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Original Research Article

Spectrum-effect relationship between HPLC fingerprints and inhibitory activity in MUC5AC mucin of *Pinelliae Rhizoma Praeparatum Cum Alumine*

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Abstract

Purpose: *To investigate the spectrum-effect relationship between HPLC fingerprints and the inhibitory effect on MUC5AC mucin of Pinelliae Rhizoma Praeparatum Cum Alumine (PRPCA).*
Methods: *The fingerprints of 20 PRPCA batches were established using HPLC and their similarities or differences were analyzed using hierarchical cluster analysis (HCA) and principal component analysis (PCA). The inhibitory effects of MUC5AC mucin were evaluated in LPS-treated NCI-H292 cells. The spectrum-effect relationship between common chromatographic peaks and MUC5AC inhibition was established using a partial least squares-discriminant analysis (PLS-DA).*
Results: *Fifteen common chromatographic peaks were identified by analyzing HPLC fingerprints, with uridine, tyrosine, uracil, and inosine found as possible markers to distinguish the PRPCA from different sources. Spectrum-effect relationship analysis showed that the chromatographic peaks 5, 6, 10 (vernine), 12 (5-hydroxymethylfurfural), 14 (tryptophan) and 15 (adenosine) were closely associated with the inhibitory effect on MUC5AC mucin.*
Conclusion: *The spectrum-effect relationship between HPLC fingerprints and the inhibitory effect on MUC5AC mucin of PRPCA was successfully established in the present study. Our findings further reveal the material basis of PRPCA and provide an effective method for its quality control.*

Keywords: *Pinelliae Rhizoma Praeparatum Cum Alumine, HPLC fingerprint, spectrum-effect relationship, MUC5AC mucin*

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INTRODUCTION

Airway mucus is important in protecting the respiratory system from harmful factors, including irritant chemicals, invasive particles, and pathogenic microorganisms [1]. However, mucus hypersecretion is related to many chronic airway diseases, including chronic bronchitis, asthma, and chronic obstructive pulmonary disease, *etc.* [2]. Mucins are the major components and contributors to the viscoelastic properties of mucus, and play an important role in maintaining the airway environment and function [3]. Prior studies have shown that regulation of mucin expression is an important strategy for the

treatment of airway mucus hypersecretion in chronic inflammatory airway diseases [4].

The dried tuber of *Pinellia ternata* (Thunb.) Breit (TPB) is a famous traditional Chinese medicine (TCM). The Chinese name of TPB is *Banxia*, and it is mainly used for the treatment of cough and inflammation [5, 6]. PRPCA is an important traditional processed product of TPB, and is used for treating phlegm syndrome (mucus hypersecretion) in TCM [7]. Our previous study showed that PRPCA exhibited significant therapeutic effects in allergic asthmatic rats by downregulating MUC5AC mucin [8].

However, the active ingredients related to the regulation of the MUC5AC mucin effect of PRPCA need to be thoroughly identified. Thus, the quality of PRPCA cannot be evaluated comprehensively using the evaluation method recorded in the Chinese pharmacopoeia (2020 Edition). The spectrum-effect relationship (between chemical compositions and efficacy) analysis has been widely applied to explore the active substances in TCM [9].

Therefore, the present study aimed to establish a spectrum-effect relationship between HPLC fingerprints and inhibitory effects on MUC5AC mucin in PRPCA. This investigation provides a simple and effective method for identifying the material basis for resolving phlegm effect and quality evaluation of PRPCA. The findings will help identify a safer and more effective alternative medicine for treating mucus hypersecretion.

EXPERIMENTAL

Sample collection

The 20 baches of PRPCA samples (S1 to S20) were collected from different Traditional Chinese Medicine manufacturers, local drug stores or processed by the TCM processing laboratory in Chengdu University of Traditional Chinese Medicine (Chengdu, China). All the PRPCA samples were authenticated by Professor Chunjie Wu (School of Pharmacy, Chengdu University of Traditional Chinese Medicine). Chromatographic acetonitrile was purchased from Sigma Aldrich (St. Louis, MO, USA). Roswell Park Memorial Institute (RPMI)-1640 culture medium and PBS buffer were purchased from Sigma Aldrich, St. Louis (MO, USA).

