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Monosaccharide analysis and fingerprinting identification of polysaccharides from *Poria cocos* and *Polyporus umbellatus* by HPLC combined with chemometrics methods

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ABSTRACT

Objective: *Poria cocos* and *Polyporus umbellatus* are similar medicinal fungi in traditional Chinese medicines. A method for fingerprint analysis of monosaccharide composition of polysaccharides by HPLC combined with chemometrics methods has been developed for characterization and discrimination of them in this research.

Methods: The polysaccharides were extracted by decocting in water, and then completely hydrolyzed with hydrochloride. Monosaccharides in the hydrolyzates were derivatized with 1-phenyl-3-methyl-5-pyrazolone (PMP) for HPLC analysis. More than 20 batches of *P. cocos* and *P. umbellatus* from different regions were analyzed.

Results: The fingerprints of *P. cocos* showed five common characteristic peaks, which were identified by comparing with the reference substances. The five peaks corresponded to the derivatives of mannose, ribose, glucose, galactose, and fucose. At the same time, the fingerprints of *P. umbellatus* showed eight common characteristic peaks, of which seven were identified as the derivatives of mannose, ribose, rhamnose, glucose, galactose, xylose, and fucose. Moreover, the similarity of their fingerprints was respectively calculated by the Similarity Evaluation System for Chromatographic Fingerprint of TCM published by China Pharmacopoeia Committee (Version 2004A). And the data were further processed by hierarchical cluster analysis (HCA) and principal component analysis (PCA). The similarity evaluation and HCA indicated that there were no significant difference in *P. cocos* or *P. umbellatus* samples from different geographical regions, but PCA was performed to characterize the difference in monosaccharide constituents between *P. cocos* and *P. umbellatus*, and linear discriminant analysis (LDA) showed the overall correct classification rate was 100%.

Conclusion: The fingerprint analysis method of monosaccharide composition of water-soluble polysaccharides can distinguish *P. cocos* and *P. umbellatus*, and can be applied for the authentication or quality control for *P. cocos* and *P. umbellatus*.

1. Introduction

Poria cocos (Schw.) Wolf (Fuling in Chinese) and *Polyporus umbellatus* (Pers.) Fries (Zhuling in Chinese) are well-known medicinal fungi and widely used in traditional Chinese medicine (Wang et al., 2013; Zhao, 2013). They have been listed in different versions of Chinese Pharmacopoeia (Chinese Pharmacopoeia Commission, 2015). Their healthcare function and bioactivities are similar but not exactly the same. The Compendium of Meteria Medica, *Bencao Gangmu*, described that the efficacy of *P. umbellatus* is similar to that of *P. cocos*, but not as good as *P. cocos* when as a tonic.

The quality standard of *P. cocos* and *P. umbellatus* such as the basic description (color, odor, shape, etc.), morphological identification, loss on drying, total ash, extract content, and so on are included in Chinese Pharmacopoeia (2015 edition). But, these test items cannot be applied to distinguish them in powdered crude drugs or extracts. Many researches have shown that the major active substances in *P. cocos* are polysaccharides and triterpenes (Wang et al., 2013), while the major active substances in *P. umbellatus* are polysaccharides and steroids (Zhao, 2013). The studies about the assay and fingerprint of triterpenes in *P. cocos* (Fu et al., 2018; Zhu et al., 2018) or steroids in *P. umbellatus* (Zhao et al., 2010; Chen, Zhou, Wang, Yang & Guo, 2017) had been reported. However, the content of triterpenoids in *P. cocos* and steroids in *P. umbellatus* are very low: two (pachymic acid and dehydrotumulosic

