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Chinese Journal of Natural Medicines 2016, **14**(3): 0236–0240

Chinese Journal of Natural Medicines

Sources of varieties and quality of circular Fructus Ligustri Lucidi

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Available online 20 Mar., 2016

[ABSTRACT] This study aimed to trace sources and quantitatively analyze the specnuezhenide content of circular Fructus Ligustri Lucidi for clinical use. Different specifications of Fructus Ligustri Lucidi were identified using DNA barcoding technology and the specnuezhenide content was analyzed by High Performance Liquid Chromatography (HPLC). The ITS sequence of circular Fructus Ligustri Lucidi was identical to that of standard privet, which was determined through botanical identification. ITS sequence similarity between circular Fructus Ligustri Lucidi and Fructus Ligustri Lucidi which was registered in NCBI ranged from 99.5% to 100%. The sequences of circular and other Fructus Ligustri Lucidi were clustered in a Neighbor-Joining tree with bootstrap value of 95, and these sequences could be distinguished from adulterants. Conforming to pharmacopoeia standard, the average specnuezhenide content of circular Fructus Ligustri Lucidi was higher than that of chicken waist Fructus Ligustri Lucidi. Circular Fructus Ligustri Lucidi derived from *Ligustrum lucidum* Ait. and the specnuezhenide content was higher in circular Fructus Ligustri Lucidi than that in chicken waist Fructus Ligustri Lucidi.

[KEY WORDS] Fructus Ligustri Lucidi; Specifications; DNA barcoding; Specnuezhenide

[CLC Number] R917 **[Document code]** A **[Article ID]** 2095-6975(2016)03-0236-05

Introduction

Fructus Ligustri Lucidi is the dried ripe fruit of *Ligustrum lucidum* Ait. belonging to Oleaceae family, which is used in traditional Chinese medicine to nourish liver and kidney and brighten eyes and hair [1]. However, Fructus Ligustri Lucidi can be confused and faked with other species, including *L. quihoui* Carr., *L. japonicum* Thunb., *L. gracile* Rehd., *L. sinense* Lour., *L. obtusifolium* Sieb., *Brucea javanica* (L.) Merr., and *Ilex chinensis* Sims [2]. At present, two specifications of Fructus Ligustri Lucidi are in the market. Chicken waist Fructus Ligustri Lucidi is characterized by brownish and kidney-shaped. The exocarp of chicken waist Fructus Ligustri Lucidi is shriveled and close to the skin, which cannot be easily peeled. Circular Fructus Ligustri Lucidi is characterized by ovate or elliptic and dark purple and the exocarp of circular Fructus Ligustri Lucidi is expanded and can be easily peeled. Survey of locality has shown that these different characteristics can be attributed to different harvest times. Triterpenes (such as oleanolic acid), iridoid glycosides (such as salidroside [3]), total flavonoids, and polysaccharides [4] have been detected in different harvest times and the contents of these substances differ. However, differences in terms of characteristics and contents of specnuezhenide ($C_{31}H_{42}O_{17}$) in different harvest times have not been investigated. The chicken waist is better, based on the traditional Chinese medicine theory, which is more common in the market and slightly more expensive than the circular. However, whether the heteromorphic circular Fructus Ligustri Lucidi and traditional common Fructus Ligustri Lucidi are obtained from the same source remain unknown, and their different qualities as well as effects in medicine industry and clinical use have not been reported yet.

The characteristics of medicinal materials differ because of origin, harvest, processes, and other factors. As such, studies should investigate whether medicinal materials with different characteristics have identical sources and yield different qualities. Thus, medicinal materials can be classified in detail and used selectively and appropriately in pharmaceutical industries and in the clinic. The present study

[Received on] 28-Apr.-2015

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These authors have no conflict of interest to declare.

aimed to trace the source of circular Fructus Ligustri Lucidi through DNA barcoding molecular identification technology and to determine specnuezhenide content using High Performance Liquid Chromatography (HPLC). With the combined molecular and chemical techniques, authenticity and quality of the two specifications of Fructus Ligustri Lucidi could be identified simultaneously for the first time. The present study provided a basis for the clinical application of Fructus Ligustri Lucidi and a reference for the development of an identification system to determine the authenticity and quality of heteromorphic medicinal materials as well.

