Anti-glycated and antiradical activities *in vitro* of polysaccharides from *Ganoderma capense*

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ABSTRACT

Background: *Ganoderma capense* is a *Ganoderma* species and is widely used, especially in Asia, as a well-known medicinal mushroom for health-promoting effect and for treatment of chronic diseases, such as diabetes, aging, etc. *G. capense* is rich of polysaccharide. **Objective:** To isolate the polysaccharides from *G. capense* and evaluate their anti-glycated and antiradical activities *in vitro*. **Materials and Methods**: The dried powder of submerged fermentation culturing mycelium of *G. capense* was defatted, extracted with water/ alkaline water followed by ethanol precipitation and deproteinated. And four crude polysaccharides, named as GC50, GC70, GC90 and GCB, were obtained. For the first time, the *in vitro* anti-glycated activities of the four samples were studied by non-enzymatic glycation reaction. Then, the DPPH radical and hydroxyl radical assays were established to estimate the antiradical capacity of the four samples. Meanwhile the contents of polysaccharides were determined by phenol-sulphuric acid colorimetry. **Results and Conclusion**: Preliminary antiradical *in vitro* studies indicated that the four crude polysaccharides showed concentration-dependent scavenging abilities on DPPH and hydroxyl radicals. The evaluation of anti-glycation activity suggested that GC70 had good potential for inhibiting the formation of advanced glycation end products. Time- and dose-dependent effects were also observed for all GC70 samples.

Keywords: Anti-glycated activity, antiradical activity, *Ganoderma capense* (Lloyd) Teng, *in vitro*, polysaccharide

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INTRODUCTION

Glycation has been confirmed to have a significant role in the development of chronic diabetic complications including cataract[1] and normal aging.[2] Non-enzymatic glycation of proteins by reduction with carbohydrates and further rearrangements, eliminations and oxidations would result in the formation of advanced glycation end products (AGEs),[3] which might alter peptide structures, functions and stability. There have been many reports on the accumulation of AGEs under several pathophysiological conditions such as diabetes and aging.[4] Therefore, searching a potential anti-glycated agent has drawn much attention from researchers. Till now, publications on the anti-glycated activity of polysaccharides derived from natural sources are very limited.

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Oxidation is essential to many organisms for the production of energy fuel biological processes.[5] However, the uncontrolled production of oxygen-derived free radicals is involved in the onset of many diseases, such as cancer, rheumatoid arthritis and atherosclerosis, as well as in degenerative processes associated with aging.[6] Almost all organisms have natural antiradical properties and are able to repair an oxidative damage in their systems. However, but these systems are unable to prevent the damage completely.[7] Antioxidants and radical scavengers are substances that can delay or prevent the oxidation of oxidizable substrates. Published data indicate that some plant polysaccharides have strong antiradical activities and can be explored as novel potential antioxidants.[5,8]

Ganoderma strains belonging to the family Polyporaceae were one of the most important medical fungi of basidiomycetes.[9,10] In many Asian countries, *Ganoderma*, or 'Lingzhi' in Chinese and 'Reishi' in Japanese, including *G. capense*, *G. alucidum* and *G. japonicum*, that have been collected, cultivated and used for hundreds of years are

being evaluated as edible and medicinal resources.[11] Active compounds in *Ganoderma* included polysaccharide, steroids, lignins, lectin, ganomycins, vitamins C, nucleosides, nucleotides, alkaloids, amino acids, triterpenes, etc.[12] Especially, the content of polysaccharide in *G. capense* was the highest among the twelve samples.[13] *G. capense* is a *Ganoderma* species that morphologically resembles *G. lucidum* to a certain extent.[14] *G. capense* is widely used, especially in Asia, for the treatment of chronic diseases, such as diabete, aging, cancer, hepatitis, bronchitis, asthma, haemorrhoids, hereditary cerebellar ataxia, etc.[9,14-16] In contrast to *G. lucidum*, there is a dearth of information about the biochemical constituents of *G. capense* other than some simple ring compounds and lectin.[14] Polysaccharides are the best known and most potent mushroom-derived substances with a number of medicinal properties. In order to investigate the bioactive of *G. capense* polysaccharide in anti-glycated and antioxidant effects, the present investigation was under taken to isolate the polysaccharides from *G. capense* and evaluate the anti-glycated and antiradical activities *in vitro*.

MATERIALS AND METHODS

Plant materials and reagents

Submerged fermentation culturing mycelia powder of *G. capense* was denoted by Huai'an Yutu Ganoderma Co. Ltd (Jiangsu Province, China). Voucher specimens were deposited in College of Pharmacy, Guangdong Pharmaceutical University.