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acid) of the highest content of triterpenoids in *P. cocos* were just 0.022% and 0.020% (Fu et al., 2018). And three (ergosterol, polyporusterone A, and polyporusterone B) of the highest content of steroids in *P. umbellatus* were just 0.068%–0.238% (Zhao et al., 2010), 0%–0.0175%, and 0%–0.0380% (Chen et al., 2017), which is difficult to detect especially in the traditional decoction. Polysaccharides were thought to be important bioactive ingredients, which showed the activities of antitumor (Ke, Lin, Chen & Ji, 2010; Zhang et al., 2015; Tan, Guo & Wang, 2016), immune-modulating (Dai et al., 2012; Sun & Zhou, 2014), anti-oxidant (He et al., 2016; Wang et al., 2016), liver protecting (Wu, Fan, Huang, Wu & Guo, 2018), and others (Khan et al., 2018; Song et al., 2018; Wu et al., 2016). At present, the analysis of total polysaccharide content often determined by colorimeter with phenol-sulfuric acid (Takashi, Hiroyuki & Kazuaki, 2009), which could not characterize the polysaccharide. Therefore, it is necessary to develop a specific method to characterize the polysaccharides of *P. cocos* and *P. umbellatus*.

Fingerprint profiling has been recognized as an efficient technique for quality control of traditional Chinese medicine (Zou & Yan, 2018). In the present study, we collected more than 20 batches of *P. cocos* and *P. umbellatus* from different geographical regions. The polysaccharides were extracted by decocting in water, respectively, of which the monosaccharide constituents were analyzed by HPLC. Subsequently, the similarity of these fingerprints was calculated and the data were carried out with hierarchical cluster analysis (HCA), principal component analysis (PCA), and linear discriminant analysis (LDA).

2. Materials and methods

2.1. Materials

Twenty-two batches of *Poria cocos* (Schw.) Wolf and 20 batches of *Polyporus umbellatus* (Pers.) Fries (Table 1) were collected from different regions of China. They were identified as authentics by Dr. Mo-li Hua (State Institute of Pharmaceutical Industry, Shanghai, China), according to the descriptions in Chinese Pharmacopoeia (2015 edition).

Glucose (Glc, Lot #20070115) and *D*-mannose (D-Man, Lot #F20120107) were purchased from Sinopharm Chemical Reagent Co., Ltd (SCRC, Shanghai, China). *D*-ribose (D-Rib, Lot #1451793) and *D*-fucose (D-Fuc, Lot #050M1909, Sigma) were obtained from Singma-Aldrich Co. Ltd. (St. Louis, MO, USA). *L*-rhamnose (L-Rha, Lot #839801), *D*-xylose (D-Xyl, Lot #820115), and *D*-galactose (D-Gal, Lot #840215) were all purchased from Shanghai Chemical Reagent Second Factory (Shanghai, China). 1-Phenyl-3-methyl-5-pyrazolone (PMP, Lot #H1718064) was obtained from Shanghai Aladdin Bio-Chem Technology Co., Ltd (Shanghai, China). Ethanol (AR), hydrochloric acid (AR, Lot #20160930), sodium hydroxide (AR, Lot #20130327), and potassium dihydrogen phosphate (AR, Lot #20140901) were all purchased from SCRC. Acetonitrile (HPLC grade, Damas-beta) was obtained from Shanghai Titan Scientific Co., Ltd. The water for HPLC analysis was purified by a Milli-Q water purification system (Merck & Co Inc., USA).

2.2. Extraction of polysaccharides

According to traditional preparation method of decoction (Zhao et al., 2018; Zhou et al., 2018; Zhu et al., 2017), 50 g of *P. cocos* and *P. umbellatus* was accurately weighed and extracted twice: firstly soaked in 450 mL distilled water for 1 h at room temperature and then decocted for 30 min. After being filtered, 350 mL distilled water was added into the residues and boiled for 25 min. The filtrate were combined and concentrated at 60 °C under reduced pressure, then precipitated adding ethanol to a final concentration of 80% (Fang et al., 2017). After being placed at 4 °C overnight, the mixture was centrifuged at 4000 rpm for 10 min and the precipitates were dissolved in 10 mL water for freeze-drying.

