Pharmaceutical Evaluation of Glycyrrhiza uralensis Roots Cultivated in Eastern Nei-Meng-Gu of China

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To clarify the feasibility of medicinal use of the cultivated Glycyrrhiza resources, the equivalency between the G. uralensis roots cultivated in eastern Nei-Meng-Gu of China and medicinal licorice (Glycyrrhizae Radix, Gancao in Chinese and Kanzo in Japanese) was examined. The HPLC fingerprint including glycyrrhizin (GL) of the cultivated roots was similar to that of medicinal Gancao, but different from that of non-medicinal Xinjiang-Gancao (Shinkyo Kanzo in Japanese). Similarity between the cultivated roots and two medicinal Gancao was confirmed quantitatively by hierarchical cluster analysis on the basis of HPLC-7-peak-area data. Moreover, the 4-year-old adventitious roots conformed to the five standards described in the Japanese Pharmacopoeia XIV (JP XIV). The 4-year-old adventitious roots had similar pharmaceutical properties to those of medicinal Dongbei-Gancao (Tohoku Kanzo in Japanese) as determined by examining IgE-mediated triphasic skin reaction in mice and pharmacokinetic profile of glycyrrhetic acid, an anti-allergic metabolite of GL. The present pharmaceutical study suggests that the 4-year-old adventitious roots of G. uralensis cultivated in eastern Nei-Meng-Gu of China are comparable to medicinal Gancao conforming to the JP XIV, and may be a potential medicinal source to compensate for the insufficiency of wild Glycyrrhiza plants caused by collection restriction in China.

Key words Glycyrrhiza uralensis; HPLC fingerprint; allergic inflammation; pharmacokinetics; glycyrrhizin; hierarchical cluster analysis

Recent environmentally irresponsible over-collection of wild Glycyrrhiza plants, which produce a well-known medicinal licorice (Glycyrrhizae Radix,甘草, Gancao in Chinese and Kanzo in Japanese), is one of the factors inducing desertification. It is reported that the World Health Organization (WHO) plans to introduce Good Field Collection Practice (GFCP) to conserve the natural environment. In 2000, the Chinese government imposed restrictions on the collection of wild Glycyrrhiza plants. Gancao is most frequently used in a famous traditional Chinese medical formulary Shan-Han-Lun (傷寒論) and has been used for allergic-inflammatory, gastrointestinal and liver disorders in both traditional Chinese and modern medicine.5,4,3

With the increasing demand for Gancao and the decline of wild Glycyrrhiza resources, cultivated Glycyrrhiza roots have attracted attention as an additional source of Gancao. This growing interest in a new cultivated source requires a clarification of the equivalency between the cultivated Glycyrrhiza roots and concurrent Gancao. We previously reported on glycyrrhin (GL)-content in G. uralensis roots cultivated in the eastern Nei-Meng-Gu (内蒙古自治区) of China, which is the habitat of G. uralensis and the major source of Dongbei-Gancao (東北甘草, Tohoku-Kanzo in Japanese). It was the first report that the GL-content of 4-year-old G. uralensis adventitious roots exceed the Japanese Pharmacopoeia XIV (JP XIV) standard (2.5% or more). Our cultivation study has been continued and we have obtained higher GL-content and yield of roots as well as adequate ingredients-composition similar to concurrent medicinal Gancao prepared from wild G. uralensis roots.

In the present study, we describe the pharmaceutical properties of the cultivated G. uralensis roots. The overall gross similarities in chemical ingredients between the cultivated roots and Dongbei-, Xibei- (西北甘草, Seihoku-Kanzo in Japanese), Xinjiang-Gancao (新疆甘草, Shinkyo-Kanzo in Japanese) and wild G. uralensis roots were examined by comparing the HPLC fingerprints (chromatograms) including the GL peak. The analysis of HPLC fingerprints was further examined quantitatively by hierarchical cluster analysis (HCA) and principal component analysis (PCA).

In addition to the chemical study, pharmacological and pharmacokinetical studies play an important role to assure safety and effective clinical use of a new medicinal candidate. The pharmacological properties were examined using IgE-mediated triphasic skin reaction in mice.7) The plasma concentration–time profile of glycyrrhetic acid (GA), an anti-allergic8) metabolite biotransformed from GL by intestinal bacteria, was also examined.