Fetal bovine serum (FBS) and trypsin were obtained from Gibco-BRL Life Technologies (Grand Island, NY). ELISA kit for MUC5AC mucin was purchased from ECOSAI Biotechnology (Taicang) Co., Ltd (Suzhou, China).

HPLC fingerprints

Chromatographic conditions

A CAPCELL PAK MG II S5 C_{18} column (250 × 4.6 mm, 5 μm) were used for chromatographic separations at 30 °C on an Agilent 1260 HPLC (Agilent, USA). The mobile phase consisted of acetonitrile (solvent A) and water (solvent B), and the HPLC elution conditions were optimized as follows: 0 – 15 min, 99.9 % B; 15 – 20 min, 99.9 – 95 % B; 20 – 30 min, 95 % B; 30 – 50 min, 95 – 85 % B. The flow rate was 1.0 mL/min, and the detection wavelength was set to 265 nm.

Preparation of sample solutions

Dried PRPCA powder was extracted using the method of ultrasound-assisted extraction with deionized water (1:10, w/v). The supernatants were collected after centrifugation (10000 rpm, 5 min), and filtered through a membrane (0.22 μm) to obtain a PRPCA sample solution for HPLC analysis. For the cell experiments, PRPCA supernatants were obtained according to the above method, concentrated using a rotary evaporator, and dried at 50 °C to obtain the PRPCA extract.

Chemometric analysis

The peak areas and fingerprint similarity of PRPCA were analyzed using a traditional Chinese medicine software similarity evaluation system for chromatographic fingerprinting (Version 2004A, Beijing, China). The obtained data matrix of the peak area was imported into SIMCA-P14.1 software (Umetrics, Umea, Sweden). Then, models were established using HCA and PCA to detect similarities and differences between 20 PRPCA batches.

Inhibitory effect of PRPCA on MUC5AC mucin in NCI-H292 cells treated by LPS

Cell culture

The NCI-H292 human airway epithelial cell line was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in RPMI-1640 culture medium supplemented with 10 % fetal bovine serum, penicillin (100 units/mL), and streptomycin (100 μg/mL) in an incubator with a 5 % CO_2 atmosphere at 37 °C. The adherent cells were cultured every 3 – 4 days by treatment with trypsin-EDTA solution.

MUC5AC assay

NCI-H292 cells were inoculated into six-well plates (5×10^5 cells/well) until confluent. After serum-starved for 24 h, cells were treated with PRPCA extract (400 µg/mL) for 30 min. LPS (1 µg/mL) was added and incubated for another 24 h. The model group was treated with LPS alone, and the cells without any treatment served as the control group. The MUC5AC content was determined using ELISA. The inhibition of MUC5AC in the PRPCA group compared to the model group was used to estimate the inhibitory effects on MUC5AC mucin production, which was calculated using the following formula:

Inhibition rate of MUCSAC (%) = $(1\text{-}C_p/C_m) \times 100$

where C_p and C_m represent the MUC5AC content in the PRPCA group and model groups, respectively.

Spectrum-effect relationship analysis

The spectrum-effect relationship between the inhibition rate of MUC5AC expression and common peak area in the HPLC fingerprints was analyzed. The inhibition rate of MUC5AC expression and the peak area data of PRPCA were taken as the dependent variable Y and X value. The X value was sequentially labelled as X1, X2, X3, X4 …. A PLS-DA was used to construct the spectrum-effect relationship using SIMCA 14.1 software.

