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NOTE

Seasonal variation of mogrosides in Lo Han Kuo (*Siraitia grosvenori*) fruits

Dianpeng Li · Tsuyoshi Ikeda · Yonglin Huang · Jinlei Liu · Toshihiro Nohara · Tatsunori Sakamoto · Gen-Ichiro Nonaka

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Abstract Mogrosides at different growth stages of Lo Han Kuo (*Siraitia grosvenori*) fruits were analyzed qualitatively and quantitatively using TLC and HPLC. The results show that we can clearly discriminate the seasonal variation of the main mogrosides in Lo Han Kuo fruits: mogroside V is the main constituent of ripe fruits, while mogrosides III and II E are the leading components in unripe fruits. A comprehensive validation (sensitivity, linearity, reproducibility and recovery) of an HPLC method that can simultaneously determine the content of mogrosides V, III and II E was conducted. This method is proposed as a simple, rapid and accurate method for quantitative determination of the mogroside V, mogroside III and mogroside II E content in various samples of Lo Han Kuo (*S. grosvenori*) fruits.

Keywords *Siraitia grosvenori* · Lo Han Kuo · Mogrosides · HPLC · TLC · Seasonal variation

D. Li · T. Ikeda · T. Nohara (✉)
Faculty of Medical and Pharmaceutical Science, Kumamoto University, 5-1 Oe-honmachi, Kumamoto 862-0973, Japan
e-mail: none@gpo.kumamoto-u.ac.jp

Y. Huang · J. Liu
Guanxi Institute of Botany, Chinese Academy of Science, Guilin 541006, China

T. Sakamoto
Sakamoto Limestone Industrial Co. Ltd, 273-1 Tamana, Kumamoto 865-0013, Japan

G.-I. Nonaka
Nonaka Usaien Pharmaceutical Co. Ltd, 1-3-17 Zaimoku, Saga 840-0055, Japan

Introduction

The fruit of *Siraitia grosvenori* Swingle (formerly *Momordica grosvenori* Swingle), which belongs to the family Cucurbitaceae, has long been used in traditional Chinese medicine as a pulmonary demulcent and emollient for the treatment of dry cough, sore throat, dire thirst and constipation [1]. Recently, some additional interesting pharmacological characteristics, such as anti-cancer and anti-hyperglycemic effects and inhibition of oxidative modification of low-density lipoprotein, have beenreported [2–4]. Many cucurbitane-type triterpene glycosides have been isolated from the fruits and characterized [5–12]. Among them, mogroside V and mogroside IV are extremely sweet, but the fruit also contains some tasteless glycosides, as well as bitter-tasting glycosides such as mogroside III and mogroside II E. Ripe fruits contain mainly mogroside V, so are very sweet. On the basis of these characteristics, *S. grosvenori* fruit extract is utilized commercially as a sweet component in sugar substitutes, and is widely used as an additive and ingredient in some health foods and beverages. Because of cold weather during winter, some fruits cannot mature naturally. The unripe fruits have a bitter taste, and at some sites of cultivation unripe fruit may amount to about one-quarter of total production. If fruits at different stages of ripening are mixed in the raw materials, it is impossible to avoid influencing the quality of the extract. One problem is that ripe and unripe fruit cannot be distinguished by their appearance. The shape of the fruit is fixed after about 50 days of growth, thus distinction of fruit stages by shape is difficult during vegetative growth stages. Therefore, a scientific method to control the quality of Lo Han Kuo

(*S. grosvenori*) fruits is required. In this study, we collected Lo Han Kuo fruits at different growth stages, and selectively extracted and isolated the main chemical constituents. A qualitative analysis of the main mogrosides in the extracts was performed by TLC. Moreover, we developed a new HPLC quantitative method to analyze the different growth stages of Lo Han Kuo fruit. As a result, we have conducted a qualitative and quantitative analysis of the seasonal variation of mogrosides in Lo Han Kuo fruits. This method can also be used to determine the mogroside content of different species of Lo Kan Kuo fruits and samples from different sites of cultivation.

