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Enhance the flavonoids content and α-glucosidase inhibitor efficiency of mulberry leaves by fermented with *Ganoderma lucidum*

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Abstract: *Diabetes is a kind of chronic disease that seriously threatens human health. α-glucosidase inhibitors are an effective way to inhibit the increase of postprandial blood glucose. Mulberry leaf flavonoids can be used as highly effective α-glucosidase inhibitors, but usually in low concentrations. Microbial fermentation is an effective method to increase the content of flavonoids in plants. In this paper, the fermentation of mulberry leaves with Ganoderma lucidum was conducted to investigate the cell growth of microbial, the content and α-glucosidase inhibitory efficiency of flavonoids in mulberry leaves. The results indicated that mulberry leaves can satisfy the nutritional needs of G. lucidum, and the maximum cell dry weight of 393.32 mg was obtained after a 10 d of fermentation. The flavonoid content in mulberry leaves was increased significantly with the fermentation time. Compared to unfermented mulberry leaves, the flavonoid content in mulberry leaves fermented for 8 d increased by 56.5%. The inhibitory efficiency of the fermented flavonoids was also significantly improved. The IC_{50} of α-glucosidase of flavonoids extracted from 8 d fermented mulberry leaves and unfermented mulberry leaves were 0.041 mg/mL and 0.01 mg/mL, respectively. This article will provide a reference for the enrichment of functional active ingredients in plants through microbial fermentation technology.*

Keywords: *Mulberry leaves, α-glucosidase inhibitor, Ganoderma lucidum, Flavonoids, Microbial fermentation technology*

1. Introduction

Diabetes is a growing threat to human health. According to the data released by the International Diabetes Federation (IDF), the number of adults with diabetes has reached 465 million worldwide in 2020, while this figure was only 151 million in 2000, and the number is expected to increase to 578 million by 2030 [1]. Therefore, the drug development and research of diabetes has become a research hot spot in the world. α-glucosidase inhibitors are a class of oral hypoglycemic drugs, which can be used to treat diabetes by slowing down the absorption of carbohydrates in the intestine. And currently, α-glucosidase inhibitors have become the drug of first choice for type 2 diabetic patients with poor dietary control alone and the drug of adjuvant choice for insulin therapy in patients with type 1 diabetes [2]. This kind of inhibitors prevent breakdown of disaccharide and oligosaccharide substrates into absorbable monosaccharides, which, in turn leads to a delayed intestinal carbohydrate digestion/absorption and reduced postprandial hyperglycemia [3]. At present/Presently, the major α-glucosidase inhibitors that used in clinical treatment include acarbose, voglibose, miglitol, etc. However, it was reported that these drugs might lead to various side effects such as hypoglycemia, weight gain, lactic acidosis, gastrointestinal intolerance and even adverse liver events [4, 5], which were caused by abnormal fermentation of carbohydrates under the action of intestinal flora [6].

The conventional therapy of diabetes in ancient China is traditional Chinese medicine treatment, which utilizes a variety of active ingredients in different plants to achieve the purpose of multi-target treatment, not only the effect is significant, but also has low side effects [7, 8]. Mulberry leaves are a

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kind of Traditional Chinese medicine. By virtue of its wide distribution in Asia and rich bioactivities in its extract, mulberry leaves are widely used as herbal medicine and food resource. The treatment of diabetes using mulberry leaves has a long history. It has been revealed that mulberry leaves are rich in polysaccharides, flavonoids, alkaloids, volatile oil and other active components, which endows mulberry leaves with hypoglycemic, lipid-lowering, antioxidant and anti-aging effects. More and more researches have focused on the therapeutic potentiality of mulberry leaf flavonoids (MLF) on type II diabetes, which indicated that MLF can reduce the serums levels of glucose and lipids, increase the consumption of glucose and skeletal muscle mitochondrial function in IR mice, and have a positive impact on insulin resistance (IR) mice [9, 10]. Fan et al found that in db/db mice, MLF attenuates insulin resistance and activate AMPK to improve skeletal muscle mitochondrial function and glucose uptake [2]. Furthermore, flavonoid intake not only decreases DM incidence rate, it also reduces cardiovascular risk factors DM type 2 populations [6]. Therefore, mulberry leaf extract is widely used in the treatment of diabetes [11-13].