RESULTS

Method validation for HPLC fingerprints

The method validation of the HPLC fingerprint results is presented in Figure 1. The similarity values for the method precision, reproducibility, and storage stability of the six sample solutions were > 0.99. The results indicated that the experimental instrument had good precision. The established method had good repeatability and the sample was stable for 24 h.

Similarity analysis of HPLC fingerprints

Under optimized conditions, chromatograms of all batches of PRPCA were obtained, and the fingerprints are shown in Figure 2. Notably, the different PRPCA batches had similar chemical characteristics. As depicted in Figure 3, 15 common peaks were observed by analyzing the HPLC chromatograms of the 20 PRPCA batches. Using UPLC-MS technology, nine common chromatographic peaks were identified in previous studies [8]. The peaks included uridine (3), tyrosine (4), uracil (7), adenine (8), inosine (9), vernine (10), 5 - hydroxymethylfurfural (12), tryptophan (14), and adenosine (15). The similarity values and common peak areas of 20 PRPCA batches are presented in Tables 1 and 2, respectively. The peak areas of the chemical constituents varied in the different PRPCA batches of different origins and processing methods.

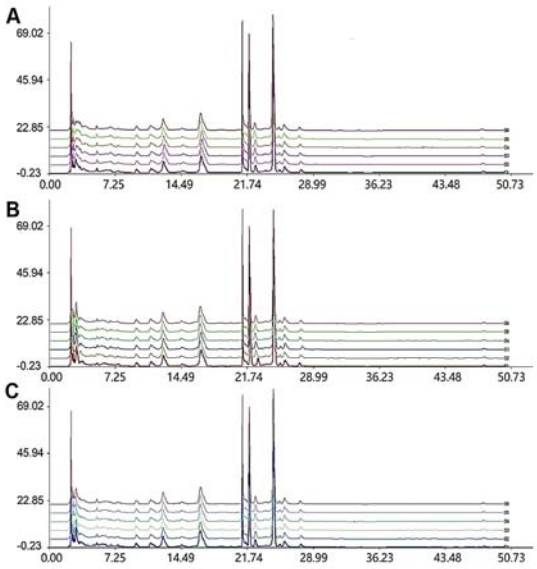

Figure 1: Overlay chromatograms of precision (A), stability (B) and repeatability (C) test.

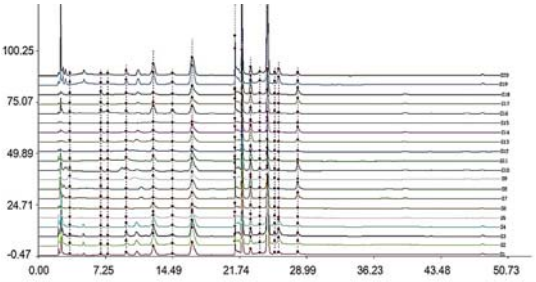

Figure 2: HPLC fingerprint of PRPCA

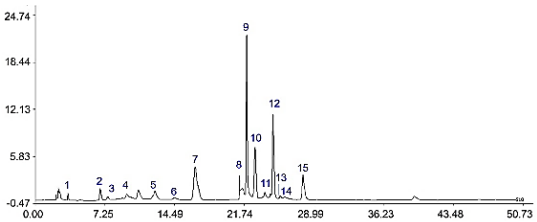

Figure 3: Common peaks of HPLC fingerprint. Uridine (3), tyrosine (4), uracil (7), Adenine (8), inosine (9), vernine (10), 5 - hydroxymethylfurfural (12), tryptophan (14), adenosine (15)

