HPLC fingerprinting and pattern recognition of Brazilian green propolis and Chinese propolis

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Abstract. In order to improve the quality control method for propolis, HPLC fingerprinting and pattern recognition of 12 batches of Brazilian green propolis and 12 batches of Chinese propolis were carried out. HPLC fingerprinting was performed on a C18 column (250 mm × 4.6 mm, 5μm) using gradient elution with methanol-water (containing 0.2% formic acid) as the mobile phase. Similarity analysis was executed on similarity evaluation system for chromatographic fingerprint of TCM (2012A). Pattern recognition was performed by SIMCA-P 13.0 software. As a result, as for the two groups of propolis samples, 21 common peaks were calibrated and 11 of them were identified as chlorogenic acid, caffeic acid, isochlorogenic acid B, isochlorogenic acid C, quercetin, kaempferol, apigenin, pinocembrin, caffeic acid phenylethyl ester, galangin and artepillin C. The similarity of the fingerprint profiles of the tested Brazilian propolis is more than 0.92, and that of the tested Chinese propolis is more than 0.91. However, the two groups of fingerprints’ similarity are from 0.523 to 0.693. The results of PCA and OPLS-DA showed that chemical profiles of the tested Brazilian green propolis were different from those of Chinese propolis. A set of compounds, namely, artepillin C, chlorogenic acid, pinocembrin, isochlorogenic acid B and caffeic acid phenylethyl ester can act as difference marker of the two groups of propolis. Moreover, HPLC fingerprint combined with pattern recognition could be developed into one new method for quality control of propolis.

1. Introduction

Propolis (bee glue) is a kind of natural substance collected and processed by honey bees (Apis mellifera) from plants leaves, flower buds, plant resin. While transporting, bees usually mix it with their own secretions, such as beeswax, saliva. Finally, propolis, a resinous substance, is produced as a sealant to protect their hives against invaders, heat, humidity and wind [1].The chemical composition of propolis is very complicated. Flavonoids, phenolic acids and terpenes are proved and regarded as its functional components [2-3]. A growing body of studies have shown that the biological activities of propolis are closely related to its chemical components [3-4]. However, its chemical composition is varied greatly with the origin location [5].

Now, in China, pharmaceutical and cosmetic products containing propolis in the public markets come from all around the world. Among them, Brazilian green propolis and Chinese propolis are dominated for their relatively adequate supply. However, up to now, there are few literature which conducts in-depth comparative studies on the chemical composition of the two kinds of propolis. Nowadays, fingerprint combined with pattern recognition technology have been widely used in quality control of propolis.
control of food and herbal drugs [6-8]. Herein, hypothesizing that it is also suitable for propolis quality control, in the present study, low molecular weight metabolite profiles of 12 batches of Brazilian green propolis and 12 batches of Chinese propolis were recorded by HPLC. Successively, we evaluated the similarity of their fingerprint profiles using professional software. At the same time, we also performed multivariate statistical analysis to screen the significantly different compounds, which could act as the markers reflecting the difference of the two groups of propolis. Moreover, the suitability and feasibility of HPLC fingerprint and pattern recognition act as a new method for quality control of propolis were discussed.

2. Experimental

2.1 Samples
Propolis samples were supplied friendly by By-health Co. Ltd, which were collected during summer 2017 from different manufacturers (Figure 1) and stored at -20℃ until analysis.

2.2 Reagents and chemicals
Methanol (HPLC grade), formic acid (HPLC grade) and ethanol (95%) were purchased from Merck Chemicals (Darmstadt, Germany).

2.3 Preparation of reference substance solution
Reference substance of chlorogenic acid, caffeic acid, isochlorogenic acid B, isochlorogenic acid C, caffeic acid phenethyl ester, myricetin, quercitrin, kaempferol, apigenin, pinocembrin and galangin were supplied by Chengdu Alfa Biotechnology Co., Ltd. (Chendu, China). Artepillin C was isolated in our lab from the ethanolic extract of Brazilian green propolis, and the purity of which was determined as 98.2% by analytical HPLC with PDA detector while the chemical structure was confirmed by HRMS, 1H-NMR and 13C-NMR.

Figure 1. Tested samples of Brazilian green propolis (A) and Chinese propolis (B).

