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Research Article

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The extract optimization and identification study of bioactive total triterpenoids from the rare traditional Chinese medicine Qinling Polyporusumbellatus

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ABSTRACT

Herein, we have provided a facile, simple and feasible extraction process of Qinling Polyporustriterpenoids, which can set solid research foundation and scientific basis for the further study of Qinling Polyporus , a rare traditional Chinese medicine located in Shaan'xiprovice. Our objective is to study the extraction and identification of Qinling Polyporustriterpenoids as well as the comprehensive utilization of traditional Chinese medicine Polyporusumbellatus found densely in clusters among Qinling Mountains. The content of polyporus total polyporustriterpenes was measured by UV spectrophotometry within vanillin - glacial acetic acid - perchloric acid solution as color developing agent; Various solvent systems were tested in the extraction of triterpenoid compounds to select the best solvent system, such as Different proportions of alcohols, esters and alcohol esters mixtures. The optimum extraction condition was evaluated both by single factor test and orthogonal test, while the structure of the triterpenoids was identified based on the physicochemical constants and spectral data evidences. The optimum conditions for this extraction were identified as adding six times amount of 95% alcohol and refluxing for three times, 2h each time.

Key words: Qinling Polyporus; Triterpenoids; Traditional Chinese medicine; Extraction process; Identification

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INTRODUCTION

The Qin Mountains (simplified Qiling) are a major east-west mountain range in southern Shaanxi province, China. The mountains provide a natural boundary between the North and South of the country, and support a huge variety of plant and wildlife, some of which is found nowhere else on Earth, Such as wild Pandas and Rhinopithecus.

Polyporusumbellatus(**Figure 1**) is a rare, edible species of mushroom, found growing on roots of old beeches or oak[1]. Polyporusumbellatus mainly shall be found in Asia and also can be found in the forests of German but very rare.It is also a traditional chinesemedcine sources found densely in clusters among Qin Mountains[2]. The fruiting body of Polyporusumbellatus is composed of numerous (sometimes several hundred) caps[3]. They are 1–4 cm in diameter, deeply umbilicate, light brown, and form the extremities of a strong, many branched stalk. The compound fungus can be up to 40 cm in diameter. The pores are narrow and white. The stalk is whitish grey, and originates from a strong, tuber like nodule that is underground. The flesh is white, rather soft when young, although hardens with age[4].

As we all know, triterpenoids contain triterpene molecules have antiretroviral, antimalarial, and anti-inflammatory properties, as well as a more recently discovered potential as an anticancer agent, by inhibition of topoisomerase[5]. which can be listed as, Betulinic acid (a naturally occurring pentacyclictriterpenoid), Araloside A, Astragaloside IV, bacoside A, Cucurbitacin, Eleutheroside A (daucosterol)(**Figure 2**)[6-8], additionally, Ginsenosides or Panaxosides are a class of triterpenesaponins, and also steroid glycosides, found exclusively in the plant genus Panax (ginseng)[9]. Polyporusumbellatus contains a wide range of bioactive compounds with immunostimulating, anticancer, anti-inflammatory, and hepatoprotective properties[10]. Triterpenoids and terpenoid derivatives are major pharmacologically active ingredients which have been found in Polyporusumbellatus[11].

Li X. et al has reported that Polysaccharide purified from Polyporus umbellatus induces the activation and maturation of murine bone-derived dendritic cells via toll-like receptor 4[12]. Zhao Y. Y. et al have published articles on new anti-inflammatory ergostane-type ecdysteroids from the sclerotium of Polyporus umbellatus[13]. Studies on constituents of fruit body of Polyporusumbellatus and their cytotoxic activity have never been terminated, especially several cytotoxic steroids from Polyporusumbellatus have also been investigated. Continuously, two new polyporusterones have been isolated from the sclerotia of Polyporusumbellatus by Zhou WW et al.[14-17].

Figure 1, Qinling Polyporusum bellatus

For the comprehensive utilization of traditional chinesemedcinePolyporusumbellatus found densely in clusters among Qinling Mountains, our research purpose focused on the extraction technology of QinlingPolyporustriterpenoids as well as practical identificcation. We want to provided a facile, simple and feasible extraction process of QinlingPolyporustriterpenoids, which can afford a solid scientific basis for the further systematically application of traditional chinesemedcineQinlingPolyporus located in Shaan'xiprovice.