Table 1: The similarity of HPLC from 20 samples

ID	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12	S13	S14	S15	S16	S17	S18	S19	S20
S1	1.000	0.989	0.990	0.989	0.943	0.953	0.791	0.828	0.787	0.788	0.712	0.680	0.632	0.856	0.699	0.936	0.814	0.855	0.956	0.954
S2	0.989	1.000	0.992	0.991	0.936	0.944	0.775	0.815	0.781	0.775	0.709	0.671	0.628	0.847	0.689	0.926	0.808	0.847	0.948	0.946
S3	0.990	0.992	1.000	0.997	0.934	0.942	0.763	0.802	0.773	0.763	0.694	0.658	0.611	0.838	0.672	0.919	0.798	0.840	0.949	0.946
S4	0.989	0.991	0.997	1.000	0.935	0.941	0.764	0.803	0.774	0.764	0.693	0.659	0.612	0.839	0.673	0.922	0.797	0.840	0.950	0.947
S5	0.943	0.936	0.934	0.935	1.000	0.976	0.812	0.849	0.820	0.807	0.770	0.777	0.705	0.883	0.787	0.962	0.852	0.880	0.930	0.931
S6	0.953	0.944	0.942	0.941	0.976	1.000	0.819	0.853	0.830	0.817	0.776	0.782	0.720	0.888	0.809	0.958	0.853	0.885	0.932	0.932
S7	0.791	0.775	0.763	0.764	0.812	0.819	1.000	0.954	0.840	0.908	0.874	0.813	0.853	0.937	0.798	0.835	0.931	0.937	0.816	0.820
S8	0.828	0.815	0.802	0.803	0.849	0.853	0.954	1.000	0.857	0.912	0.872	0.818	0.842	0.946	0.807	0.877	0.934	0.943	0.859	0.862
S9	0.787	0.781	0.773	0.774	0.820	0.830	0.840	0.857	1.000	0.891	0.911	0.866	0.875	0.888	0.836	0.855	0.862	0.887	0.783	0.792
S10	0.788	0.775	0.763	0.764	0.807	0.817	0.908	0.912	0.891	1.000	0.905	0.850	0.883	0.931	0.816	0.814	0.933	0.931	0.782	0.786
S11	0.712	0.709	0.694	0.693	0.770	0.776	0.874	0.872	0.911	0.905	1.000	0.903	0.924	0.904	0.856	0.796	0.897	0.911	0.730	0.738
S12	0.680	0.671	0.658	0.659	0.777	0.782	0.813	0.818	0.866	0.850	0.903	1.000	0.880	0.862	0.871	0.777	0.848	0.870	0.686	0.694
S13	0.632	0.628	0.611	0.612	0.705	0.720	0.853	0.842	0.875	0.883	0.924	0.880	1.000	0.887	0.865	0.716	0.886	0.883	0.625	0.630
S14	0.856	0.847	0.838	0.839	0.883	0.888	0.937	0.946	0.888	0.931	0.904	0.862	0.887	1.000	0.853	0.891	0.984	0.992	0.858	0.862
S15	0.699	0.689	0.672	0.673	0.787	0.809	0.798	0.807	0.836	0.816	0.856	0.871	0.865	0.853	1.000	0.799	0.841	0.848	0.707	0.714
S16	0.936	0.926	0.919	0.922	0.962	0.958	0.835	0.877	0.855	0.814	0.796	0.777	0.716	0.891	0.799	1.000	0.848	0.888	0.946	0.950
S17	0.814	0.808	0.798	0.797	0.852	0.853	0.931	0.934	0.862	0.933	0.897	0.848	0.886	0.984	0.841	0.848	1.000	0.978	0.813	0.816
S18	0.855	0.847	0.840	0.840	0.880	0.885	0.937	0.943	0.887	0.931	0.911	0.870	0.883	0.992	0.848	0.888	0.978	1.000	0.858	0.862
S19	0.956	0.948	0.949	0.950	0.930	0.932	0.816	0.859	0.783	0.782	0.730	0.686	0.625	0.858	0.707	0.946	0.813	0.858	1.000	0.993
S20	0.954	0.946	0.946	0.947	0.931	0.932	0.820	0.862	0.792	0.786	0.738	0.694	0.630	0.862	0.714	0.950	0.816	0.862	0.993	1.000