2.3. Analysis of monosaccharide in polysaccharides by HPLC

The monosaccharide composition analysis was followed previous reports with some modification (Sun et al., 2014). The reference standard solutions for each monosaccharide, including Man, Rib, Rha, Glc, Gal, Xyl, and Fuc were dissolved in water at the concentration of 1 mg/mL. The derivatization of each monosaccharide was as following: 100 μL of the reference standard solution were mixed with 50 μL of 0.3 mol/L sodium hydroxide and 60 μL of 0.5 mol/L methanolic solution of 1-phenyl-3-methyl-5-pyrazolone (PMP), then kept at 70 °C for 30 min. After cooling, the mixture was neutralized with 50 μL of 0.3 mol/L hydrochloric acid and diluted to 1 mL with distilled water. Then, 1 mL of chloroform was added and the organic phase was discarded after vigorous shaking

Table 1
Origin and similarity evaluation results of *P. cocos* samples and *P. umbellatus* samples.

Batches	Geographical regions	Similarity	Batches	Geographical regions	Similarity
FL-R		1	ZL-R		1
FL-001	Anhui	0.929	ZL-001	Shaanxi	0.969
FL-002	Anhui	0.947	ZL-002	Sichuan	0.977
FL-003	Yunnan	0.976	ZL-003	Sichuan	0.953
FL-004	Yunnan	0.972	ZL-004	Sichuan	0.853
FL-005	Yunnan	0.910	ZL-005	Jilin	0.995
FL-006	Anhui	0.999	ZL-006	Aba, Sichuan	0.997
FL-007	Bozhou, Anhui	0.955	ZL-007	Shaanxi	0.988
FL-008	Dabieshan, Anhui	0.981	ZL-008	Shaanxi	0.993
FL-009	Yunnan	0.991	ZL-009	Shaanxi	0.944
FL-010	Hubei	0.996	ZL-010	Shaanxi	0.921
FL-011	Yuexi, Anhui	0.999	ZL-011	Shaanxi	0.990
FL-012	Yuexi, Anhui	0.994	ZL-012	Shangluo, Shaanxi	0.977
FL-013	Yuexi, Anhui	0.890	ZL-013	Shangluo, Shaanxi	0.989
FL-014	Yuexi, Anhui	0.948	ZL-014	Shangluo, Shaanxi	0.965
FL-015	Yuexi, Anhui	0.981	ZL-015	Shangluo, Shaanxi	0.929
FL-016	Yuexi, Anhui	0.986	ZL-016	Shangluo, Shaanxi	0.953
FL-017	Shaanxi	0.974	ZL-017	Hanzhong, Shaanxi	0.982
FL-018	Anhui	0.947	ZL-018	Shanxi	0.962
FL-019	Yunnan	0.962	ZL-019	Shaanxi	0.995
FL-020	Dabieshan, Anhui	0.999	ZL-020	Henan	0.994
FL-021	Jinzhai, Anhui	0.994			
FL-022	Yunnan	0.977			

and layering. The extraction was performed for three times. Finally, the upper aqueous solution at the concentration of 0.1 mg/mL was passed through a 0.45 μm syringe filter for HPLC analysis.

20 mg of prepared polysaccharides was accurately weighed and hydrolyzed with 5 mL of 4 mol/L hydrochloric acid in an ampoule at 90 °C water-bath for 6 h. After cooling, 2 mL of methanol was added into the hydrolyzates and the mixture was evaporated at 60 °C under vacuum to remove hydrochloric acid. The procedure was performed for several times. The dried hydrolyzates were dissolved with 1 mL of water and derivatized as above of monosaccharides.

Analysis of monosaccharide derivative was performed on a Dionex U3000 HPLC (Thermo Fisher Scientific, Massachusetts, USA) equipped with a quaternary pump, auto-sampler manager, diode array detector (DAD), and Chromeleon 7 Chemstation. Chromatographic separation was carried out on an Agilent Extend C_{18} column (5 μm, 4.6 × 250 mm), eluted with a mobile phase of 0.05 mol/L phosphate buffer (PH 6.8) and acetonitrile (82.5:17.5), the flow rate was 1 mL/min, the injection volume was 10 μL, and the column temperature was 30 °C. The UV detection wavelength was set at 250 nm. The monosaccharides were identified by comparing the retention time with those of the reference substances.

