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Phytochemicals, Nutritional Analysis and *In vitro* Antioxidant Activities of Pickled *Perilla frutescens* Ethanolic Leaf Extract

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Authors' contributions

This work was carried out in collaboration between all authors. Authors JW and ZW designed the study. Authors YL and JG managed the analyses of the study. Author YW performed the statistical analysis and author YG managed the literature searches. Authors CL and YL wrote the manuscript. All authors read and approved the final manuscript.

Original Research Article

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ABSTRACT

Aims: With pickled perilla leaves as raw materials, this paper proposed the optimal ethanol extraction conditions and made a profound analysis for extract in the compositions of major active ingredients, nutrients, mineral elements and amino acid to characterize the nutritional and biological properties of pickled perilla leaves, which could aid its finely processing and future application in the development of functional food.
Methods: The optimum ethanol extraction process for preparing freeze-dried powder from pickled perilla was studied by means of orthogonal experiments, with the concentration of ethanol, extracting temperature and extracting time as factors. Meanwhile, the contents of the activity components, such as polysaccharide, flavones and rosmarinic acid, as well as the mineral elements and nutritional contents in the freeze-dried powder were determined according to the methods reported by relevant literatures without or with a few modifications.
Results: The optimal extracting conditions as follows: 50ºC of temperature, 60 min of

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extraction time and 80% of ethanol concentration. Under the optimal extracting conditions, the extraction rate of the freeze-dried powder was 1.71%. Moreover, perilla leaf extract contained rich biological and nutritional ingredients, including 33.39% of flavonoids, 9.24% of polysaccharides, 22.79% of rosmarinic acid, 5.47% of protein, 7.61% of fat, 2354 mg/kg of Ca, 111.4 mg/kg of Fe, 5.045 mg/kg of Zn, 1817 mg/kg of K , 12.66 mg/kg of Mn and nine of essential amino acids. In addition, perilla leaf extract exhibited obvious scavenging effects on the DPPH•, •OH and O_2^-•.

Conclusion: In summary, pickled perilla leaf ethanol extract was rich in biological ingredients as well as a variety of nutrients, and showed antioxidant activities in vitro, thus it is valuable and promising in the development of functional foods in the future.

Keywords: Perilla freeze-dried powder; nutritional contents; mineral elements; activity components; antioxidant activity.

1. INTRODUCTION

Perilla frutescens (L.) Britt. belonging to the family Lamiaceae (Labiatae) is native to mountainous areas of China and India and is grown mainly in Asia [1]. *Perilla frutescens* with red coloured leaves is an edible plant, frequently used as one of the most popular spices and food colorants in some Asian countries such as China, Japan and India [2]. Moreover, *Perilla frutescens* has been used as a traditional medicinal herb in China for centuries to treat various diseases including cough, bacterial and fungal infections, allergy [3], depression, anxiety, tumor [4] and some intestinal disorders [5]. Thus, *Perilla frutescens* is a kind of “drug homologous food” and is one of the first batch materials which are identified as both food and medicine by Chinese Ministry of Health. Since the various beneficial properties ascribed to *Perilla frutescens* are associated with consumption of the leaves and seeds, their functional constituents have been found, such as flavonoids, rosmarinic acid, triterpene acids and other constituents [6,7].

The leaves of *Perilla frutescens* have a unique flavor and several studies have also suggested that the extract of *Perilla frutescens* leaves is useful in preventive treatment of several human diseases [8]. Since the picking fresh leaves are easy to decay, the *Perilla frutescens* leaves are usually salted for a long storage. Salted vegetable are also appropriate for extraction of plant nutrients or bioactive compounds. However, there are little research which focuses on investigating the component characteristics and biological activities of alcohol extractive from salted *Perilla frutescens* leaves.

Thus, in this study with pickled perilla as raw materials, we proposed the optimal extraction process with ethanol and made a complete detailed analysis of main nutrients, mineral elements and major active ingredients (flavonoids, polysaccharide and rosmarinic acid) for the alcohol-extract. We also did further research on the antioxidant capacity of the alcohol-extract, in order to get a better understanding of the potential health value of it. Our study is useful for understanding the nutritional and biological properties of salted *Perilla frutescens* leaves and aids its finely processing.