MATERIALS AND METHODS

Cultivated G. uralensis Roots and Comparative Samples As shown in Fig. 1, 4- and 5-year-old roots cultivated in the same field in eastern Nei-Meng-Gu as in the previous report5) were used. The botanical origin of the cultivated plant was identified previously9) to be G. uralensis by referring to the morphological characteristics of flowers and fruits described.9) The same Dongbei-Gancao, Xibei-Gancao and wild G. uralensis roots (collected in eastern Nei-Meng-Gu, one of the major sources of Dongbei-Gancao of China) as in the previous report10) were used. The botanical origin of part of Dongbei-Gancao collected in eastern Nei-Meng-Gu and Xibei-Gancao collected in Shanxi province (陕西省), Ningxia (宁夏回族自治区), and western Nei-Meng-Gu was identified as G. uralensis by our on-the-spot investigations10) in their producing districts of China by referring to the reported characteristics.9) Xinjiang-Gancao (collected in Xinjiang in 2000) possessing lichochalcone A was selected by examining TLC11) in advance. Its botanical origin was estimated as G. inflata by comparing its HPLC fingerprint with the reported
one,12,13) but was not confirmed pharmacognostically. The voucher samples have been deposited in the Department of Pharmacognosy, Institute of Natural Medicine, Toyama Medical and Pharmaceutical University.

**Chemicals** GL standard (control: No. 992) was purchased from the National Institute of Health Sciences, Japan, and GA and liquiritin were purchased from Wako Pure Chemical Industries Ltd., Osaka. Liquiritin apioside and isoliquiritin apioside were kindly provided by Mr. Takayuki Nakada of Alps Pharmaceutical Ind., Co., LTD. Isoliquiritin was kindly provided by Dr. Kazuo Koike of Toho University, School of Pharmaceutical Sciences. Lichochalcone A was isolated from peels of Xinjiang-Gancao by preparative TLC using the conditions mentioned previously,11) and identified by comparison of the reported HPLC chromatogram.12,13) Dinitrofluorobenzene (DNFB) and dinitrophenol (DNP) were purchased from Nacalai Tesque, Kyoto. Prednisolone-21 acetate was purchased from Sigma Chemical Co., St. Louis. All other chemicals and solvents were of analytical and/or HPLC grade.

**Animals** Female specific pathogen-free BALB/c mice (6 weeks old) and male Wistar rats (8 weeks old) were purchased from Japan SLC Inc. (Hamamatsu, Japan), and used for the triphasic skin reaction and the pharmacokinetic study, respectively. All animal experiments were carried out in accordance with the Guidelines of the Animal Care and Use Committee of Toyama Medical and Pharmaceutical University and approved by the Japanese Association of Laboratory Animal Care.

**HPLC Fingerprint Analysis** About 18 whole 4- and 5-year-old cultivated *G. uralesis* roots and 400 whole roots of the same Dongbei- and Xibei-Gancao as analyzed in the previous report10) were used. Seven and 4 whole roots of Xinjiang-Gancao and wild *G. uralesis* roots collected in northeast Nei-Meng-Gu10) were used, respectively. The EtOH soluble portion of 90% aqueous MeOH extracts of each sample was analyzed by HPLC under the conditions12) described in the legend of Fig. 4.

**HCA and PCA Based on HPLC-Peak-Area Data** Whole roots of *G. uralesis* cultivated for 4—5 years, Dongbei-, Xibei-, Xinjiang-Gancao and wild *G. uralesis* were used. The 7-peak-area (from A to G shown in Fig. 4) data of HPLC fingerprints of each sample were treated using multivariate analysis software (Pirouette,14) Infometrix Co., Woodinville, U.S.A.). The 7-peak-area data (*A*: *n* = 7) were calculated from autoscale preprocessing with the following equation: (*A* − *A*<sub>mean</sub>)(standard deviation: S.D.), *A*<sub>mean</sub> represents the mean value of each peak-area.

**Anti-allergic Study** The freeze-dried extracts of the 4-year-old adventitious roots and Dongbei-Gancao were prepared by boiling in water (3 g Gancao in 600 ml water) for 40 min, then were filtered and freeze-dried into powder. To obtain a constant quality of extract, a mixture of extracts prepared from five extractions was used. The IgE-mediated triphasic skin reaction in mice was carried out as previously reported. Briefly, BALB/c mice were given i.v. injection of a 1 ml aliquot of anti-DNP monoclonal antibody (mAb) IgE mAb-containing fluid 24 h before the DNFB challenge. A skin reaction was elicited by applying 10 μl of 0.1% DNFB/EtOH to each side of each ear of the sensitized mice. Ear swelling was determined using a dial thickness gauge (G-1A type, Peacock) immediately before and at appropriate times after the challenge.

