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ORIGINAL ARTICLE

Content Determination of Phenylpropanoids and Enhancing Exercise Ability of Effective Fractions in Pedicularis densispica

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ABSTRACT

Background: Most researches were focused on chemical constituents and bioactivities of *Pedicularis*. However, there were a few reports on simultaneous determination of the series phenylpropanoids compounds in *Pedicularis* by High Performance Liquid Chromatography (HPLC). **Objective:** To establish an HPLC method for simultaneous determination of salidroside, verbascoside, iso-verbascoside, leucoseptoside A, jionoside D and martynoside in *Pedicularis densispica* (PD), and to assess the enhancing exercise ability of effective fractions of phenylpropanoids (EFP). **Materials and Methods:** The separation was performed on C_{18} column with step-wise gradient elution with water (A)-methanol (B) as the mobile phase at a flow rate of 1.0 mL/min, with detection wavelength at 275 nm (0–4 min) and 330 nm (4–40 min). The EFP were obtained from extracts of PD by resin gradient dilution. The enhancing exercise ability of EFP was exerted in exhaustive swimming and anoxia endurance tests *in vivo*. **Results:** The contents of six marker compounds had good linear relationship in the ranges of 2.10–8.40, 13.60–54.40, 0.93–3.72, 0.53–2.12, 1.50–6.00, 0.37–1.28, respectively, and the average recoveries of the six phenylpropanoids were all in the range of 98–103%. Total contents of phenylpropanoids in EFP were more than 60%. Three medicine groups of exhaustive swimming and anoxia endurance time were higher than those of the water group. **Conclusion:** The analytical method is reliable, simple and accurate, and can be used for the comprehensive quality control of PD. This experiment suggests that PD has the effect of promoting the recovery and elimination of fatigue and improving the exercise capacity.

Key words: Anoxia endurance, effective fractions of phenylpropanoids, enhancing exercise ability, exhaustive swimming, HPLC, *Pedicularis densispica*,

SUMMARY

- A simple, practical and low-cost RP-HPLC method has been developed for the simultaneous determination of six marker phenylpropanoids *in Pedicularis densispica*.
- Three effective fractions of phenylpropanoids groups of exhaustive swimming and anoxia endurance time were higher than those of the water group.
- The separation was performed on C18 column with stepwise gradient elution with water-methanol. The enhancing exercise ability was exerted in exhaustive swimming and anoxia endurance tests *in vivo*.
- This plant has the effect of promoting the recovery and elimination of fatigue and improving the exercise capacity.

Abbreviation used: PD: Pedicularis densispica, EFP: Effective fractions of phenylpropanoids, DAD: Diode array detector, HPLC: High performance liquid chromatography, LOD: Limits of detection, LOQ: Limits of quantification, RSD: Relative standard deviation, BV: Bed volumes

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INTRODUCTION

The *Pedicularis* (Scrophulariaceae) is a very large genus of *ca* 400 species of hemiparasitic herbs.[1] Of these, many are used in the traditional Chinese system of medicines as a tonic for the treatment of general debility, collapse, exhaustion, as well as to invigorate the circulation of blood, to aid digestion, and to improve vitality.[2] Up to now, several major classes of compounds, phenylpropanoids, lignans, iridoids, flavonoids and alkaloids have been reported as bioactive compounds from *Pedicularis*.[3] Among these components, phenylpropanoids glycosides, such as salidroside, verbascoside, jionoside D, and martynoside have been extensively studied for their pharmacological properties because of their high abundance in medicinal herbs.[4-6] Pharmacological studies on phenylpropanoids from *Pedicularis* showed that they had strong scavenging effects on superoxide and anti-oxidant effects.[7-10]

In previous paper,[11] we have reported the isolation and structural elucidation of chemical components from *Pedicularis densispica* (PD) including eight phenylpropanoids. Anti-sports anaemia effects of verbascoside and martynoside from *Pedicularis* in mice indicated that both had the potential of antagonizing sports anaemia, and the mechanism of this effect might be related to preventing red blood cell from free radical damage.[12] In addition, salidroside also showed similar anti-fatigue activities and mechanism.[13,14]

