Evaluation of glycyrrhizin variation in Gancao collected from 1986 to 2000 at drug markets in Japan and discrimination of Daitou-Gancao and Dongbei-Gancao

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Abstract

Quality variation of 337 lots of medicinal Gancao (Kanzo in Japanese) collected at drug markets in Japan from 1986 to 2000 was evaluated by measuring glycyrrhizin (GL)-content and roots specific gravity (RSG). The present results clearly indicated that GL-content (5.16±1.00%, 109 lots) of Dongbei-Gancao (Tohoku-Kanzo in Japanese) is significantly higher (4.36±1.45%, 228 lots) than that of Xibei-Gancao (Seihoku-Kanzo in Japanese). Furthermore, the RSG of Dongbei-Gancao (0.51±0.09 g / cm^3, 66 roots) is significantly lower than that of Xibei-Gancao (0.65 ±0.11 g / cm^3, 66 roots). This finding indicates that the RSG is useful indicator in distinguishing Dongbei-Gancao with flexible roots from Xibei-Gancao with hard and heavy roots. During the experimental process, it was found that there have been some odd Dongbei-Gancao with high RSG in drug markets in Japan from about 1976. Our market research in China indicated that Gancao with knot-like upper roots collected at Xibei (northwest) region in China has been under circulation designated as Daitou (or Youtou)-Gancao from early 1990's in China. The result of RSG examination on Daitou-Gancao (Taito- or Yuto-Kanzo in Japanese) allows us to consider that some Dongbei-Gancao with high RSG seems to correspond to Daitou-Gancao. This is the first report to characterize Daitou-Gancao which has a similar knot-like upper root and high GL-content to Dongbei-Gancao and also has RSG as high as Xibei-Gancao.

Key words licorice, Gancao, *Glycyrrhiza uralensis*, glycyrrhizin, specific gravity, HPLC.

Introduction

Gancao (Kanzo in Japanese) has been used as an agent to coordinate the tone of "zhong-jiao" (chu-sho in Japanese) which means the function of the digestive system according to the traditional Chinese medicine. Gancao, Glycyrrhizae Radix, is also a well-known modern drug, licorice, used as a remedy for inflammatory, allergic and gastric disorders.[1] Glycyrrhizin (GL) is known as one of the active principles of Gancao and is widely used in patients with chronic liver diseases in Japan.

Gancao is prepared from underground parts, especially from the roots, of wild *Glycyrrhiza* species (Leguminosae). In drug markets in Japan, there are three types of Gancao such as Dongbei-Gancao (Tohoku-Kanzo in Japanese, Dongbei in Chinese means northeast), Xibei-Gancao (Seihoku-Kanzo in Japanese, Xibei in Chinese means northwest) and Xinqian-Gancao (Shinkyo-Kanzo in Japanese, Xinqian is Chinese province name). The former two Gancao for medicinal use are examined in this report.

Comparative studies of some commercial Gancao have already been reported by means of HPLC method[2,3] and histological analysis.[4] With the increasing demand of Gancao and decline of wild resources of the traditional region, Gancao collected at other sources

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became an additional resource. Due to the change of the production and distribution a variation in Gancao quality was caused and the continuous quality control study of commercial Gancao is necessary to assure safety and effective clinical use.

In the present study, we collected 337 lots of medicinal Gancao (109 lots of Dongbei-Gancao and 228 lots of Xibei-Gancao) at drug markets in Japan from 1986 to 2000 to determine the GL-content and dilute ethanol-soluble extract (EtOH ext.) content. Furthermore, roots specific gravity (RSG: weight / volume) is examined to distinguish Dongbei-Gancao from Xibei-Gancao. RSG examination confirmed that Dongbei-Gancao with soft and flexible roots is significantly lower RSG than that of Xibei-Gancao with hard and heavy roots. Among the experimental process, some odd Dongbei-Gancao with higher RSG and knot-like upper root are detected in drug markets in Japan from about 1976.

This paper deals with characterization of the odd Dongbei-Gancao by means of RSG examination and market research together with the GL-content variation in 337 lots of Gancao collected from 1986 to 2000 at drug markets in Japan.