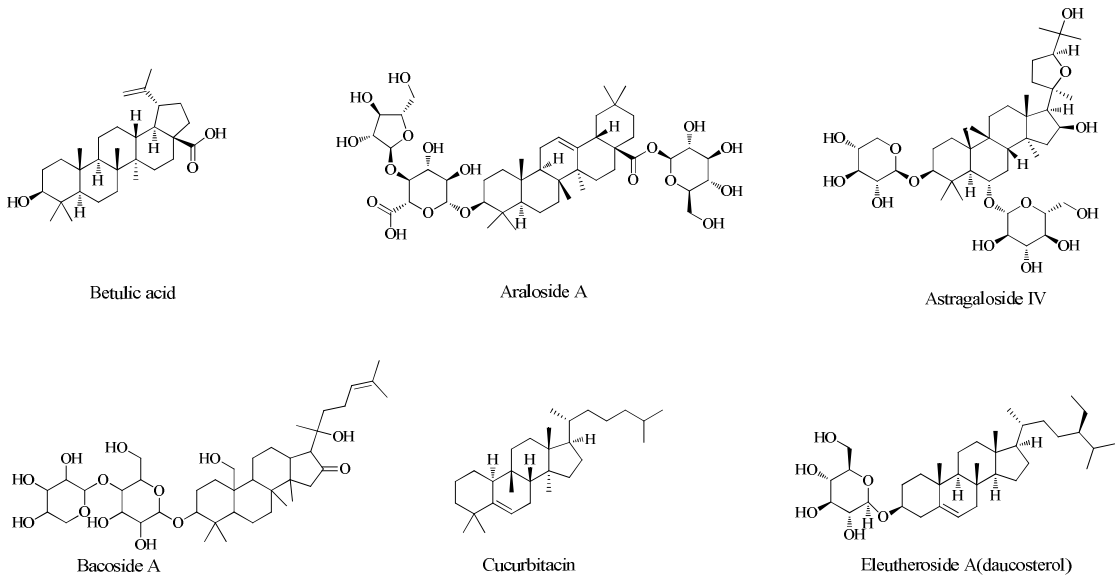

Figure 2,Some naturally occurring pentacyclic triterpenoids.

EXPERIMENTAL SECTION

1.1 Materials

Polyporus were purchased from Xi'an medicine market of Shaanxi Province and identified as the QinlingPolyporus by pharmacognosia experts. the roots of QinlingPolyporus were cutted while the stem and corona were retained, dried, crushed, over 20 mesh sieve.

1.2 Reagents

Standard oleanolic acid for the use of content determination were purchased from National Institute of Standard Pharmaceutical and Biological Products, Beijing, China. distilled water, glacial acetic acid, chloroform, ethanol, perchloric acid, ethyl acetate and petroleum ether, etc. were supplied by commercial chemical companies scuh as Sinopharm Reagents and Sigma-Aldrich (analytical pure).

1.3 Instruments

Shimadzu UV-2550 UV-visible spectrophotometer (supplied by Shanghai International Trade Co., Dan Ding) SZFJ herbal grinder (Guangzhou Xu Long Machinery Co., Ltd.) DK-S26 electric heated water bath (Shanghai Electronic Technology Co., Ltd. Kai ago) R210 rotary evaporator (Switzerland BUCHI Company).

2. Experimental Section

2.1 Extraction of the triterpenoids in QinlingPolyporus

Two kilograms of QinlingPolyporus powder were accurately weighted and added 6 times amount ethanol (95%). The mixture was refluxed, filtrated and extracted for 3 times respectively, 2 hours each time. After filtration, the total filtrate were concentrated to extract under decompression, dried at 80℃, appropriate amount of distilled water was added to wash products, then standing and centrifuged 5min at the speed of 2500r/min, the supernatant was discarded, the precipitate was dried at 80℃ meanwhile. Then 3 times amount of petroleum ether was added, after ultrasonic extraction and filtration, the extract was retained to recover the petroleum ether. The precipitate was washed with chloroform for two times,each time by three times amount of the chloroform, and the chloroform was distilled off by concentration under reduced pressure to obtain relatively pure triterpene compounds, which can be dissolved in the three times amount of ethyl acetate and stored in a conical flask for usage.

