



Characterization of botanical origin of selected popular purple *Eleutherococcus* tea grown in Yunnan province of China and quantification of Its anthocyanins using spectrophotometric method

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Abstract

Purple *Eleutherococcus* is a selected plant grown in high mountain in Puer region of Yunnan province, which is a rich source of anthocyanins with great potential as local vegetables and tea. However, the unknown botanical origin of purple *Eleutherococcus* restricted its edible value. Our objectives were to identify the botanical origin of purple *Eleutherococcus* by DNA marker combined with the morphological characteristics, optimize the extraction method of anthocyanins by orthogonal experiment method and determine the contents of anthocyanins in purple *Eleutherococcus* tea. The results revealed that the purple *Eleutherococcus* was more closely related to *Eleutherococcus simonii* through comparing the sequence alignment of ITS2. Combined with the morphological characteristics of plants, purple *Eleutherococcus* was finally identified and classified into *Eleutherococcus simonii*. Through orthogonal experiment, the optimal extraction conditions of anthocyanins from purple *Eleutherococcus* leaves were determined: the volume fraction of ethanol was 60%, the pH of ethanol solution was 3, and the water bath temperature was 60 °C. The ultrasonic extraction was conducted for 30 min. Our study provides scientific theoretical basis for the industrial extraction of anthocyanins and provides reference value for the further development of purple *Eleutherococcus* in Puer region of Yunnan province in China.

Keywords: purple *Eleutherococcus*; botanical identification; extraction optimization; determination of anthocyanins.

Practical Application: Origin of purple *Eleutherococcus* tea and quantification of its anthocyanins.

1 Introduction

As a perennial shrub plant, purple *Eleutherococcus* had a light violet stem and spherical fruit with special aroma, which was mainly distributed in Puer region of Yunnan province and southeast Asia. Its characteristic of the tender tip was purple, sweet aftertaste and no bitter taste (Li et al., 2016). As well known, *Eleutherococcus* plants have anti-inflammatory, anti-rheumatism, anti-arrhythmia, tonic and other medicinal value (Zhao et al., 2014). In addition, the purple *Eleutherococcus* can improve the quality of sleep, benefit vital energy, tonify spleen and relieve mental strain (Wang et al., 2018). Through related content determination, purple *Eleutherococcus* was rich in amino acids, crude fiber, vitamin C and minerals (Zhao et al., 2014). Previous study had shown that purple *Eleutherococcus* was considered as *Eleutherococcus senticosus* (Rupr. et Maxim.) Maxim. However, morphologically characteristic (Figure 1) showed purple *Eleutherococcus* had branches glabrous, usually with a coarsely recurved spine, while *Eleutherococcus senticosus* had branches with dense to scattered, slender, terete, bristlelike prickles (<http://www.iplant.cn/frps>) (Zhai et al., 2017). Therefore, the origin of purple *Eleutherococcus* still needs to be further determined.

Anthocyanins was a class of water-soluble natural pigment that widely present in plants which belongs to flavonoids (Harakotr et al., 2015; Timberlake & Bridle, 1977). The leaves and stem bark of purple *Eleutherococcus* was pale purple, which contained large amounts of anthocyanins. At the same time, anthocyanins were also dozens of times of *Eleutherococcus senticosus*. The complex contents of saccharides, sugar alcohol, organic acids, amino acids, proteins, and other components in purple *Eleutherococcus* affects the stability of anthocyanins, and its liquid viscosity was relatively high, resulting in difficulty in concentration and drying (Abdel-Aal et al., 2014; Fuleki & Francis, 1968). Therefore, it is necessary to study the method of extracting and purifying anthocyanins from purple *Eleutherococcus*.

To reach our objectives, we compared the ITS2 sequence of purple *Eleutherococcus* with other known *Eleutherococcus* species identified by Yook Changsoo professor (Liu et al., 2015), and combining with the plant morphological characteristic analysis to determine the origin of purple *Eleutherococcus* (Bae et al., 2001; Park et al., 2005). In addition, anthocyanins were extracted from the purple *Eleutherococcus* tea by ultrasonic-assisted