Materials and Methods

Commercial samples of Dongbei- and Xibei-Gancao: 109 lots of Dongbei-Gancao and 228 lots of Xibei-Gancao were collected from 1986 to 2000 at drug markets in Osaka (Japan). Dongbei-Gancao was selected for examination according to morphological characteristics such as the head-like knot of upper root. Commercial samples of two types of Gancao are classified by means of length and thickness of root into 4 grades of each Gancao (1-, 2-, 3- and 4-go-Kanzo in Tohoku-Kanzo and Ko-, Otsu-, Hei-, and Tei-kyu Kanzo in Seihoku-Kanzo in Japanese) by the primary wholesale dealers at the producing districts as standardized in China. Furthermore, out-of-grade of Gancao such as Hun-jie (Kon-setsu in Japanese) and Mao-cao (Mo-so in Japanese) was also collected and analyzed.

Commercial sample of Daitou (Youtou)-Gancao: 5 lots of commercial Daitou-Gancao (collected in Heshui of Gansu province of China) were collected at drug markets in Osaka in 2000.

Plant material of Glycyrrhiza uralensis Fisch.: Wild specimens of *G. uralensis* with fruits and flowers were collected in the northeastern area of NeiMong-gol (Wengniuteqi Toudaogou, one of the major sources of Dongbei-Gancao) of China in Aug. 2000. They were identified as *G. uralensis* Fisch. by means of characteristic of undulate leaf-lets and falcate-fruits with prickle hairs as described in the report.[5] *G. uralensis* has a knot-like upper root as shown in Fig. 1. The specimens (No. 000815WN) have been deposited in 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. All other chemicals and solvents were of analytical and/or HPLC grade.

Sample preparations: In order to investigate the GL-, EtOH ext.-content, and RSG variations in each individual sample, one piece of root was dried at 60 ℃ and cut into five 15 cm parts from the head-like knot of the upper root (part A) to the root end part (E) as shown in a diagrammatic representation (Fig. 1). In order to investigate the variations in each lot of Gancao, about 6 to 10 pieces of whole roots were cut together, powdered and analyzed. The operations were further repeated more than 3 times to obtain the representative analytical data of each lot (more than 18 whole roots analyzed per one lot, thus more than 3000 and 9500 roots analyzed from Dongbei- and Xibei-Gancao, respectively).

GL- and EtOH ext.-content: HPLC determination of GL (% to dry root weight) was measured as described in Japanese Pharmacopoeia XIII. Briefly, powdered Gancao (0.5g) prepared from 6-10 pieces of whole root was extracted with 50% ethanol (70 ml) for 15 min under shaking for 2 times and the solution was passed though a 0.45 μm filter. HPLC analyses were carried out by LC-6A and C-R7A (Shimadzu) equipped with a reverse-phase column YMC-Pack ODS-A (A-312: 150 × 6 mm, YMC Co. Ltd.), UV detector (SPD-6A, Shimadzu) set at 254 nm and mobile phase of diluted AcOH (1 in 15) and CH_3CN (3 : 2). EtOH ext.-content (% to dry weight of root) was also measured as described in Japanese Pharmacopoeia XIII.

RSG: RSG was calculated from dried weight (g) divided by volume (cm^3) of each cylindrical as shown in Fig. 1. RSG of Dongbei- and Xibei-Gancao were also measured with part B's from 66 roots (6 roots per year

for 11 years: 1990-2000). RSG of Daitou-Gancao was measured with part B's from 17 roots obtained in 2000.

Statistics: Results were presented as the mean±S. D. Student's *t*-test was performed for a comparison of the means. Correlation coefficient and regression formula calculated by software Excel 2000 (Microsoft corporation). To obtain the property of the universe in Dongbei-Gancao by statistical method, Shapiro-Wilk test is carried out using multivariate data analysis software STATISTICA (StatSoft JAPAN Co. Ltd.).