2.4. Standard fingerprint profiles and evaluation of similarity

HPLC data of all samples were submitted for analysis by professional software named Similarity Evaluation System for Chromatographic Fingerprint of Traditional Chinese Medicine (Version 2004A, China Pharmacopoeia Committee, Beijing, China).

2.5. Statistical analysis

Hierarchical cluster analysis (HCA), principal component analysis (PCA), and linear discriminant analysis (LDA) were carried out based on the relative peak areas of monosaccharides in fingerprint chromatograms using software IBM SPSS (Version 21.0, SPSS Inc., Chicago, Ill., USA).

3. Results

3.1. Validation of methods

The precision, repeatability, and stability were validated respectively, which were evaluated by relative peak retention time and relative peak area. Galactose (peak 4 in fingerprint of *P. cocos*; peak 7 in fingerprint of *P. umbellatus*) was assigned as the reference peak to calculate relative peak retention times and relative peak areas.

Precision of chromatographic separation and detection was investigated. The relative standard deviations (RSDs) of relative retention time (RRT) and relative peak area (RPA) of common peaks were less than 3%.

Stability of the derivatives solution stored at room temperature for 0, 2, 4, 8, 12, and 24 h was tested. The RSDs of RRT and RPA of common peaks were also less than 3%, it was revealed that the derivatives of hydrolyzates were stable.

The test of repeatability was done, showed that RSDs of RRT were below 3% and RSDs of RPA were below 3.73%. All results as above indicated that the method was able to meet the requirement of fingerprint analysis.

3.2. Standard fingerprint profiling and similarity evaluation

Monosaccharide composition of polysaccharides from 22 batches of *P. cocos* and 20 batches of *P. umbellatus* was analyzed by HPLC. And the fingerprints (Fig. 1A and C) were obtained and then analyzed by software "Similarity Evaluation System for Chromatographic Fingerprint of Traditional Chinese Medicine (Version 2004A)". The standard fingerprint (Fig. 1B) of *P. cocos* samples was established and there were five kinds of monosaccharide in all common peaks, which identified as the derivatives of mannose (1), ribose (2), glucose (3), galactose (4), and fucose (5) by comparing the retention times with those of the reference substances. The standard fingerprint (Fig. 1D) of *P. umbellatus* samples showed eight kinds of monosaccharide in all common peaks, seven of which were identified as the derivatizes of mannose (1), ribose (2), rhamnose (3), glucose (5), galactose (6), xylose (7), and fucose (8) by comparing with the reference substances.

The similarity of each fingerprint was evaluated by "Similarity Evaluation System for Chromatographic Fingerprint of Traditional Chinese Medicine" with the corresponding standard fingerprints and the similarity values were calculated in Table 1. The results showed that the similarity values were almost larger than 0.900 except for sample FL-013 (0.890) and sample ZL-004 (0.853), which indicated that the fingerprint characteristics of the polysaccharides in *P. cocos* or *P. umbellatus* from different geographical regions were highly similar, respectively.

3.3. HCA of polysaccharides in P. cocos and P. umbellatus

In order to further analyze the fingerprint characteristics of polysaccharides in *P. cocos* and *P. umbellatus* from different geographical regions, HCA was done with the software IBM SPSS (Version 21.0). The relative area of each monosaccharide peaks to galactose peak in each fingerprint of polysaccharides from *P. cocos* or *P. umbellatus* formed the data matrix. Then the data matrix was imported into the software for HCA, respectively. The dendrograms of *P. cocos* and *P. umbellatus* were shown in Fig. 2, which indicated the whole process of clustering intuitively. The dendrograms showed that the samples from different geographical regions could not be classified as a different category, which indicated that there was no significant difference in the fingerprint of polysaccharides from *P. cocos* cultivated in four different geographical regions (Yunnan, Anhui, Hubei, and Shaanxi Provinces) and from *P. umbellatus* cultivated in five different geographical regions (Shaanxi, Sichuan, Shanxi, Jilin, and Henan Provinces). The results were consistent with the results of similarity evaluation of the fingerprints. Therefore, the fingerprint of polysaccharides by HPLC could reflect common characteristic in *P. cocos* or *P. umbellatus* from various regions.

