**Title:**

Analysis of nutrient composition and determination of active substances of *Craterellus cornucopioides.*

**Abstract:**

**Keywords:**

*Craterellus cornucopioides*

Nutritional composition

Polysaccharides

Antidiabetic activity

**Abstract**

1. **Introduction**

Diabetes is a group of metabolic diseases characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both, and caused a series of health problems1. Diabetes is the result of an integrated metabolic disorder that leads to higher cardiovascular disease morbidity and mortality2. At the same time, diabetes can lead to many complications, including retinopathy that may lose vision, kidney disease leading to renal failure, neuropathy, etc1. Epidemiological studies have shown that the risk of diabetes and its complications is mainly affected by daily diet3. Reasonable supplementation of edible fungi in the diet will be of great benefit to the prevention and treatment of diabetes and vascular diseases4.

Edible fungi are an ideal dietary supplement for people with diabetes because they contain very low fat and cholesterol and are rich in protein, vitamins and minerals5. Many edible fungi with hypoglycemic activity have been reported so far, and many edible fungi have also been reported to have other medicinal activities, such as anti-tumor, blood pressure lowering and immunity enhancement6.

Although there have been some reports on the nutrient composition of *Craterellus cornucopioides*, such as protein content, amino acid composition and so on7. However, there is still a lack of systematic and complete coverage of its nutrients.

Therefore this study will systematically evaluate the nutrient composition and anti-diabetic activity of *C.cornucopioides*, determine the main active substances in the extract, and separate and purify the active substance and preliminary structure identification. Finally, the results of in vitro and animal experiments are combined to establish the theoretical basis of the structure-activity relationship. This study not only provides an important reference for nutritious diet, but also has important academic and practical significance for the development and utilization of wild edible fungi resources.

1. **Materials and methods**
   1. *Materials and chemicals*

The fruiting body of *Craterellus cornucopioides* was purchased from Sichuan Provinces of Southwest China. The obtained materials were centrifuged, lyophilized, and a part of the material was passed through a 60 mesh sieve to obtain a dry powder of the gray *Craterellus cornucopioides*. Sreptozocin (STZ) and glibenclamide were purchased form Sigma-Aldrich Co.LLC. (USA). All other chemicals used in this study were analytical reagent grade and purchased from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China).

* 1. *Chemical composition*
     1. *Proximate analysis*

The moisture content was obtained by heating fresh samples at 105 ° C until the weight was constant. The ash content was obtained by weighing the residue after 24 hours of incineration at 550 ° C. The crude protein content obtained by using Kjeldahl method. The crude fat content was obtained by Soxhlet extraction using petroleum ether as a solvent. The total carbohydrate content is obtained by calculating the total mass of the sample minus the mass of crude protein, crude fat and ash. Finally, the total energy of the sample is calculated by the following formula:

Total energy (kJ) = 17 × (g crude protein + g total carbohydrate) + 37 × (g crude fat).

* + 1. *Amino acid analysis*

Amino acid was determined by reversed-phase high performance liquid chromatography (HPLC, Agilent 1100) equipped with a Hypersil ODS C18 column (4 mm × 125 mm, Agilent) with a gradient elution at a flow rate of 1 mL/min. The column temperature was 40 °C. O-Phthalaldehyde (OPA, Sigma) was used as a derivatization reagent, and the detection wavelength was set to 338 nm (262 nm for detection of proline). Standard amino acids were all configured with 0.1 M HCl (except for tryptophan in ultrapure water) to a stock of 1 mM concentration. The content of each amino acid in the sample is calculated from a standard curve drawn from a standard. All samples were analyzed three times and averaged.

* + 1. *Mineral composition*

1 g of the sample powder was placed on a porcelain crucible and placed in a muffle furnace (500 ° C, 24 h) to completely ash the sample. After cooling, 2 mL of concentrated hydrochloric acid and 25 mL of distilled water were added, and the mixture was filtered through a filter paper, and the filtrate was collected and stored for use. The concentrations of Fe, Zn, K, Na, Ca, Mn, Cu and Mg were determined by flame atomic absorption spectroscopy (FAAS) using SpectrAA 220 (Varian, USA); and the contents of Pb, As and Cd were measured by graphite furnace atomic absorption spectroscopy (GFAAS) using SpectrAA 220Z (Varian, USA); the concentration of P was measured by molybdenum blue spectrophotometry.

* 1. Determination of major hypoglycemic substance
     1. *Preparation of extract*

The extraction method of edible fungus alcohol extract, aqueous extract and crude polysaccharide is slightly modified as described in Wang et al. Approximately 25 g of the fresh sample after lyophilization was placed in 250 mL of 95% ethanol for overnight so as to prepare an alcohol extract; Two portions of the ground fresh 25 g sample were treated in 250 mL boiling water for 2 hours (continuous stirring), one portion to prepare an aqueous extract, and the other portion to separate the polysaccharide in the aqueous extract by a Sevage method to obtain a crude polysaccharide extract.. The above there extracts were filtered through a filter paper, and then rotary evaporated at 50 ° C to obtain a corresponding concentrate. Finally, the concentrate was lyophilized, and the lyophilized powder was stored at -80 ° C until use.