DPPH and Sodium azide were purchased from Sigma-Aldrich (St. Louis, USA). Vitamin C and BSA (Bovine Serum Albumin) were purchased from the Puboxin Biotechnology Co., Ltd. (Beijing, China). Glucose, phenol and sulphuric acid were obtained from Guangzhou Reagent Co. (Guangzhou, China). All other reagents used in this study were of analytical grade.

Extraction of crude polysaccharides from *G. capense*

Preparation of crude polysaccharide was carried out according to the method of Yu[16,17] with some modifications. The dried powder of submerged fermentation culturing mycelium of *G. capense* (2000g) was defatted with petroleum ether twice, each time for 2h. Then, the powder was extracted three times with hot water (80°C), each time for 2h. The combined aqueous extracts were filtered and concentrated under reduced pressure and three fractions of crude polysaccharides, termed as gc50, gc70 and gc90, were obtained by graded ethanol precipitation, at final concentration of 50%, 70% and 90% of ethanol, respectively. The residues were further extracted with 0.3mol/l NaOH three times at room temperature. Subsequently the alkaline extracts were filtered, combined, adjusted to pH 7 with diluted HCl and concentrated under reduced pressure. The aqueous fraction was precipitated with 75% ethanol for 24h at 4ºC. The precipitate was collected, named as gcB. All of the four fractions of polysaccharides, gc50, gc70, gc90 and gcB, were deproteinated by the Sevag reagent[18] and dialyzed against distilled water for 48h to remove low molecular weight materials (exclusion limit 3.5 kDa), respectively. Then, the polysaccharides were lyophilized to obtain four crude polysaccharides, termed as GC50, GC70, GC90 and GCB.

The content of polysaccharides was determined by the phenol-sulphuric acid method of Xie[8] with D-glucose as standard at 490 nm, wherein glucose was prepared in a series of concentrations to make a standard curve for the polysaccharide calculation. Protein content was estimated by the Lowry's method[19] with bovine serum albumin as standard.

Preparation of standard curve

0.2, 0.4, 0.6, 0.8 and 1.0mL glucose standard solutions (0.1mg/ml) were put into separate tubes, and diluted with water till 1mL. Then 1 mL of phenol and 5 mL of sulphuric acid were poured into each tube respectively. After fully mixing for 30 min, average Abs value of each tube was examined in 490nm with 1 mL distilled water as control by using UV-2550 spectrophotometer (Shimazu, Japan). The regression equation was $Y = 10.815x + 0.0635$ ($r = 0.998$), linear range was 0.02-0.10mg/mL.

In vitro assay for anti-glycated activity

Experiments were performed as described by Zhao with a minor modification.[20] 2%(w/w) bovine serum albumin in phosphate buffer (20mM, pH 7.4) containing 0.05% (w/w) sodium azide was preincubated with polysaccharide or aminoguanidine at concentrations of 0.1, 0.3 and 0.5 mg/ml for 10 min at room temperature. 1.0 M glucose solution was added to the reaction mixture. Bacteria were removed by membrane filtration with a pore size of 0.22μm. Then, the solutions were incubated in dark at 37 ºC for 3 weeks. The control group was prepared using distilled water instead of polysaccharide. Fluorescence was determined every week using UV-2550 spectrophotometer (Shimazu, Japan) with an excitation wavelength of 370 nm and an emission wavelength of 450 nm. The percentage of anti-glycated activity (G%) was calculated as:

$$G\% = (A_c - A_s)/A_c \times 100\%$$

Where A_c represents the fluorescent determination of control group, and A_s represents the fluorescent determination of sample group.

In vitro assays for antiradical activities

Scavenging activity of DPPH radical

The free radical scavenging activity of the polysaccharides

was measured by DPPH(1,1-diphenyl-2-picryl-hydrazyl) test according to the method of Xie *et al*, with some modifications.[8] Solution of DPPH in methanol (0.1mM) was prepared the day before Ultraviolet (UV) measurements. 2 ml of the polysaccharides in different concentrations (25-250μg/ml) dissolved in ultra-pure water was thoroughly mixed with 2 ml of freshly prepared DPPH. The mixture was shaken thoroughly and subsequently, allowed to stand for 30 minutes in the dark. Afterwards, the absorbance was then measured at 517 nm against a blank. Lower absorbance of the reaction mixture indicated higher free radical-scavenging activity, which was analyzed from the graph plotted of inhibition percentage against compound concentration. Ascorbic acid was used as positive control. The experiment was carried out in triplicate. The capability to scavenge the DPPH radical was calculated using the following formula:

Scavenging rate (%) = $[1 - (A_i - A_J)/A_0] \times 100\%$

Where, A_0 is the absorbance of DPPH solution without sample or positive controls; A_i is the absorbance of the test sample mixed with DPPH solution; and A_J is the absorbance of the sample without DPPH solution.

