Study of the extraction, purification and antidiabetic potential of ursolic acid from *Cornus officinalis* Sieb. et Zucc.

**Background:** *Cornus officinalis* Sieb. et Zucc. (CSZ) has been used in traditional Chinese medicine for over 1000 years, owing to its immunoregulation, antishock, antiarrhythmia, antioxidation and antidiabetes properties. The purpose of this study was to optimize the extraction method of ursolic acid (UA), and to find the main hypoglycemic ingredient of CSZ, then evaluate the toxicity of UA isolated from CSZ. **Methods:** UA was extracted by the methods of ethanol infusion, methanol infusion, reflux ethanol and sonication from CSZ; solvent extraction and macroporous resin column chromatography were used for purification; thin-layer chromatography and visible spectrophotometry were applied for the qualitative and quantitative identification. UA isolated from CSZ in the 100 and 200 mg/kg body weight doses was administered to alloxan-induced diabetic mice to determine the hypoglycemic effects. **Results:** The yield was the highest using ultrasonic extraction and macroporous resin purification. UA isolated from CSZ had a significant hypoglycemic effect compared with the diabetes control mice since day 10 (p < 0.05 or 0.01). The result of LD50 test and single-cell gel electrophoresis demonstrated that UA was not toxic. **Conclusion:** These results indicated that ultrasonic extraction was best among these extraction methods. Meanwhile, UA isolated from CSZ is the main hypoglycemic ingredient, and resulted in a good hypoglycemic effect in alloxan-induced diabetic mice. UA was testified to be nontoxic in the LD50 test (*in vivo*) and single-cell gel electrophoresis (*in vitro*).
Factory (Qingdao, China), and macroporous resin was obtained from the chemical plant of Nankai University (Tianjin, PR China). UA standards were obtained from Yousi Biotechnology Inc. (Shanghai, China). Alloxan was purchased from Sigma Chemicals Co. (MO, USA). Glucose Analyzer (GT-1640) and strips were purchased from ARKRAY Inc. (Kyoto, Japan).

Preparation of experimental animals
Male ICR mice weighing 24–28 g were provided by the Animal Department of the Beijing Institute of Traditional Medical and Pharmaceutical Sciences (Beijing, China). The animals were housed in cages under laboratory conditions at 22 ± 2°C and 60–65% relative humidity with a normal 12-h light and dark cycle. A total of eight mice were chosen randomly as a normal control (NC) group, while the rest were fasted overnight with free access to water and injected intraperitoneally with alloxan dissolved in normal sterile saline solution. The dosage of alloxan was 120 mg/kg of body weight. The same treatment was carried out as above after the mice were fasted overnight, except the dose of alloxan was changed to 100 mg/kg body weight. A total of 72 h after injection, the fasting blood glucose level of the mice was determined according to the glucose oxidase method [13], using a glucose analyzer. A blood glucose level above 16.7 mmol/l was defined as diabetic.

Extraction of plant material
Ethanol infusion extraction
This was prepared by extracting 0.5 kg of dried and powdered CSZ using ethanol in a ratio of 1:10. The extraction was carried out at 30°C for 24 h, stirring at regular intervals. It was then filtered and evaporated to dryness under reduced pressure, finally giving the dried extraction.

Methanol infusion extraction
A total of 0.5 kg of powdered CSZ was infused in methanol for 24 h. Following concentration, the solution was dissolved in methanol-dH2O (2:3) and then extracted in ethyl acetate. The upper ethyl acetate phase was separated by column chromatography using silica gel and eluted with dichloromethane. The supernatant was evaporated to dryness under reduced pressure.

Refluent ethanol extraction
Cornus officinalis Sieb. et Zucc. (0.5 kg) was soaked in 70% ethanol for 2 h, and then was boiled twice in 70% ethanol under reflux for 1 h. The combined infusion was filtered through filter paper and concentrated under reduced pressure; the solution was separated by column chromatography using macroporous resin (AB-8) and eluted with dH2O, 50%, 75% and anhydrous ethanol, respectively. The anhydrous ethanol phase was collected to dryness, resulting in the dried extraction [14].

Sonication extraction
The powdered CSZ (0.5 kg) was soaked in 70% ethanol for 2 h, and then extracted for 30 min with ultrasonic (40 kHz, 500 W, Kunshan Ultrasonic Instrument Ltd, Kunshan, China). The supernatant was filtered and evaporated to dryness under reduced pressure, then separated by column chromatography using macroporous resin (AB-8); all conditions were the same as in refluent ethanol extraction [15].