Materials and methods

Reagents and plant materials

Acetonitrile and methanol were of HPLC grade and were purchased from Tedia Chemicals (Fairfield, OH). Purified water was prepared by a Millipore Simpli Lab UV (Millipore, Bedford, MA). Silica gel (200—30 mesh) was purchased from Qingdao Haiyang Chemical Industry (Qingdao, China), Diaion HP-20from Mitsubishi Chemical (Tokyo, Japan), Sephadex LH-20 (25–100 mm) from Pharmacia Fine Chemicals (Uppsala, Sweden), and Chromatorex ODS (30–50 μm) from Fuji Silysia Chemical (Aichi, Japan). Seasonal plant samples of *S. grosvenori* fruits were collected at the same field sites at the town of Longjiang, Guilin, Guangxi province, China, from July to October 2004. The fresh fruits was dried to powder dryness immediately under freezing vacuum conditions. For different cultivation sites, samples of *S. grosvenori* fruits were collected from their original habitats during October 2004.

Apparatus and chromatography conditions

An Agilent 1100 series HPLC-DAD system (Hewlett-Packard, Palo Alto, CA) consisting of a G1311A quaternary pump, a G1322A vacuum degasser, a G1315A diode-array detector (Hewlett Packard) and a 7725i manual sampler was employed in this research. Detection was carried out at a wavelength of 203 nm. The column used in this study was a ZORBAX SB-C18 (4.6 mm × 150 mm, 5 μm) (Agilent, Wilmington, DE)) with a compatible guard column (C18, 5 μm, 4.6 mm × 7.5 mm), The mobile phase was acetonitrile:water (75:25) and the flow rate was maintained at 1 ml/min. The column temperature was controlled at 25°C. Data collection and manipulation were performed using HP Chem Station software for HPLC analyses. TLC was performed on precoated silica gel 60 F_{254} plate (Merck, Darmstadt, Germany) in developing solvent $CH_3(CH_2)_2$–CH_2OH:HAC:H_2O (4:1:1); detection was by spraying with 10% aqueos H_2SO_4 heated to 105°C for about 3–5 min.

Isolation of mogroside V, mogroside III and mogroside II E from *S. grosvenori*

Fresh fruits (5 kg, 60 days growing time) of *S. grosvenori* were extracted with methanol at room temperature for 10 days. The extract was evaporated under reduced pressure to yield a methanol extract. The extract was resolved chromotographically on Diaion HP-20, with successive elution with H_2O, 30%, 80%, and 100% methanol. The 80% methanol fraction was appliedto a silica gel column, and eluted with a $CHCl_3$–MeOH–H_2O (8:2:0.2; 7:3:0.5; 6:4:1), gradient, giving ten fractions. Fractions 3, 4, 5 were further purified on Chromagtrex ODS (55–65% MeOH), yielding mogroside V, mogroside III and mogroside II E, respectively. The purity of each compound was confirmed by HPLC. The structures were characterized by spectroscopic analyses [5–10].

Preparation of standard solutions

Stock solutions of mogroside V, mogroside III and mogroside II E were prepared in methanol at a concentration of 1.0 mg/ml; these solutions were used directly for TLC analysis. The HPLC solutions were diluted with mobile phase to obtain a series of standard solutions with concentrations of 0.02, 0.06, 0.10, 0.20, 0.40 and 0.80 mg/ml. Linearity of response was determined for six concentrations with three injections for each level. The calibration curve was based on the relationship of concentration (mg/ml, *x*-axis) to peak area (*y*-axis).

Sample preparation for TLC

Sample powder (0.1 g) was extracted with 5 ml methanol by means of sonication at room temperature for 0.5 h. The extraction was repeated three times. The total extracts were combined, and evaporated at reduced pressure. The residue was suspended in H_2O, and partitioned with chloroform and 1-butanol, consecutively. The 1-butanol partitioned fraction was evaporated at reduced pressure, and the syrup was then dissolved in methanol and transferred to a 10 ml

volumetric flask, with the volume being made up with methanol. About 2 μl of the solution was injected for TLC analysis.

Sample clean-up for HPLC

Accurately weighed 1.0 g sample powder was introduced into a 100 ml volumetric flask and extracted with 20 ml methanol in a reflux bath for 1 h. The extractions were repeated three times. The total extracts were combined, and evaporated at reduced pressure. The residue was dissolved in H_2O by means of sonication. The solution was resolved by chromotography on Diaion HP-20 (1 × 10 cm). The column was first washed with H_2O to remove organic acids, sugars and pigments; mogrosides were completely retained. After washing, mogrosides were eluted with 80% methanol. The eluate was collected, evaporated to dryness in vacuo, and the residue dissolved in 5 ml mobile phase. Extracts were then filtered through a syringe filter (0.2 μm, Alltech, Beerfield, IL). An aliquot of 10 μl solution was injected for HPLC analysis.