Several publications have reported the analysis of salidroside or verbascoside in other raw materials using HPLC method.[15,16] However, the simultaneous determination of a series of phenylpropanoids in plant extracts is a difficult task due to their structural similarity. In this article, a simple, rapid and accurate HPLC method coupled with a diode array detector (DAD) is described for the analysis of salidroside, verbascoside, iso-verbascoside, leucoseptoside A, jionoside D and martynoside from PD [Figure 1]. The optimal conditions for the analytical method were also investigated for the best resolution and highest sensitivity of detection.

Extraction of compounds from plant materials is one of the most important steps prior to their determination by HPLC. Conventional extractions are usually time consuming and require relatively large quantities of solvents. Reflux and leaching extraction are usually used. In recent years, some novel extraction methods of phenolic compounds

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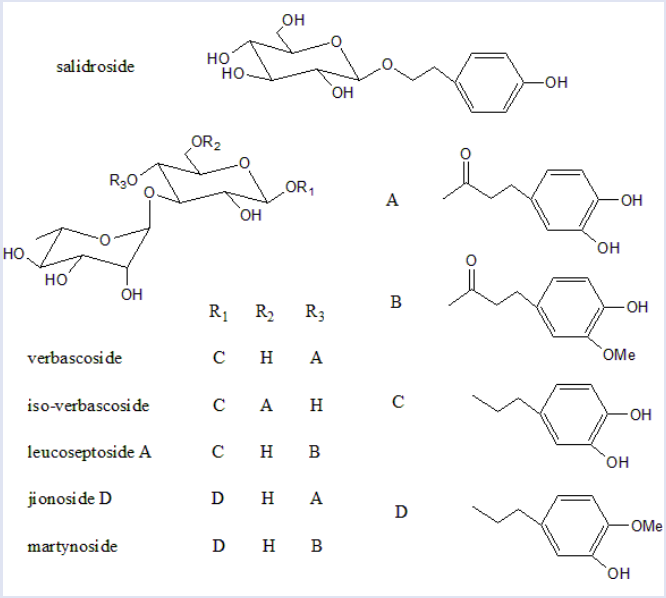

Figure 1: Structures of six marker compounds

have been developed including enzyme-assisted extraction,[17] ultrasound-assisted extraction,[18,19] microwave-assisted extraction,[20] smashing tissue extraction.[21] To compare ultrasound, reflux and smashing tissue extraction efficacy, the contents of each and total marker phenylpropanoids of PD in this experiment were analyzed by HPLC. The efficient method consists of smashing tissue extraction (6 min), followed by the simultaneous determination of a series of phenylpropanoids, which is the first time that this novel method has been investigated.

Fatigue is known to be accompanied by a feeling of extreme physical or mental tiredness, resulting from severe stress and hard physical or mental work.[22] Physical fatigue is thought to be accompanied by deterioration in performance.[23] In the past few decades, health scholars and athletic physiologists have been looking for natural antioxidant components that can not only improve athletic ability, postpone fatigue and accelerate the elimination of fatigue in human beings, but also have few side effects.[24] On the basis of above HPLC analytical method, the effective fractions of phenylpropanoids (EFP) were obtained from extracts of this plant by D_{101} macroporuous resin gradient dilution. The enhancing exercise ability of EFP was exerted in exhaustive swimming and anoxia endurance tests *in vivo*.

MATERIALS AND METHODS

Plant materials

The plant material was collected in Xianggelila, Yunnan Province of China in August 2013 and identified (voucher specimen No. JU2011090) by Prof. Zhaochang Liang (School of Medicine, Jinggangshan University, Ji'an, China). The dried whole herb of PD was powdered and sieved through a number 80 mesh and set aside.