Results and Discussion

Variation of GL- and EtOH ext.-content in each root of G. uralensis and commercial Gancao (Figs. 1-2)

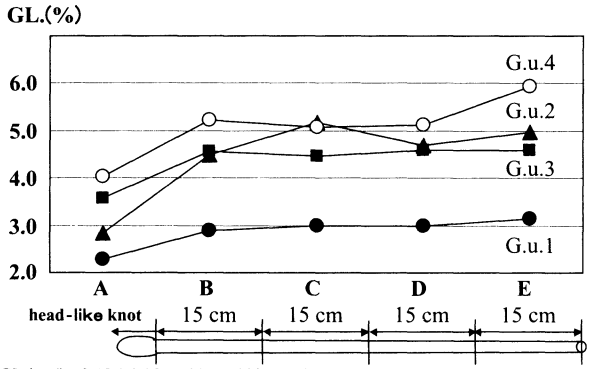
In order to investigate the variations of GL- and EtOH ext.-content in each part of *G. uralensis* root, it was cut into five parts as shown in Fig 1. GL-content in each part varied from 2.84% (G.u. 2: A part) to 5.16% (C part). Although GL-content is reported to be higher in the thicker parts of the stolon[6] and root[7] than in the thinner parts, no correlation between GL-content and root diameter was observed in this study. GL-content in each root also varied from 2.86% (G. u. 1) to 5.08% (G. u. 4). EtOH ext.-content in each root also showed a relative variation from 36.4% to 40.2%.

Significant ($p<0.05$) differences were also found in the GL- and EtOH ext.-contents among the parts in commercial two Gancao (Fig.2). GL-content also varied from 3.59% to 4.86% in each root of Dongbei-Gancao and from 1.54% to 4.54% in Xibei-Gancao, respectively. EtOH ext.-content varied from 27.6% to 39.0% in each root of Dongbei-Gancao and from 30.3% to 37.0% in Xibei-Gancao, respectively. Therefore, in order to investigate an average variation within individuals, about 6 to 10 pieces of commercial Gancao were cut together, powdered and analyzed as described in the materials and methods.

Variation of GL- and EtOH ext.-contents in commercial Gancao (Fig. 3, Table I)

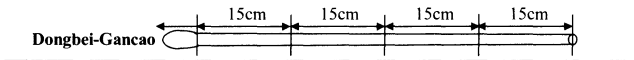
GL- and EtOH ext.-contents in Dongbei- and Xibei-Gancao collected at drug markets in Osaka during 15 years (1986-2000) are shown in Fig. 3. GL-content varied from 4.43±0.41% (sample of 1991) to 6.27±0.84 % (sample of 1997) in Dongbei-Gancao and 3.09± 0.59% (sample of 1998) to 5.42±1.53% (sample of 1992) in Xibei-Gancao, respectively. The mean values of GL- and EtOH ext.-contents in commercial Dongbei-Gancao were similar to those of wild *G. uralensis* root.

As shown in the bar-graph in Fig. 3, GL-contents in Dongbei-Gancao were usually found to be higher than those in Xibei-Gancao except in the samples of 1992. To ascertain the significance of difference, the average GL-contents in two types of Gancao were examined. The results were as follows: GL content of Dongbei-Gancao (5.16±1.00%, 109 lots) is significantly higher ($p<0.05$) than that of Xibei-Gancao (4.36±1.45%, 228 lots). Although the results obtained this time were similar to those already reported using 19 and 15 samples of



GL.(n=4)	3.18±0.77	4.30±0.99*	4.42±1.01*	4.35±0.93*	4.65±1.17*
Ex. (n=4)	32.4±3.0	37.1±1.3*	39.5±2.3*	39.5±2.6*	40.4±3.2*
RSG (n=4)		0.47±0.01	0.51±0.02	0.55±0.08	0.60±0.11

