The medicinal characteristics of alcohol-extraction-water-precipitation fraction from *Swertia mussotii* Franch.

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The alcohol-extraction-water-precipitation fraction of *Swertia mussotii* Franch. (SME-d) had been proved to be hepatoprotective without obvious toxicity in previous report. In this article, high performance liquid chromatography (HPLC), rat experiment and P450 model tests were employed for studying the pharmacology characteristics of SME-d. The results showed that the contents of sweroside, swertiamarin, mangiferin, gentiopicroside, and isoorientin were 0.24, 3.96, 12.30, 13.53, 16.85 mg/g in SME-d, respectively. SME-d could reduce the CCl₄-induced exaltation of alanine transaminase (ALT), aspartate aminotransferase (AST), total bilirubin (TBIL), total bile acid (TBA) significantly in rat, and the protective activity showed dose-dependent in 0.9 and 1.8 g/kg body weight (BW). The hepatoprotective activity of SME-d was different to positive drug bifendate, which only did on ALT value significantly. Bifendate could inhibit 66.17 ± 2.12% of CYP3A4 activity, while SME-d showed 99.0 ± 0.267% reductions on CYP1A2. The different medicinal characteristics of SME-d to bifendate, which are used widely to cure hepatitis in China, can give more choices for hepatitis.

Key words: *Swertia mussotii* Franch, the alcohol-extraction-water-precipitation fraction, pharmacology characteristic, hepatoprotective activity.

INTRODUCTION

Liver is an important organism for the metabolism and detoxification of various components entering into the body, and is hurt usually by the toxins and drugs, viral infections (Hepatitis A, B, C, D, etc.) and microbial infections (Sharma and Ahuja, 1997). Hepatitis is a big challenge to the modern medicine always. Plant-based traditional medicines were widely and successfully used in the treatment of liver disorders, for example, *Picrorhiza kurroa* (Chander et al., 1992), *Phyllanthus emblica* (Gulati et al., 1995), *Silybum marianum* (Flora et al., 1998). Bifendate, coming from herb *Schisandra chinesis*, had been the common drug for hepatitis in China (Pan et al., 2006).

*Swertia mussotii* Franch., referred to as “Zang Yin Chen” in Chinese, is a biennial herb of the family Gentianaceae that has been widely used in Tibetan folk medicine. *S. mussotii* is often used to remedy diseases in liver (Yang, 1991). Inventing a new medicine for hepatitis is always a big objective and challenge to plant chemist from *S. mussotii* Franch..

The alcohol-extraction-water-precipitation fraction from *S. mussotii* Franch. (SME-d) had been proved to be hepatoprotective without obvious toxicity in mice (Lv et al., 2010). If we wanted to devise a new medicine in China, 50% composition should be identified for injection medicine, its function should be proved in rat, the dose relationship should be illustrated, and the effects on P450 activity should be clarified. So high performance liquid
chromatography (HPLC), hepatoprotection evaluation in rat and P450 model test were employed for answering these questions.

MATERIALS AND METHODS

The preparation of SME-d

The whole plant of *S. muscatus* Franch. (SM) was collected from Sichuan province, China, and at the full-blooming stage in July, 2008. It was authenticated by the Centre of Tibetan Medicine, Northwest Institute of Plateau Biology, Chinese Academy of Sciences. 1 g crude SM was subjected to 10 ml ethanol (75% v/v) in hot water bath for three times, and the ethanol was removed by distillation under reduced pressure. Then the ethanol extract was dissolved in distilled water (1:8 v/v) for 24 h, and centrifuged (8000 rpm/min) for 10 min. At last, the sedimentation was taken as SME-d.

Chemicals

Carbon tetrachloride (CCl₄), olive oil and other solvents were purchased from XinXin Glass & Reagent Co. (Xining, China). Bilendate was supplied as a positive control sample by Zhejiang Wanbang Pharmaceutical Co., Ltd (Wenling, China). The references of swertiamarin, gentiopicroside, sweroside, mangiferin, and isoorientin were purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China).

Chemical analysis with high performance liquid chromatograph (HPLC)

The chemical profile of SME-d was recorded by high performance liquid chromatography (Agilent 1100) with diode array detection (DAD). Samples were dissolved by methanol, and the solutions were filtered with 0.45 μm Millipore filters. A reverse phase C₁₈ column (Agilent Eclipse XDB-C₁₈, 250 mm × 4.6 mm, 5 μm) was eluted with the gradient phase (0 min, 18% methanol → 25 min, 55% methanol → 47 min, 80% methanol → 60 min, 100% methanol) at the flow rate of 1 ml/min. The eluate was monitored at the wavelength of 210, 230 and 254 nm, and the column temperature was kept at 25°C. Swertiamarin, gentiopicroside, sweroside, mangiferin, and isoorientin were used as the reference standard.