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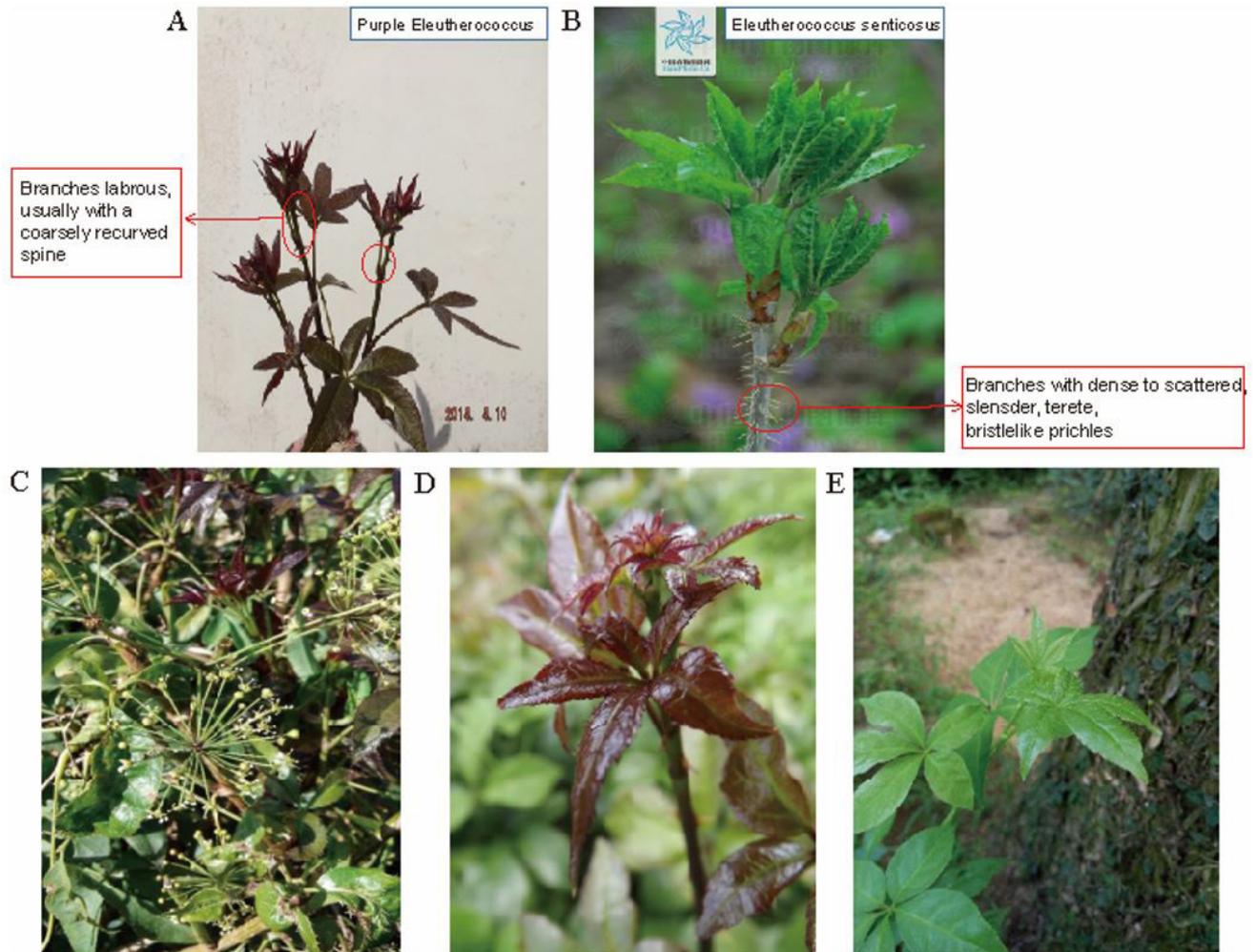


Figure 1. A: Flowers and leaves of purple Eleutherococcus. B: Leaves of *Eleutherococcus simonii*. C: Flowers of purple-Elletherococcus. D: Leaves of purple-Elletherococcus. E: Leaves of *Eleutherococcus trifoliatus* (Linn.) Merr. var. *setosus* Li.

acidification (Jiang et al., 2017). The effective extract conditions of anthocyanins were determined by establishing orthogonal table and related validation experiments (Hiranrangsee et al., 2016). Our study provides a scientific theoretical basis for the industrial extraction of anthocyanins and provides a reference value for the further development of the medicinal value of anthocyanins from the purple Eleutherococcus.