Fig. 1 Glycyrrhizin-, EtOH ext.-contents and RSG in different parts of *Glycyrrhiza uralensis* roots
Each value represents the mean±S.D. (n = 4).
G.u.1-4: 4 samples of *Glycyrrhiza uralensis* Fisch.
GL.: glycyrrhizin-content (%); Ex.: EtOH ext.-content (%); RSG.: roots specific gravity (g / cm^3) *: $p<0.05$ vs. part A



sample		A(root head)	B	C	D	E	C.V.
D.	GL.	3.45±0.87	4.41±0.66*	4.31±0.39*	4.29±0.62*	4.54±0.56*	7.60
(n=6)	Ex.	31.7±2.7	35.9±5.1*	36.0±6.1*	35.8±6.1	36.5±4.9*	2.86
	RSG	.....	0.49±0.07	0.50±0.06	0.49±0.08	0.54±0.09	4.98
X.	GL.	.....	3.11±0.95	3.19±1.00	3.26±1.11	.....	5.64
(n=6)	Ex.	.....	34.5±3.0	33.9±2.6	32.4±3.2	.....	5.25
	RSG	.....	0.67±0.11	0.71±0.10	0.76±0.08	.....	7.76

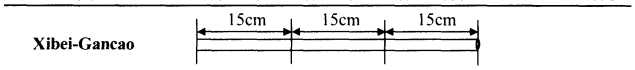


Fig. 2 Glycyrrhizin-, EtOH ext.-contents and RSG in different parts of commercial Dongbei- and Xibei-Gancao roots
Each value represents the mean±S.D. (n = 6).
D.: Dongbei-Gancao; X.: Xibei-Gancao
GL.: glycyrrhizin-content (%); Ex.: EtOH ext.-content (%); RSG: roots specific gravity (g / cm^3) *: $p<0.05$ vs. part A

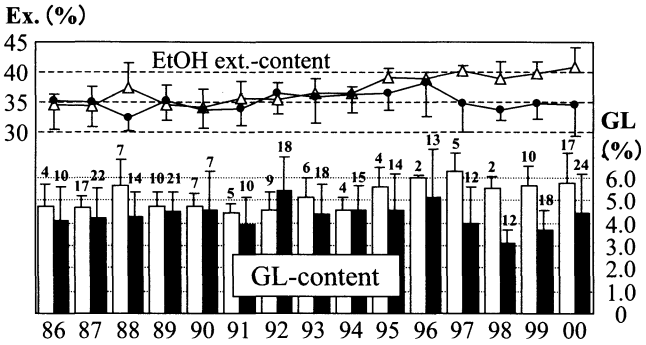


Fig. 3 Variation of Glycyrrhizin- and EtOH ext.-contents in commercial Dongbei- and Xibei-Gancao collected from 1986 to 2000 at drug markets in Japan

Each value represents the mean±S.D. (Figures described above each bar represent the number of lots examined).

Line graph: variation of EtOH ext.-content (Ex. %); △: Dongbei-Gancao, ●: Xibei-Gancao. EtOH ext.-content varied from 34.1±3.1% (samples of 1990) to 40.8±3.4% (samples of 2000) in Dongbei-Gancao and 32.5±2.1% (samples of 1988) to 38.2±5.6% (samples of 1996) in Xibei-Gancao, respectively.

Bar graph: variation of glycyrrhizin-content (GL. %); open and solid column: Dongbei- and Xibei-Gancao, respectively.

86-00: sampling year from 1986 to 2000

Table. I Variation of Glycyrrhizin content in each grade of Dongbei- and Xibei-Gancao collected from 1986 to 2000 at drug markets in Japan

	Dongbei-Gancao (%)	Xiebei-Gancao (%)
total	5.16±1.00* (n= 109)	4.36±1.45 (n= 228)
1 grade	4.81±0.73 (n= 15)	4.40±1.30 (n= 5)
2 grade	5.34±1.02* (n= 63)	3.91±1.09 (n= 42)
3 grade	5.05±1.00 (n= 19)	5.18±1.45 (n= 47)
4 grade	5.08±1.41 (n= 6)	4.29±1.47 (n= 115)
out-of-grade	4.50±0.15* (n= 6) (Hun-jie)	3.72±1.23 (n= 19) (Mao-cao)

Each value represents the mean±S.D.