Table 2: Common peak area of fingerprints for 20 batches of PRPCA

ID	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	P11	P12	P13	P14	P15
S1	1028069	2094472	1865233	4198583	15195690	5802801	25111690	7530425	40341720	7861618	4714319	67703270	3647260	6452114	10600560
S2	1692137	4393710	3221802	6679899	21818320	7111949	36345080	4909580	56646880	11164050	6358846	96545380	6203798	15354300	12622600
S3	1042851	1660178	1524227	5196731	17777600	5197984	30403080	2719343	50615130	8939477	4832739	86980370	4707970	12688630	8456236
S4	1090313	1677886	1471081	5277560	19399690	6086027	32367960	3219070	52503400	9442898	4639239	90996350	5060840	13404740	9022368
S5	494409	1707258	1012178	1964792	12599860	9068572	12840530	2114573	20252660	5196736	3415006	27397450	2812444	2223846	4657461
S6	377687	1361889	505425	1383770	9565961	5703830	9050985	2135187	15831030	4228242	2668581	20830790	2226517	1512335	3853265
S7	1005630	2020297	1124153	1324111	2745957	1037924	12161380	4293881	16745640	10130020	1490282	10713790	795094	974743	7506207
S8	3725772	3265809	1700226	1782421	5001104	1926323	18150750	7209348	24095960	13816110	2650752	18279430	1517696	1929608	9862270
S9	4789645	1496680	1088897	2609427	7783705	1836103	15378320	3000062	27726910	3719390	4038660	10600850	1858346	1051839	1075672
S10	2081681	2012845	1258072	6104854	4726589	1756217	25106830	6324889	31140940	12175140	4342553	15388340	2663686	1566654	12909920
S11	1009475	5044275	4981824	8980456	7832077	1679979	20239100	1232842	27956460	10088120	3583160	7581638	2411510	1768543	3426864
S12	539991	1246813	1395077	3339020	5792713	6537969	10119100	1038158	16918200	6086442	3498667	4244850	1387672	1277676	2397336
S13	1073282	3104334	1457609	3330342	10829390	2336208	19051030	2065937	28546320	18097960	2476269	3020927	1656550	1319152	4941862
S14	834072	2230650	1101964	2506267	5977308	2248470	15763480	3303499	23652030	13269390	4166920	16668530	1588264	2195144	8184979
S15	78215	406807	289642	1366162	7358852	2218801	4914775	2029364	11402200	4373288	2059434	3561387	1214008	2242663	2829344
S16	3735616	4131294	3692744	3637990	15040840	7582041	14742060	8614456	26870080	6337620	4120171	32568030	2696462	2470979	3771341
S17	758248	1692993	805211	2196123	3854291	2011978	13384780	1664651	19407120	11419400	3485233	12148210	1546041	2169887	8911191
S18	895582	2712986	1564439	3797054	5717207	2485908	17641420	2964844	26444580	14334510	4073801	18450930	1586563	2001736	8699803
S19	1700518	3435624	9775614	7403278	22762390	8385050	40367420	16165550	57546990	11583180	7505090	94961990	4869560	12836920	9331745
S20	1660901	3415523	10418720	7959734	21174760	7481719	37695700	16837230	55412860	10440330	12587370	88179870	4496130	11530970	8250409
RSD (%)	83.50	48.55	112.31	58.07	59.00	60.85	49.71	90.69	47.86	39.84	55.03	97.87	56.36	104.98	48.76

Chemometric analysis

The HCA results are shown in Figure 4 and were divided into three categories, as follows: Group 1 (S1-S4, S16, S19, and S20), Group 2 (S5, S6, S9, S12, S15), and Group 3 (S7, S8, S10, S11, S13, S14, S17, S18). The PCA score and load plots of PCA are shown in Figure 5. The PCA score plot result was consistent with that of HCA, and the samples were also divided into three categories. The loading plot (Figure 5B) showed that the 15 common chromatographic peaks had different contributions, which were disproportionate the classifications. The coefficients of 3, 4, 7, and 9 in the loading plot were larger than those of the other chromatographic peaks, which indicates that the four chemical compositions had a greater influence on the classification. Therefore, uridine (3), tyrosine (4) uracil (7), and inosine (9) could be used as markers to distinguish PRPCA from different sources.