Hepatoprotective evaluation in rats

KM rats (male and female) 20 to 25 g, were purchased from Laboratory Animal Center, Gansu College of Traditional Chinese Medicine. The animals were maintained at a constant temperature of 23 ± 2°C and fed with tap water and standard laboratory chow (Beijing Ke-Ao-Xie-Li feed. Co., LTD, Beijing, China). Normal group and control group were fed with 20 ml/kg BW distilled water for 8 days orally. Test groups were fed with SME-d at doses of 0.9 and 1.8 g/kg BW for 8 days orally, and positive group was fed with 80 mg/kg BW bifendate for 8 days. Each group contained eight rats. At the 8th day, 10 ml/kg BW CCl₄ (0.1%, v/v in olive oil) was administrated by intraperitoneal injection to all groups except normal group. At 22 h after the last dose, all rats were sacrificed. Serum was separated by centrifuging at 3000 rpm for 10 min and used for the measurement of the alanine aminotransferase (ALT), aspartate aminotransferase (AST), bilirubin (TBIL), bile acid (TBA) value (Drotman and Lawhorn, 1978). The ALT and AST activities were measured with the ALT and AST Elisa Kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The TBIL and TBA contents were determined by the TBIL and TBA Test Kits (Kehua, Shanghai, China).

The effects of SME-d on CYP1A2 and CYP3A4 activities

CYP1A2 and CYP3A4 belonged to the cytochrome P450 superfamily. They are plentiful in liver and responsible for catalyzing the oxidation of organic substances. For drug discovery, researchers need to determine how drug candidate alter P450 activity. In this experiment, all samples were analyzed by P450-GloTM CYP1A2 Screening System (Cat.#V9970) and P450-GloTM CYP3A4 Screening System (Cat.#9910) (Promega, America). CYP1A2 or CYP3A4 enzymes were incubated with their substrates, or and sample for 10 to 30 minutes at 37°C. Reactions were initiated by the addition of a nicotinamide adenine dinucleotide phosphate regenerating solution. P450 activity was stopped and luminescence was initiated by adding luciferin detection reagent. Luminescence was read directly on the FLUOstar OPTIMA microplate reader (BMG LABTECH, Germany) in luminescence mode. 1 μM α-naphthoflavone (sigma, America) was positive control in CYP1A2 screening experiment, and 5 μM Ketoconazole (sigma, America) was in CYP3A4 screening experiment. Firstly, samples were tested in 20 μg/ml dose. If the inhibition of some sample was bigger than 50%, the half maximal inhibitory concentration (IC₅₀) was measured in seven gradients.

Statistical analysis

All statistical analyses were performed by using Microsoft Excel 2000 (Guo, 2000) or the SPSS 10.0 (Mo, 2004) for windows software package. The date were analyzed by Student's t-test to assess the significance of the differences between two means or by one-way analysis of variance (ANOVA) followed by least-significant-difference (LSD) test for more than two means (Milton and Tsokas, 1983). Statistical significance was considered at p < 0.05.

RESULTS

The contents of five hepatoprotective chemical compounds

The contents of sweroside, swertiamarin, mangiferin, gentiopicroside, isoorientin were 0.24, 3.96, 12.30, 13.53, 16.85 mg/g in SME-d, respectively (Table 1).

The hepatoprotective activity in rat

Compared with normal group, CCl₄ induced serum ALT and AST activity in control group significantly (P < 0.01). High and low dose of SME-d and bifendate could inhibit the exaltation of the serum ALT activity induced by CCl₄. The inhibition effect of 0.9 g/kg SME-d < 80 mg/kg bifendate < 1.8 g/kg SME-d (Table 2). High and low dose of SME-d could also inhibit the exaltation of the serum AST activity induced by CCl₄, but the inhibition of bifendate was not obvious statistically. The effect of 0.9 g/kg SME-d < 1.8 g/kg (Table 2). CCl₄ also induced the serum TBIL and TBA content in control group significantly...


Table 1. The contents of five hepatoprotective chemical compounds (mg/g).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>SME-d</th>
<th>Sweroside</th>
<th>Swertiamarin</th>
<th>Mangiferin</th>
<th>Gentiopicroside</th>
<th>Isoorientin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sweroside</td>
<td>0.24</td>
<td>3.96</td>
<td>12.30</td>
<td>13.53</td>
<td>16.85</td>
<td></td>
</tr>
</tbody>
</table>

Table 2. The ALT and AST, TBA and TBIL value (mean ± s, n = 6).

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Dose</th>
<th>ALT (U/L)</th>
<th>AST (U/L)</th>
<th>TBIL (µmol/L)</th>
<th>TBA (µmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>-</td>
<td>48.67±5.72**</td>
<td>154.33±15.33**</td>
<td>2.65±0.10**</td>
<td>19.84±3.74**</td>
</tr>
<tr>
<td>Control</td>
<td>-</td>
<td>1952.96±771.75</td>
<td>3130.67±1598.88</td>
<td>6.65±2.71</td>
<td>257.90±95.16</td>
</tr>
<tr>
<td>SME-d</td>
<td>0.9 g/kg</td>
<td>842.33±467.94*</td>
<td>1143.33±493.11*</td>
<td>3.95±0.62*</td>
<td>141.36±24.64**</td>
</tr>
<tr>
<td></td>
<td>1.8 g/kg</td>
<td>605.54±185.45*</td>
<td>1050±253.24*</td>
<td>3.32±0.35*</td>
<td>103.87±24.64**</td>
</tr>
<tr>
<td>Bifendate</td>
<td>80 mg/kg</td>
<td>648.05±177.48**</td>
<td>2222.5±857.93</td>
<td>8.40±2.49</td>
<td>193.38±65.21</td>
</tr>
</tbody>
</table>

*P < 0.05, **P < 0.01 vs control.