*: $p<0.05$ vs. Xiebei-Gancao

Grade: Gancao are classified by means of length and thickness of root into 4 grades of each Gancao (1-, 2-, 3- and 4-go-Kanzo in Tohoku-Kanzo and Ko-, Otsu-, Hei-, and Tei-kyu Kanzo in Seihoku-Kanzo in Japanese) by the primary wholesale dealers at the producing districts as standardized in China.
Gancao with thick and long roots is classified into upper grade.

Dongbei- and Xibei-Gancao,[2] it is the characteristic that the data in this paper was confirmed by analyzing every grade of more than 300-lots of trial samples collected from 1986 to 2000.

Furthermore, GL-content of each grade in Dongbei-Gancao was also higher than those of Xibei-Gancao, except for the value of 3 grade as shown in Table I.

Correlation between GL- and EtOH ext.-contents in commercial Gancao (Fig. 4)

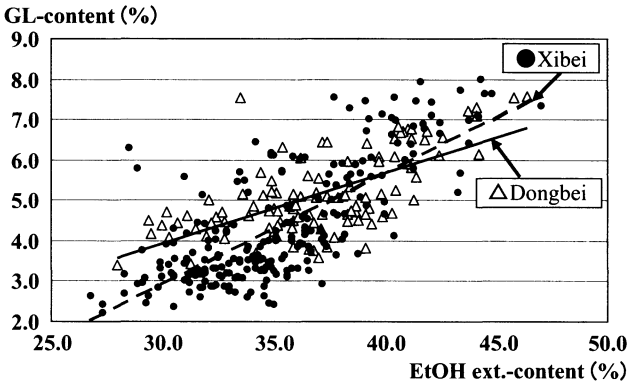


Fig. 4 Correlation between Glycyrrhizin- and EtOH ext.-contents in commercial Dongbei- and Xibei-Gancao collected from 1986 to 2000 at drug markets in Japan

△: Dongbei-Gancao (109 lots).; ●: Xibei-Gancao (228 lots)

Regression equation (Y: GL-contents, X: EtOH ext.-content):
Y = 0.18 X - 1.3 (r = 0.654 for Dongbei-Gancao) ;
Y = 0.28 X - 5.4 (r = 0.751 for Xibei-Gancao)

By the result of investigation of 337 lots of Gancao as shown in Fig. 4, it is evident that a positive correlation exists between GL- and EtOH ext.-content both in Dongbei- (r= 0.654) and Xibei-Gancao (r= 0.751). Therefore, even if the analytical instruments such as HPLC are not available at the production region of Gancao, GL-content could be simply predicted by the evaluation of EtOH ext. content.

RSG of Gancao and detection of Daitou (or Youtou)-Gancao (Figs. 5-6, Table II)

RSG of Dongbei- and Xibei-Gancao are examined to reassure the general knowledge that Dongbei-Gancao has more light, soft and flexible root than Xibei-Gancao. The data shown in Fig. 5 reveals that the mean RSG of Dongbei-Gancao (0.51±0.09 g / cm^3, 66 roots) is significantly ($p<0.05$) lower than that of Xibei-Gancao (0.65±0.11 g / cm^3, 66 roots).

As shown in Fig. 5, there are 3 odd samples of Dongbei-Gancao collected in 1997, 1998 and 2000, which have higher RSG (0.69 g / cm^3). The result of the Shapiro-Wilk test to examine the distribution curve of RSG data in Dongbei-Gancao indicated that distribution of 66 samples including the 3 odd samples do not fit the normal distribution curve, but 63 samples except for 3 odd samples fit in the normal distribution (Fig 6). Therefore, it is estimated statistically that the 3 odd samples with high RSG belong to another source out of Dongbei-Gancao.