Chemicals and reagents

Methanol of HPLC grade from Fisher (USA) and pure water from Wahaha Co. (Hangzhou, China) were used in the analysis. D_{101} macroporous adsorption resin was purchased from Xingnan YN Co. (Tianjin, China), while Yangke Tibetan Hongtian capsules and soda lime were supplied by Tibet Yangke Biotech Co. (Lhasa, China) and Nahui Dry Reagent Factory (Shanghai, China), respectively. Other reagents were of analytical grade. The standards of salidroside, verbascoside, iso-verbascoside, leucoseptoside A, jionoside D and martynoside (purity >98%) were isolated in our laboratory, and their purity and structures were confirmed by HPLC and by comparison of spectral data to those published in the literature. Structures of the standards were shown in Figure 1.

Animals

Male Kunming mice, weighing between 18 and 22 g, were purchased from Hunan SJA Laboratory Animal Co., Ltd (Changsha, China.), and were fed a commercial diet and water ad libitum. The animals were housed under a 12/12 h light/dark cycle at a temperature of 25 ± 1°C and moderate humidity (50 ± 5). The mice were allowed to acclimate to the laboratory environment for at least one week before the experiments. Ethical clearance for performing the experiments on animals was obtained from Institutional Animal Ethics Committee of Jinggangshan University (20140315).

Preparation of sample solution

The dried and powdered plant sample (50 g) was added with 500 mL of methanol and weight was accurately measured. Then, mixture was sonicated for 30 min at room temperature and repeated twice. The solution was weighed again, and the loss in weight was made up with methanol. The solution was filtered through a 0.45-μm membrane filter, and the filtrated was used as the test solution. Sample solution of 10 μL was injected into the HPLC system. Sample (50 g) was added with 500 mL of methanol and weight was accurately measured. Then, mixture was refluxed for 60 min and repeated twice. The solution was weighed again, and the loss in weight was made up with methanol. The solution was filtered through a 0.45-μm membrane filter, and the filtrated was used as the test solution. The plant material (50 g) was extracted with methanol (500 mL) under smashing tissue extraction for 6 min and repeated twice. Similarly, smashing tissue extraction test solution was prepared for HPLC analysis.

HPLC/DAD Conditions

The HPLC analysis of salidroside, verbascoside, iso-verbascoside, leucoseptoside A, jionoside D and martynoside was performed on a Agilent 1260 HPLC system (Agilent, USA) with a ZORBAX C_{18} analytical column (4.6 mm × 150 mm, 5-μm particle size) (Merck, Darmstadt, Germany). The separation was performed on C_{18} column with stepwise gradient elution with water (A)-methanol (B) (0 ~ 10 min, 63% A; 10 ~ 10.5 min, 63% →58% A; 10.5 ~ 40 min, 58% A) as the mobile phase at a flow rate of 1.0 mL/min, with detection wavelength at 275 nm (0 ~ 4 min) and 330 nm (4 ~ 40 min). The temperature of the column was maintained at 35 °C, volume of injection was 10 μL.

Standard solutions and calibration graphs

Stock solutions of individual reference substance were prepared by dissolving each compound in methanol. The mixed working solutions of the six compounds were prepared by diluting the stock solutions with methanol at a concentration of 420 μg/mL for salidroside, 2720 μg/mL for verbascoside, 186 μg/mL for iso-verbascoside, 106 μg/mL for leucoseptoside A, 300 μg/mL for jionoside D and 74 μg/mL for martynosid. All the solutions were stored at approximately 4C. To construct the calibration curve, six mixed working solutions (5, 7.5, 10, 15, 17.5, 20 μL) were injected into the column. The peak areas were plotted against the corresponding concentrations to obtain the calibration graphs. Linear calibration curves were generated using least-squares linear-regression analysis.

Analytical method validation

The precision was performed by six replicate determinations of six standard solutions, and the percentage relative standard deviation (RSD) of area was calculated. The limits of detection (LOD) and limits of quantification (LOQ) of the analysis method were determined as the analyte concentrations giving rise to signal-to-noise ratios of 3 and 10, respectively. The analysis repeatability was examined by injecting six different samples obtained through the same sample preparation procedure. For the stability test, the same real sample used for analysis repeatability was analyzed after 24 h at room temperature.