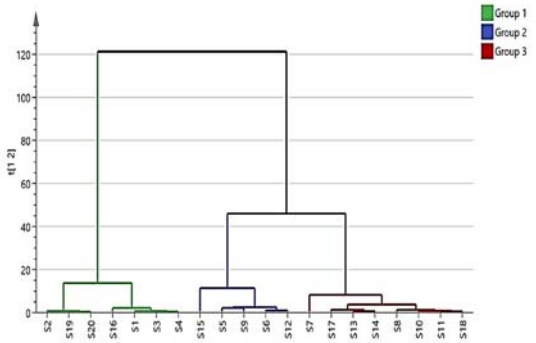

Figure 4: Hierarchical cluster analysis (HCA) of HPLC fingerprint

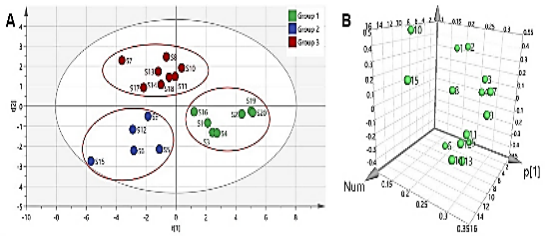

Figure 5: Principal component analysis (PCA) of HPLC fingerprint. (A) score plot, (B) 3D loading plot

Inhibitory effects on MUC5AC mucin production

The effects of the 20 PRPCA batches on the MUC5AC content of LPS-treated NCI-H292 cells are presented in Table 3. Notably, PRPCA decreased the expression of MUC5AC in LPS-treated NCI-H292 cells, and the inhibition rate of MUC5AC expression significantly differed among different PRPCA batches.

Table 3: The inhibitory effects of different PRPCA batches on MUC5AC mucin

Sample No.	Inhibition rate of MUC5AC (%)	Sample No.	Inhibition rate of MUC5AC (%)
S1	52.04 ± 3.16	S11	46.12 ± 3.60
S2	53.16 ± 5.47	S12	35.20 ± 3.24
S3	50.37 ± 4.63	S13	30.12 ± 2.09
S4	54.55 ± 3.85	S14	40.65 ± 3.83
S5	47.90 ± 5.88	S15	26.02 ± 3.95
S6	45.82 ± 2.29	S16	51.18 ± 6.47
S7	32.41 ± 3.72	S17	32.21 ± 3.29
S8	43.33 ± 4.95	S18	44.90 ± 5.52
S9	45.69 ± 4.06	S19	50.71 ± 4.76
S10	49.55 ± 5.51	S20	58.27 ± 6.11

Spectrum-efficacy relationship analysis

After selecting characteristic markers from 15 common peaks of HPLC and inhibition of MUC5AC in NCI-H292 cells of 20 batches of PRPCA, the score plot and classification verification results are shown in Figure 6. As shown in Figure 6A, the PRPCA samples were divided into three categories, as labelled with different colors. Then, the classification results were verified using the extracted variables. A VIP graph is shown in Figure 7. Chromatographic peaks of 5, 6, 10 (vernine), 12(5-hydroxymethylfurfural), 14 (tryptophan) and 15 (adenosine) were selected using VIP. Notably, these samples can be used as pharmacodynamic evaluation indicators for evaluating the inhibitory effects of PRPCA on MUC5AC mucin.

DISCUSSION

The chemical components of TCM are complicated, and affected by their origins and processing methods. The HPLC fingerprint plays an important role in identifying the authenticity, assessing quality, and distinguishing the components of TCM [10].