Qualitative identification
Thin-layer chromatography
Thin-layer chromatography was performed on a TLC silica gel 254 plate (10 × 20 cm). All the extracted samples and the UA standards were determined on the TLC plate, all of which were separated with the n-butyl alcohol:distilled water (19:1) system before sprinkling with 10% sulphuric acid/acetic acid. Developed plates were allowed to dry at 105°C for 10 min.

Quantitative identification
Standard curve of ursolic acid
Ursolic acid standards were dissolved in 3 ml anhydrous methyl alcohol to produce the different concentrations (3.33, 6.67, 9.99, 13.32 and 16.65%), each of which were detected three times using visable spectrophotometry, giving the mean value. The wavelength was 550 nm [16].

Detection of properties of ursolic acid
The linear relationship, precision, stability and recurrence of UA were investigated. Based on these results, the concentration of all samples was measured. The samples of the extracts (0.01 g) were dissolved in 3 ml anhydrous methyl alcohol, then were detected three times using visible spectrophotometry at 550 nm [17].
Hypoglycemic effect
The experimental mice were randomly divided into four groups: the NC group, diabetes control (DC) group, diabetes + UA low-dose (UA LD) group and diabetes + UA high-dose (UA HD) group. Each group consisted of eight mice. In the diabetes + UA-treated groups, each mouse received UA extract, dissolved in 0.5% CMC solution, at a dose of 100 mg/kg body weight (for the UA LD group) and 200 mg/kg of body weight (for the UA HD group) daily by gavage for 40 days. By contrast, the control mice (NC and DC groups) received the same volume of 0.5% CMC solution only. Blood glucose levels of the mice were measured in the trace blood samples from the tail vein every 10 days.

For detecting the effects of UA on normal mice, the acute hypoglycemic test was designed. A total of 16 normal mice were divided into two groups randomly, with each group assigned eight mice (NC and UA groups). After fasting for 12 h, the NC mice were fed with the same volume of 0.5% CMC solution, and the UA mice were treated with UA 0.5% CMC solution (100 mg/kg body weight). The blood glucose levels of the mice were recorded at 1, 2, 4 and 8 h following oral administration of the test compound to the animals.

Toxicity detection
LD50 experiment
A total of 24 normal mice were divided into four groups, each group included six animals (three females and three males) weighing approximately 18–22 g. The mice were orally administered UA isolated from CSZ in doses of 400, 600, 800 and 1000 mg/kg body weight, respectively. The animals were then observed for gross behavioral, neurologic, autonomic and toxic effects at short intervals of time for 24 h. Food consumption, feces and urine were also examined at 2 h, and then at 6-h intervals for 24 h.

Single-cell gel electrophoresis experiment
Single-cell gel electrophoresis (SCGE) was performed according to the method of Singh et al., with some modification [18]. Blood was collected from ten mice, and lymphocytes were separated from whole blood using a Ficoll–Paque lymphocyte separation medium and then suspended in phosphate-buffered saline (PBS). Cells were incubated in RPMI 1640 medium and exposed to the test compounds, which included dH2O control (same volume), UA (50, 100 µg/ml, final concentration) or H2O2 (5 µmol/l), cultured at 37°C in a 5% CO2 incubator for 1 h. The cells were then centrifuged and suspended in PBS. The cells were mixed with 0.5% low-temperature agarose, and then placed on microscope slides covered with a thin layer of 0.5% normal-temperature agarose. The slides were immersed in an alkaline solution for 1 h to break the cells and permit DNA unfolding. Electrophoresis was conducted at 25 V for 20 min. Following electrophoresis, the slides were washed gently to remove alkali and detergents with Tris buffer, rinsed with distilled water, and then stained with ethidium bromide (EB). The slides were evaluated under a fluorescence microscope (Nikon). Four different cultures were analyzed, and the tail lengths of 200 cells per culture were evaluated and categorized. The assay was repeated three times to avoid selection bias.

Statistical analysis
Statistical analyses were performed using the SPSS statistical software package. Data are expressed as a mean with standard error (SE). The effects of UA on blood glucose levels were determined using analysis of variance (ANOVA) for repeat measures. Results were considered significantly different with a p-value less than 0.05.

Results
Chromatogram of TLC
The UA in all samples was identified by comparing the color and retention factor (Rf) value with the standard solution. As shown in Figure 1, under the same conditions, a good separation and identification was possible under the described conditions. All the samples appeared blackish purple in colour, and presented the same Rf value corresponding to the UA standards, which confirmed that they all contained the UA ingredient. However, stains appeared in the samples of ethanol as well as methanol infusion, while the sample of refluuent ethanol was a little bit better. This demonstrated that the extracts were not pure, and so identified that the three methods were not without fault. The sample of sonication was longer than others, while the color was also deeper, which demonstrated its higher purity and concentration as compared with the others. Therefore, the sonication extraction and column chromatography using macroporous resin (AB-8) were the better candidates to extract and purify UA from CSZ.