Extraction recovery

To assess the extraction recoveries of six marker phenylpropanoids from PD, 6 dried and powdered plant samples (25 g) were added with equal mixed standard solutions before extraction. The follow-up extractions and HPLC analyses were performed in the same manner as described above.

Preparation of EFP and content analysis

The dried and powdered plant material (300 g) was extracted with methanol (2000 mL) under smashing tissue extraction for 6 min and repeated twice. The resulting solvent was eliminated under reduced pressure to obtain a dried extract, with a yield of 12.6 g/100 g of the starting crude material. The dried extract was dissolved with distilled water to get 1000 mL sample solutions. Newly purchased D_{101} macroporous resins were soaked in two column bed volumes (BV) of 95% ethanol for 24 h; after fully swollen, the resins were eluted with ethanol, and then with distilled water until there was no smell of ethanol and 500 mL of pretreated resin was taken and loaded on the column by wet packing method. Sample solutions were added into column loading-treated D_{101} macroreticular resin for adsorption at 5 mL/min. Subsequently, the resin was washed with water and 20% methanol (each 2000 mL), and the eluent was discarded. The resin was then washed with 30%, 50% methanol (each 2000 mL). The eluent was concentrated using the rotary vacuum evaporator and vacuum-dried to obtain EFP. According to above mentioned chromatography method, total phenylpropanoids in EFP were determined using the HPLC.

Exhaustive swimming test

After adaptation, 60 mice were randomly divided into five groups each containing 12 mice. The first group designated as water group (Vehicle) was administered with distilled water by gavage every day. The second group designated as Yangke Tibetan Hongtian capsules group (Control, 180 mg/kg). The third (High), fourth (Medium) and fifth (Low) groups designated as EFP treatment groups were administered with EFP of 40, 20 and 10 mg/kg body/weight day, respectively. The administration of all groups was continued for 7 days. The doses of EFP and 7 days treatment time used in this study were confirmed to be suitable and effective in tested mice, according to preliminary experiments. The mice were allowed to rest for 30 min after the last feeding. Then, tin wire weighing 5% of the body weight of a mouse was attached to the end of each mouse's tail. The mice were put into a swimming tank with water at a depth greater than 30 cm at 25 ± 1.0 °C. The water was agitated to keep the mice swimming until the endpoint of the test, which was defined as the time point when the mice secondly failed to rise to the surface for breathing within 4 s. Time period from the beginning of the swimming to the endpoint was recorded as the exhaustive swimming time.

Anoxia Endurance Test

After adaptation, 60 mice were randomly divided into five groups each containing 12 mice. The Vehicle, Control, High, Medium and Low groups were administered with distilled water, Yangke Tibetan Hongtian capsules (180 mg/kg), EFP of 40, 20 and 10 mg/kg body/weight day. The administration of each group was continued for 7 days. The mice were allowed to rest for 30 min after the last feeding. Then, the mice in the groups were placed into the enclosed 125 mL ground and wide mouthed bottles separately, which contained 7.5 g soda lime for absorption of carbon dioxide and water, and the soda lime was covered with filter paper to absorb urine. There was one mouse in each bottle, and the mouth of bottle was smeared with Vaseline to avoid air leak. The survival time of the mice was observed by taking the last breath as the index.

RESULTS AND DISCUSSION

Optimization of HPLC conditions

HPLC conditions were selected to obtain good resolution on the chromatograms within a short retention time. For optimization of the chromatographic conditions, the effects of the composition of the mobile phase on separation were examined. A mobile phase of water–methanol proved satisfactory for separating the structurally similar, but different solar compounds and resulted in good resolution of all compounds, as well as satisfactory peak symmetry and shape. The main components, such as verbascoside and salidroside, showed maximum absorption at 275 nm. Besides these, other compounds could also be detected well at about 330 nm. The typical chromatograms of the standard mixtures and sample are shown in Figure 2, from which it can be seen that all target compound peaks were clearly separated within 35 min. Therefore, this HPLC system was simple, easy to use, and effective for the identification and quantification of six marker compounds in PD.