3.4. PCA of polysaccharides in P. cocos and P. umbellatus

In order to compare the fingerprint features of polysaccharides from *P. cocos* and *P. umbellatus* and further analyze the major components contributing to the discrimination, PCA was done by software IBM SPSS (Version 21.0). The data matrix as mentioned before was submitted into the software for PCA and the 3D score plot was shown in Fig. 3A. The first three principal components PC1 (47.308%), PC2 (25.919%), and PC3 (14.777%) visually showed the obvious differentiation between *P. cocos* and *P. umbellatus*. Samples of *P. cocos* (FL-001−FL-022) were all converged on the left side of the score plot, while samples of *P. umbellatus* (ZL-001−ZL-020) were all gathered on the right side of the score plot, i.e. it could be easy to distinguish between *P. cocos* and *P. umbellatus* by PCA based on chromatographic fingerprint data. The loading plot of PCA was presented in Fig. 3B. The farther the origin point was away from, the greater the contribution of the component to distinguish between *P. cocos* and *P. umbellatus*. Obviously, the points representing mannose and ribose were the farthest from the origin, which hinted that mannose and ribose were the main two kinds of monosaccharide to distinguish polysaccharides from

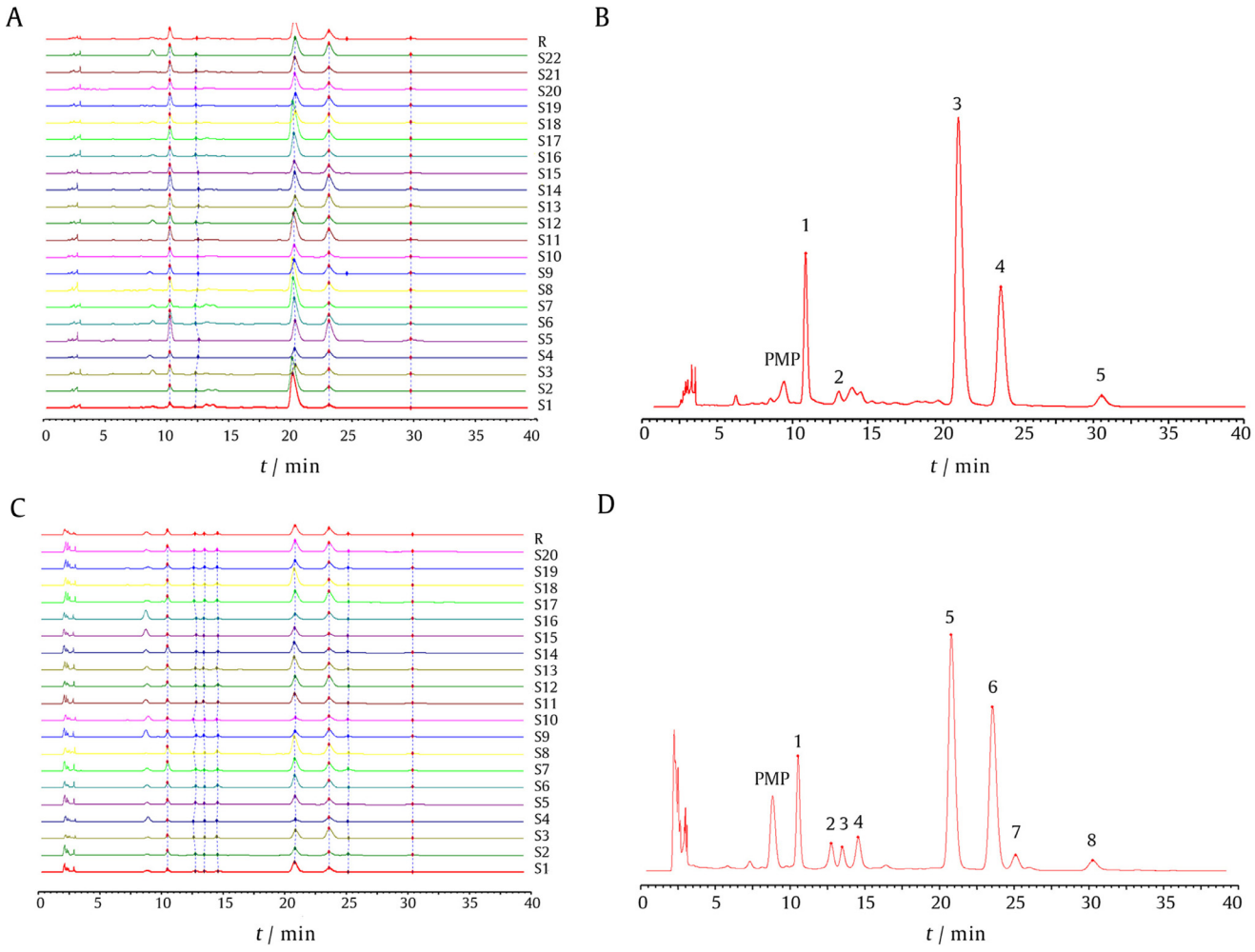

Fig. 1. Fingerprints of mixed standards (B, 1-Man, 2-Rib, 3-Glc, 4-Gal, and 5-Fuc) and monosaccharides from *P. cocos* polysaccharides (A, S1–S22 are samples FL-001–FL-022), and fingerprints of mixed standards (D, 1-Man, 2-Rib, 3-Rha, 5-Glc, 6-Gal, 7-Xyl, and 8-Fuc) and monosaccharides from *P. umbellatus* polysaccharides (C, S1–S20 are samples ZL-001–ZL-020).

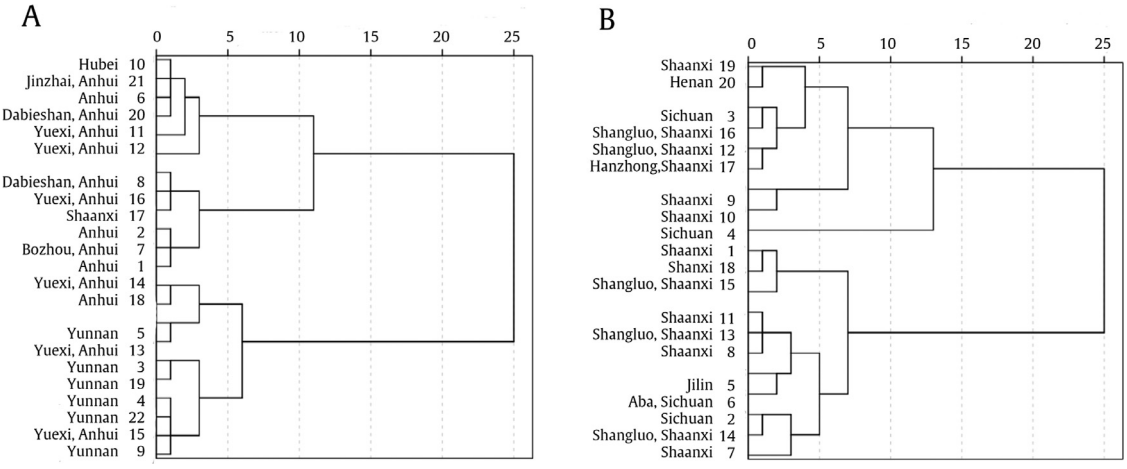

Fig. 2. Dendraogram of 22 batches of *P. cocos* polysaccharide (A) and 20 batches of *P. umbellatus* polysaccharide (B).