Linearity of calibration curve
The standard curve of UA is shown in Figure 2. Correlative coefficients (0.9882) were obtained. The standard curve of UA was
Y = 0.0093X + 0.0158, where Y was absorbance and X was the concentrations of UA standard solution. Based on the data obtained in the experiment, a good linear correlation was obtained in correlation and regression analysis.

Extraction yields of all samples

All samples of the extracts were measured by visible spectrophotometry as described above, and the extraction yields are summarized in Table 1. The mean absorbance values and theoretical concentrations for all samples were calculated.

Hypoglycemic effect of ursolic acid

All experimental animals were treated as above for 40 days, and blood glucose levels were monitored in the trace blood samples from the tail vein at 10-day intervals. The results are presented in Table 2. On day one, there was no significant difference in blood glucose levels among the groups except the NC group. Blood glucose levels in the two UA-treated groups (LD and HD) were all lower than that in the DC group (p < 0.05) after 10 days of UA treatment, and significantly different compared with the DC group, which continued to exhibit elevated glucose levels throughout the study period. However, a more pronounced activity was recorded in the later stages. The glucose levels of UA-treated mice were significantly decreased at 20, 30 and 40 days as compared with the blood glucose concentrations of the fasted DC animals (p < 0.01). On day 40, the blood glucose levels in the UA LD and HD groups decreased by 48.4 and 58.3%, respectively. The NC and DC mice did not show any significant variation in blood glucose level throughout the experimental period. These results indicate that UA has a significantly antihyperglycemic effect in the alloxan-diabetic mice, and it is the main ingredient of CSZ. During the acute hypoglycemic experiment, there was no significant difference between the NC and UA groups until 8 h after oral feeding (p > 0.05, Table 3), which indicated that UA did not affect the blood glucose level of normal mice.

LD50 experiment

The behavior of the treated mice appeared normal during the experiment. No toxic effect was found at up to ten-times the effective dose, and no death occurred in any of these groups. Only the consumption of water was increased two- and four-times in the high-dose groups, but remained normal after 5 h.

Single-cell gel electrophoresis

Cultured lymphocytes from the four groups were assayed with SCGE. The results are shown in Figure 3 and Table 4. The percentage of DNA in
the tail \(\left(\frac{2.5 \times \text{cells}0 + 12.5 \times \text{cells}1 + 30 \times \text{cells}2 + 60 \times \text{cells}3 + 90 \times \text{cells}4}{\Sigma \text{cells}}\right)\) was calculated to express the amount of DNA damage [19]. The results indicated that UA-treated lymphocytes were not damaged, and the images were similar in dH2O-treated cultures. However, H2O2-treated cells were heavily damaged.

**Discussion**

Currently, there has been an increased interest globally to identify antidiabetic compounds from natural products that have low or no side effects. This study was aimed at optimizing the extraction of UA from CSZ. UA is one of the main constituents of CSZ. It was also desired to investigate the hypoglycemic properties of the UA extracted from CSZ.

In this study, we have investigated the optimum extraction conditions employing ethanol infusion, methanol infusion, refluent ethanol and sonication methods to extract UA from CSZ. The highest yields were obtained with sonication and the lowest with methanol infusion. In comparison, yields from refluent ethanol were lower than sonication extraction conditions, while similar results were demonstrated for ethanol infusion. The purity and concentration of UA with sonication extraction conditions was superior to those of others, which suggested that the ultrasonic extraction method possessed advantages when compared with other extraction methods. The results suggest that ultrasound-assisted extraction is a well-established method in the processing of plant material, particularly to extract low-molecular-weight substances, and in the extraction of bioactive substances from plants [20].

The traditional separation process, including ethanol infusion, methanol infusion and refluent ethanol, is not effective with regards to reagents, energy consumption and labor intensiveness. Alternatively, the adsorption–desorption process of macroporous resins is an efficient method for moderate purification. Furthermore, there is a high adsorption capacity in macroporous polymers. In recent years, the macroporous resins have been extensively applied to separate and purify the bioactive components of herbs [21] due to their unique adsorption properties, including ideal pore structure and the availability of various surface functional groups, less solvent consumption and easy regeneration [22]. In the study, TLC provided the qualitative identification of UA, and visible spectrophotometry provided the quantitative identification by means of concentration. These results strongly illustrated that it was a feasible way to purify the UA component with macroporous resins.