Method validation

Calibration curves were plotted using six marker phenylpropanoids peak arearatios for HPLC. The calibration curve constructed was evaluated by its correlation coefficient with an excellent linearity. The analytical procedure was also sensitive with respect to the LOD and LOQ for six marker compounds. All LOD and LOQ values obtained for these six standards were low enough to allow detection of traces of these compounds in either a crude extract or its EFP. Regression data, LODs, and LOQs for six standard substances are presented in Table 1.

The precision was performed by six replicate determinations of six standard solutions. The analysis repeatability was examined by injecting 6 different samples obtained through the same sample preparation

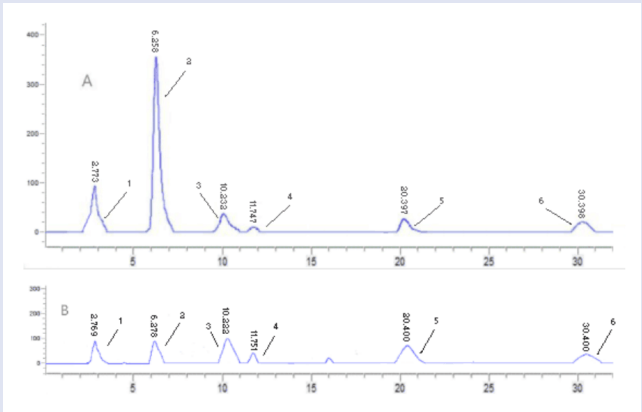

Figure 2: HPLC Chromatograms of reference (A) and sample (B) in Pedicularis densispica

Table 1: Regression data, limit of detections (LODs) and limit of quantifications (LOQs) for six standard substances

Standard substances	Regression equation Y=aX+b[a]	R[b]	Linear range / mg.mL^{-1}	LODs μg.mL^{-1}	LOQs μg.mL^{-1}
salidroside	Y=1301.8X+3.602	0.9998	2.10~8.40	0.07	0.16
verbascoside	Y=5014.2X-922.79	0.9996	13.60~54.40	0.06	0.46
iso-verbascoside	Y=5952.6X-87.106	0.9998	0.93~3.72	0.21	0.63
leucoseptoside A	Y=7760.5X-63.727	0.9998	0.53~2.12	0.10	0.38
jionoside D	Y=2212.1X+30.245	0.9999	1.50~6.00	0.38	0.80
martynoside	Y=15307X-50.802	0.9997	0.37~1.28	0.05	0.19

[a] Y and X stand for the peak area and the injection quantity (μg) of each standard substance, respectively;
[b] R = correlation coefficient.

Table 2: Precision, repeatability, stability and recovery of six standard substances

Standard substances	Precision RSD (%)	Repeatability RSD (%)	Stability RSD (%)	Recovery (%)[a] Mean	RSD (%)
salidroside	0.5	2.5	1.8	98.3	1.4
verbascoside	0.6	2.3	2.4	99.3	1.8
iso-verbascoside	0.8	1.8	1.4	101.2	1.5
leucoseptoside A	0.7	2.2	1.7	98.1	2.2
jionoside D	1.1	1.3	2.3	99.7	1.1
martynoside	0.2	1.5	1.3	102.2	2.1

[a] Recovery (%) = 100 × (amount found-original amount)/amount spiked.

procedure. To evaluate the stability, the sample solution was injected at 0, 4, 8, 12, and 24 h after preparation. The validation data are shown in Table 2. The extract recovery tests of all six markers were performed by adding the standards to the powder, and then extracted and analyzed according to the described procedures. The results indicated that the recovery for all six markers were in the range of 98–103% [Table 2].