The stock solutions of chlorogenic acid, caffeic acid, isochlorogenic acid B, isochlorogenic acid C, caffeic acid phenethyl ester, myricetin, quercitrin, kaempferol, apigenin, pinocembrin, galangin and artepillin C, were prepared by accurately weighing 15, 18, 15, 17, 19, 22, 20, 28, 20, 17, 12, 13 mg of corresponding powder of reference substance and dissolving it in 5 mL of methanol. The stock solutions were stored at 4 ℃. From the stock solutions, working mixed reference substance solution was prepared by mixing and diluting with methanol appropriately. The working solution was filtered through 0.45μm membrane filter (Millipore, USA).

2.4 Preparation of sample solution
Sample solution was prepared as previously described[9]. In brief, about 20 gram of propolis was grinded to a fine powder with a pre-cooled mini-grinder. Subsequently, 100 mg of the fine powdered smaples were extracted with methanol two times in a total time of one hour with the sonication. The final volume was fixed to 5mL. The extract was centrifuged at 3,000 rpm for 10 min and 0.1mL of the
supernatant was diluted into 1 mL with methanol. Then the dilution was filtered with 0.45 µm membrane, the filtered solution was subjected to HPLC analysis.

2.5 Chromatographic Condition
The HPLC fingerprint profiles were acquired using DIONEX 3000 HPLC system (Thermo Fisher, Dreieich, Germany), equipped with a diode array detector and a Chromelon™ Chromatography Data System (Thermo Fisher, Dreieich, Germany). Chromatographic separation was performed on a Kromasil C18 column (250 mm × 4.6 mm, 5 µm). The mobile phases were composed of methanol (A) and water with 0.2% formic acid (B) at a flow rate of 1.0 mL·min⁻¹, and monitored at 310 nm. The gradient profile was as follows: 0~22 min, linear 75% → 55% of B; 22~45 min, linear 55% → 45% of B; 45~58 min, linear 45% → 30% of B; 58~75 min, linear 30% → 20% of B; 75~80 min, 20% → 5% of B; 80~88 min, 5% of B; 88~95 min, linear 5% → 75% of B; 95~105 min, linear 75% of B.

2.6 Multivariate analysis
Multivariate statistical analysis of HPLC fingerprint data from all the tested propolis samples was performed as previously described[10-11]. Briefly, similarity analysis of fingerprint was performed on the similarity evaluation system for chromatographic fingerprint of TCM (Chinese Pharmacopoeia Commission, Version 2012A). PCA and OPLS-DA analysis were carried out with the integral area of fingerprint peaks using SIMCA-P 13.0 software (Umetrics, Umeå, Sweden).

3. Results

3.1 Precision, repeatability and stability of the analytical method
One of the tested propolis samples was randomly selected and the sample solution was prepared according to the method described above. Then, the sample solution was continuously injected for six times and the HPLC fingerprint data were recorded and compared. Relative standard deviation (RSD) values of relative integral area and relative retention time of each main peak, which accounted for more than 2% of the total area, were calculated. The results showed both of them were less than 2.0% and 3.0% respectively. Meanwhile, the similarity of fingerprints were all above 0.990, indicating that the instruments had good precision.

To confirm the repeatability, six different working solutions prepared from the same propolis sample which was randomly selected, were analyzed in parallel by the abovementioned method. The similarity of the recorded 6 fingerprint profiles were all above 0.960, which revealed high repeatability of the method.

Stability of sample solution was tested at room temperature. One of the tested propolis samples was randomly selected and the sample solution was prepared. Then, the sample solution was injected at 0, 3, 6, 12, 18, 24h. The similarity of HPLC fingerprint profiles acquired at above six different time spots were all above 0.980, which demonstrated a good stability in tested propolis methanol solution within 24h.

3.2 Similarity evaluation
All the tested 24 batches of Brazilian and Chinese propolis samples were subjected for fingerprint data collecting according to abovementioned method. All the data were processed and analysed by the above softwares. The corresponding fingerprint profiles with referring chromatography were shown as Figure 2 and Figure 3. Among them, 21 characteristic peaks were marked, and 11 marked peaks were identified and shown as Figure 4.

Compared with their corresponding referring chromatography, the similarity of the fingerprint of 24 batches of propolis was evaluated. The results showed that the similarity of the fingerprint of 12 batches of Brazilian propolis was greater than 0.92, meanwhile that of 12 batches of Chinese propolis was greater than 0.91. However, the similarity between Brazilian propolis and Chinese propolis was from 0.523 to 0.693. The results showed that the internal difference between the two groups of
propolis samples was smaller than the external difference, indicating that the original location had a great influence on the chemical composition of propolis.