Table 3. The effects of SME-d on CYP1A2 and CYP3A4 activity.

<table>
<thead>
<tr>
<th>Samples</th>
<th>CYP1A2</th>
<th>CYP3A4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% Inhibition (20 µg/ml)</td>
<td>IC50 (µg/ml)</td>
</tr>
<tr>
<td>SME-d</td>
<td>99.0±0.267</td>
<td>0.79±0.040</td>
</tr>
<tr>
<td>Bifendate</td>
<td>5.3±6.76</td>
<td>-</td>
</tr>
<tr>
<td>α-naphthoflavone (1 µM)</td>
<td>99.5±0.267</td>
<td>-</td>
</tr>
<tr>
<td>Ketoconazole (5 µM)</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

The effects of SME-d on P450 (CYP1A2 and CYP3A4) activities

SME-d could decrease 99.0 ± 0.267% activity of CYP1A2 in liver cell, and did little influences on CYP3A4. On the contrary, bifendate could inhibit 66.17 ± 2.12% activity of CYP3A4, but affected the activity of CYP1A2 hardly.

DISCUSSION

Some constituents of *S. mussotii* have been proved to be hepatoprotective (Sun et al., 1991), which contain swertiamarin (Singh, 2008), gentiopicroside (Li et al., 2001), sweroside (Singh, 2008), mangiferin (Liao et al., 2005), isoorientin (Orhan et al., 2003). Measuring these compounds contents in SME-d was a shortcut for understanding its hepatoprotective mechanism. The contents of sweroside, swertiamarin, mangiferin, gentiopicroside, isoorientin were 0.24, 3.96, 12.30, 13.53, 16.85 mg/g in SME-d, respectively. The extract procedure had enriched isoorientin, because the isoorientin content had been measured to be between 2.46 and 7.4 mg/g in *S. mussotii* (Bao et al., 2006; Li et al., 2008). Moreover, isoorientin could show obvious hepatoprotective function in 15 mg/kg BW (Orhan et al., 2003), and 1.5 mg/kg BW isoorientin should be given in 0.9 g/kg BW of SME-d, so isoorientin should be take parted in the hepatoprotective activity of SME-d. Of course, more works should be done for understanding the hepatoprotective mechanism of SME-d.

Carbon tetrachloride-induced liver injury model was the common model for studying the hepatoprotective medicine. ALT and AST were the sensitive index for the acute liver injury (Fu and Wei, 2005), and TBIL and TBA were the sensitive index for jaundice of liver injury (Zhang et al., 1989). Therefore, the serum ALT, AST, TBA and TBIL were taken as liver injury indicators in rats. CCl4 could induced the ascension of the serum ALT and AST activity, TBA and TBIL content significantly (P < 0.01), which showed the CCl4-induced liver injury model was constructed successfully. SME-d could cut down the exaltation of the ALT, AST, TBA and TBIL induced by CCl4 significantly, which could be deduced that SME-d can perform the hepatoprotective function in rat. Bifendate could decrease the serum ALT activity significantly, and did no obviously influence on the serum AST activity, the serum TBA and TBIL contents, which was similar to (P < 0.01). High and low dose of SME-d could inhibit significantly the exaltation of the serum TBIL and TBA content induced by CCl4 (Table 2). The inhibition effect of SME-d showed dose-dependent in 0.9 g kg and 1.8g/kg (Table 3). Bifendate showed no significant effect on the serum TBIL and TBA content (Table 2).

**The effects of SME-d on P450 (CYP1A2 and CYP3A4) activities**

SME-d could decrease 99.0 ± 0.267% activity of CYP1A2 in liver cell, and did little influences on CYP3A4. On the contrary, bifendate could inhibit 66.17 ± 2.12% activity of CYP3A4, but affected the activity of CYP1A2 hardly.
previous results (Liu, 2006). The difference of SME-d and bifendate should give SME-d more chance to be a new medicine.

The CYP3A4 and CYP1A2 belonged to the cytochrome P450 superfamily. They are plentiful in liver and often employed for evaluating the possibility of the interactions in medicines, especially for new medicine. SME-d could inhibit the CYP1A2 activity without effects on CYP3A4, which should give some suggestions for using SME-d correctly. Its difference to bifendate would give hepatitis more choices.

ABBREVIATIONS

HPLC, High performance liquid chromatography; ALT, aspartate transaminases; AST, alanine transaminases; TBIL, total bilirubin; TBA, total bile acid; CYP1A2, cytochrome P450 1A2; CYP3A4, cytochrome P450 3A4; SME-d, the alcohol-extraction-water-precipitation fraction of *Swertia mussotii* Franch.; BW, body weight.

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