P. cocos and *P. umbellatus*. For a clearer explanation of the difference, the area ratio of mannose peak to ribose peak, which presented opposite value between *P. cocos* and *P. umbellatus*, was shown in Fig. 4, displaying that samples with the value more than 6.0 could be considered as *P. cocos* samples (FL), and those with the value no more than 6.0 could be considered to be *P. umballetus* samples (ZL). Therefore, in our study, PCA was performed to characterize the difference in fingerprint of polysaccharides by HPLC combined with chemometrics methods between *P. cocos* and *P. umbellatus*.

3.5. LDA of polysaccharides in P. cocos and P. umbellatus

To evaluate the discriminatory ability of the discriminant functions for *P. cocos* and *P. umbellatus*, LDA was done with the software IBM SPSS (Version 21.0), and the original and cross validation

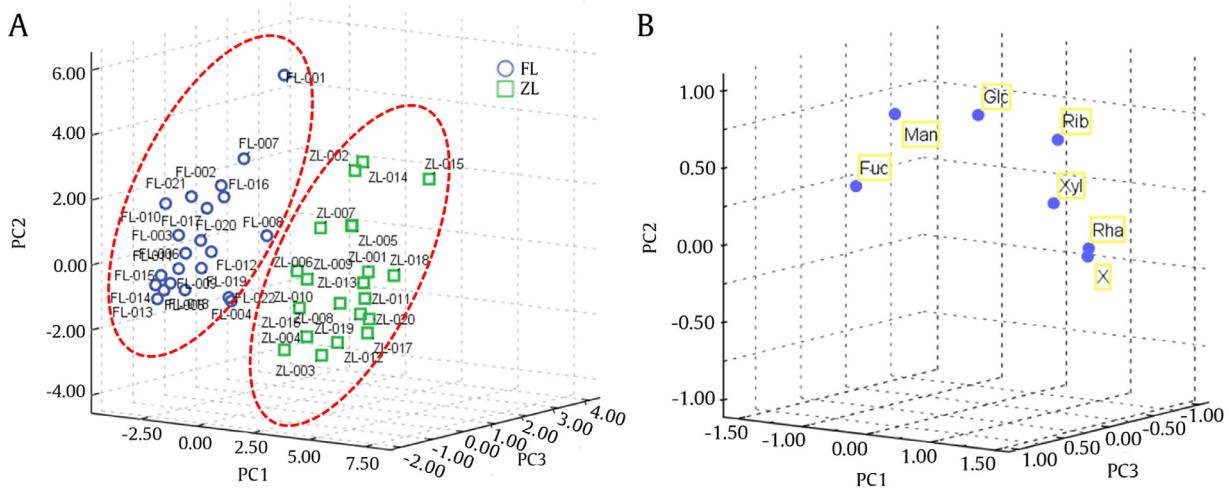

Fig. 3. 3D score plot (A) and loading plot (B) of polysaccharides of *P. cocos* (FL-001–FL-022) and *P. umballetus* (ZL001–ZL-020) on first three principal components.

