Pharmaceutical Evaluation of Cultivated *Glycyrrhiza uralensis* Roots in Comparison of Their Antispasmodic Activity and Glycycoumarin Contents with Those of Licorice

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In China, the collection of wild *Glycyrrhiza uralensis*, one of the raw materials of Chinese licorice, has been restricted to prevent desertification. To compensate for the reduced supply of wild *Glycyrrhiza* plants, cultivation programs of *G. uralensis* have been initiated in eastern Inner Mongolia. The goal of the present study was to compare the chemical and pharmacological properties of cultivated *G. uralensis* roots to those of licorice prepared from wild *Glycyrrhiza* plants. The antispasmodic effect of boiled water extract of 4-year-old cultivated *G. uralensis* roots and licorice on carbachol-induced contraction in mice jejunum was similar (ED₅₀: 134±21 μg/ml vs. 134±16 μg/ml). In addition, glycycoumarin content, which is an antispasmodic and species-specific ingredient of *G. uralensis*, was similar when comparing the boiled water extracts of 4-year-old cultivated roots and licorice (0.10±0.02% vs. 0.10±0.06%). These data suggest that cultivated *G. uralensis* roots may be an adequate replacement for the generation of licorice in the context of the restriction of wild *Glycyrrhiza* plant collection.

Key words  *Glycyrrhiza uralensis*; antispasmodic activity; glycycoumarin; licorice; HPLC-profile

Since licorice is a frequently used crude drug in traditional Chinese formulations, a stable supply of licorice is an essential issue. However, because of the risk of desertification in Northern China, collection of wild *Glycyrrhiza* plants, which serve as raw materials of licorice, have recently been restricted. In an effort to compensate for the reduced supply of wild *Glycyrrhiza* plants, cultivation programs of *G. uralensis* have been initiated in eastern Inner Mongolia. In fact, this program has already yielded 4-year-old cultivated roots that meet the Japanese Pharmacopoeia XV (JP XV) standard requirements and can be used to the acquisition of licorice with adequate glycyrrhizin content, which is an important pharmacologically active glycoside of licorice. Further, we previously demonstrated that oral administration of the boiled water extract of 4-year-old roots had similar anti-allergic actions and glycyrrhizin bioavailability when compared with those of licorices.

In Japanese clinical practice, Shao-yao-Gancao-Tang (shakuyakukanzoto in Japanese), a traditional Chinese formula composed of Shao-yao (peony) and Gancao (licorice), efficiently treats various abdominal pains. Recently, glycycoumarin (Fig. 1) has been isolated as an antispasmodic ingredient of licorice, as demonstrated by its ability to relax carbachol (carbachol)-induced contraction of isolated mice jejunum. Thus, the goal of the present study was to clarify the bio-equivalency between the cultivated *G. uralensis* roots and concurrent licorice by assessing glycycoumarin contents and the relaxant effects on isolated mice jejunum of each preparation.

**MATERIALS AND METHODS**

**Plants and Reagents** Four-year-old (gathered on October in 2001) and 5-year-old roots (October in 2002), cultivated in eastern Inner Mongolia (the suburbs of Chifeng), and licorice (Dongbei-Gancao in Chinese, and Tohoku-kanzo in Japanese; collected in Inner Mongolia in 1999) were used for these experiments. The cultivation conditions have been described previously. The botanical origin of these samples was identified as *G. uralensis*. Voucher samples have been deposited in the Department of Kampo-Pharmaceutics, Institute of Natural Medicine, University of Toyama.

Glycycoumarin was kindly provided by Tsumura Co., Ltd. Peppermint oil of JP XIV grade (Yamada Pharmaceutical Co., Ltd.) and l-menthol (Wako Pure Chemicals Industries, Ltd.) was used as a positive control. The grade of acetylcholine, carbachol, and all other chemicals used in Tyrode solution and HPLC analysis were identical to those used in previous reports.

**HPLC-Profile and Glycycoumarin Content** Respective pieces of 5 different roots from 4- and 5-year-old cultivated roots and Dongbei-Gancao were cut severally. Freeze-dried extracts were prepared by boiling 6 g of their pieces in water (600 ml) for 40 min, followed by filtering and freeze-drying into powdered. The extract powders (5 mg each) were dissolved with MeOH (10 ml) for 30 min under ultrasonication and filtrated through a 0.45 μm membrane filter. An aliquot (10 μl) of the filtrate was injected into the HPLC system under the conditions described in the legends for Fig. 2 (HPLC-profile analysis) and Table 1 (glycycoumarin quantitative analysis).