Sample analysis

Using optimized experimental conditions, the developed HPLC method was applied for the simultaneous quantitation of salidroside, verbascoside, iso-verbascoside, leucoseptoside A, jionoside D and martynoside in the extracts of PD. Because different extraction methods potentially affect the process and results, in this study ultrasonic, reflux, smashing tissue extraction samples were analyzed by HPLC. The calculated content of the six components expressed as percentage of the label claim are shown in Table 3. The results indicated that main components salidroside, venbascoside, jionoside D and total phenylpropanoids in smashing tissue extraction sample were the highest among three samples. The application of smashing tissue extraction significantly reduced extraction time, extraction temperature, and achieved superior six marker phenylpropanoids yields. Smashing tissue extraction is a new method for extracting and separating active ingredients by smashing tissue extractor, which is effective, rapid, energy-saving, easy to operate, and can be operated at room temperature. These results clearly demonstrated that the method provided a good alternative for the extraction of six phenylpropanoids analogues from PD. In present study, we performed the ultrasonic, reflux and smashing tissue extraction for the establishing optimal method. However, previous reports demonstrated that microwave-assisted extraction is superior to ultrasound-assisted extraction method.[25] The better extraction yield for phenylpropanoids in PD need to be investigating for a broader extraction method.

Preparation of EFP and content analysis

Increasingly, there are good possibilities that natural analogues in a plant extract with variable bioactivity and potency can be retained altogether to exert bioactivity. These compounds may be termed as leading compounds of a bioactive extract. Biologically, a class of analogues may be more bioavailable to reach target receptors. In a bioactive botanical extract, the whole series of natural analogues of a core structure in the extract itself could be used as leading compounds to exert a defined bioactivity. This may be exactly where botanical extracts

Table 3: Each and total contents of phenylpropanoids in *Pedicularis densispica*

Standard substances	Ultrasonic extraction		Reflux extraction		Smashing tissue extraction	
	Each content (%)	Total content (%)	Each content (%)	Total content (%)	Each content (%)	Total Content (%)
salidroside	0.047	0.463	0.060	0.587	0.079	0.808
verbascoside	0.070		0.088		0.117	
iso-verbascoside	0.066		0.083		0.111	
leucoseptoside A	0.003		0.004		0.005	
jionoside D	0.275		0.350		0.466	
martynoside	0.002		0.002		0.003	

as novel therapies have an advantage over single entity drugs.[26] On the contrary, there are several conventional methods, such as polyamide chromatography, gel chromatography, and silica gel column, available for the enrichment of active constituents. However, these methods have several disadvantages, including long time consuming poisonous residual solvents and low recoveries. Recently, growing attention has been taken to enrich and purify targeted components from crude biological samples using macroporous resins for their convenience, low operating costs, low solvent consumption, high chemically stability, and easy regeneration.[27,28] In this study, the absorption and desorption on D_{101} macroporous resins were utilized for the enrichment of EFP from PD.

The eluent, washed with 30%, 50% methanol D_{101} macroreticular resin, was concentrated using the rotary vacuum evaporator and vacuum-dried to obtain EFP 1.68 g. The extraction rate was 0.56% (EFP: 1.68 g/raw plant material: 300 g). Table 4 shows the each marker compound ratio and total ratio in the EFP from PD. The higher amount of compounds were jionoside D, salidroside, verbascoside, which were no less than 38.45%, 5.67%, 4.72% in EFP, respectively. The amount of total six marker phenylpropanoids was more than 60% in EFP extract. This result suggested that EFP enrichment using D_{101} macroreticular resin, resulted in a satisfying phenylpropanoid analogues content.

In-vivo exhaustive swimming of EFP

Anti-fatigue activities of EFP were evaluated on exhaustive swimming model. The model representative of muscular exercise endurance is a reliable model adopted in the study of the anti-fatigue test which gives a high reproducibility. Reduced susceptibility to fatigue is correlated with longer swimming time. As shown in Table 5, the exhaustive swimming time of Yangke Tibetan Hongtian capsules treatment group (Control group) and EFP treatment groups (High, Medium, Low groups) were higher ($P < 0.01$) than that of the water group (Vehicle group), indicating EFP possesses an anti-fatigue activity. Mice of Low, Medium and High group swam longer than that of Control group, and high dose groups are significantly more effective than those of low dose, suggesting phenylpropanoids content might be critical in exerting anti-fatigue activity.