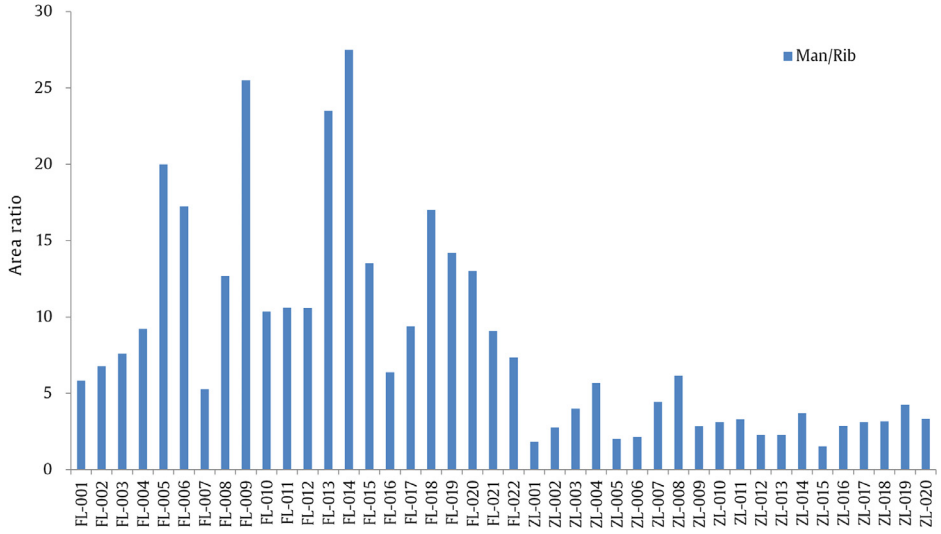

Fig. 4. Area ratio of Man peak to Rib peak of *P. cocos* samples (FL-001–FL-022) and *P. umballetus* samples (ZL-001–ZL-020).

Table 2
Discriminatory ability of LDA for classification of *P. cocos* and *P. umbellatus*.

Groups	Items	Samples	Predicted group membership[c]		Total
			FL	ZL	
Original[a]	Count	FL	22	0	22
		ZL	0	20	20
	%	FL	100.0	0.0	100.0
		ZL	0.0	100.0	100.0
Cross-validated[b]	Count	FL	22	0	22
		ZL	0	20	20
	%	FL	100.0	0.0	100.0
		ZL	0.0	100.0	100.0

[a] 100.0% of cross-validated grouped cases correctly classified.
[b] 100.0% of original grouped cases correctly classified.
[c] Cross validation is done only for those cases in the analysis. In cross validation, each case is classified by the functions derived from all cases other than that case.

methods were considered. In the cross validation method, each case is classified by the functions derived from all cases other than that case. Finally, the statistical criterion of Wilk's Lambda was 0.048 ($P < 0.05$). The discriminant functions in Table 2 showed that the overall correct classification rate was 100% for the original and 100% for the cross validation method, which was considered a very satisfactory discrimination rate for this method.

4. Discussion

P. cocos and *P. umbellatus* are similar medicinal fungi, widely used in clinical Chinese medicine and health food in China. The major active substances in *P. cocos* are polysaccharides and triterpenes, while the major active substances in *P. umbellatus* are polysaccharides and steroids. However, the content of triterpenoids in *P. cocos* and steroids in *P. umbellatus* are very low, which is difficult to detect, especially in the traditional decoction.

In recent years, polysaccharides have been normally thought to be major active substances of *P. cocos* and *P. umbellatus*. However, it has been a difficult problem to identify and distinguish the polysaccharide from *P. cocos* and *P. umbellatus*. In the study, a fingerprint analysis of polysaccharides has been developed by HPLC combined with chemometrics methods and the fingerprint, which could indicate monosaccharide compositions of the polysaccharides. More than 20 batches of *P. cocos* and *P. umbellatus* from different regions were analyzed. The fingerprints of *P. cocos* showed five common characteristic peaks, which were identified as mannose, ribose, glucose, galactose, and fucose. At the same time, the fingerprints of *P. umbellatus* showed eight common characteristic peaks, of which seven were identified as mannose, ribose, rhamnose, glucose, galactose, xylose, and fucose. Moreover, the similarity evaluation and HCA indicated that there were no significant

difference in *P. cocos* or *P. umbellatus* samples from different geographical regions, but PCA indicated the difference in monosaccharide constituents of polysaccharides between *P. cocos* and *P. umbellatus*. And LDA showed that the overall correct classification rate was 100% for the original and 100% for the cross validation method, which is considered as a very satisfactory discrimination rate for this method.

5. Conclusion

In conclusion, the fingerprint analysis of polysaccharides by HPLC combined with chemometrics methods could demonstrate not only the common characteristic in *P. cocos* or *P. umbellatus* from different geographical regions but also the difference in monosaccharide compositions of polysaccharides between *P. cocos* and *P. umbellatus*. Therefore, the method can be applied to the authentication or quality control for *P. cocos* and *P. umbellatus*.

Declaration of Competing Interest

The authors have declared that there is no conflict of interest.

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