**GA Pharmacokinetic Study** The same freeze-dried extracts of the 4-year-old cultivated *G. uralesis* roots and Dongbei-Gancao as used in the skin reaction were used. Pharmacokinetic study of GA after oral administration of two extracts was carried out as previously reported.14) Briefly, rats were fasted overnight prior to the administration of two extracts containing 45 mg/kg GL (756 mg/10 ml/kg of cultivated *G. uralesis* roots extract and 452 mg/10 ml/kg Dongbei-Gancao extract). Blood samples (about 0.3 ml) were collected from the tail vein at each specified time after the administration. The plasma GA levels were determined using the same HPLC system and conditions as in our previous report.14) The area under the mean concentration of GA versus time curve from zero to 24 h (∑AUC<sub>0-24h</sub>), peak plasma concentration of GA (C<sub>max</sub>) and the time to reach C<sub>max</sub> (T<sub>max</sub>) of GA were determined by assessing the actual GA levels in the plasma.

**Statistical Analysis** The results are shown as the mean ± S.D. of the number (*n*) of experiments. Data of the triphasic skin reaction were analyzed for statistical significance using Mann–Whitney’s *U* test and the data of other examinations were analyzed by Student’s *t*-test. Probability (*p*) values less than 0.05 were considered to be significant.

**RESULTS**

**Root Growth and GL-Content (Figs. 2, 3, Table 1)** The underground part of cultivated Glycyrrhiza was composed of a taproot and 1—4 pieces of adventitious roots. Basal diameter of 4- and 5-year-old adventitious roots was 0.8±0.2 cm (*n* = 7) and 1.2±0.3 cm (*n* = 7), respectively, which corresponds to the mean diameter of “3-go-Kanzo (3号甘草, 0.5 cm or more),” which is the trading standard of root diameter of Dongbei-Gancao. The root specific gravity (RSG), a term describing solidity of roots calculated from dry weight divided by cylindrical volume as previously described,10) of 4- (0.66±0.07 g/cm<sup>3</sup>, *n* = 15) and 5-year-old adventitious roots (0.59±0.11 g/cm<sup>3</sup>, *n* = 15) was similar to that
of Xibei-Gancao (0.65±0.11 g/cm³, n=66), and slightly larger than that of Dongbei-Gancao (0.51±0.09 g/cm³, n=66). As shown in Fig. 2, the maximum dry weight per root (71.9±41.4 g, n=18) was observed in the 4-year-old root (Oct. 2001). The yield of taproots and adventitious roots (4-year-old, Oct. 2001) was about 21 and 29 kg/are.

As shown in the left panel of Fig. 3, GL-content (%) of 4- and 5-year-old adventitious roots except the sample collected in Aug-2001 exceeded the JP XIV standard. Taking the root growth into account, the GL-contents (g/root) of the adventitious roots collected in 4- (Oct. 2001) and 5-year-old roots (Oct. 2002) were almost equal (right panel of Fig. 3). Moreover, the 4-year-old adventitious roots and 5-year-old roots, which are externally dark-brown to red-brown, have a slight odor and sweet taste, conformed to the JP XIV standards (Table 1).

HPLC Fingerprint Analysis (Figs. 4, 5) As shown in Fig. 4, the major peaks B (liquiritin) and F (GL) of the 4- and 5-year-old cultivated roots were shown in fingerprints detectable at 254 nm. Their fingerprints are similar to those of two medicinal Dongbei- and Xibei-Gancao (and wild G. uralensis roots, data not shown) and also comparable with those previously reported. On the other hand, the fingerprint of Xinjiang-Gancao is characterized by the presence of peaks E, which is believed to be lichochalocone B by referring to the reported HPLC profile, and G (lichochalcone A). Peaks E and G are absent in other samples including the cultivated roots.

In order to discriminate quantitatively the similarity of fingerprints between the cultivated roots and other Gancao, a multivariate analysis was conducted. The HCA dendrogram (Fig. 5) obtained using 7-peak-area data (peaks A to G in Fig. 4) indicates that the 4-year-old adventitious roots (s1 to s6 in Fig. 5) belong to cluster I, which contains two medicinal Gancao and wild G. uralensis roots. Cluster II contains non-medicinal Xinjiang-Gancao and can be distinguished from medicinal Gancao. The same results were also found in the PCA scatter diagram (data not shown) obtained using the same data as in HCA.