Results and discussion

Mogroside V, mogroside III and mogroside II E (Fig. 1), which are not commercially available, were isolated from *S. grosvenori* (60 days fruits) by our laboratory with purities of 99.1, 98.1 and 98.5%, respectively, as confirmed by HPLC. The structures were determined by spectroscopic analyses.

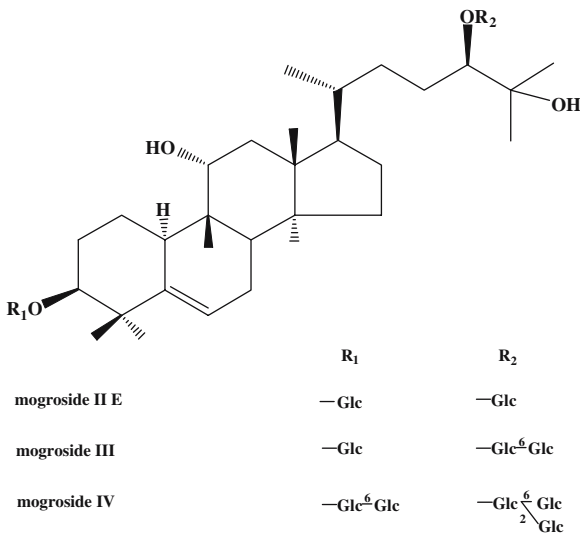

Fig. 1 Structure of mogroside V, mogroside III and mogroside II E

TLC qualitative analysis for mogrosides

From a TLC chromatogram obtained with extracts of fruit from different growth stages together with mogroside standards (Fig. 2), we could clearly discriminate the seasonal variation in the mogroside content. The content of mogrosides is different at different growth stages of Lo Han Kuo fruits. The main constituent of ripe fruits (85 day growth stage) is mogroside V, while in unripe fruit (45–60 days growth stage) the main constituents are mogroside II E and mogroside III.

Development and validation of HPLC method

The method determined for quantitative analysis of mogroside V, mogroside III and mogroside II E was validated in terms of linearity, precision, accuracy and recovery. Due to the use of the high effective resolution ODS column and a photodiode array detector (DAD), better baseline stability and chromatogram resolution were obtained than previously reported [13], and we can detect three mogrosides in one chromatographic system rapidly and simultaneously. Mogroside V, mogroside III and mogroside II E were well resolved in the HPLC chromatogram and eluted at 6.08, 7.31 and 8.45 min retention time (Fig. 3), respectively.

Linearity was examined with standard solutions prepared in the concentration range of 0.02–0.8 mg/ml. The linear relationship between the concentrations (mg/ml, *x*-axis) of mogroside V, mogroside III and

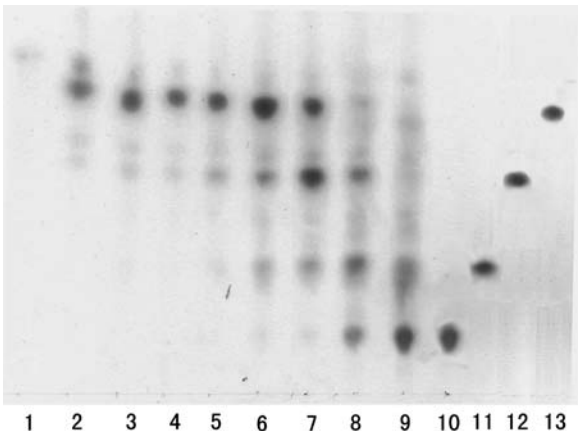

Fig. 2 TLC chromatogram of mogrosides in *Siraitia grosvenori* fruits at different growth stages (days). Lanes: *1* Embryonic fruit, *2* 5-day-old fruit, *3* 10-day-old fruit, *4* 20-day-old fruit, *5* 30-day-old fruit, *6* 40-day-old fruit, *7* 55-day-old fruit, *8* 70-day-old fruit, *9* 80-day-old fruit, *10* mogroside V, *11* mogroside IV A and mogroside IV E, *12* mogroside III, *13* mogroside II E