### Table 1. The extraction yields of all samples under different extraction conditions.

<table>
<thead>
<tr>
<th>Extraction methods</th>
<th>Ethanol infusion</th>
<th>Methanol infusion</th>
<th>Ethanol reflux</th>
<th>Sonication</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absorbance (OD)</td>
<td>0.227</td>
<td>0.201</td>
<td>0.279</td>
<td>0.309</td>
</tr>
<tr>
<td>Theoretical conc. (%)</td>
<td>1.362</td>
<td>1.194</td>
<td>1.697</td>
<td>1.891</td>
</tr>
<tr>
<td>RSD (%)</td>
<td>0.945</td>
<td>1.190</td>
<td>0.760</td>
<td>0.886</td>
</tr>
</tbody>
</table>

*OD: Optical density; RSD: Relative standard deviation.

### Table 2. Hypoglycemic effect of ursolic acid from *Cornus officinalis* Sieb. et Zucc. on alloxan-induced mice.

<table>
<thead>
<tr>
<th>Day</th>
<th>Groups</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NC</td>
</tr>
<tr>
<td>0</td>
<td>4.788 ± 0.132</td>
</tr>
<tr>
<td>10</td>
<td>4.988 ± 0.134</td>
</tr>
<tr>
<td>20</td>
<td>5.063 ± 0.084</td>
</tr>
<tr>
<td>30</td>
<td>4.912 ± 0.091</td>
</tr>
<tr>
<td>40</td>
<td>4.85 ± 0.104</td>
</tr>
</tbody>
</table>

*Each value represents mean ± SE of eight mice per group.
*Statistical significance versus diabetes control (p < 0.05).
**Statistical significance versus diabetes control (p < 0.01).

CSZ: *Cornus officinalis* Sieb. et Zucc.; DC: Diabetes control; NC: Normal control; SE: Standard error; UA: Ursolic acid; UA HD: Diabetes plus ursolic acid high dose (200 mg/kg/day); UA LD: Diabetes plus ursolic acid low dose (100 mg/kg/day).
Our results demonstrated that UA is the main active ingredient in CSZ extracts to decrease the blood glucose level of the alloxan-induced diabetic mice. In addition, the results indicated that the blood-glucose-lowering effect of UA in diabetic mice occurs within 10 days of the onset of UA treatment. However, UA did not affect the blood glucose levels of normal mice, which suggested that UA has a two-directional regulated function on blood glucose. Significant reduction of blood glucose levels in streptozotocin-induced diabetic rats treated with CSZ extracts was confirmed [23]. Zhang and colleagues discovered that UA and its derivative can inhibit protein tyrosine phosphatase 1B, enhance insulin receptor phosphorylation and stimulate glucose uptake [24,25]. Insulin receptor activators of UA were identified by culturing Chinese hamster ovary cells and 3T3-L1 primary adipocytes. The results showed that UA greatly enhanced the effects of insulin on insulin receptor signaling. It induced the substantial increase of insulin-mediated tyrosine autophosphorylation of the insulin receptor β-subunit, as well as GLUT4 translocation. UA was an effective insulin-mimetic agent at doses over 50 µg/ml, and an insulin sensitizer at doses as low as 1 µg/ml in a cellular model of insulin-sensitive adipose tissue [26]. However, the more detailed mechanisms were not revealed.

Alloxan is cytotoxic to the pancreatic β cells and thus is an effective diabetes-induction agent. It has been widely used to induce DM animal models, allowing the investigation of hypoglycemic agents in the treatment of diabetes [27]. Alloxan and the product of its reduction, dialuric acid, establish a redox cycle with the formation of superoxide radicals. These radicals undergo dismutation to hydrogen peroxide. Thereafter, highly reactive hydroxyl radicals are formed by the Fenton reaction. The action of reactive oxygen species with a simultaneous massive increase in cytosolic calcium concentration causes rapid destruction of β cells [28]. Alloxan injection consistently produced symptoms of DM, including hyperglycemia, decreased insulin levels, polyuria and weight loss [29]. Alloxan treatment causes destruction of β cells, resulting in a decrease in endogenous insulin secretion, which then decreases the utilization of glucose by the tissues [30].

Toxicity and adverse effects are important concerns for drug development. Thus we evaluated the toxic effect of UA through the LD50.
experiment and DNA damage in the SCGE assay, which is a rapid, simple, visual and sensitive technique for measuring DNA breakage in individual mammalian cells [31]. The damage is quantified by measuring the displacement between the genetic material of the nucleus (comet head) and the resulting tail. The SCGE of UA-treated cells and LD50 test of UA-treated animals were performed, and no obvious adverse effects were observed.