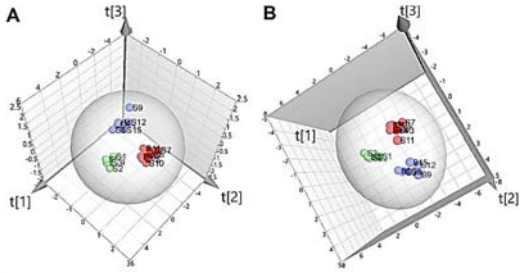

Figure 6: PLS-DA between HPLC common peaks and inhibition rate of MUC5AC in NCI-H292 cells. (A) score plot, (B) classification verification result

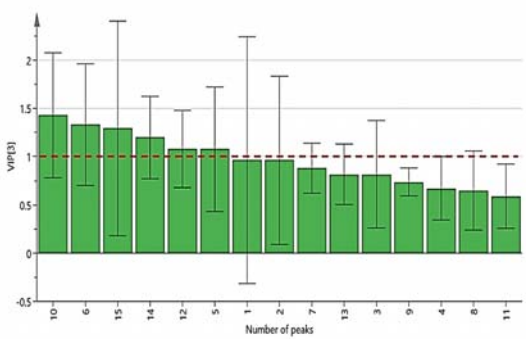

Figure 7: VIP plot of PLS–DA between HPLC common peaks and inhibition % of MUC5AC in NCI-H292 cells

However, HPLC fingerprinting has some limitations, because it cannot be associated with efficacy. The spectrum-effect relationship analysis indicated both the chemical features and the activities of the components [9]. Thus, research on the spectrum-effect relationship is very important for the quality control of TCM [11], and has been widely used to investigate the material basis of TCM. Therefore, in the present study, the spectrum-effect relationship of PRPCA was established to further study the material basis for the inhibitory effect of PRPCA on MUC5AC mucin production.

Chemometric methods, such as HCA and PCA, are typically used to estimate similarities and differences between samples [12]. Certain chemometric methods have also been used to analyze the spectrum-effect relationship, such as PLS-DA, PCA, and grey correlation analysis, *etc.* [9, 13]. Notably, the HPLC fingerprints of 20 PRPCA baches were established and analyzed using HCA and PCA in the present study. The results revealed that four chemical components, uridine (3), tyrosine (4) uracil (7), and inosine (9), could be used as markers to distinguish samples from different origins and processing methods. In addition, PLS-DA showed that peaks 5, 6, 10 (vernine), 12 (5-hydroxymethylfurfural), 14 (tryptophan) and 15 (adenosine) in the fingerprints were closely related to the inhibitory effect on MUC5AC mucin production, and could provide pharmacodynamic evaluation indicators for resolving the phlegm effect of PRPCA.

NCI-H292 cells, a human airway mucus-secreting epithelial cell line, provide a model system for studying lung and bronchial mucus hypersecretion diseases [14,15]. LPS stimulation significantly increases the expression of mucin in NCI-H292 cells, which can be used in the preparation of mucus hypersecretion models to evaluate its inhibitory effect on mucin production [16]. Excessive production of airway mucin, especially the type of MUC5AC, is a typical feature of human asthma and in mouse asthma model [17]. Thus, the regulation of MUC5AC expression in airway epithelial cells provides an important target for resolving phlegm [18]. In this study, the phlegm resolution effects of 20 PRPCA batches were evaluated by the inhibition of MUC5AC in LPS-treated NCI-H292 cells. The results showed that PRPCA decreased the MUC5AC expression in LPS-treated NCI-H292 cells, and MUC5AC inhibition significantly differed among the different PRPCA batches.

CONCLUSION

The spectrum-effect relationship between HPLC fingerprints and the inhibitory effect on MUC5AC mucin of PRPCA was successfully established in the present study. The results further reveal the material basis of PRPCA and provide an effective method for its quality control.

DECLARATIONS

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Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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