**Evaluation of Relaxant Effects on Isolated Mice Jejunum** The freeze-dried extracts powder was dissolved in MeOH to obtain the sample solution (50 mg/ml) for use in carbachol-induced contractile experiments. Evaluation of carbachol-induced contractile response of isolated mice jejunum was performed as previously described. Briefly, a

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1 cm segment of jejunum was isolated from male ICR mice (7—10 weeks old). After maximal contraction was evoked by acetylcholine (1 μM), the segments were washed and allowed to equilibrate for another 45 min, and then were exposed to carbachol (1 μM). When the contraction reached a steady state level, samples were administered in a cumulative manner every 5 min. The relaxant effects of the extracts, the HPLC-profile of the MeOH soluble portion of two extracts was analyzed. The peak area of glycycoumarin in MeOH extract from 4-year-old cultivated roots was 0.09 ± 0.01% (n=3), indicating that glycycoumarin extracted with boiled water from 4-year-old cultivated roots was 33.3%.

All animal experiments and care were conducted in a manner conforming to the Guidelines of the Animal Care and Use Committee of University of Toyama and were approved by the Japanese Association of Laboratory Animal Care.

**Statistical Analysis** Data are expressed as mean±S.D. of the number (n) of experiments. Multiple comparison test analysis of variation (ANOVA) was used for comparisons among groups. A correlation between glycycoumarin contents and relaxant effects was assessed using Pearson’s correlation coefficient. Differences were considered statistically significant at p<0.05.

**RESULTS**

**HPLC-Profile and Glycycoumarin Content (Fig. 2, Table 1)** In order to assure the homogeneity of the boiled water extract of samples used in pharmacological examinations, the HPLC-profile of the MeOH soluble portion of two extracts was analyzed. The peak area of glycycoumarin in the HPLC-profile was similar when comparing the 4-year-old cultivated roots extracts to the licorice (Fig. 2). Further, the extract yield (%) and glycycoumarin contents (approximately 0.10%) of the 4- and 5-year-old cultivated roots extracts were comparable to those of the licorice extract (Table 1).

**Relaxant Effects (Figs. 3, 4)** Contractile tracings of the isolated mice jejunum are illustrated in Fig. 3. Peppermint oil (positive control) and the boiled water extracts of the 4-year-old cultivated G. uralensis roots (from 25 to 250 μg/ml).

**Table 1. Characteristics of Cultivated G. uralensis Roots and Licorice**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Boiled water extract yield (%)</th>
<th>EC50 (μg/ml) of extract for relaxant effect</th>
<th>Glycycoumarin in extract (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cultivated root</td>
<td>4-year-old 31.8±3.4</td>
<td>134±21</td>
<td>0.10±0.02</td>
</tr>
<tr>
<td></td>
<td>5-year-old 32.7±3.0</td>
<td>149±10</td>
<td>0.10±0.03</td>
</tr>
<tr>
<td>Dongbei—Ga cans</td>
<td>29.2±4.2</td>
<td>134±16</td>
<td>0.10±0.06</td>
</tr>
</tbody>
</table>

Each value represents the mean±S.D. (n=5). HPLC system and conditions: column, YMC-Pack ODS-A (250×4.6 mm I.D.); column temperature, 25°C; pump, 880-PU (Jasco); detector, UVIDEC-100-IV (Jasco); guard column, Develosil Packed Column ODS-MG-5 (4.0/10); mobile phase, 0.05 M ammonium acetate (pH 3.6): CH3CN=50:50; flow rate, 1.0 ml/min; wavelength, multi (200—650 nm); column oven, CO-1565 (Jasco); degasser, DG-1580-54 (Jasco). GC, glycycoumarin; IG, isoliquiritigenin.
induced relaxation in carbachol-induced contracted jejunal segments to a similar degree (Fig. 4). The maximal relaxant effects of the cultivated roots (72.7±3.2%) and the licorice (68.5±5.2%) were observed at 250 μg/ml.

The concentration–response curve of the peppermint oil was shifted to the left in comparison to those of the cultivated roots and licorice. The maximum relaxant effect of the peppermint oil on carbachol-induced contractions was 77.0±9.6% at 40 μg/ml.

Comparison of Relaxant Effects (Fig. 5) The boiled water extracts (50—200 μg/ml) of 4- and 5-year-old cultivated roots and the licorice induced similar degrees of relaxation in a concentration-dependent manner in carbachol-induced contracted jejunal segments (Fig. 5). Further, the EC$_{50}$ was similar for the 4- and 5-year-old cultivated roots and the licorice (134±21, 149±10, 134±16 μg/ml, respectively) (Table 1). However, their potencies were less than that of peppermint oil (EC$_{50}$: 13.4±6.0 μg/ml). The ED$_{50}$ of glycyccoumarin (1.08±0.35 μg/ml, 2.95±0.94 μg) was lower than that (14.6±6.7 μg/ml, 93.3±42.7 μg) of $l$-menthol (an active component of peppermint oil).

Fig. 4. Effect of 4-Year-Old Cultivated $G.$ uralensis Roots and Licorice (Dongbei-Gancao) on Carbachol-Induced Contracted Mice Jejunum

Each point represents mean±S.D. (n=5). After contraction was induced by carbachol (1 μM), extracts were added cumulatively. The relaxant effects (%) were calculated and expressed as the percentage of inhibition of the plateau level contraction (the maximum contraction) induced by carbachol.

