**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 250 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 250 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*

The proximate chemical content of *C.cornucopioides* is presented in Table 1. The results of this study are very similar to the previous reports2. It is worth noting that *C.cornucopioides* has a higher protein and total sugar content, while the ash and crude fat content is lower. Therefore, the total energy of *C.cornucopioides* is low, so the edible fungus is important for the prevention of cardiovascular disease.

**Table 1** Moisture (g/100 g fw), macronutrients (g/100 g dw) and total energy (kJ/100g dw).

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Moisture | Ash | Crude protein | Crude fat | Total carbohydrate | Total energy |
| 86.56 ± 0.58 | 8.44 ± 0.32 | 21.57 ± 0.62 | 2.06 ± 0.07 | 67.93 ± 0.63 | 1579.71 ± 6.35 |

Each value is expressed as mean ± SD (n = 3). And fw and dw represent fresh weight and dry weight respectively.

* + 1. *Amino acid composition*

The amino acid composition and content of edible fungi are shown in Table 2. According to previous reports, general edible fungi contain 7-17 known amino acids. This is basically consistent with the results of this study. The results of the study showed that the ratio of essential amino acids to non-essential amino acids of *C.cornucopioides* was 0.82. According to the recommendations made by the FAO, the standard for the intake of protein in foods with a ratio of essential amino acids to non-essential amino acids of 0.6 or higher is required to meet the standard. Studies have shown that the protein composition of edible fungi is more nutritional than most plant proteins.

**Table 2** Free amino acid composition of *Craterellus cornucopioides*

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Amino acidA | Content | Amino acid | Content | Amino acid | Content |
| ValB | 0.41±0.16 | PheB | ND | Ser | 16.79±0.98 |
| ThrB | 6.37±0.58 | LysB | 8.09±0.61 | Cys | 0.34±0.07 |
| MetB | 12.74±1.03 | Gly | 5.71±0.22 | Arg | ND |
| LeuB | 17.52±0.48 | His | 22.07±2.89 | Ala | 0.86±0.07 |
| IleB | 0.08±0.02 | Tyr | 0.82±0.05 | Glu | 7.86±0.56 |
| TrpB | ND | Asp | 0.54±0.09 |  |  |

A Valine (Val), Threonine (Thr), Methionine (Met), Leucine (Leu), Isoleucine (Ile), Tryptophan (Try), Phenylalanine (Phe), Lysine (Lys), Glycine (Gly), Histidine (His), Tyrosine (Tyr), Asparagine (Asp), Serine (Ser), Cysteine (Cys), Arginine (Arg), Alanine (Ala), Glutamate (Glu)

|  |
| --- |
|  |

B Essential amino acid; C Each value is expressed as mean ± SD; D ND indicates that it has not been detected.

* + 1. *Mineral composition*

The mineral content of the gray horn fungus is shown in Table 3. The content of the mineral elements to be tested in the sample from large to small is: K>P>Ca>Mg>Na>Fe>Zn>Cu>Mn>Gd>Pb>As (μg/g dw). Edible fungi are considered to be one of the ideal sources of supplemental minerals in the human body. All minerals measured by the Institute are in compliance with NRC/NAS recommended dietary intake standards. It can be seen from Table 3 that K is the highest content mineral element. The ratio of Na element to K element is one of the important indicators for measuring physical health. According to previous reports, the Na/K ratio of edible fungi is between 0.01 and 0.06. Lower Na/K ratios are more in line with nutritional requirements, as excessive intake of sodium chloride or high-salt foods may increase the risk of cardiovascular disease. Gd, Pb, As are toxic heavy metals, in which the content of Gd and As are lower than the highest level of heavy metals in the body proposed by the European Economic Community Committee (Gd 2.0μg/g, As 2.0 μg/g), and the Pb element is not even Was detected.