Figure 2. HPLC fingerprints of 12 batches of Brizilian propolis.

Figure 3. HPLC fingerprints of 12 batches of Chinese propolis.

Figure 4. Reference HPLC chromatogram of tested Brazilian and Chinese propolis samples. chlorogenic acid(1), caffeic acid(2), isochlorogenic acid A(5), isochlorogenic acid C(6), quercitrin(9), kaempferol(10), apigenin(11), pinocembrin(14), caffeic acid phenethyl ester(15), galangin(17), artepillin C(20).

3.3 Pattern recognition analysis
All data obtained from 24 batches of propolis samples were processed and imported into SIMCA-P software for PCA analysis with integral area as the variates. The result was shown as Figure 5. Obviously, the Brazilian green propolis samples and the Chinese propolis samples were divided into two categories in the score plot of PCA. Furtherly, OPLS-DA was used to establish the difference model of two groups of propolis HPLC fingerprint profile, which was shown as Figure 6.
Taking the VIP value >1 and p value < 0.05 of the variates as the threshold, the main chemical components causing the difference between the two groups of Brazilian green propolis and Chinese propolis were screened out. The result (Figure 7) showed that twelve peaks, namely, peak 20, 14, 15, 1, 3, 7, 19, 5, 8, 1, 12 and 21, were significant difference markers of Brazilian green propolis and Chinese propolis.

3.4 Chemical markers
Confirmed by the accurate molecular weight, the relative retention time and the MS/MS fragmentation pattern, chlorogenic acid (peak 1), isochlorogenic acid B (peak 5), caffeic acid phenethyl ester (peak 15), pinocembrin (peak 14), artepillin C (peak 20), were identified from the above screened out twelve chemical markers. Shown as Figure 8, the content of artepillin C, chlorogenic acid and chlorogenic acid A are much higher in Brazilian green propolis, while the contents of caffeic acid phenethyl ester and pinocembrin are higher in Chinese propolis. There existed significant difference between the two groups of propolis samples. Especially for artepillin C, it can nearly not be detected in Chinese propolis.
Figure 8. A set of chemical markers standing for the difference between Brazilian green propolis and Chinese propolis.

4. Discussion
Effort focusing on quality control of food and herbal medicine is always worthwhile. Herein, we reported the HPLC fingerprint and pattern recognition of Brazilian green propolis and Chinese propolis. In the present study, by making use of the optimized HPLC-DAD analysis methods, fingerprint data of 24 propolis samples were collected. During the data processing, fingerprint profiles of six different detection wavelengths, namely, 254, 276, 290, 310, 330 and 360nm, were extracted and parallelly analysed. Among them, 310nm was the best choice. So, in the following experiments, the detection wavelength was fixed at 310nm. Moreover, it’s also suitable for HPLC system only equipped with UV detector.

Usually, different geographical origins and production processing protocols lead to different quality of herbal or agricultural products[12-14]. It’s believed that the different chemical profiles supported the different phenotypes. In the present study, HPLC fingerprints of the two groups of samples showed obvious difference. However, except the obvious difference, the two groups of propolis had a few of common components. Further multivariate analysis revealed the different chemical markers between different original propolis. In our study, a set of small molecules were screened out as the different markers of these two kinds of propolis. However, it would be better if we can run a larger size samples verification. Nevertheless, HPLC fingerprinting and pattern recognition can cluster samples with common characteristics in terms of origin and production process. So, a new quality control method for propolis can be developed based on our results. It’s noteworthy that our results also validated artemillin C as a unique and characteristic component of Brazilian green Propolis. Meanwhile, chlorogenic acid, caffeyalquinic acids and some other phenolic acids should contribute a lot for the special quality formation of Brazilian green propolis[15]. As for Chinese propolis, quite a few of low molecular weight flavonoids play an active role for their bioactivities[5,15].

5. Conclusions
As a gift presented by the nature, Brazilian and Chinese propolis are both precious. Our results showed that the chemical composition of Brazilian and Chinese propolis were different from each other, but
the two types of propolis still had many common components. We also proved that HPLC fingerprinting combined with pattern recognition could act as a tool for distinguishing Brazilian green propolis and Chinese propolis. Meanwhile, a set of compounds, chlorogenic acid, isochlorogenic acid B, caffeic acid phenethyl ester, pinocembrin, artepillin C, should be regarded as the chemical markers of the two groups of propolis.

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