2. MATERIALS AND METHODS

2.1 Materials

Pickled perilla leaves were provided by Qianwei Trade Co., Ltd. (Shanghai). Rosmarinic acid standard (Aladdin's reagent). The standard rutin was purchased from Sigma-Aldrich, Shanghai, China. Other chemical reagents employed were of the analytical reagent grade.

2.2 Preparation of Perilla Freeze-dried Powder

300 g of pickled perilla leaves was mixed with deionized water in proportion of 1：10 (w/v, g/mL) and stirred for 15 min, then the liquid was removed by using an 80 mesh sieve. The drained perilla leaves was desalinated again according to the above same desalination steps and the salinity of the perilla leaves was determined by salinity meter and its value was reduced to 0.2.

Ethanol-water solution was added into desalted perilla leaves in proportion of 1:2 (w/v, g/mL) and the mixture was ground by using a beater machine. The serosity was stirred at a speed of 170 rpm/min in a water bath. Then the serosity was filtered with an 80 mesh sieve, the filtrate was centrifuged at 6000 rpm for 20 min and the supernatant was collected, concentrated in vacuum and finally lyophilized. The obtain freeze-dried perilla powder was closed packed with moisture-proof plastic bag or aluminum foil bag inside and stored at 4ºC for futher analysis.

2.3 Optimization of Alcohol Extraction Conditions by Orthogonal Experiment

Based on the preliminary experiments, the main affecting factors for the yield of perilla alcohol extract were found to be concentration of ethanol solution, extraction time and extraction temperature. In order to optimize the extraction conditions, the orthogonal experiment ($L_9(3^4)$), including three-factors and three-levels (Table 1) was used to get the best extraction conditions.

Table 1. Factors and levels of the orthogonal experiment

Level	Factor		
	A	B	C
	Ethanol concentration (%)	Extraction time (min)	Extraction temperature(°C)
1	40	20	40
2	60	40	50
3	80	60	60

2.4 Determination of Phytochemical Constituents of Perilla Extract Powder

The functional phytochemical constituents of perilla extract powder, including flavonoids, polysaccharides and rosmarinic acid were analyzed in this paper.

The content of total flavonoids in freeze-dried perilla powder was determined at 500 nm by ultraviolet spectrophotometry with rutin as reference material according to the procedures described by Chen et al. [9].

Total polysaccharides content in freeze-dried perilla powder was determined using the phenol-sulfuric acid assay by ultraviolet spectrophotometry with glucose as a standard according to the procedures described by Pierre et al. [10].

The spectrophotometric method by Lopez-Arnaldos et al. [11] enables selective quantitation of rosmarinic acid in plant extracts by the formation of a blue-colored complex with ferrous ions. In this assay, 0.2 mL of perilla extract at a concentration of 1 mg/mL was mixed with 4 mL HAC-NaAC buffer solution (pH 6.0) at a concentration of 0.1 M. The mixture was added with 30 µL FeSO4 solution and 0.77 mL of deionized water to a final concentration of ferrous ions of 1 mM. The dye reaction was developed for 1 h at 25ºC in dark and the absorbance was determined at 572 nm. The concentration of rosmarinic acid in perilla extract powder was evaluated from a calibration curves of rosmarinic acid reference material plotted in a concentration range of 50-300 µg/mL.

2.5 Determination of Nutrients of Perilla Extract Powder

A traditional Soxhlet extraction method with diethyl ether (AOAC Method 960.39) was used for crude fat analysis. Crude protein content was analyzed by the Kjeldahl method (AOAC Method 978.04). The moisture content was determined according to AOAC Method 945.15. Mineral elements were determined by using Inductively Coupled Plasma Atomic Emission Spectrometry (ICPAES, USA) according to the method of Wang et al. [12]. The composition of amino acids was analyzed by an L-8900 amino acid automatic analyzer (Hitachi, Japan) according to the same procedure described in our previous research paper [13].