* + 1. *α-glucosidase inhibitory activity*

The method for measuring the α-glucosidase inhibitory activity is slightly modified based on the method of Palanisam et al. α-glucosidase was dissolved in 2 mL of phosphate buffer (0.1 M, pH 6.8) to achieve an enzyme concentration of 0.2 IU/mL, followed by 50 μL of glutathione (1 mg/mL) and 50 μL of p-nitrophenol glucopyranoside (pNGP, 0.1 M), and finally add the appropriate amount of the alcohol extract, aqueous extract, crude polysaccharide, negative control (blank) or positive control (acarbose). The mixed solution was placed in a 37 ° C water bath (15 min). The reaction was stopped by the addition of 10 mL of 0.1 M sodium carbonate, and the final reaction solution was placed on a spectrophotometer (400 nm). All samples were analyzed three times and averaged. Calculated according to the following formula:

EC=[(Anegative - Asample )/Anegative]×100%

Wherein, EC represents the inhibition rate; Anegative is the absorption value of the negative control; Asample is the absorption value of the sample. The final experimental result is expressed as EC50: when the inhibition rate of α-glucosidase is 50%, the effective concentration (μg/mL) of the inhibitor is added.

* + 1. *α-amylase inhibitory activity*

The method for determining the α-amylase inhibitory activity is described in the method of Palanisam et al., with minor modifications. The α-amylase was dissolved in 0.5 mL of 20 mM phosphate buffer (pH 6.9) to bring the enzyme concentration to 2 IU/mL, and various dilutions of the alcohol extract, aqueous extract, crude polysaccharide, negative control (blank) or positive control (acarbose) were added. The mixed solution was placed in a 37 ° C water bath and warmed for 15 min. Then 0.5 mL of potato starch solution (1.5%) was added to the solution, and a constant temperature bath at 37 ° C for 5 min. Finally, 1 mL of DNS reagent was added, and the reaction solution was placed in boiling water at 100 ° C (10 min), and after rapid cooling, the absorbance of the final product was measured with a spectrophotometer (520 nm). All samples were analyzed three times and averaged.Calculated according to the following formula:

EC=[(Anegative - Asample )/Anegative]×100%

Wherein, EC represents the inhibition rate; Anegative is the absorption value of the negative control; Asample is the absorption value of the sample. The final experimental result is expressed as EC50: when the inhibition rate of α-amylase is 50%, the effective concentration (μg/mL) of the inhibitor is added.

* 1. *Preparation of crude Polysaccharides*

The water soluble polysaccharide was prepared by slightly modifying the previous method. The powder (8 g) was immersed in 95% (v/v) ethanol for 12 hours to remove residual low molecular weight components. The materials were then extracted with hot water (1:20, w/v) at 85 ° C for 3 hours. The supernatant was evaporated under reduced pressure at 45 ° C using a rotary evaporator, and the protein was removed using a Sevag reagent (chloroform: n-butanol, 4:1 (v/v)), and the resulting liquid was dialyzed against tap water for 24 hours, and dialyzed (Mw cutoff 3000 Da) against distilled water for 12 hours. Finally, the liquid was concentrated by precipitation with 4 volumes of 95% (v/v) ethanol at 4 ° C for 24 hours. The precipitate obtained by centrifugation (2654 × g, 10 min, 4 ° C) was finally lyophilized to obtain a crude polysaccharide.

* 1. *Preliminary characterization of Polysacchrides*
     1. *Molecular weight determination*

The Molecular weight determination was measured by high-performance gel permeation chromatography (HPGPC) with an Agilent 1100 HPLC system equipped with Waters 2410 refractive index detector and a TSK-GEL G5000 PW x 1 column (7.8 × 300 mm, Tosoh Corp, Japan). Ultrapure water as the mobile phase, it flowed at a rate of 0.8 mL/min and a temperature of 30 °C. A 20 μL material of polysaccharide solution (2.0 mg/mL) was injected in each run. A standard curve was created using a dextran standard in 3.0 to 670 kDa (Sigma).

* + 1. *Monosaccharide composition*

The monosaccharide composition was determined by gas chromatography (GC), and 10 mg of the polysaccharide sample was dissolved in 2 M TFA and hydrolyzed at 110 ° C for 2 h. After removing the TFA, vacuum dry. And 10 mg of hydroxylamine hydrochloride and 0.5 mL of pyridine were placed in a stoppered tube, heated in an oven at 90 ° C for 30 min, cooled to room temperature, and 0.5 mL of acetic anhydride was added. The reaction was carried out for 30 min for acetylation at 90 ° C. The obtained reaction product can be subjected to gas chromatography analysis. The type of monosaccharide of the sample is determined according to the retention time of each peak of the sample; the proportional relationship between the monosaccharides is determined according to the ratio of the area of each peak.

* 1. *FT-IR and ultraviolet*

The FT-IR spectrum of the polysaccharide was obtained using Fourier transform infrared spectroscopy (Nexus 5DXC FT-IR, Nicolet). The polysaccharide (about 1 mg) was ground with 100 mg of KBr powder, compressed into pellets, and then scanned for FT-IR measurements of the frequency range of 400-4000 cm-1. A UV-visible (UV) absorption spectrum was obtained using a UV-visible spectrophotometer (UV-2450, Shimadzu, Japan).

* 1. *Determination of antidiabetic activity in vivo*
  2. *Statistical analysis*

All data were expressed as mean ± SD, one-way ANOVA was performed by R version 3.5.0 software, and multiple comparisons of Tukey were carried out. Differences were considered to be statistically significant for P < 0.05.

1. **Results and discussion**
   1. *Chemical composition*
      1. *Proximate composition*
      2. *Amino acid composition*
      3. *Mineral composition*
   2. *Determination of antidiabetic activity in vivo*
   3. *Analysis of polysaccharides*
2. **Conclusion**

**Conflict of Interest**

**Acknowledgments**

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