Scavenging activity of hydroxyl radical

The assay was measured by the method of Sun[5] with a minor modification. Polysaccharides were dissolved in distilled water at the concentration of 0.125 to 25mg/ml. The sample solution (0.5ml) was mixed with 4.0 ml of reaction buffer [1.0 ml of 50 mM phosphate buffer (pH 7.4) and 0.75ml of 5.0mM phenanthroline, 0.5ml of 7.5mM ferrous sulfate and 1.75ml of ultra-pure water], then, 0.5ml of 0.1% H_2O_2 was added to the reaction solution and incubated for 60 minutes at 37 °C. The absorbance of the mixture was measured at 536nm against blank. The capability to scavenge hydroxyl radical was calculated using the following equation:

Scavenging rate % = $(A_{sample} - A_{negative\ control})/(A_{blank} - A_{negative\ control}) \times 100\%$

Here, ultra-pure water (1.0ml) without H_2O_2 plus reaction buffer (4.0ml) was used as a blank, 0.5ml of ultra-pure water plus 0.1% H_2O_2 (0.5ml) was experimented as a negative control, and 0.5ml of ascorbic acid plus 0.1% H_2O_2 (0.5ml) was tested as a positive control.

Statistical analysis

Tests were carried out in triplicate for three separate experiments. Statistical calculations by SPSS software were conducted to calculate the correlation. Values are presented as means ± SD (n = 3).

RESULTS AND DISCUSSION

Extraction of crude polysaccharides from *G. capense*

In this work, the powder of submerged fermentation culturing mycelium of *G. capense* (2000g) was defatted, extracted with water/alkaline followed by ethanol precipitation and deproteinated, then, four crude polysaccharides, GC50, GC70, GC90 and GCB, were obtained with yields of 20.7%, 4.6%, 3.5% and 2.1%, respectively. The total extracted rate of the polysaccharide was 30.9%. The contents of four polysaccharides were 76.98%, 43.6%, 90.07% and 82.43%, respectively.

In vitro assay for anti-glycated activity

In the present study, bovine serum albumin was chosen as the model protein and glucose was used as the glycation agent. Experiment results indicated that only GC70 showed a certain anti-glycation activity [Figure 1] among the four polysaccharides. During the three weeks, there were some positive correlation between the anti-glycated activities of GC70 and its acting concentrations. Samples of GC70 at three concentrations showed low antiglycation activities (less than 2%) at the first week. The G % of GC70 at the second week with the concentrations of 0.3 mg/ml, was higher than the first and third week. While, when a concentration of 0.5 mg/ml was used, the G % of GC70 was above 30% at the third week, significantly higher than those of the first and the second week.

In vitro assays for antiradical activities

Scavenging activity of DPPH radical

The DPPH free radical is a stable free radical, which has been widely accepted as a tool for estimating the free radical-scavenging activities of antioxidants.[21] In the DPPH test, the antioxidants were able to reduce the stable DPPH radical to the yellow-coloured diphenylpicryl

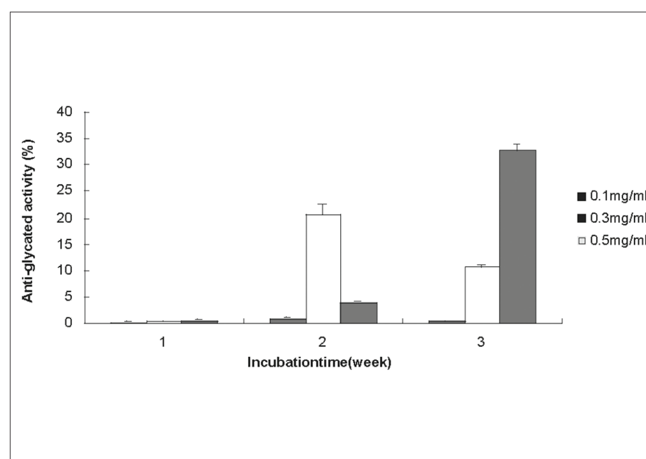

Figure 1: Anti-glycated activities of GC70 from *G. capense* during different incubation periods. Values were means ± SD of three determinations

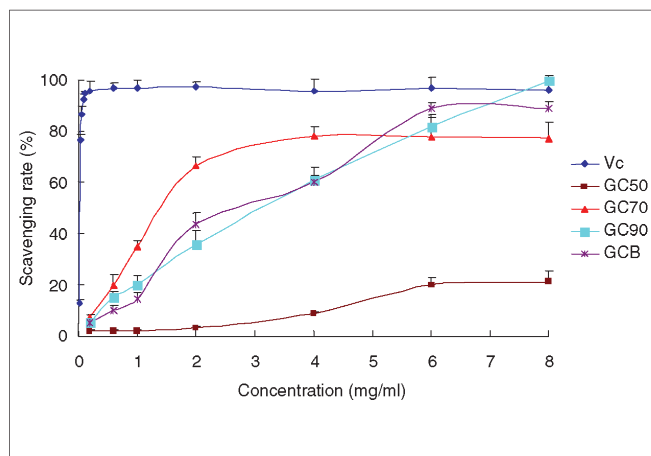

Figure 2: Scavenging effects of the four polysaccharides, GC50, GC70, GC90 and GCB, isolated from *G. capense* and vitamin C on DPPH radical. Values were means ± SD of three determinations