However, the content of flavonoids in mulberry leaves are generally low. Hence, it is important to achieve the goal of enrichment of flavonoids. The fermentation for Chinese traditional medicine has a long history in China. Nowadays, the traditional fermentation process joins the latest knowledge of biological engineering technology and microbial strains of probiotics to screening and breeding of modernization, and comprehensive digital regulation of fermentation process [14, 15]. These new technologies make the fermentation technology of Chinese herbal medicine more advantageous, such as considerably reducing the toxic and side effects of drugs, making drugs easier to be absorbed by the intestine, enhancing the efficacy and improving the flavor. In this paper, the fermentation of mulberry leaves with *Ganoderma lucidum* was performed to investigate the changes of flavonoid content in mulberry leaves during the fermentation and the effect of flavonoids on the inhibitory activity of α-glucosidase.

2. Materials and Methods

2.1. Materials and Chemicals

Ganoderma lucidum was self-isolated. Mulberry leaves were purchased from the market of Shangluo (Shanxi, China). The dried mulberry leaves were ground into powder with a blender and sieved using a 100-mesh sieve. Wheat bran was purchased from Feitian Agricultural Development Co., LTD (Henan, China). Rutin, α-Glucosidase from yeast, 4-nitrophenyl-α-D-glucopyranoside (pNPG), and glucosamine solution were all purchased from Yuanye Biotechnology Co., LTD (Shanghai, China). Other conventional reagents were purchased from Sinopharm Chemical Reagent Co., Ltd., etc., and were analytically pure without special labels.

2.2. The solid state fermentation of G. lucidum using mulberry leaves as substrate

Preparation of *G.lucidum* inoculum: *G.lucidum* was inoculated in potato dextrose broth medium and cultivated in a shaker with 180-200 rpm for 3-5 days at 28-30℃.

Preparation of mulberry leaf fermentation medium: Mixed 4 g of dry mulberry leaves with 1 g of bran evenly. Then the distilled water was added to adjust the solid-liquid ratio of the culture to 1:3. The mixture was stirred evenly under natural pH and then sterilized by autoclave at 121 ℃ for 20 min. The mulberry leaf fermentation medium was cooled and store at room temperature for use.

Solid state fermentation of *G. lucidum* using mulberry leaf medium: Inoculated 5 ml of *G. lucidum* seed solution into the mulberry leaf medium, so that the final solid-liquid ratio of the substrate was 1:4. After stirring evenly, it was cultivated statically in incubator at 30 ℃. Samples were taken from the fermentation group at 0 d, 2 d, 4 d, 6 d, 8 d and 10 d, respectively. Three repeats were set up in each experiment.

2.3. Biomass determination of G. lucidum

The glucosamine content in the fermented residues was used to monitor the biomass of *G. lucidum* during the solid state fermentation as described by Fan et al with a minor modification [16]. The pure cell of *G. lucidum* was collected by cultivated in potato-glucose medium. The dried cell was used for the determination and preparation of the standard curve of the biomass and glucosamine.

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The dried fermentation residues 1 g was socked in 10 ml concentrated HCL for 24 hours, then 40 ml distilled water was added. The mixture was hydrolyzed at 121℃ for 2 h. The supernatant was collected by centrifuged at 8000 rpm for 10 min (H2050R-1, Xiangyi Centrifuge Instrument Co., LTD, Shanghai, China). Taken 10 ml of supernatant and neutralized it with NaOH and fixed volume to 25 ml.