2 Materials and methods

2.1 Samples

Four species of *Eleutherococcus* samples (*Eleutherococcus trifoliatus* (Linn.) Merr. var. *trifoliatus*, *Eleutherococcus gracilistylus*, *Eleutherococcus simonii*, *Eleutherococcus trifoliatus* (Linn.) Merr. var. *setosus* Li) were kindly provided by the Wuhan Botanical Garden of the Chinese Academy of Sciences and identified by Yook Changsoo professor (Liu et al., 2015). *Eleutherococcus koreanum* Nakai was provided by Medicinal botanical garden of

KyungHee University in South Korea. Purple Eleutherococcus was sampled from Puer region of Yunnan province, China. All Chemicals were purchased from Hangzhou Gaojing Fine Chemical (Hangzhou Gaojing Fine Chemical Co., Ltd, Hangzhou, Zhejiang, China).

2.2 DNA marker identification of purple Eleutherococcus

Total genomic DNA was extracted from single individuals using cetyltrimethylammonium ammonium bromide (CTAB method). The nuclear rDNA fragment that included part of the 5.8S rDNA gene, the second internal transcribed spacer (ITS2), and part of the 28S rDNA gene was amplified by PCR. Amplification was performed in a T100™ Thermal Cycle (Bio-Rad Laboratories, Inc. Hercules, California, USA). The reaction mixture (DNA, PCR Forward Primer, PCR Reverse Primer, ddH₂O, PCR mix), using ITS2F, ITS3R (Supplementary Table 1) as primers, and temperature profiles (94 °C 30 s, 56 °C 30 s, 72 °C 45 s for 35 cycle) used in amplification of nuclear spacer fragment. The amplification products were separated by electrophoresis

purchased from Tanon (Tanon Science & Technology Co., Ltd. Shanghai, China) on a 1% agarose gel in 1 TAE buffer in the presence of Gel imager (Tanon Science & Technology Co., Ltd.) and photographed. The obtained PCR products were purified using a Biosilicareagent kit and sequence by using forward and reversed primers on an ABI 3130xl automated sequencer (Thermo Fisher Scientific Inc. Waltham, MA, USA) at the (<http://sequest.niboch.nsc.ru>).

2.3 Ultrasonic-assisted extraction

Five of the 1.00 g of purple *Eleutherococcus* tea powder samples were mixed with 40 mL of 60% ethanol, and the mixture was directly sonicated in the ultrasonic extraction tank (Kun Shan Ultrasonic Instruments Co., Ltd Jiangsu, China) for 30 min at 40 °C and at a frequency of 50 kHz. After extraction, suction filtration (by centrifuge for 10 min) was used to obtain the filtrate. The supernatant of 100 mL was absorbed with locomotive pipette (Eppendorf AG, Inc., Hamburg, Germany), diluted to 1 mL with two prepared buffers, and Abs was measured with enzyme-labeled instrument (BioTek Instruments, Inc. Winooski, VT, USA) at dual wavelengths (510 nm and 700 nm). The anthocyanins yield was estimated according to the method of "2.5". Measurements were done in triplicate and take the average.

2.4 Optimization of ultrasonic-assisted extraction condition

Firstly, the effects of ethanol concentration (50% to 90% v/v), extraction time (20 to 60 min), extraction temperature (30 to 70 °C) and pH of solvent (1.5, 2, 2.5, 3, 3.5 mL/g) on the extraction yield of anthocyanins from purple *Eleutherococcus* tea powder were investigated. Secondly, an orthogonal test

was designed to optimize the extraction parameters (factors) depending on the results of the above single factor experiments. The factors and levels tested in this study are presented in Table 1. The orthogonal test design consisted of nine separate experiments (Table 2). The sequence in which the experiments were performed and randomized to ensure the validity of the test results. In this study, all the experiments were performed in triplicate and IBM SPSS Statistics 20.0 was used to analyze the data in this study.

2.5 Quantification of its anthocyanins using spectrophotometric method

Use locomotive pipette to absorb 100 mL of liquid. Dilute the hydrochloric acid-sodium chloride buffer solution and acetic acid-sodium acetate buffer solution to 1 mL and mix them thoroughly. Using a 96-well plate added to each sample 200 mL into three wells for each mixed sample. Abs was measured at double wavelengths (510 nm and 700 nm) with an enzyme-labeled instrument (BioTek Instruments, Inc.) (correction optical path difference was 1 cm). We referred the method from Professor Wang Y (Ravanfar et al., 2015; Wang et al., 2016) and made appropriate improvements. The anthocyanins content of each sample solution was calculated using the Equation 1 below:

$$\text{Total anthocyanins (mg/g)} = (A \times Mt \times DF \times V) / (e \times b \times m)$$

$$A = \left\{ (A_{510} - A_{700})_{pH\ 1.0} - (A_{510} - A_{700})_{pH\ 4.5} \right\} \quad (1)$$

where

A Absorbance of sample at 510 nm and 700 nm

Table 1. Orthogonal experimental factors and levels.