2.2 Content determination of the triterpenoids in QinlingPolyporus

2.2.1 Preparation of the reference solution [18]

Oleanolic acid reference substance (10mg) was accurately weight, added in a 10ml volumetric flask, diluted with ethyl acetate to the marked line to afford a concentration of 1.0mg/ml standard solution.

2.2.2 Preparation of the test solution

The sample solution (2 ml) was precisely measured and placed in a 10ml volumetric flask, diluted with ethyl acetate to the marked line.

2.2.3 Chromogenic method [19]

The color developing agent applied on this experiment was prepared by the procedure as follows, 5% vanillin-acetic acid solution plus 2mL of perchloric acid were heated at 65℃ for 20min, then cooled in ice water and warmed up to room temperature after being shaken. Vanillin (500mg) was dissolved in acetic acid(10ml) to prepare the vanillin solution.

2.2.4 The optimum detection wavelength

Different concentrations of the oleanolic acid standard solution were taken and colored according to the chromogenic method, tested in the UV-visible region of spectrophotometer. The results indicated that the maximum absorption of standard solution was at 210nm, consequently, 210nm was chosen as the determination wavelength.

2.2.5 The standard curve

0.0,0.2,0.4,0.8,1.2,1.6,2.0 ml oleanolic acid standard solution were precisely measured, placed in a 10 ml flask with ethyl acetate to volume marked line, the mixture was then shaken, colored according to the chromogenic method.[20] The absorbance (A) of each solution was measured at 210nm wavelength, a blank solution as the control reference. Then, absorbance (A) was taken as the abscissa, the concentration (C) as the vertical axis, the standard curve (Figure 3)was based on the measurement results, the regression equation was: C = 0.1517A-0.0441, correlation coefficient r = 0.9971, which indicated the good relationship of triterpenoids within the linear range of 0.0046 ~ 0.0075mg/ml .

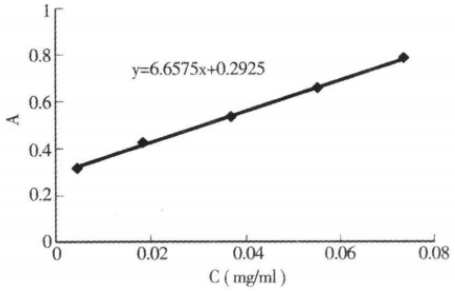

Figure 3, The standard curve of oleanolic acid standard solution

2.3 Optimization of triterpenoids extraction process

2.3.1 Single-factor test

The solid-liquid ratio, ethanol concentration, extraction time and frequency were selected as impact factors to be investigated, indexed by the extraction rate of triterpenoids, single-factor tests were undergone to determine the level of each factor before orthogonal experiment.

2.3.1.1 Solid-liquid ratio

Quadruplicate portions of Polyporus powder were weighed, each part (20 grams) was respectively added to the 4, 6, 8, 10-fold amount of ethanol, the mixture was then extracted three times (2 hours once), the results were concluded in Table 1, of which showed, except solid-liquid ratio polyporus: ethanol=1:6, other different solid-liquid ratios have no significant difference impact on the extraction rates, therefore, six times the amount of ethanol was appropriate.

Table 1, Effects of solid-liquid ratio on extraction rate of Polyporustriterpenoids

Solid-liquid ratio	A	Content (%)
4	0.167	0.35
6	0.194	0.72
8	0.178	0.61
10	0.189	0.68

2.3.1.2 Ethanol concentration

Quadruplicate portions of Polyporus powder were weighed, each part (20 grams) was respectively added to 6 times amount of ethanol, ethanol concentrations: 60%, 80%, 90%, 100%, the mixture was then extracted three times, each time for 2 hours and the results were exhibited in Table 2, which showed that the best extraction effect was conducted by the anhydrous ethanol as the extraction solvent, so the extraction rate was raised when ethanol concentration was increased.