**Table 3** Mineral composition of the *C.cornucopioides* (μg/g of dw)

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Elements | Content | Element | Content | Element | Content |
| Fe | 412±16 | Zn | 62±12 | K | 36610±10132 |
| Na | 1180±147 | Ca | 1255±241 | Mn | 27±4 |
| Cu | 43±11b | Mg | 978±34 | Pb | ND |
| Cd | 2.07±0.22 | As | ND | P | 7130±872 |

A Each value is expressed as mean ± SD (n = 3); B ND indicates that it has not been detected.

* 1. *Determination of major hypoglycemic substances*

In order to gain a deeper understanding of the hypoglycemic activity of *C. cornucopioides*, to achieve its further industrial application. Therefore, it is necessary to determine the main hypoglycemic active substance of *C. cornucopioides.* Silva et al. believe that the main active substance in edible fungi is the polysaccharide in its aqueous extract, followed by the fat-soluble substance in the alcohol extract. Therefore, in this experiment, the alcohol extract, the aqueous extract and the crude polysaccharide in the aqueous extract were separated from each other, and the blood glucose lowering activity was tested separately. By comparison, it was known. The substances play a leading role in hypoglycemic activity. As shown in Table 4, the table reflects the inhibitory activities of the alcohol extract of *C. cornucopioides*, the α-glucosidase and the α-amylase of the crude polysaccharide in the aqueous extract and the aqueous extract. It can be seen that the hypoglycemic activity of the crude polysaccharide is significantly higher than that of the alcohol extract and is similar to the hypoglycemic activity of the aqueous extract. Therefore, we believe that the main effect of *C. cornucopioides* on *hypoglycemic* activity is polysaccharide.

**Table 4** Hypoglycemic activity of ethanol extract, aqueous extract and crude polysaccharide of *C. cornucopioides*

|  |  |  |  |
| --- | --- | --- | --- |
| Biological activity | Alcohol extract | Aqueous extract | Crude polysaccharide |
| α-GI | 1.27±0.04 | 10.43±0.43 | 11.34±0.48 |
| α-AI | 4.43±0.63 | 21.63±0.87 | 24.99±0.44 |

AEach value is expressed as the mean ± standard deviation (SD); Bα-GI: α-glucosidase inhibitory activity (μg/mL); α-AI: α-amylase inhibitory activity (μg/mL)

* 1. *Analysis of polysaccharides*
     1. *Molecular weight determination*

The molecular weight of the crude polysaccharide of *C.cornucopioides* was determined by high-performance gel permeation chromatography (HPGPC). A total of two peaks were eluted, corresponding to a molecular weight of approximately 518019, 14579 Da, indicating that the crude polysaccharide has a broad molecular weight range. The molecular weight of the polysaccharide generally has a significant effect on its biological activity, and the low molecular weight polysaccharide has a higher antioxidant activity than the high molecular weight polysaccharide.

* + 1. *Monosaccharide composition*

* + 1. *FT-IR spectral analysis*

As shown in Fig. 1, the crude polysaccharide of *C.cornucopioides* has an absorption peak at about 3400 cm-1. Since the polysaccharide contains a large amount of hydroxyl groups, it will appear between 3200-3600 cm-1, and a strong absorption broad peak due to OH stretching vibration; at 2908 cm-1 The absorption peak is caused by the vibration of the CH stretching; the absorption peak at 1610 cm-1 is caused by the absorption of the β-glycosidic bond and the trace amount of water associated with the hydroxyl group; The absorption peak at 1040 cm-1, 1045 cm-1, and 1110 cm-1 is caused by the O-H angular shock; the absorption peak at 870 cm-1 indicates that the glycosidic bond of the crude polysaccharide is an β-glycosidic bond. Thus, the crude polysaccharide is mainly composed of β-pyranose.

Fig. 1. FI-IR spectra of the crude polysaccharide of *C.cornucopioides*;

* 1. *Determination of antidiabetic activity in vivo*

1. **Conclusion**

**Conflict of Interest**

**Acknowledgments**

**References Primary Sources**

**Secondary Sources**

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