Materials and Methods

Study on source of varieties

Plant materials

A total of 14 batches of Fructus Ligustri Lucidi were collected from five cities in Henan Province and Linyi in Shandong Province, China and provided by Shanghai Huqiao Company (Shanghai, China). Each batch included circular and chicken waist Fructus Ligustri Lucidi (Table 1). The standard plant was *L. lucidum* Ait. leaf, authenticated by Prof. LIU Chun-Sheng, Beijing University of Chinese Medicine.

Table 1 Plant materials investigated in the present study

Code	Location	Specification	Harvest time	Color
1	Tongbai, Henan	Chicken waist	August	green
2	Zaoyang, Hubei	Chicken waist	August	green
3	Dengzhou, Henan	Chicken waist	August	green
4	Mengzhou, Henan	Chicken waist	August	green
5	Linyi, Shandong	Chicken waist	August	green
6	Zhongmu, Henan	Chicken waist	August	green
7	Huangchuan, Henan	Chicken waist	August	green
8	Tongbai, Henan	Circular	November	dark purple
9	Zaoyang, Hubei	Circular	November	dark purple
10	Dengzhou, Henan	Circular	November	dark purple
11	Mengzhou, Henan	Circular	November	dark purple
12	Linyi, Shandong	Circular	November	dark purple
13	Zhongmu, Henan	Circular	November	dark purple
14	Huangchuan, Henan	Circular	November	dark purple

DNA extraction, PCR amplification, and sequencing

Samples were disinfected using ethanol, frozen in liquid nitrogen, and ground into fine powder. Genomic DNA was extracted using a common plant DNA extraction kit (Beijing Biomed Biotechnology Co., Ltd.). Total DNA was separated for PCR amplification and sample stored in –20 °C. General primer amplification of internal transcribed spacer (ITS) [5] was used for a small gene fragment between 18S, 5.8S, and 28S rDNA coding genes, using sense primer (5′-AGAAGTCGTAACAAGG TTTC-3′) and antisense primer (5′-TCCTCCGCTTATTGA TATGC-3′). PCR amplification was performed in a thermal cycler (Germany Biometra) with 50 μL of reaction system containing 5 μL of 10 × buffer, 4 μL of dNTPs, 4 μL of $MgCl_2$, 1 μL of sense primer, 1 μL of antisense primer, 2 μL of template DNA, and 1 μL of Taq polymerase. Amplification was performed under the following conditions: 94 °C for 5 min, followed by 35 cycles of 94 °C for 50 s, 55 °C for 50 s, and 72 °C for 1 min, and final extension of 72 °C for 10 min. Each PCR product (5 μL) was examined through electrophoresis in 1.0% agarose gel and sequenced by Shanghai Shenggong Company (Shanghai, China). Samples were sequenced forward and reverse to ensure accuracy.

Data analysis

The obtained sense and antisense sequences were joined using ContigExpress software then each completed sequence was compared in BLAST of National Center for Biotechnology Information (NCBI). The ITS sequences of samples were cut on the basis of the complete ITS sequence which showed the highest similarity among the sequences in NCBI. The sequences of species with > 99% similarity, species confused and faked as Fructus Ligustri Lucidi, as well as related species in the same genus were downloaded as reference sequences. DNAman software was used to identify similarity. A molecular phylogenetic tree was constructed using Neighbor-Joining (NJ) method in MEGA 5.0 to select reference sequences and conduct phylogenetic tree identification [6].

Content determination of specnuezhenide

Chemicals and materials

HPLC-grade methanol and ethanol were purchased from Shanghai Xingke High Purity Solvent Co., Ltd. and Shanghai Zhengxing Chemical Factory, respectively. Distilled water was supplied by Wahaha (Hangzhou, China). Specnuezhenide reference substance was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China; Batch No. 1310- 110113). The analyzed samples were shown in Table 1.