According to our market research about the distri-

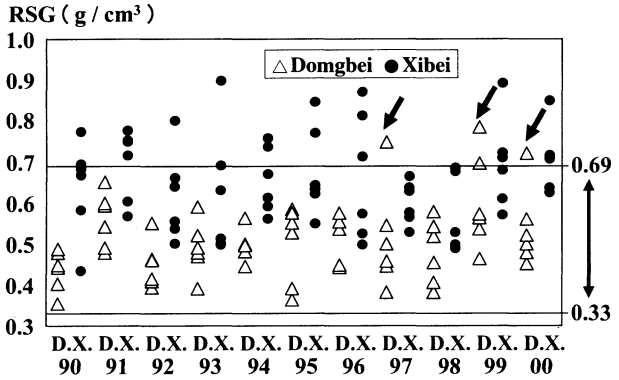


Fig. 5 Variation of RSG in commercial Dongbei- and Xibei-Gancao collected from 1990 to 2000 at drug markets in Japan
D. and △: Dongbei-Gancao (0.51±0.09 g / cm³).; X. and ●: Xibei-Gancao (0.65±0.11 g / cm³)
90-00: sampling years from 1990 to 2000
Upper and lower lines represent mean + 2S.D. (0.69) and mean - 2S.D. (0.33) of Dongbei-Gancao RSG, respectively. Arrow represents the 3 odd Dongbei-Gancao with more than mean + 2S.D. RSG.

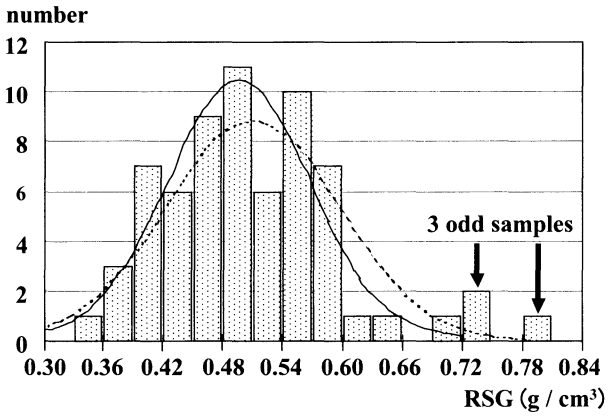


Fig. 6 Histogram and distribution curve of RSG in Dongbei-Gancao collected from 1986 to 2000 at drug markets in Japan
Solid line represents distribution curve of 63 samples (without 3 odd samples) and fits in the normal distribution examined statistically by Shapiro-Wilk test: p = 0.562.
Dotted line represents distribution curve of total 66 samples of Dongbei-Gancao and does not fit in the normal distribution examined statistically by Shapiro-Wilk test: p = 0.007.
Shapiro-Wilk test of another sample: 65 samples (without 1 odd sample): p = 0.052 (represents normal distribution), and 64 samples (without 2 odd samples): p = 0.237 (represents normal distribution). Large p value (0.562) in 63 samples represents more suitability for the normal distribution curve than small p values in 64 (0.237), 65 (0.052) and 66 (0.007) samples.

bution pattern of Gancao in China from trade companies of traditional Chinese medical materials in 1999-2000, it was found out that the Gancao with knot-like upper roots collected in the northwestern (Xibei) region of China was under circulation by the name of "Daitou (or Youtou)-Gancao" from the early 1990's. The word Daitou (or Youtou) in Chinese is "with head". This

Table. II Glycyrrhizin content and RSG of Dongbei-, Xibei-, and Daitou-Gancao

	GL-content (%)	RSG(g / cm³)
Dongbei-Gancao	5.16±1.00 * (109 lots, 3109 roots)	0.51±0.09 * (66 roots)
Xibei-Gancao	4.36±1.45 (228 lots, 9594 roots)	0.65±0.11 (66 roots)
Daitou-Gancao	6.65±0.70 * (5 lots, 165 roots)	0.61±0.10 (17 roots)

Each value represents the mean±S.D.
*: p<0.05 vs. Xiebei-Gancao

saying came from knot-like upper root of Daitou-Gancao, which has high RSG as Xibei-Gancao and has knot-like upper roots as in Dongbei-Gancao. Therefore, it was considered that some Daitou-Gancao was mixed with high-priced Dongbei-Gancao and has been exported to Japan from about 1997.