2.6 In vitro Antioxidant Capacity Assay of Perilla Extract Powder

2.6.1 DPPH• radical scavenging assay

Scavenging ability of perilla extract for the DPPH• free radical was assayed according to Roy, et al. [14] with some minor modifications. A 5 ml aliquot of perilla extract water solution sample at the concertration of 0.2 mg/mL was mixed with 1.6 mL of 3.08 x 10^{-3} mol/L DPPH• in ethanol solution and then added ethanol solution to the mixture to a final volume of 10 mL. The decrease in absorbance was determined at 517 nm using a UV800 UV-visible spectrophotometer (Beckman Coulter) when the reaction reached a plateau (after 30 min of reaction in dark). This experiment was conducted in triplicate. The DPPH• radical scavenging capacity (DRSC) was calculated as follows:

$$DRSC\ (\%) = (A_0 - A_S)/A_0 \times 100$$

Where A_0 and A_S were the absorbance of samples at 517 nm with and without perilla extract, respectively.

2.6.2 Hydroxyl radical scavenging assay

Hydroxyl radical scavenging activity of perilla extract was assayed by the method of Sudha et al. [15] with some minor modifications. The reaction mixture 10.0 mL contained 0.85 mL of 7.5 mM $FeSO_4$, 0.85 mL of 0.2% hydrogen peroxide, 0.85 mL of 7.5 mM sodium salicylate and varied concentrations of the extracts. After incubation for 30 min at 37ºC, the absorbance of the hydroxylated salicylate complex was measured at 510 nm. The scavenging activity of hydroxyl radical (SCOR) was calculated as follows:

$$SCOR\ (\%) = [1 - (A_1 - A_2)/A_0] \times 100$$

Where A_0 is absorbance of the control (without extract) and A_1 is the absorbance in the presence of the extract, A_2 is the absorbance without sodium salicylate.

2.6.3 Superoxide anion (O_2-•) radical scavenging assay

The superoxide anion scavenging ability at pH 8.2 of all test samples was determined by the method as described by Li et al. [16] with some minor modifications. Briefly, sample was dissolved in ethanol at a concentration of 0.6 mg/mL. 1.5 mL of sample was mixed with 7.3 mL Tris-HCl buffer (0.05 mol/L, pH 8.2) containing EDTA (1 mmol/L) and 0.2 mL pyrogallic acid (7 mmol/L), then shaken rapidly at room temperature. The absorbance at 325 nm of the mixture was measured (UV800 UV-visible spectrophotometer, Beckman coulter) against the Tris-HCl buffer every 30 s for 5 min. The slope of the correlation of absorbance with time was calculated. The reaction mixture without added sample was used as the control. The superoxide anion scavenging ability (SASA) was calculated as follows:

$$SASA\ (\%) = (1 - F_s/F_o) \times 100$$

Where F_s is slope of sample, F_o is slope of control.

3. RESULTS AND DISCUSSION

3.1 Ethanolic Leaf Extract Yield from Pickled Perilla

The orthogonal experiment for optimization of the extract conditions of wet pickled perilla (300g for each experiment) was carried out and the results and statistical analysis were shown in Table 2.

Table 2. Statistical analysis of the results from orthogonal experiment

Trial number	Factor level			Weight of perilla extract powder (g)
	A	B	C	
1	1	1	1	3.141
2	1	2	2	3.592
3	1	3	3	3.950
4	2	1	2	4.017
5	2	2	3	4.293
6	2	3	1	3.815
7	3	1	3	3.981
8	3	2	1	4.574
9	3	3	2	4.750
k1	3.561	3.713	3.843	
k2	4.041	4.153	4.120	
k3	4.435	4.172	4.075	
R	0.874	0.459	0.277	

The R value in Table 2 showed that ethanol concentration had the most significant effect on the yield of perilla extract powder and the order of importance that influenced yield was found to be as follows: A>B>C. According to the statistic k value at the different level, the

optimum extraction condition was $A_3B_3C_2$, namely 80% of ethanol concentration, 60 min of leaching time and 50ºC of leaching temperature.

The weight and yield of perilla freeze-dried powder under the optimal extraction condition was tested as 5.133 g and 1.71%, respectively. The perilla extract powder was purple and possesses the unique flavor of perilla.