Pentacyclic triterpenoids, such as UA and oleanolic acid, are ubiquitous in the plant kingdom and in medicinal herbs, and are integral components of the human diet. Although the detailed mechanisms of UA action need to be investigated further, our results suggest a new class of compounds to be possible hypoglycemic agents for the treatment of diabetes. UA is the main active constituent of CSZ. The results indicate that there is good reason for expanding the investigations of the active compound UA extracted with the ultrasonic-assisted method from CSZ. The results also demonstrated that UA is the main hypoglycemic compound of CSZ and has a good ability to lower blood glucose. The study also effectively identified the optimal extraction method to attain the highest purity, and demonstrated that UA had a nontoxic effect, which indicates a bright future in the therapy of DM. The results obtained from this study may provide further information on the extraction, purification and identification of UA from CSZ, and also provide scientific evidence for the main hypoglycemic compound of CSZ on alloxan-induced diabetic mice.

**Conclusion**

The various methods of extraction, purification and identification of UA from CSZ have investigated the hypoglycemic effect of UA isolated from CSZ, a potent antidiabetic compound used in the treatment of diabetes. The extraction yields were the highest by the ultrasonic-assisted extraction and the macroporous resin purification, and

| Table 4. Percentage DNA in the tail of different cultures in single-cell gel electrophoresis assay. |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Scores                         | Cell 0          | Cell 1          | Cell 2          | Cell 3          | Cell 4          | Percentage DNA in the tail |
| dH2O                           | 184             | 16              | 0               | 0               | 0               | 3.30             |
| UA (50 µg/ml)                  | 181             | 19              | 0               | 0               | 0               | 3.45             |
| UA (100 µg/ml)                 | 178             | 22              | 0               | 0               | 0               | 3.60             |
| H2O2 (5 µmol/l)                | 0               | 8               | 102             | 78              | 12              | 44.60            |

Cell 0 represents the number of undamaged cells, and Cell 4 represents the most heavily damaged cells. UA: Ursolic acid.

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**Executive summary**

- *Cornus officinalis Sieb. et Zucc.* (CSZ) has been used in traditional Chinese medicine for over 1000 years owing to its immunoregulation, antishock, antiarrhythmia, antioxidation and antidiabetes properties, but few studies have optimized the extraction method.

- Ursolic acid (UA) was extracted by the methods of ethanol infusion, methanol infusion, refluent ethanol and sonication from CSZ, and it was demonstrated that ultrasonic extraction was the best among these extraction methods.

- Ursolic acid was purified by macroporous resin column chromatography from CSZ, and has identified by thin-layer chromatography and visible spectrophotometry. The results indicated that the method could obtain a reasonably pure UA product.

- Alloxan-induced mice were treated by administering UA solution in 100 and 200 mg/kg of body weight for 40 days. Blood glucose levels were decreased by 48.4 and 58.3%, respectively.

- In an acute hypoglycemic experiment, there was no significant difference between the normal mice and UA-treated mice, which indicated that UA did not affect the blood glucose level of normal mice.

- In an LD50 experiment, UA did not have a deleterious effect for the normal mice at up to 1000 mg/kg body weight, which indicated that UA was nontoxic in an *in vivo* test.

- In a single-cell gel electrophoresis test, the UA-treated lymphocytes were not damaged, and the relative proportion of DNA in the tail was similar with dH2O-treated cultures, which showed that UA was nontoxic in an *in vitro* test.

- UA is the main hypoglycemic ingredient of CSZ, which had hypoglycemic potential for alloxan-induced diabetic mice, so it is beneficial to diabetic patients.
UA administration significantly reduced the blood glucose level in alloxan-induced diabetic mice. Compared with diabetic control mice, treatment with UA for only 10 days resulted in a significant hypoglycemic effect (p < 0.05). According to our results, UA might be somewhat beneficial to the antihyperglycemic protection system in the human body that acts against diabetes and its complications. However, the study of hypoglycemic mechanisms of UA in CSZ is in progress.

Future perspective
Most plant products are frequently considered to be less toxic and have fewer side effects than synthetic drugs, and some scientists suggest that natural products will be the main resource for diabetic treatment in the future medicine field. CSZ has been extensively used in traditional Chinese herbal medicine. The antidiabetic compounds of CSZ were isolated and identified, and the structure of the active compound was characterized. Consequently, we expect that the antidiabetic mechanisms of UA isolated from CSZ will be revealed in the coming years. Such compounds may allow for the development of novel nontoxic hypoglycemic agents in the near future.

Financial & competing interests disclosure
The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending or royalties.

No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research
The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Bibliography