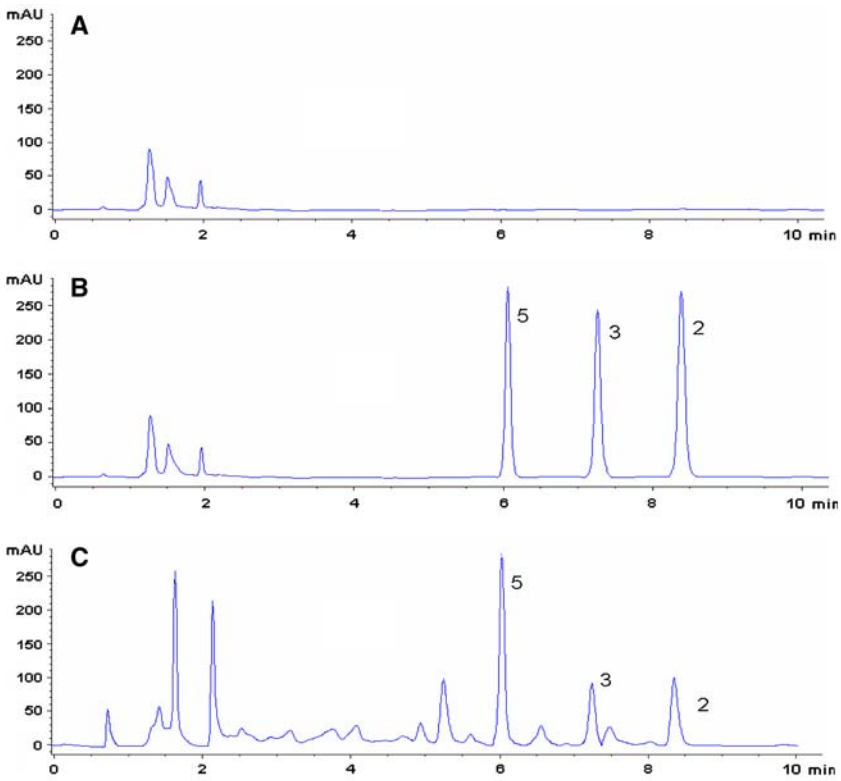

Fig. 3 **a–c** HPLC chromatograms of mogrosides. **a** Negative control, **b** mogroside standards, **c** sample. Peaks: *5* Mogroside V, *3* mogroside III, *2* mogroside II E

mogroside II E and peak area (y-axis) were expressed by the following equations: $y = 203.87x + 43.93$, $y = 250.48x + 4.39$ and $y = 313x + 34.03$, respectively. The correlation coefficients were 0.9991, 0.9992 and 0.9986, respectively. The limit of detection, defined as the amount of the compound needed to produce a signal at least three times greater than background noise ($S/N > 3$), was determined to be 100 ng for all three mogrosides.

Intra- and inter-day precision were evaluated by replicate injection of standard and sample solutions. Six injections per day were conducted on days 1, 3 and 5 after sample preparation to determine reproducibility (after measurement, the solution was stored at 6°C). The intraday precision of the standard solutions was found to have a relative standard deviation (RSD) of 0.55, 0.23, 0.33% ($n = 6$) for mogroside V, mogroside III and mogroside II E, respectively, and the corresponding interday precision had RSDs of 1.68, 1.13, or1.34% ($n = 8$). Similarly, the RSDs of measured peak areas of mogroside V, mogroside III and mogroside II E corresponding to intra- and inter-day precision in the sample solution were 1.98, 1.68, 1.74% ($n = 6$) and 2.89, 1.97, 2.21% ($n = 8$), respectively. These results show that the standard and sample solutions were stable for at least 5 days when stored at 6°C.