The extract ratio of fresh perilla extracting in boiling water was about 20% according to the standard formation process of Kampo decoctions [17]. Obviously, the extract yield in this study was lower than the literature value. The low yield could be attributed to the water-soluble material loss during the curing and leaching processings. But curing processing was useful to store the perilla leaf and give special flavor to the final power produce.

3.2 Phytochemical Constituents of Perilla Leaf Extract

Phytochemical constituents of perilla extract powder, including total flavonoids, polysaccharides and rosmarinic acid were quantitative analysis based on the standard curves with the reference material of rutin, glucose and rosmarinic acid, respectively. The standard curve and its relative coefficient value were shown in Table 3.

Table 3. The contents of flavonoids, polysaccharides and rosmarinic acid of perilla freeze-dried powder

	Standard curve	R2	Content %
Flavonoids	Y=12.252x-0.0043	0.9989	33.39±0.71
Polysaccharides	Y=10.159x-0.0186	0.9969	9.24±0.07
Rosmarinic acid	Y=0.0004x-0.0063	0.9998	22.79±0.28

Results showed that the standard curves showed a good linear relationship with the absorbance within the concentration range measured ($R^2 > 0.99$). The results of quantitative analysis suggested that perilla extract powder contained high levels of flavonoids and rosmarinic acid and the concentration was high up to 33.39% and 22.79% respectively. The concentration of polysaccharides also reached up to 9.24%. All of these showed that the active substance was very rich in perilla extract powder.

Feng et al. had pointed out that total flavonoids of perilla had hypolipidemic and antioxidant effects, which increased antioxidant enzyme activity and repressed development of hyperlipidemia in experimental rats [18]. These findings implied that the perilla extract powder with 34% flavones obtained in this study could be developed as a valuable food additive used for hyperlipidemia prevention and oxidation resistance. Moreover, polysaccharides of perilla had found to be as immunopotentiator and biological response modifiers in vitro and in vivo levels [19]. The content of polysaccharides in perilla extract powder was close to 10%, which made it beneficial to the body's immune regulation. In addition, report shown that rosmarinic acid of perilla has liver protection in D-galactosamine (d-GalN)-sensitized mice due to scavenging or reducing activities-superoxide or peroxynitirite [20]. It meant that perilla leaf extract powder with 23% of rosmarinic acid could be used as an effective antioxidant.

In a summary, the contents of above three bioactivators in perilla extract powder were relatively high. Since they had a variety of physiological functions in human health, the perilla extract powder showed important application in the development of functional food.

3.3 Nutrient Analysis of Perilla Leaf Extract

The main nutrient ingredients of perilla extract powder were analyzed and results were shown in Table 4. The content of crude fat in perilla freeze-dried powder was similar to the value reported in the literature. But the same thing was not happened in the content of crude protein, which was much smaller than the literature value. The reason why the proteins lost in extract might the chemical change during the curing process and the denaturation during ethanol extraction process. Moreover, after being freeze-dried treatment, the water content of extract was reduced to 7.68%.

Table 4. The concentration of main ingredient of freeze-dried powder from perilla (x±s)

Composition	Determined content %	Literature values%
Crude protein	5.47±0.16	23.78 [21]
Crude fat	7.61±0.11	7.62 [21]
Water	7.68±0.05	—

3.4 Mineral Element Compositions of Perilla Leaf Extract

The mineral elements of perilla extract powder were analyzed and results were shown in Table 5. Data revealed that perilla leaf extract contained some essential mineral elements highly, such as Ca, Fe, Zn, K, Mn, whose contents were high up to 2354 mg/kg, 111.4 mg/kg, 5.045 mg/kg, 1817 mg/kg and 12.66 mg/kg, respectively. However, the contents in general plant were 0.1 mg/kg of Zn, 500-1000 mg/kg of Ca, 500-1000 mg/kg of K and 1 mg/kg of Mn [22], which were significantly lower than these in perilla leaf extract.