In-vivo anoxia endurance activities of EFP

Fatigue and hypoxia are closely related. In the hypoxia environment, the body is easy to occur a series of free radical metabolism changes, resulting in a decrease in antioxidant capacity, which will increase the oxygen free radicals, which lead to fatigue.[29] The anoxia endurance effect of EFP was evaluated by observing the survival time of mice in the condition of hypoxic tolerance under normal pressure. The data were shown in Table 5. As compared with the water group (Vehicle group), the survival times in the Control group and EFP groups treated with high, medium, low dose were higher ($P < 0.01$), indicating EFP possesses an anoxia endurance activity. Mice of Low, Medium and High group swam longer than Control group, and high dose groups are significantly more effective than those of low dose. However, survival time of Medium group was the longest among all groups. The results indicated that EFP had anoxia endurance effects and these effects were not dose-dependent.

Yangke Tibetan Hongtian capsules were made of extracts of traditional Chinese medicine including Rhodiola rosea, Lycium barbarum, Panax quinquefolium and so on. Total phenylpropanoids is no less than 400 mg/100 g. The animal tests show that the capsules have the effect of improving the ability of hypoxia tolerance and enhancing the immunity. In viewing of similar properties of the capsules and EFP, which are from traditional Chinese medicine, phenylpropanoids constients and anti-fatigue activity, present study was designated as Yangke Tibetan Hongtian capsules control group (180 mg/kg). Our recent research indicated that the mechanism of verbascoside's anti-fatigue activity might be related to the inhibition of the exercise-induced synthesis of 5-HT and TPH2 expression, and to the increase of the 5-HT1B expression in the caudate putamen of exercised rats.[30] It is very interesting that verbascoside is one of the most abundant components of EFP, suggesting that both may have same mechanisms of action.

CONCLUSIONS

In this study, a simple, practical and low-cost RP-HPLC method has been developed for the simultaneous determination of salidroside, verbascoside, iso-verbascoside, leucoseptoside A, jionoside D and martynoside in PD. The results demonstrated the method was highly specific, accurate and precision, and it is promising of being used in quality control of PD. Moreover, EFP enrichment method was developed and used for extraction

Table 4: Each phenylpropanoids and total phenylpropanoids contents in EFP and raw plant material.

Standard substances	Each phenylpropanoid content in EFP (%)	Total phenylpropanoid content in EFP (%)	Each phenylpropanoid content in raw plant material (%)	Total phenylpropanoid content in raw plant material (%)
salidroside	5.67	60.07	0.032	0.34
verbascoside	4.72		0.027	
iso-verbascoside	5.78		0.033	
leucoseptoside A	2.56		0.015	
jionoside D	38.45		0.216	
martynoside	2.89		0.016	

Table 5: Effect of effective fractions of phenylpropanoids on exhaustive swimming and anoxia endurance time of mice ($\bar{x} \pm s$, n=12)

Groups	Dose / g.kg^{-1}	Swimming time / s	Anoxia endurance time / s
Vehicle	-	145.25±27.63	597.58±48.01
Control	0.180	193.50±28.13**	815.58±156.95**
High	0.040	283.75±92.60**##	907.17±178.56**
Medium	0.020	204.33±57.62**	973.58±190.77**§§
Low	0.010	194.50±51.91**	868.00±162.26**

**$p<0.01$ vs vehicle group; ##$p<0.01$ vs control group; §§$p<0.05$ vs control group.

and preconcentration series phenylpropanoids compounds from PD. The exhaustive swimming and anoxia endurance experiment suggests that the herb has the effect of promoting the recovery and elimination of fatigue and improving the exercise capacity. However, further study is needed to optimize the EFP extraction process parameters and elucidate the more exact mechanism of the anti-fatigue effect at the cellular and molecular levels.

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Nil

Conflicts of interest

There are no conflicts of interest

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