Levels	A. Ethanol volume fraction (%)	B. pH	C. Ultrasonic extraction bath temperature (°C)	D. Ultrasonic extraction time (min)
1	50	2.5	50	30
2	60	3	60	40
3	70	3.5	70	50

Table 2. Orthogonal experimental results.

Number	A. Ethanol volume fraction (%)	B. pH	C. Ultrasonic extraction bath temperature (°C)	D. Ultrasonic extraction time (min)	E. Anthocyanin content (mg/g)
1	1	1	1	1	0.494
2	1	2	2	2	0.728
3	1	3	3	3	0.334
4	2	1	2	3	1.015
5	2	2	3	1	1.476
6	2	3	1	2	1.196
7	3	1	3	2	0.975
8	3	2	1	3	0.928
9	3	3	2	1	1.069
K1	0.519	0.828	0.873	1.013	
K2	1.229	1.044	0.937	0.966	
K3	0.991	0.866	0.928	0.759	
R	0.710	0.216	0.064	0.254	

Mt Relative molecular mass of Cyanidin-3-glucoside-449.2

DF Dilution factor

V The known volume anthocyanins extract was made up to after extraction (mL)

e Extinction coefficient of Cyanidin-3-glucoside-26900

b Optical path difference (cm)⁻¹ cm

m The weight of purple *Eleutherococcus Senticosus* tea powder for extraction (g)

3 Results and discussion

3.1 Origin identified of purple *Eleutherococcus* source by DNA molecular marker

We extracted the DNA of eight species of *Eleutherococcus* (*Eleutherococcus trifoliatus* (Linn.) Merr. var. *trifoliatus*, *Eleutherococcus gracilistylus*, *Eleutherococcus simonii*, *Eleutherococcus trifoliatus* (Linn.) Merr. var. *setosus* Li, and *Eleutherococcus koreanum* Nakai.). Using ITS2F and ITS3R as amplification primers and

comparing with 2000 DNA Marker, it was found that there were obvious amplification bands around 500 bp (Figure 2).

Selected the following procedures according to the company's sequencing results. Use Geneious10 software for the *de novo* connection and alignment. K3P model was selected to construct Iqtree (Nguyen et al., 2015; Trifinopoulos et al., 2016). *Boehmeria gracilis* C. H. Wright and *Macropanax rosthornii* (Harms) C. Y. Wu ex Hoo were selected as the outer group. The composition of PCR amplification products was analyzed, and the sequences at both ends of the amplification products were removed. Use Treegraph software to modify and view the phylogenetic tree (Figure 2) (Müller & Müller, 2004). The ITS2 sequences of each sample were obtained and uploaded to GenBank. The different *Eleutherococcus* samples of each species were first grouped into one branch. On this basis, *Eleutherococcus simonii* and Purple *Eleutherococcus* were clustered as one branch, and then together with *Eleutherococcus trifoliatus* (Linn.) Merr. var. *setosus* Li were clustered as one branch, indicating that these three *Eleutherococcus* were closely related. The ITS2 sequence of No.1-6 purple *Eleutherococcus* samples were identical, with a sequence length of 433bp. The homology of purple *Eleutherococcus*