Table 5. The content of mineral elements in perilla leaf extract (mg/kg, ppm)

Elements	Content	Elements	Content
Al	15.35	K	1817
Ca	2354	Mg	48.36
Co	0.4387	Mn	12.66
Cr	2.551	Na	611.6
Cu	55.35	P	1341
Fe	111.4	Zn	5.045

The high contents of above-mentioned mineral elements in perilla leaf extract play many important roles in keeping human body health [23]. For instance, Ca is one of the most abundant elements in human body and an important part of human skeleton. It plays a very important role in physiological process such as nerve, muscle stress, nerve impulse sensing etc. Intake deficiency of Ca would cause a series of diseases including the reduction in serum calcium and crisp bone. K is also an essential element of life, which helps maintaining a stable blood pressure and nerve conduction activities. Zn is essential to almost every organization in the human body and it is related to the composition and metabolism of more

than 80 kinds of enzymes in the human body. It can also participate directly in the synthesis of nucleic acid and protein.

3.5 The Amino Acids Composition of Perilla Leaf Extract

Amino acids are the basic unit of protein and constitute the basic material that animal nutrition desired. They play an important role in human nutrition and physiology. Experiments showed that perilla leaf extrat was rich in amino acids and assay results were shown in Table 6. Data showed that as many as 16 kinds of amino acids were detected in perilla leaf extrat, among them nine of essential amino acids were found, including Isoleucine, Leucine, Lysine, Methionine, Phenylalanine, Threonine, Valine Histidine and Arginine. So, the protein derived from perilla had a high nutritional value.

Table 6. The concentration of amino acid of perilla leaf extract (%)

Amino acids	Content	Amino acids	Content	Amino acids	Content
Aspartic acid	0.170	Valine *	0.092	Lysine *	0.082
Threonine *	0.078	Methionine *	0.016	Arginine *	0.053
Serine	0.101	Isoleucine*	0.060	Histidine *	0.020
Glutamic acid	0.254	Leucine *	0.117	Cysteine	0.040
Glycine	0.160	Tyrosine	0.081	Tryptophan *	—
Alanine	0.094	Phenylalanine *	0.118	Proline	—

**The human body essential amino acids*

3.6 The Scavenging Ability for the DPPH• of Perilla Leaf Extract

DPPH• is a relatively stable aromatic free radical, which needs resonance stabilizing effect and steric hindrance of 3 benzene rings, that keeps the unpaired electrons folded in the mid-carbon atoms from playing its due role of electronic pairs. DPPH• has single electron which has strong absorption at 517nm and whose ethanol solution was purple. And if other material can provide an electron to be paired with the single one, the absorption will disappear and solution would be reduced to light purple. Thus DPPH• is currently widely used in the study of antioxidants [24,25]. So this paper directly evaluated the strength of the antioxidant activity of perilla leaf extract by determinating the extent of decline that the reaction system at 517nm.

The results of determination were shown in Fig. 1, perilla leaf extract (freeze-dried powder) showed obvious the DPPH• scavenging ability within the measured concentration range. The ability of scavenging significantly increased with the increase of the amount. But the clearance rate was lower than that of Vitamin C at the same concentration.

IC_{50} is generally used to measure the scavenging ability of samples. The smaller the value of IC_{50} is the stronger the ability of scavenging free radicals. The IC_{50} of Vitamin C was 0.0226 mg and that of perilla freeze-dried powder was 0.1482 mg.

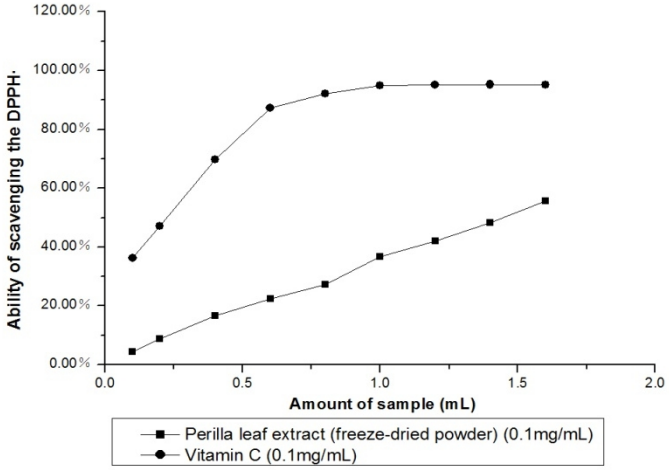

Fig. 1. Scavenging effects of perilla leaf extract (freeze-dried powder) and Vitamin C on DPPH•