- 4-year-old $G.$ uralensis roots cultivated in the eastern region of Inner Mongolia autonomy of China; • licorice (Dongbei-Gancao collected in the Inner Mongolia of China in 1999); ■ peppermint oil (positive control).

Fig. 5. Effect of Cultivated Roots and Licorice on Carbachol-Induced Contracted Mice Jejunum

Each value represents mean±S.D. (n=5). EC$_{50}$ concentrations of boiled water extracts of 4-year-old (134±21 μg/ml), and 5-year-old cultivated roots (149±10 μg/ml) were comparable to those of licorice (Dongbei-Gancao: 134±16 μg/ml) currently in use in Japan.

- 4-year-old $G.$ uralensis roots; △ 5-year-old cultivated $G.$ uralensis roots; • licorice (Dongbei-Gancao). Regression equation: $Y=0.68X+0.44$ (Y: relaxant effects at 150 μg/ml, X: glycyccoumarin contents).

Fig. 6. Relationship between Relaxant Effect and Glycyccoumarin Content

We have cultivated $G.$ uralensis roots as a replacement for wild Glycyrrhiza plants. Since cultivated $G.$ uralensis roots are new licorice resources, they should be compared chemically and pharmacologically with conventional licorice prepared from wild Glycyrrhiza plants.

As shown in Fig. 2, the chemical composition, including glycyccoumarin content, of the cultivated roots was similar to that of licorice prepared from wild Glycyrrhiza plants, which is consistent with results from previous reports by comparing the chemical composition including glycyrhrizin content. As suggested by Food and Drug Administration, the three-dimensional HPLC-profile analysis is a useful method of characterizing and comparing the composition of multi-component crude drugs.

Licorice is often used clinically to treat various abdominal spasmodyc symptoms as a component of Shaoyao-Gancao-Tang, whose efficacy has been demonstrated in a placebo-controlled double-blind parallel study. Further, the smooth muscle relaxing activity of licorice has been shown in acetylcholine-induced contracted guinea pig ileum. Recently, glycyccoumarin has been characterized as the antispasmodic ingredient of licorice through experiments with carbachol-induced contracted mice jejunum. Based on these data, the ex vivo method of using isolated jejunum to screen for bioactive properties of extracts appears to be of utility in defining the active ingredients of licorice.

As shown in Figs. 3 and 4, the smooth muscle relaxing effects of the boiled water extracts of the cultivated roots on carbachol-induced contracted jejunal segments are comparable to those of the licorice extracts. Data illustrated in Fig. 6 suggest that the relaxation activity of Glycyrrhiza roots and...
licorice was directly dependent on their glycy coumarin contents. Although glycy coumarin is a minor ingredient in the hot water extracts of licorice (approximately 0.10%), it is an important species-specific ingredient of *G. uralensis*.\(^{13}\) Therefore, glycy coumarin content is a parameter when considering quality control of *Glycyrrhiza* roots and licorice. As shown in Table 1, the pharmaceutical properties (represented by the relaxant ED\(_{50}\) values) and the glycy coumarin contents of the cultivated roots are comparable to those of licorices. These results suggest that cultivated *G. uralensis* roots can provide an alternate to wild *Glycyrrhiza* plants as a source of licorice.

In the present examination, peppermint oil was used as a positive control, which has been shown to relax human colon spasm,\(^{14}\) guinea pig ileum,\(^{15}\) and rabbit jejenum.\(^{16}\) Peppermint oil resulted in significant relaxation in the present contracted segments of mice jejunum. Although species-specific and regional-based differences in gastrointestinal smooth muscle has not been well characterized, the present *ex vivo* method of using segments of mice jejunum may be a good strategy to establish biological tenderization of *Glycyrrhiza* plants.

Based on the glycy coumarin content (0.10±0.02%) of the extracts of cultivated roots, the ED\(_{50}\) dose (134±21 μg/ml) contains 0.134 μg/ml of glycy coumarin. This is a relatively small dose and is equivalent to 10% of the ED\(_{50}\) dose (3.6 μM; 1.32 μg/ml) of glycy coumarin. Since the licorice fraction containing isoliquiritigenin (IG shown in Fig. 2) also modulated contractile responses (data not shown), further investigations to identify relaxant ingredients of other than glycy coumarin would be of benefit.

In summary, the present study demonstrated that the relaxant effects of the cultivated *G. uralensis* roots on carbachol-induced contracted mice jejunum were comparable to those of licorice prepared from wild *Glycyrrhiza* plants. In addition, content of the effective relaxant ingredient, glycy-

coumarin, was similar when comparing cultivated roots and licorices. These data suggest that cultivation of *G. uralensis* roots represent an adequate alternative to collection of wild *Glycyrrhiza* plants.

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**REFERENCES AND NOTES**

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