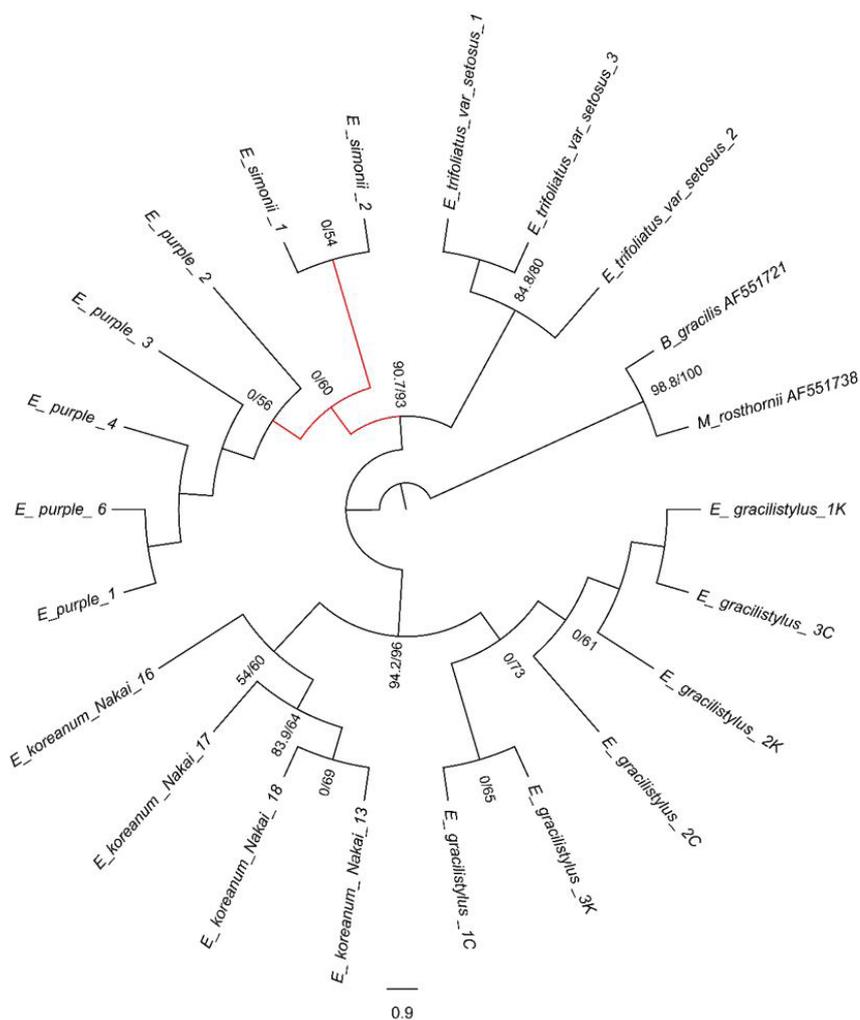


Figure 2. Phylogenetic tree of purple *Eleutherococcus* compared with other *Eleutherococcus* plants.

(No. 1-6) and the corresponding sequence of *Eleutherococcus simonii* in GenBank (No: GQ434775) was 100% identical.

We observed and compared the leaves and flowers of purple *Eleutherococcus*, there were 5-loculeds of the ovary, when it is fruiting the stigma lobes are separated with the styles united into columns. The umbel forms a compound umbel with longer petiole, strong and curved branches. Leaf blade of purple *Eleutherococcus* was larger with densely setae on bilateral veins, doubly serrate of margin. The morphological feature was similar to *Eleutherococcus simonii* (Figure 1). Meanwhile, with the phylogenetic relationship analysis of phylogenetic trees, we classified purple *Eleutherococcus* as *Eleutherococcus simonii*.

3.2 Optimize extraction of anthocyanins in purple *Eleutherococcus* tea by orthogonal experimental method

Ethanol volume fraction

Pictures was drawn by Graph Pad Prism 6 software (Graph Pad Software Inc, CA). As shown in Figure 3A, the anthocyanins yield increases first when the ethanol concentration changes from 50% to 60% (v/v) and goes down when the ethanol concentration is above 60%. When the volume fraction of ethanol

above 60%, some impurities will also be extracted, affecting the dissolution of anthocyanins and the final determination of anthocyanins content. Therefore, the optimal ethanol volume fraction determined as 60%.

pH in ethanol solution

In Figure 3B, when pH increased from 2 to 3, anthocyanins yield increased with the increase of pH of the extracted solution. When pH was 3, anthocyanins yield reached the maximum value, which was 1.335 mg/g. Anthocyanins contain phenolic hydroxyl groups, which are more water-soluble. When the acidity is too strong, the hydrogen bond formed between the phenolic hydroxyl group and the water molecule is easy to break, thus affecting the solubility of anthocyanins and reducing the extracted anthocyanins. When the acidity is relative weak, anthocyanins change to the colorless chalcone form, reducing anthocyanins content. Therefore, the most moderate pH determined to be 3.