1 ml of Erhlich's reagent was added to 1 ml of the above glucosamine extract and then reacted at 90 ℃ for 1 hour. After cooling, 6 ml ethanol was added and then reacted at 65 ℃ for 10 minutes. Then, the colorimetric analysis was performed at 530 nm using a UV-Visible Spectrophotometer (754PC, Jinghua Technology Co., LTD, Shanghai, China). The biomass of *G. lucidum* in the fermented mulberry leaves residues could be calculated according to the standard curve between the cell dry weight of *G. lucidum* and glucosamine content.

2.4. The extract and determination of mulberry leaf flavonoids

The flavonoids extract of mulberry leaves was prepared with the following procedure. Precisely weighed 4.0 g of mulberry leaves medium mixed with 100 ml 75/25 water/methanol, and extracted by ultrasonic at 50℃ for 40 min. The liquid phase was collected by filtration, and the solid phase was exacted one more time using the above method. Combined the liquid phases to obtain the mulberry leaf flavonoid extract [17].

Two milliliter of flavonoids extract of mulberry leaves was added with 0.3 ml of 5% sodium nitrite solution. It was shook well and was standing for 6 min at room temperature. Then 0.3 ml 10% aluminum nitrate solution was added. After reacted at room temperature for 6 min, 4 ml of 4% sodium hydroxide solution was added and diluted with 70% ethanol to a fixed volume of 10 ml. The solution was mixed intensively and reacted at room temperature for 15 min. With rutin as standard and 70% ethanol solution as blank, the absorbance (A) was measured at 510 nm.

2.5. The extract and determination of polysaccharides in mulberry leaf

The extraction and determination of mulberry leaf polysaccharides as conducted as describe as below [18]. Mulberry leaf samples were mixed evenly with distilled water with a solid-to-liquid ratio of 1:30 (g/mL). Then the mixture was ultrasonically extract in a water bath at 80 ℃ for 20 min. Collect the supernatant as the mulberry leaf polysaccharide extract. The polysaccharide extract was hydrolyzed with 6 mol/L hydrochloric acid in a boiling water bath for 30 min. Adjust the pH to 8.0 after cooling to obtain the mulberry leaf polysaccharide hydrolysate. Using glucose as standard, the reducing sugar content in the hydrolysate was determined by the Dinitrosalicylic acid method (DNS). The content of the reducing sugar in the mulberry leaf polysaccharide hydrolysate was calculated according to the standard curve.

2.6. Assay of α-glucosidase inhibitory rate

The analysis of α-glucosidase inhibitory rate of flavonoids extracted from fresh mulberry leaves was performed on the 96 well plate [19, 20]. The experiment was put into 4 groups which are blank group, control group, sample blank group, and sample group. The reagents and adding order of each group are shown in Table-1. At the end of the reaction, 100 μl 1 mol/L $NaCO_3$ solution was used to terminate the reaction. After that, absorbance of the solutions were measured at 405 nm using a microplate reader (MB-96B, Chenghuai technology Co., LTD, Suzhou, China). The concentration of the inhibitor required for inhibiting 50% of the enzyme activity under the assay conditions was defined as the IC_{50}.

Table 1: The analysis procedures of α-glucosidase inhibitory

Reagent	B (Blank)	C (Control)	SB (Sample Blank)	S (Sample)
PBS buffer (μl)	80	70	60	50
Flavonoid extract from mulberry leaves (μl)	0	0	20	20
α-glucosidase(μl)	0	10	0	10
Mix up completely, activate under 37℃ for 15 min				
pNPG (μl)	20	20	20	20
Mix up completely, react under 37℃ for 30 min				
Na_2CO_3 (μl)	100	100	100	100

The inhibition rate was calculated as the following formula.

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$$\text{Inhibition rate}\% = \frac{(A_C - A_B) - (A_S - A_{SB})}{A_C - A_B} \times 100\% \quad (1)$$

Where, A_B: the absorbance of pNPG caused by self-decomposition of pNPG; A_C: the total absorbance caused by self-decomposition and enzymatic degradation of PNPG; A_S: the total absorbance caused by pNPG self-decomposition, enzymatic degradation of sample under the existence of inhibitor; A_{SB}: the total absorbance caused by sample and self-decomposition of pNPG.