We got the Daitou-Gancao from the northwestern region of the Gansu province in China to examine its characteristics. As shown in Table II, RSG (0.61±0.10 g / cm³, 17 roots) of Daitou-Gancao is significantly greater than that of Dongbei-Gancao and almost the same as that of Xibei-Gancao. From the examination of RSG, GL-content and morphological characteristics as the knot-like upper root, the 3 odd samples of Dongbei-Gancao with high RSG as shown in Fig. 5 may correspond to Daitou-Gancao.

Since the GL-content in Daitou-Gancao was at satisfactory levels (6.65 ± 0.70%, 5 lots) as those in Dongbei-Gancao, Daitou-Gancao might become a promising source of Gancao for clinical use. Further pharmacognostical investigations on botanical origin, chemical constituents and pharmacological properties of Daitou-Gancao to compare with those of Dongbei- and Xibei-Gancao are in progress.

In summary, the characteristic of this paper is that the results were confirmed by analyzing 337 lots (more than 18 roots per lot were examined) of Gancao collected from 1986 to 2000. The following results were obtained:

1) Variation in GL-content: Although GL-content of Gancao varied every year, GL-content of Dongbei-Gancao (5.16±1.00%, 109 lots) is significantly higher (p<0.05) than that of Xibei-Gancao (4.36±1.45%, 228 lots).

2) Variation of RSG: RSG of Dongbei-Gancao (0.51±0.09 g / cm³, 66 roots collected from 1990 to

2000) is significantly lower than Xibei-Gancao (0.65±0.11 g / cm^3, 66 roots for the same 11 years). This result supports the empirical distinctions between Dongbei-Gancao with soft and flexible roots and Xibei-Gancao with hard and heavy roots.

3) Detection and characterization of Daitou (Youtou)-Gancao: RSG examination and market research indicated that Daitou-Gancao was under circulation from the early 1990's in drug markets in China and that it was mixed to make up the deficit of Dongbei-Gancao in markets in Japan from about 1997.

This is the first report to characterize Daitou-Gancao (Taito-Kanzo in Japanese) collected in the northwestern (Xibei) region of the Gansu province in China. It has a similar knot-like upper root and high GL-content (6.65±0.70%, 5 lots) to Dongbei-Gancao and also has similar high RSG (0.61±0.10 g / cm^3, 17 roots) to Xibei-Gancao (Table II).

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和文抄録

本論文の特徴は，日本市場甘草の品質変動を1986年から2000年まで15年間にわたって集めた多数の試料（337 lots）に基づいて検討した点にある。比較のために同定可能な花期・果実期に中国内蒙古自治区の東部（翁牛特旗頭道溝：東北甘草の産地）で採集した *Glycyrrhiza uralensis* Fisch. の根を用いた。

市場品甘草の glycyrrhizin 含量は年度によって変動するが，15年間の平均値において東北甘草の glycyrrhizin 含量は西北甘草より有意に高いことを確認した。見掛け比重（RSG）は根を円柱とみなして重量と体積を量り算出した。東北甘草の RSG は西北甘草より有意に低いことを確認した。今回確立した RSG は，柔軟な東北甘草と質の充実した堅い西北甘草を判別する経験的な選品法を裏付ける指標となる。

なお1997年頃以降に集めた東北甘草の中に RSG の高い（mean＋2 S.D. を超える）個体が散見された。中国における市場調査の結果，甘粛省など中国西北地方で産し根頭部に「こぶ」のある甘草が帯頭甘草あるいは有頭甘草という名で出回り，日本に輸出されていることが明らかになった。市場品の東北甘草中に散見された RSG の高い甘草は，帯頭甘草の可能性があることを Shapiro-Wilk 検定で統計学的に推定した。

この帯頭甘草（甘粛省産）を入手して分析したところ，根頭部に「こぶ」のある形態と glycyrrhizin 含量の高い点は東北甘草に類似し，RSG が高い点は西北甘草に類似することが明らかになった。帯頭（有頭）甘草（中国市場名）の流通状況や形質を明らかにしたのは本論文が最初である。

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