Ultrasonic extraction temperature

The Figure 3C reflects the trends that the temperature rises from 30 °C to 60 °C, the yield of anthocyanins increased from 1.303 mg/g to 1.798 mg/g, and with the continued rise

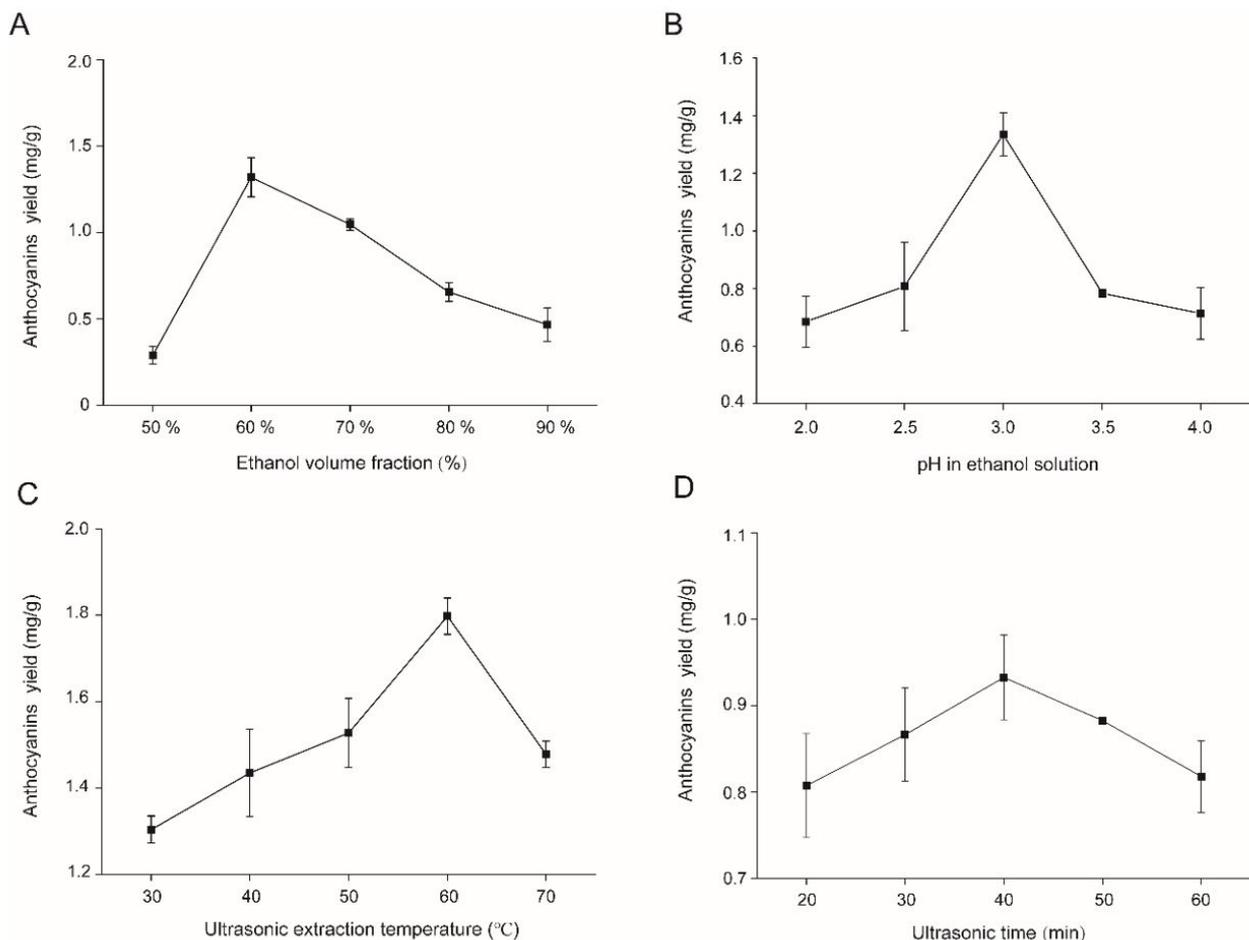


Figure 3. The line chart of ultrasonic-assisted extraction affected the anthocyanin content of each sample.

Table 3. Verification test results.

Number	Abs	Anthocyanin content (mg/g)	Average (mg/g)	RSD
1	0.276	1.844	1.797	2.27%
2	0.265	1.770		
3	0.266	1.777		

of temperature the yield inversely decreased. The temperature affects the random motion of molecules, the rising temperature accelerates the dissolution rate, and more anthocyanins dissolved. Moreover, high temperature damage the chemical structure of anthocyanins, reducing the active components, leading to oxidation and degradation phenomenon. Therefore, the optimal ultrasonic extraction temperature determined under the experimental conditions is 60 °C.