Table 2, Effects of ethanol concentration on extraction rate of Polyporustriterpenoids

Ethanol concentration (%)	A	Content (%)
60	0.151	0.27
80	0.348	1.95
90	0.495	2.69
100	0.583	3.26

2.3.1.3 Extraction time

Triplicate portions of Polyporus powder were weighed, each part (20 grams) was respectively added to six-fold amount of anhydrous ethanol, the mixture was extracted three times, the extraction time was conducted under 1, 2, 3 hours respectively, and the results were shown in Table 3. The highest extraction rate was achieved when the extraction time was 3h, but the extraction rate of 3h had no obvious difference compared with the extraction rate of 2h, taking the efficiency and energy consumption of the experiment into account, 2h was selected in the subsequent experiment.

Table 3, Effects of extraction time on extraction rate of Polyporustriterpenoids

Extraction time (h)	A	Content (%)
1	0.205	0.80
2	0.298	1.65
3	0.314	1.69

2.3.1.4 Times of extraction

Triplicate portions of Polyporus powder were weighed, each part (20 grams) was added respectively to six-fold amount of anhydrous ethanol, the mixture was respectively refluxed for1, 2, 3 times, the extraction time was 2 hours equally, and the results were shown in Table 4. the highest extraction rate was achieved when the times of extraction was three, thus three times extraction was selected in the subsequent experiment.

Table 4, Times of extraction affect on extraction rate of Polyporustriterpenoids

Extraction times (times)	A	Content (%)
1	0.235	0.94
2	0.261	1.35
3	0.293	1.61

2.3.2 Orthogonal experiment

According to the results of single factor test, orthogonal experiment $L_9(3^4)$ was designed for the etraction of QinlingPolyporustriterpenoids, in which solid-liquid ratio (A), ethanol concentration (B), extraction time (C) and extraction times (D) were selected as the four impact factors, each impact factor has three levels, and the extraction rate of triterpenoids was designed as the index, factors and levels were shown in Table 5, the experimental program and the results were concluded in Table 6.

Table 5, Factors and levels of orthogonal experiment

Levels	Factors			
	Solid-liquid ratio **A**	Concentration of ethanol **B** /%	Extraction time **C** /h	Times ofextraction **D** /times
1	4	60	1	1
2	6	80	2	2
3	8	100	3	3

Table 6, Results and analysis of orthogonal test $L_9(3^4)$

Test No.	Factors				Triterpenoids yield/%
	A	B	C	D	
1	1	1	1	1	0.178
2	1	2	2	2	0.332
3	1	3	3	3	0.453
4	2	1	2	3	0.983
5	2	2	3	1	0.191
6	2	3	1	2	1.212
7	3	1	3	2	0.701
8	3	2	1	3	0.560
9	3	3	2	1	0.657
Ⅰ	0.918	1.862	1.950	1.026	
Ⅱ	2.386	1.083	1.972	1.996	
Ⅲ	1.963	2.322	1.345	2.245	
R	0.902	1.205	0.338	1.453	

On the basis of the orthogonal experiment: $A_2>A_3>A_1$,$B_3>B_1>B_2$,$C_2>C_1>C_3$,$D_3>D_2>D_1$, the optimum condition of Polyporustriterpenoids reflux extraction was A_2 B_3 C_2 D_3, in which six-fold amount of anhydrous ethanol was used as the solvent to extract for three times and 2 hours each time to obtain the highest extraction rate. The variance analysis of orthogonal experiment were shown in Table 7, analysis of variance showed that the concentration of ethanol and extraction times had significant impacts on the extraction of triterpenoids in Polyporus, while, extraction time and solid-liquid ratio had no obvious impacts.