3.7 The Scavenging Ability for •OH of Perilla Leaf Extract

•OH is currently known as the most harmful free radical and has the strongest organism toxicity. It can react with most of intracellular organic compounds and leads to series of chain reactions in order to destroy nucleic acids, amino acids, proteins and lipids, farther damage the cell structure and function. However, this damage is the basis of the initiation and promotion of cancer and other diseases [26]. So, the scavenging ability for •OH of perilla leaf extract was determined and results were shown in Fig. 2. Data showed that the scavenging ability of perilla leaf extract freeze-dried powder significantly increased with the increase of the sample amount. But the scavenging effect is lower than that of Vitamin C at the same concentration. The IC_{50} of Vitamin C was 0.267mg and that of perilla freeze-dried powder was 1.099mg. It could be seen that perilla freeze-dried powder have a stronger ability of scavenging DPPH• than scavenging •OH according to the value of IC_{50}.

3.8 The Scavenging Ability for O2-• of Perilla Leaf Extract

The superoxide radical (O_2^-•) is a kind of main free radicals in human body and studies have shown that O_2^-• is related to aging, cancer, inflammation and mutation [27]. The results from Table 7 shown that perilla leaf extract (freeze-dried powder) displayed somewhat antioxidant capacity on O_2^-•. But the antioxidant capacity was much lower compared with Vitamin C. The antioxidant capacity on O_2^-• of Vitamin C was 10 times as large as perilla freeze-dried powder at the same concentration.

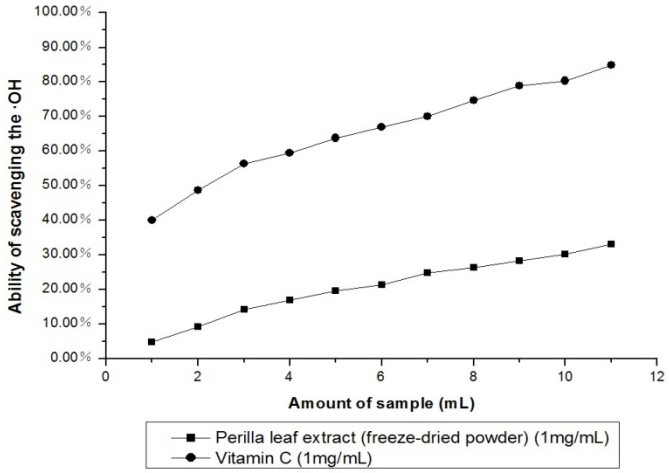

Fig. 2. Scavenging effects of perilla leaf extract (freeze-dried powder) and Vitamin C on hydroxyl radicals

Table 7. Scavenging effects of perilla leaf extract (freeze-dried powder) and Vitamin C on superoxide radical

Item	Scavenging rate（%）	
Vitamin C（0.1mg/ ml）	48.93±3.06	$p<0.05$
Perilla solution（0.1mg/ ml）	4.67±0.35	

4. CONCLUSION

Ethonal extract from pickled perilla leaf was rich in nutrients and active ingredients, mainly including flavonoids, polysaccharides, rosmarinic acid, protein, amino acids and a variety of beneficial minerals. Both the freeze-dried powder and solution of perilla leaf extract were gorgeous purple and possess the unique fragrance flavor of perilla, and could be processed into nutritional tasty foods. The vitro studies found that perilla leaf extract had a certain scavenging effects on the DPPH•, •OH and O_2^-•. But the scavenging capacity was weaker than Vitamin C at the same concentration. The scavenging activity of perilla leaf extract was different towards different free radicals, which illustrated it's selectively scavenged among different free radicals. Since free radicals could lead to a range of diseases such as cancer, arteriosclerosis, diabetes, cataracts and so on, perilla leaf extract had obvious antioxidant capacity, a unique flavor and nutrient-rich, it can be applicative to the development of functional foods in the future.

CONSENT

Not applicable.

ETHICAL APPROVAL

Not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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