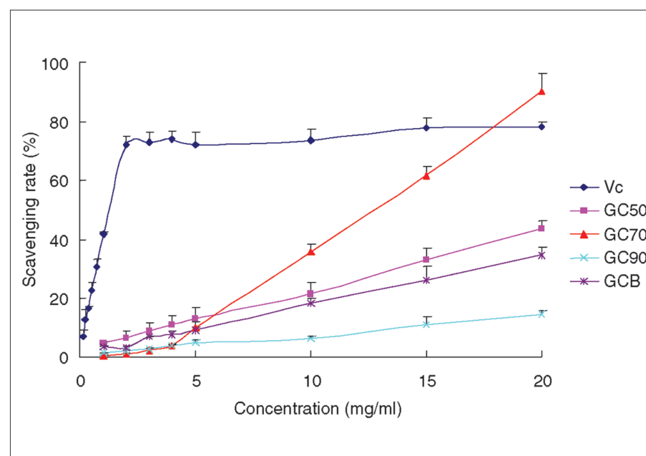

Figure 3: Scavenging effects of the four polysaccharides, GC50, GC70, GC90 and GCB, isolated from *G. capense* and vitamin C on hydroxyl radical. Values were means ± SD of three determinations

hydrazine. The effect of antioxidants on DPPH radical-scavenging was conceived to be due to their hydrogen-donating ability. The DPPH radical-scavenging activities of polysaccharides from *G. capense* and vitamin C used as a positive control were determined, and the results are plotted in Figure 2. As illustrated, GC70, GC90 and GCB exhibited scavenging activity towards DPPH radicals in concentration-dependent manners. GC70 showed higher activity than other samples when the concentration was less than 5mg/ml. While, GC90 and GCB had better effects than GC70 at high concentration, especially the scavenging rate of GC90 at a dose of 8mg/ml was up to 99.4%, which was slightly stronger than vitamin C. Under the experimental conditions, GC50 showed poor antiradical activity.

Scavenging activity of hydroxyl radical

It is well-known that reactive oxygen species (ROS), such as hydroxyl radicals, are related to the pathogenesis of various diseases.[16,22,23] Hydroxyl radical is the most reactive among the oxygen radicals and induces severe damage to the adjacent biomolecules. Thus, to evaluate the *in vitro* antiradical activities of the four polysaccharides, the hydroxyl radical-scavenging ability was measured in the present study.

The hydroxyl radical-scavenging activities of the four polysaccharides and vitamin C used as a positive control were determined, and the results were plotted in Figure 3. As illustrated in the figure, all of the four samples exhibited the scavenging activity towards hydroxyl radicals in concentration-dependent manners. Overall, GC70 exhibited the strongest activity when the concentration was above 5mg/ml, followed by GC50 and GCB, with GC90 displaying the weakest activity. All of the polysaccharides displayed similar trends in antioxidant activity. These results indicated that GC70 had a noticeable effect on the scavenging free radical, especially at high concentration.

CONCLUSION

On the basis of the previously mentioned studies, four polysaccharides (GC50, GC70, GC90 and GCB) were isolated from the dried powder of *G. capense* by water/alkaline extraction and ethanol precipitation. In the present work, we firstly studied the *in vitro* anti-glycated activity of the polysaccharides from *G. capense*. Only GC70 exhibited an anti-glycated activity, and there was positive correlation between the activities and their acting concentrations within the three weeks. Meantime, the antiradical activities of the four samples were studied. DPPH radicals scavenging assay *in vitro* indicated that the scavenging rates of four polysaccharides were increased along with increasing concentrations, and GC90 and GCB have noticeable effect at a high concentration, which was similar closed to the positive control (vitamin C). However, GC50 was lower than that of vitamin C. Hydroxyl radical scavenging activities *in vitro* indicated that the four crude polysaccharides exhibited significant scavenging effect at the high dose, in which GC70 was stronger than vitamin C at the concentration of 20mg/ml. In this study, GC70 showed good anti-glycated activity and antiradical activity towards hydroxyl radical *in vitro* among the four polysaccharides. However, the composition and structural characterization of GC70 is still not clear. At present, the exact structure of 90P and the relationship between structure and activities are in progress.

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