Table 7, Variance analysis of orthogonal experiment

Factors	Sum of squared deviations	DOF	F value	P
A	0.050	2	1.000	>0.05
B	0.263	2	19.875	<0.05
C	1.061	2	2.571	<0.05
D	0.685	2	12.498	<0.05

Note: $F_{0.1}(2,2) = 9.00$； $F_{0.05}(2,2) = 19.00$； $F_{0.01}(2,2) = 99.00$

2.4 Identification of triterpenoids

2.4.1 Qualitative analysis of triterpenoids[21]

Appropriate amount of Polyporustriterpenoids, obtained by ethanol extraction, was weighed and dried under 80℃, respectively reacted under the conditions of anhydride - concentrated sulfuric acid (20:1), trichloroacetic acid, glacial acetic acid - ethyl chloride and chloroform - concentrated sulfuric acid for the qualitative identification of triterpenoids, operations and phenomena were as follows:

Acetic anhydride-concentrated sulfuric acid: appropriate amount of sample was dissolved in acetic anhydride,then added concentrated sulfuric acid - acetic anhydride (1:20) , the solution was observed from yellow to red gradually , then to blue and finally faded ;

Trichloroacetic acid: appropriate amount of the sample solution was dropped on the filter paper, spraying 25% alcohol solution of trichloroacetic acid, heated to 100 ℃,the spraying area was observed from red to purple gradually [22];

Glacial acetic acid-ethyl chloride: appropriate amount of sample was dissolved in glacial acetic acid, added a few drops of acetyl chloride, zinc chloride and a few drops of crystal, heated slightly, pale red solutionwas found [23];

Chloroform-concentrated sulfuric acid: appropriate amount of sample was dissolved in chloroform, after addition of concentrated sulfuric acid, was found to exhibit blue with fluorescent in the chloroform layer [24].

2.4.2 UV absorption spectrum of triterpenoids

Preparation of the sample solution (0.05mg/mL): Polyporustriterpenoids extracted by ethanol (0.005g) was dried and weighed at 80℃, then dissolved in ethanol, poured in a 100mL volumetric flask, diluted with ethanol to the marked line. The sample solution was scanned at 190nm~700nm wavelength by UV-2550 UV-visible spectrophotometer to determine the UV absorption changes of the extract. The UV absorption spectrum (Figure 4) showed that the extract obtained from Polyporus by using ethanol as the extraction solvent had absorption between the wavelength range of 200~260nm, and the maximum absorption wavelength was at 240~245nm. It can be inferred that the extract had significant UV spectral features of triterpenoids, and vice versa the absorption spectrum indicated that the extract of QinlingPolyporus involve triterpenoids.

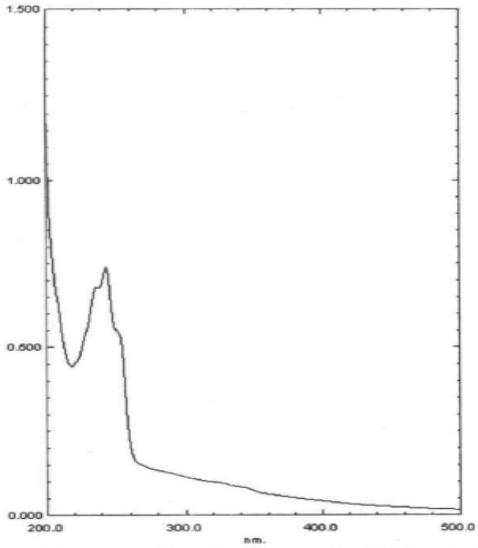

Figure 4. UV spectrum of Polyporustriterpenoids

CONCLUSION AND DISCUSSION

In this study, single factor and orthogonal experiments were applied to investigate the effects of solid-liquid ratio, ethanol concentration, extraction time and extraction times on the extraction of QinlingPolyporustriterpenoids. The optimum extraction condition can be concluded as adding six times amount of anhydrous alcohol and refluxing three times, 2h each time. The extraction process was confirmed to be efficient, feasible, stable and suitable for

polyporustriterpenoids production.
The content of total triterpenoids of QinlingPolyporus was determined by ultraviolet spectrophotometry, the preparation process and usage of chromogenic system (5% vanillin - acetic acid - perchloric acid) should be within 1 hour, otherwise the color effect will fade, which would affect the result of the experiments.

The triterpenoids extract was purely obtained with refluxed ethanol, which provided a theoretical basis for rational development and utilization of Polyporus. However, there are a wide variety of exist triterpenoids, only the determination of total triterpenoids and preliminary qualitative identification is not enough for fully understanding of QinlingPolyporus. It is necessary to do successive isolation and further identification research of triterpenoids ingredients. Sequential investigations has also been carried on in our laboratory for further study of QinlingPolyporustriterpenoids .

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