2.7. Statistical analysis

Experiments were conducted in three replicates and results were presented as the mean ±SD. The data were analyzed with Origin 8.0 software package. One-way analysis of variance (ANOVA) at probability level (P)≤0.05 was used to determine the statistically significant differences between the mean samples.

3. Results and discussions

3.1. The growth of G. lucidum on mulberry leave medium

Ganoderma lucidum is a medicinal and edible fungus that has been specified by the Health Ministry of China as one of the industrial fermentation microbial [21]. With the development of the application of natural medicinal plants in China and the continuous expansion of the demand for health products, the use of medicinal fungi for the fermentation of medicinal and edible plants has broad application prospects [22]. Studies have found that the fermentation of *G. lucidum* would change the content of active substances in raw materials, such as polysaccharides, ganoderic acid, and saponins, even generate new substances [21, 23]. In this paper, *G. lucidum* was used for the fermentation mulberry leaves. The fermentation process of *G. lucidum* when grown on mulberry leaves medium was shown in Fig.1. It was indicated that *G. lucidum* grew well on mulberry leaves medium, entered a rapid growth period from 4 d to 8 d. The cell dry weight of *G. lucidum* was reached the maximum value of 393.32 mg on the 10 d of fermentation, and from then on kept stabilized (Fig.1). Meanwhile, with the growth of *G. lucidum*, the weight loss rate showed the same trend with cell dry weight. On the 10 d of fermentation, the weight loss rate was 23.55%. It means that the fermentable substance in mulberry leave medium, such as cellulose, hemicellulose, protein and polysaccharides, might be degraded by *G. lucidum*. Wu et al [24] also reported that the contents of insoluble dietary fiber and total dietary fiber contents in soybean processing residues were significantly decreased during the fermentation of *G. lucidum* 60229.

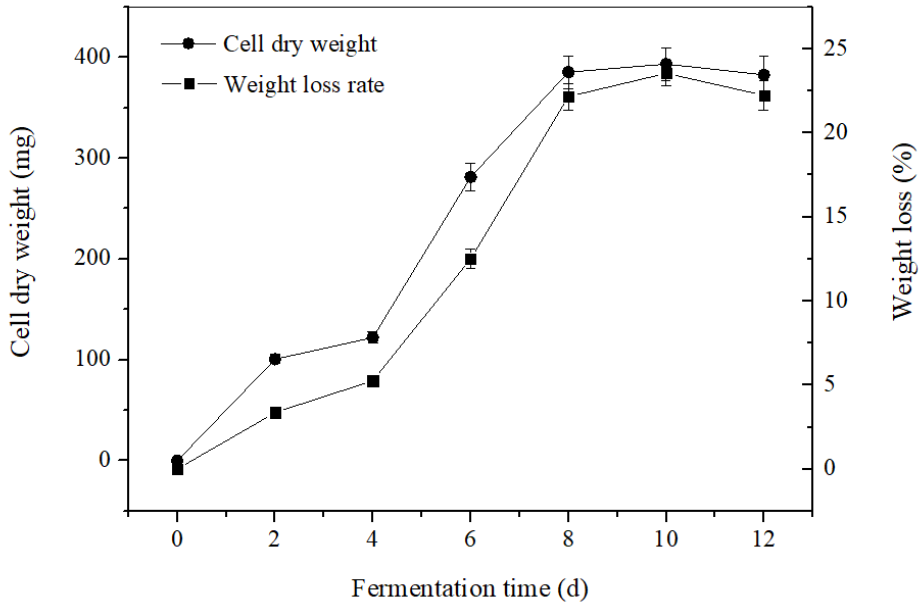

Fig.1 The growth curve of G. lucidum and weight loss rate of mulberry leave during the fermentation

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3.2. The flavonoids content in mulberry leaf after been fermented by G. lucidum

