), (D) EtOAc Fr. (17.2 mg/kg). After administration for 4 weeks, the mice were sacrificed and pancreatic tissues were harvested. Decrease of pancreatic islets numbers and sizes, ambiguity of their verges, karyopyknosis and degranulation of cells, vacuolation and invasion of connective tissues were detected in diabetic mice. These abnormal histological signs changed obviously in all test article-dosing groups compared to the diabetic control group.
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Table 4. DPPH Radical-Scavenging Activities of EtOAc Fr. and its Some Isolated Compounds

<table>
<thead>
<tr>
<th>Test Materials</th>
<th>IC$_{50}$ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EtOAc. Fr.</td>
<td>15.56 µg/ml</td>
</tr>
<tr>
<td>Protocatechuic Acid</td>
<td>30.23</td>
</tr>
<tr>
<td>Catechin</td>
<td>11.80</td>
</tr>
<tr>
<td>p-Hydroxybenzoic Acid</td>
<td>&gt; 100</td>
</tr>
<tr>
<td>Vanillic Acid</td>
<td>&gt; 100</td>
</tr>
<tr>
<td>Quercetin</td>
<td>6.17</td>
</tr>
<tr>
<td>Naringenin</td>
<td>&gt; 100</td>
</tr>
<tr>
<td>Kaempferol</td>
<td>17.46</td>
</tr>
<tr>
<td>3, 5-Dimethoxy-4-Hydroxybenzoic Acid</td>
<td>&gt; 100</td>
</tr>
</tbody>
</table>

**Effects of EtOAc Fr. and its Some Isolated Compounds on DPPH Radical-Scavenging**

To reveal the potential mechanism of EtOAc Fr., scavenging free radical *in vitro* was performed. The results of Table 4 show the DPPH radical-scavenging activities of EtOAc Fr. and some isolated compounds. The IC$_{50}$ of EtOAc. Fr. was 15.56 µg/ml. Some compounds also presented the activities of scavenging DPPH radicals in the order as quercetin (6.17 µM) > catechin (11.80 µM) > kaempferol (17.46 µM) > protocatechuic acid (30.23 µM).

**Discussion**

Our study evaluated the anti-diabetic activity of *E. alatus* in mice, identified EtOAc Fr. as a major active fraction and indicated flavonoids and phenolic acids as active components. Different fractions of *E. alatus* were screened for glucose-lowering activity and EtOAc Fr. was validated as an active fraction because of its ability to significantly decrease both normal and alloxan-induced blood glucose. In addition, components were analyzed and presented by three-dimensional HPLC profile. Flavonoids were major components in the active fraction. Subsequently, the active fraction was evaluated systematically for biological activity using alloxan-induced diabetic mice with high-fat diet.

Diabetes mellitus is a metabolic disorder characterized by increased levels of blood glucose. In our experiment, EtOAc Fr. of *E. alatus* could significantly reduce the plasma glucose in both normal mice and alloxan-induced diabetic mice (Fig. 1), which also confirmed in oral glucose tolerance test (Fig. 2). Based on the three-dimensional HPLC profile, flavonoids such as quercetin, kaempferol, and catechin are major components of EtOAc Fr. The anti-diabetic activity of some of these compounds have been reported. In streptozotocin-induced diabetic model, quercetin could ameliorate diabetic status after oral treatment (Shetty *et al.*, 2004). Meanwhile, Kaempferitrin, glycoside of kaempferol isolated from *Bauhinia forficata* as an antidiabetic herbal, can stimulate glucose uptake in rat soleus muscle (Jorge *et al.*, 2004). In addition, some isoflavones isolated from
Astragalus membranaceus and Pueraria thomsonii could activate PPARα and PPARγ and induce adipocyte differentiation in vitro, especially biochanin A and genistein (Shen et al., 2006). It has also been reported that (-)-epicatechin has insulin-like activity and could increase insulin secretion from isolated rat islets (Ahmad et al., 1989; Hii and Hovell, 1984).

Reduction in food intake and resulting body weight loss alone can ameliorate hyperglycemia in diabetics. However, our results showed that there were slight, but no significant changes in body weight gain and food intake for EtOAc Fr.-treated mice as compared with the control group.

There is considerable evidence that hyperglycemia results in the generation of reactive oxygen species (ROS), ultimately leading to increased oxidative stress in a variety of tissues. Several biomarkers for oxidative stress, including SOD, lipid peroxidation (Table 2) and nonenzymatic glycosylated proteins (Fig. 1) significantly improved in the treatment group (EtOAc Fr. 17.2 mg/kg) compared to the control. The flavonoids and some phenolic acids are characterized by a special structure, well-known to scavenge oxygen-derived free radicals (Nijveldt et al., 2001). In vitro experiment of DPPH radical-scavenging activity, quercetin, kaempferol and catechin reveal their special properties. The levels of TC and TG in blood are in line with that of free fatty acid, which could impair insulin action by increasing the level of oxidative stress. Indeed, EtOAc Fr. treatment led to a reduction of TC and TG in alloxan-induced diabetic mice with high-fat diet. This was in agreement with the report that E. alatus could remarkably decrease the levels of TC and TG in diabetic ICR mice induced by high-fat diet (Park et al., 2005).

Histological analysis of pancreatic sections revealed that the improvement of glucose control as measured by lowering of fed and fasting plasma glucose as well as the improvement of glucose tolerance could be accounted for by the beneficial effects of EtOAc Fr. on pancreatic islets. β-cells expressed low level free-radical quenching enzymes such as SOD, glutathione peroxidase, and catalase; therefore they are particularly susceptible to the oxidative stress caused by hyperglycemia in diabetics. Indeed, there were obvious changes in histological signs, such as decrease of pancreatic islets number and size, karyopyknosis and degranulation of cells, vacuolation and invasion of connective tissues (Fig. 6B). These pathological phenomena were ameliorated by the treatment of EtOAc Fr. (Fig. 6D), which was in line with improving oxidative stress, including scavenging free-radicals (Table 4), increasing SOD, and reducing MDA (Table 2).

A sequential metabolic correlation for reducing the high levels of blood glucose and ameliorating lipid abnormality from E. alatus suggests the possible biochemical mechanism through decreasing in the level of ROS and increasing insulin release and agonizing PPARα/γ. Traditional Chinese medicine is like a “cocktail,” and includes several bioactive components as well as therapeutic targets. Our data indicate E. alatus targets multiple pathways in the process of type 2 diabetes and phenolic components may play a major role in glucose- and lipid-lowering activity. Further studies will focus on identifying active compounds and underlying molecular mechanisms.
References


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