Anti-allergic Effect (Fig. 6) Since many traditional Chi-

| Table 1. Quality Profiles of Cultivated G. uralensis Roots in Relation to JP XIV Standard |
|-----------------|----------|-------------|-------------|-------------|-------------|-------------|
|                 | GL-content (%) | EtOH-ext. content (%) | Loss on drying (%) | Total ash (%) | Acid-insoluble ash (%) | Dry weight (g/root) |
| 4-year-old (Jun) adventitious roots | 2.58±0.62 | 32.2±4.2 | 8.5±0.5 | 4.1±0.5 | 1.0±0.3 | 12.0±5.1 |
| 4-year-old (Oct) adventitious roots | 2.81±0.76 | 29.6±3.6 | 4.9±0.4 | 4.3±0.7 | 1.3±0.5 | 41.3±24.6 |
| 5-year-old (Jun) whole roots | 3.28±0.60 | 29.2±4.0 | 7.1±0.9 | 4.5±0.7 | 1.0±0.3 | 35.4±16.9 |
| 5-year-old (Jun) adventitious roots | 4.14±0.79 | 34.1±5.0 | 7.5±1.0 | 4.9±0.8 | 1.2±0.5 | 19.2±11.2 |
| 5-year-old (Aug) adventitious roots | 2.97±0.62 | 33.7±3.2 | 7.4±0.7 | 3.9±0.5 | 0.6±0.2 | 20.6±9.2 |
| 5-year-old (Oct) adventitious roots | 2.88±0.72 | 32.6±4.5 | 7.0±1.5 | 3.8±0.6 | 0.7±0.3 | 30.6±11.9 |
| JP XIV standard | 2.5 | 25.0 | 12.0 | 7.0 | 2.0 | — |

Each value represents the mean±S.D. (n=18) measured as described in JP XIV. The 6 samples fitted the tests of “description and identification” described in JP XIV.
nese formulations containing Gancao such as Xiao-Feng-San (消風散),\(^\text{19}\) Baihu-Tang (白虎湯)\(^\text{17}\) and Taobe-Chengqi-Tang (桃核承気湯)\(^\text{18}\) have been used clinically for atopic dermatitis, we examined the anti-allergic effect of the 4-year-old cultivated adventitious roots. As shown in Fig. 6, oral administration of both extracts of the cultivated roots and Dongbei-Gancao extracts significantly inhibited the IgE-mediated triphasic ear swelling.\(^\text{17}\) There were no significant differences in the value of each dose between them.

**GA Pharmacokinetics (Fig. 7)** It is well known that orally taken GL in Gancao extracts is biotransformed into an anti-allergic\(^\text{17}\) GLA, which is absorbed into the blood.\(^\text{19}\) As shown in Fig. 7, the GA plasma concentration–time profile for oral administration of the 4-year-old adventitious roots and Dongbei-Gancao extracts are almost similar in appearance. Furthermore, GA reached a maximum concentration at 9 h and there was no significant difference between \(C_{\text{max}}\) (1.90±0.40 \(\mu g/ml\)) and \(AUC_{\text{0-24h}}\) (16.34±2.39 \(\mu g h/ml\)) of GA from the cultivated roots and those (\(C_{\text{max}}\) : 1.51±0.91 \(\mu g/ml\); \(AUC_{\text{0-24h}}\) : 13.60±4.62 \(\mu g h/ml\)) from Dongbei-Gancao.

**DISCUSSION**

In the present study, pharmaceutical properties of 4- and 5-year-old \(G.\ uralesis\) roots cultivated in the eastern Nei-Meng-Gu of China were examined to clarify how many cultivation years are necessary to conform to the JP XIV standard (GL-content: 2.5% or more). The yields (21 and 29 kg/are) of cultivated taproots and adventitious roots (4-year-old, Oct-2001) are satisfactory in comparison with the reported yield (75 kg/are, about twice the weight of \(G.\ uralesis\) of 3-year-old \(G.\ glabra\) stolon and roots cultivated in Japan\(^\text{20}\), whose GL-content (%) was barely met the JP XIV standard.\(^\text{21, 22}\) The left panel of Fig. 3 shows that 4 or 5 cultivation years are necessary to conform to the JP XIV standard.
necessary to fit the GL-content standard of JP XIV.

As shown in Table 1, the 4-year-old adventitious roots conform to the JP XIV standard. From a practical point of view, to reduce cultivation expenses, the roots cultivated for 4 years (especially the adventitious roots collected in autumn) could be an additional source of medicinal Gancao. The cultivation of G. uralensis could stabilize the quality and supply of Gancao, however, the curtailment of cultivation expenses is a subject for a future study.