Table 1 Content of mogroside V, mogroside III and mogroside II E as determined by HPLC in samples of Lo Han Kuo (*Siraitia grosvenori*) fruit at different growth stages (values are the average of three samples)

Fruits sample at different growth stages	Content of mogroside V (mg/g)	Content of mogroside III (mg/g)	Content of mogroside II E (mg/g)
Flower	0.00	0.00	0.00
Embro	0.00	0.00	0.00
5-day-old fruit	0.00	1.25	12.06
10-day-old fruit	0.00	2.61	28.21
20-day-old fruit	0.00	3.26	19.95
30-day-old fruit	0.00	4.04	12.40
40-day-old fruit	1.04	4.79	5.50
50-day-old fruit	5.50	6.06	1.17
60-day-old fruit	8.80	4.00	0.61
70-day-old fruit	10.50	1.15	0.00
80-day-old fruit	16.50	0.00	0.00
85-day-old fruit	16.30	0.00	0.00

In order to examine the accuracy of the method as well as the recovery of extraction, samples of powdered fruits (1.000 g) were spiked with 1.0 mg mogroside V, mogroside III and mogroside II E before being subjected to the extraction procedure described above. The average recoveries of mogroside V, mogroside III and mogroside II E were 96.3% (RSD = 3.5%, $n = 3$),

Table 2 Content of mogroside V, mogroside III and mogroside II E determined by HPLC in samples of Lo Han Kuo (*S. grosvenori*) fruits from different habitats (values are the average of three samples)

Collection location of Lo Han Kuo fruit	Content of mogroside V (mg/g)	Content of mogroside III (mg/g)	Content of mogroside II E (mg/g)
Longjiang town, Yongfu county, China	16.50	0.00	0.00
Baishou town, Yongfu county, China	14.50	0.90	0.00
Wannian town, Linggui county, China	17.50	1.25	0.00
Liangjiang town, Linggui county, China	11.30	6.61	2.21
Fengmu town, Zhiyuan county, China	16.10	1.26	0.00
Xingan town, Xingan county, China	15.50	0.00	0.00
Nongshen town, Nongshen county, China	10.40	4.70	1.50
Lipu town, Lipu county, China	14.50	2.20	1.00
Yanshan town, Guilin city, China	15.50	0.00	0.00
Shantang town, Guilin city, China	10.50	1.15	0.00

98.2% (RSD = 1.8%, $n = 3$), and 97.8% (RSD = 1.7%, $n = 3$), respectively.

Seasonal variation in mogroside content at different growth stages of Lo Han Kuo fruits

Using the HPLC method developed above, we simultaneously determined the contents of mogroside V, mogroside III and mogroside II E at different growth stages of Lo Han Kuo fruits (Table 1). The samples were collected from the same habitat and the same species, and only the growth stages were different. The results showed that the content of mogrosides exhibit marked differences at different growth stages. Unripe fruit (young fruit) contains mainly mogroside III and mogroside II E (both indicated relatively high content at this growth stage), and mogroside V is the major component of ripe fruits. The highest accumulation of mogroside V was observed after 80 days of growth. This conclusion was in accordance with the TLC qualitative analysis results.

Comparison of mogroside content in samples from different habitats

Recently, due to the increasing demand for Lo Han Kuo fruits, many *S. grosvenori* fruits have been collected from different habitats. It is known that diversity of growing environments introduces variability into the quality of the final herbal preparation. Therefore, we collected ten commercial samples from the main habitats of Lo Han Kuo and evaluated their quality. The results are shown in Table 2. It can be concluded that the content of mogrosides depends not only on the collecting time but also on the habitat. Therefore, it is necessary to control the quality of Lo Han Kuo from different habitats.

Conclusion

In this study, the qualitative and quantitative analyses of mogrosides at different growth stages in fruit of *S. grosvenori* were established using TLC and HPLC methods. The results demostrate that we can clearly discriminate the seasonal variation of mogrosides in Lo Han Kuo (*S. grosvenori*) fruits, and provide scientific evidence for timing of harvesting Lo Han Kuo (*S. grosvenori*) fruits. At the same time, a rapid, reverse phase HPLC assay for quantitative analysis of mogroside V, mogroside III and mogroside II E in Lo Han Kuo (*S. grosvenori*) fruits was developed and validated. The method is rapid and reproducible. Since sample preparation is very simple, the method is suitable for analysis of numerous samples. The HPLC method established in the present study can be presumed to be a reliable method for the analysis of the mogroside content in different fruits of Lo Han Kuo (*S. grosvenori*). It has been confirmed that this method could be used as a routine analytical tool for the quantitative analysis of mogrosides in Lo Han Kuo (*S. grosvenori*) fruits.

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