Ultrasonic time

The effects of the ultrasonic time on the extraction yield of anthocyanins from purple *Eleutherococcus* tea were investigated. The results are shown in Figure 3D. The yields significantly increased when the extraction time increased from 20 to 40 min and then decreased from 40 to 60 min. A longer extraction time indicated a positive effect on the extraction yield, but the yield increased slightly. This phenomenon may be due to the active ingredients will not be dissolved when the solubility of dissolving-out substances became saturate 40 min with the increase of extraction time, while the loss of anthocyanins was increased with the viscosity of extracts increased when extraction time increased. Therefore, in view of time consumption, extraction time ought not to exceed 40 min.

Orthogonal experimental method

Through the establishment of orthogonal table and the relevant experiments, we found that the influence of four single factors on the anthocyanins content of each sample in purple *Eleutherococcus* was quite different manifest as follows: ethanol volume fraction > ultrasonic time > pH > water bath temperature, which the influence degree of pH and ultrasonic time is similar. In this study, the best combination to extract anthocyanins from purple *Eleutherococcus* was determined as A2B2C2D1.

Moreover, the orthogonal experiment was carried out as Table 2 to verify the content of anthocyanins in purple *Eleutherococcus* tea. Table 3 shows that the content of anthocyanins in the validation test is higher than that in the extraction scheme designed by orthogonal test, which indicates that the optimal extraction technology obtained by orthogonal test is feasible. The results obtained have a small standard deviation and high precision with three repeats.

4 Conclusion

In this study, DNA molecular marker technology was performed to determine the origin of purple *Eleutherococcus*, combining the morphological characteristics of plants. As a result, the species of purple *Eleutherococcus* was identified

and classified as *Eleutherococcus simonii*. The application of DNA marker identification provides technical support for the development of Chinese medicine identification and makes an important contribution to complete the Flora of China.

Anthocyanins of purple *Eleutherococcus* was a class of water-soluble natural pigment in plants belongs to flavonoids. The toxicity and economic benefits of the solvent should be considered in the experimental process, ethanol was chosen as the extraction solvent. The efficiency of the experiment was greatly improved because the enzyme-labeled instrument could simultaneously measure the two wavelengths. This method was more rapid and accurate in measurement, providing reference for the determination of anthocyanins.

Through relevant tests, we found that 60% ethanol had the best extraction effect on anthocyanins with the best permeability and least impurities under such conditions. According to relevant literatures, we found pH = 3 is the mildest acidity to extract anthocyanins, pH breaks the balance between the four structures of anthocyanins, making them transform into each other. As temperature influences effective components of anthocyanins by affecting the stability of anthocyanins, 60 °C is the optimal extraction temperature of extract anthocyanins. The optimal ultrasonic time determined by the experiment was 40 min which affecting the oxidation ability of anthocyanins. In conclusion the optimal technology to extracted anthocyanins from purple *Eleutherococcus* as follows: 60% ethanol volume fraction, pH 3, 60 °C for ultrasonic extraction and water bath temperature, 30 min for ultrasonic extraction time. The content of anthocyanins from purple *Eleutherococcus* is 1.797 mg/g, which was much higher than 935.14 µg/g that Jin-wen Liu (Liu et al., 2015) extracted anthocyanins from *Eleutherococcus senticosus* fruit. Therefore, we speculated that purple *Eleutherococcus* had higher contents of anthocyanins, which indicated greater underlying application for medical and food industry.

Conflict of interest

There are no conflicts of interest to declare.

Author contributions

Xiaodan Zhang and Dongfeng Yang designed the study and interpreted the results. Yue Chen collected test data, drew diagrams and drafted the manuscript. Majie Gong, Xianxian Nie and Yue Chen cooperatively completed the experiments in this study. Zhechen Qi assembled ITS2 sequence and constructed Iqtree. Xiangqian Liu and Qinghao Jin sampled *Eleutherococcus* samples.

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Supplementary Material

Supplementary material accompanies this paper.

Supplementary Table 1 Sequencing primer.

Supplementary Table 2 Submitted Genebank ID.

This material is available as part of the online article from <https://www.scielo.br/j/CTA>