Cultivation trials of Glycyrrhiza plants have been performed since the early 1990’s in northwestern China. Most of the 3- and 4-year-old cultivated roots obtained in our on-the-spot-research in the producing districts (Shanxi province and western Nei-Meng-Gu of China) did not fit the JP XIV standard. Although some 11-year-old cultivated samples were obtained (Shanxi in 2001) which barely conformed to the JP XIV standard, an 11-year cultivation period is not practical. These samples were straight taproots, which seemed to be cultivated without transplantation. Although the cause of the low GL-content of these samples was not confirmed, from our results obtained by transplanting them one year after seeding, the transplantation itself may be a contributing factor to the increased GL-content.

The HPLC fingerprint is one of the indexes reflecting gross chemical composition of the samples as a whole. Comparison of the present HPLC fingerprints has enabled differentiation of the cultivated G. uralensis roots and non-medicinal Xinjiang-Gancao. It is a useful method to obtain a constant quality of Gancao as well as of other traditional Chinese drugs and formulations possessing many ingredients. The Food and Drug Administration (FDA) of the U. S. Department of Health and Human Services has already recommended that the chromatographic fingerprints should be used as part of the quality control of botanical products. As shown in Fig. 5, the HCA dendrogram means that the fingerprint of the 4-year-old adventitious roots is similar to that of medicinal Gancao and is different from that of non-medicinal Xinjiang-Gancao. The present multivariate analysis indicates the potential value of HPLC fingerprint recognition combined with HCA (or PCA) for this aim. The HCA and PCA has already been used for discrimination of crude drugs possessing various morphological, chemical and genetic characteristics.

Together with chemical quality analysis, a pharmacological study is also an important and useful approach to evaluate the efficacy and toxicity of a new medicinal resource. The result shown in Fig. 6 suggests that the 4-year-old cultivated adventitious roots are bio-equivalent to medicinal Gancao, although additional studies on their inflammatory and hepatic protective effects are necessary. The present anti-allergic effect is compatible with the reported evidence of some traditional Chinese formulations containing Gancao as Baihu-jia-Renshen-Tang (白虎加入参汤) and Taohe-Chengqi-Tang in the same triphasic skin reaction. The animal method gave...
an immediate phase response (IPR), late phase response (LPR) and very late phase response (vLPR) peaking at 1 h, 24 h and 7 or 8 d after the challenge, respectively. The vLPR is believed to be reflected in chronic allergic inflammatory diseases as atopic dermatitis.\(^7\) It is worth stating that Gancao inhibited IPR, LPR and vLPR in the same manner as prednisolone, but not as an anti-histaminic agent (H1 receptor antagonists).\(^3\)

A pharmacokinetical study was also necessary to clarify the bio-equivalency between the cultivated roots and concurrent medicinal Gancao. The present GA pharmacokinetic profile (Fig. 7) suggests that the 4-year-old adventitious roots are almost bio-equivalent to medicinal Dongbei-Gancao.

In summary, the present study was set up to examine the feasibility of medicinal use of \textit{G. uralensis} roots cultivated in eastern Nei-Meng-Gu of China. Several important pieces of evidence can be drawn from the present pharmaceutical studies of \textit{G. uralensis} roots cultivated for 4—5 years: (a) the cultivated roots have HPLC fingerprints detected at 254 nm similar to those of concurrent medicinal Gancao (Fig. 4); (b) the similarity of the HPLC fingerprints between the cultivated roots and medicinal Gancao was confirmed by HCA (Fig. 5); (c) the 4- and 5-year-old roots fitted the JP XIV standard (Table 1); (d) the anti-allergic effect of the 4-year-old adventitious roots on the IgE-mediated triphasic ear swelling was comparable to that of medicinal Dongbei-Gancao (Fig. 6); (e) the 4-year-old adventitious roots were bio-equivalent to Dongbei-Gancao based on examination of GA pharmacokinetics after oral administration in rats (Fig. 7).

In conclusion, the present chemical, pharmacological and pharmacokinetical studies indicate that the 4-year-old adventitious roots of \textit{G. uralensis} cultivated in Nei-Meng-Gu of China are equivalent to Dongbei-Gancao and conform to the JP XIV. The new Gancao candidate could make up for a shortage of wild \textit{Glycyrrhiza} resources caused by collection restrictions in China.

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**REFERENCES AND NOTES**


6) Since these are roots newly growing from injured taproots after transplantation, instead of the term lateral roots used in the previous report\(^6\) we use here adventitious roots.


24) Food and Drug Administration (FDA) of U. S. Department of Health and Human Services: (http://www.fda.gov/cder/guidance/1221dft.htm)