The flavonoids content of mulberry leaves during the fermentation of *G. lucidum* was investigated. In the un-fermented mulberry leaves, 1.821 (mg/g dried mulberry leave) flavonoids and 196.7 mg/g polysaccharides was detected. Along with the fermentation, the content of flavonoids kept increasing and reaching the maximum content of 2.849 (mg/g dried mulberry leave) at 8 d of fermentation, which was increased by 56.5% compared to that in the unfermented mulberry leaves. Wu et al [24] reported that the content of soluble dietary fiber, protein, free amino acid and total phenolic were higher than that of the non-fermented. In addition, microorganisms may promote the production of flavonoids through biotransformation during the fermentation process. As reported by Shen et al [25], in the early stage of *G.lucidum* fermentation for bean dregs, glycoside isoflavones in bean dregs would be converted into aglycon-based isoflavones. As for polysaccharides, the content of polysaccharides in mulberry leave was decreased rapidly during the whole fermentation period. It was only 76.3 (mg/g dried mulberry leave) of polysaccharides was detected on the 10 d of fermentation. The degradation rate of polysaccharides was 61.2%. The degradation of polysaccharides might be the major reason of the weight loss of mulberry leave medium, which consequently resulted in the increase of relative content of flavonoids in the fermentation residues.

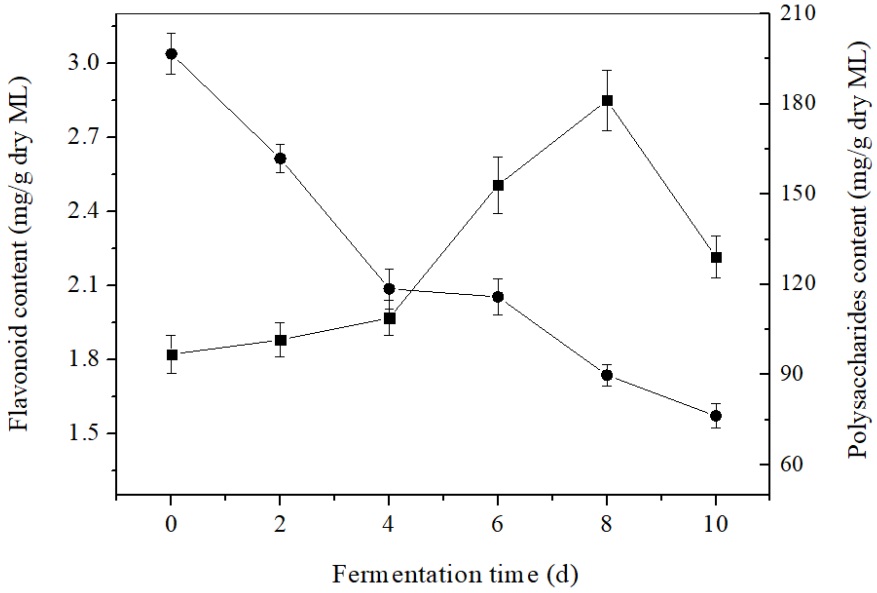

Fig.2 The changes of flavonoids and polysaccharides content of mulberry leaf during the fermentation of G. lucidum

3.3. Effect of G. lucidum fementation on α-glucosidase inhibitory rate of MLF

In this paper, through in vitro test evaluation, the effects of flavonoids extracted from unfermented mulberry leaves and 8d fermented mulberry leaves on the inhibitory efficiency of α-glucosidase was investigated. It can be seen from Figure 3 that in the concentration range of 0.02-0.075 mg/ml, the inhibitory efficiency of flavonoids extract unfermented mulberry leaf (MLF_0) on α-glucosidase increased slowly with the increase of the concentration. However, after been fermented for 8 d, in the concentration range of 0.0025~0.02 mg/ml, the inhibitory efficiency of α-glucosidase of flavonoid extracted from the fermented mulberry leave (MLF_8) was increased rapidly with the concentration. At concentration of 0.02 mg/ml, the α-glucosidase inhibition rates of MLF_0 and MLF_8 were 25.478% and 65.155%, respectively. Wu et al [24] also reported that the fermentation of *G. lucidum* could enhance the α-glucosidase inhibition rate of okara extract by 116.6%. Zhu et al [26] found that microbial fermentation could promote the release of isoflavones, saponins and other biologically active ingredients in okara, thereby improving the α-glucosidase inhibitory efficiency of fermented okara.

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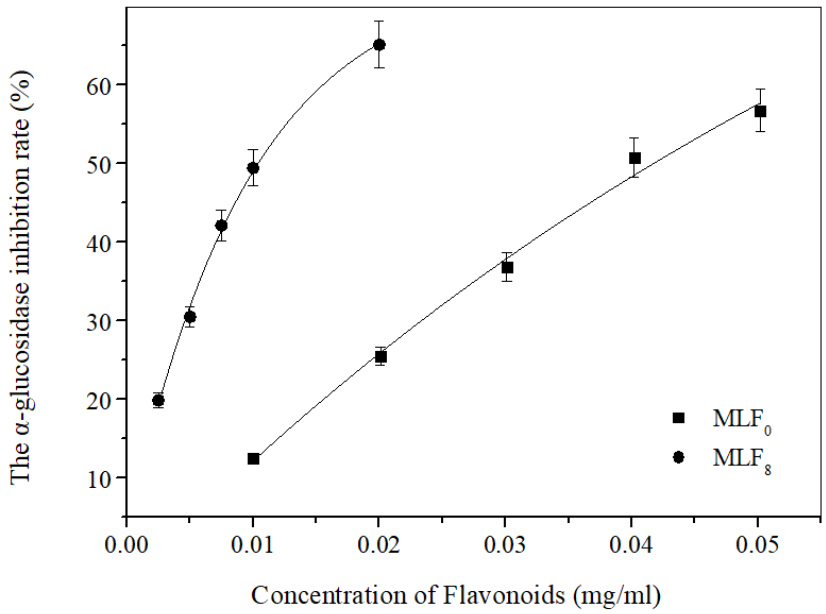

Fig.3 Effect of G. lucidum fementation on α-glucosidase inhibitory rate of MLF

The relationship between MLF concentration and α-glucosidase inhibition rate was fitted using ExpDec1 model. The fitting equation and R^2 were shown in Table 2. The IC_{50} on α-glucosidase was significantly reduced from 0.041 mg/ml of MLF_0 to 0.010 mg/ml of MLF_8, which indicated that *G. lucidum* fermentation can significantly improve the inhibitory ability of MLF on α-glucosidase.

Table 2: The α-glucosidase inhibition efficiency of mulberry leaves flavonoids

MLF (x)	Fitting equations	R^2	IC_{50} (mg/ml)
MLF_0	y=-101.69*exp(-x/0.023) +67.035	0.971	0.041
MLF_8	y=-69.65*exp(-x/0.0087) +71.34	0.994	0.010

4. Conclusion

The effects of *G. lucidum* fermentation on the content of flavonoids in mulberry leaves and α-glucosidase inhibitory efficiency of were investigated. The following conclusions were obtained:

(1) Mulberry leaf can be used as the medium for the fermentation of *G. lucidum*, and the maximum cell dry weight of *G. lucidum* was obtained on 10 d of fermentation.

(2) The fermentation of *G. lucidum* would benefit for increasing the content of flavonoids in mulberry leaves. The content of flavonoids in mulberry leaves fermented for 10 d increased by 56.5% compared with that of unfermented mulberry leaves.

(3) At the same concentration, the α-glucosidase inhibitory efficiency of the flavonoids extracted from 8 d fermented mulberry leaves was significantly higher than that extracted from unfermented mulberry leaves. The IC_{50} values of flavonoids extracted from 8 d fermented mulberry leaves and unfermented mulberry leaves were 0.041 mg/mL and 0.01 mg/mL, respectively.

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