

# Effect of *Ganoderma lucidum* fermentation on the content and $\alpha$ -glucosidase inhibitory activity of mulberry leaves flavonoid

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**Abstract.** Diabetes is now a worldwide disease that has a negative impact on human health. As a result, the search for low-cost, safe, and effective diabetes treatment drugs derived from natural medicinal plants has become a research priority. Mulberry leaf (ML) flavonoids have been proven to be a highly effective  $\alpha$ -glucosidase inhibitor and have a good effect on the treatment of diabetes. The solid state fermentation of *Ganoderma lucidum* utilizing ML as substrate was explored in this study with the goal of increasing the concentration and  $\alpha$ -glucosidase inhibitory effect of mulberry leaf flavonoid (MLF). The results indicated that fermented by *G. lucidum* for 8 d could increase the content of MLF from 1.596 mg/ml to 4.736 mg/ml. Meanwhile the polysaccharide in ML would be degraded and utilized as nutrients to support the cell growth of *G. lucidum*. The inhibitory rate of MLF on  $\alpha$ -glucosidase was significantly enhanced by fermentation. During the concentration of 10  $\mu$ g/ml, the  $\alpha$ -glucosidase inhibition rate of MLF was enhanced from  $45.31 \pm 2.24\%$  to  $73.46 \pm 3.28\%$  when fermented by *G. lucidum* for 8 d. Moreover, it was found that the inhibition type of MLF would change from competitive inhibition to mixed-type inhibition. This study will serve as a reference for microbial fermentation and enrichment of medicinal plant active components, as well as a contribution to the high-value-added use of mulberry leaf resources.

**Keywords:** Mulberry leaves, Diabetes mellitus, Flavonoid,  $\alpha$ -Glucosidase inhibitory activity, *Ganoderma lucidum*, Microbial fermentation.

## 1. Introduction

Diabetes is a metabolic disorder caused by genetic factors, immune disorders, microbial infections, and other pathogenic factors that cause hypoplasia and insulin resistance in the body [1]. It was reported that type II diabetes (T2DM), mainly caused by the insulin resistance and  $\beta$ -cell dysfunction accounted for more than 90% of diabetes cases and affected more than 400 million people worldwide [2,3]. For decades, the global diabetes situation has been severe, and the prevalence and mortality have been slowly increasing [4]. According to the International Diabetes Federation (IDF), more than 463 million people in the world have diabetes, and that number is expected to climb to 700 million by 2045 [5,6].

With the rising prevalence of diabetes, many anti-diabetic drugs (ADMs) have been developed. These include insulin secretagogues (sulfonylureas and non-sulfonylureas), biguanides,  $\alpha$ -glucosidase inhibitors, and thiazolidinediones (insulin sensitizers) [7]. By decreasing the activity of  $\alpha$ -glucosidase on the small intestine mucosa,  $\alpha$ -glucosidase inhibitors prevent glucose absorption [8]. Acarbose, miglitol, and voglibose are the most commonly used  $\alpha$ -glucosidase inhibitors in clinical practice today. However, their efficacy is still controversial due to their possible adverse side effects such as bloating, flatulence, and asthma or diarrhea [9]. Compared with synthetic inhibitors, natural compounds originate from medicinal plants have advantages in terms of safety, diversity and economy, and thereby received increasing attention world widely [10].

Presently, the  $\alpha$ -glucosidase inhibitory activity of several medicinal and edible homologous plants such as astragalus, mulberry leaves, angelica, ginseng, tea and pumpkin have been confirmed [9]. Among them, mulberry leaf (ML) is a Chinese herbal medicine with huge resources, which has been used for treatment of diabetes since ancient times. Therefore, it has a good development prospect to extract hypoglycemic active substances from ML and make hypoglycemic products [11].

Modern research indicates that ML contains a variety of active compounds with-glucosidase inhibitory activity,such as polysaccharides,flavonoids,and alkaloids[12],which have a variety of biological activities,including hypoglycemic,hypolipidemic,antioxidant,and anti-aging properties[13].Mulberry leaf is a popular traditional Chinese medicine[14]that has anti-diabetic and anti-inflammatory properties[15].The annual harvest of mulberry leaves can exceed 20 tons per hectare due to the rapid development of mulberry plants.Mulberry leaves,on the other hand,were only used 1-3 percent of the time[16].As a result,increasing the amount of active chemicals in mulberry leaves is critical.Microbial fermentation technology is widely used for enhancing the content and activity of active ingredients in the medicinal plants[17,18].Solid state fermentation(SSF)is often used in the processing of traditional Chinese medicine[19].*Ganoderma lucidum* is a kind of porous fungi and its polysaccharide is a good hypoglycemic active component[20].Therefore,the SSF of *Ganoderma lucidum* was examined utilizing ML as a substrate in order to improve the flavonoids content and improve the-glucosidase inhibitory activity of mulberry leaves in this work.

## 2. Materials and approaches

### 2.1. Materials and reagents

The China Center of Industrial Culture Collection sold *Ganoderma lucidum*(CICC 14029).(CICC,Beijing,China).Mulberry leaves were purchased from Shangluo City,Shaanxi Province.Aladdin Biochemical Technology Co.,Ltd provided the acarbose(Shanghai,China).Yuanye Biotechnology Co.,Ltd provided-glucosidase(from yeast),rutin standard,and pNPG(4-nitrophenyl—D-glucopyranoside)(Shanghai,China).Other traditional reactans were acquired from Sinopharm Chemical Reagent Co.,Ltd.(Beijing,China)and were analytical grade unless otherwise noted.

### 2.2. Mulberry leaf fermentation in solid state

Preparation of *G.lucidum* seed liquid:potato juice-glucose liquid medium,shake cultured for 3-5 days(28-30°C,180-200rpm),and then used for fermentation inoculation.The inoculum of *G.lucidum* was prepared by cultivated at 28-30°Cfor 3-5 d.

Mulberry leaf medium was prepared by mixing 0.5 g maize flour and 0.5 g bran into 8 g fresh ML,then adding distilled water to achieve a solid-to-liquid ratio of 1:3.The mixture was autoclaved for 20 minutes at 115°C after being thoroughly mixed.

The SSF of *G.lucidum* on mulberry leaf medium:10%of the *G.lucidum* inoculum was inoculated into mulberry leaf medium to make the final S/L to 1:4.The mixture was then statically cultured at 28°C,with samples obtained at 0,2,4,6,and 8 days.The control group was those who were not injected with *G.lucidum*.Each experiment was run in three different ways.

### 2.3. Extraction and purification of flavonoids

Total flavonoids were extracted and assayed in ML according to the instructions below[21].Two gram(accurated to 0.001 g)of the samples were accurately added with 20 ml of 70%ethanol solution.After that,the mixture was ultrasonically extracted for 20 minutes at 50°C,and the filtrate was collected by filtration.The precipitate was extracted again,and the filtrations were combined to obtain the crude mulberry leaf total flavonoid extracts.The pH of the aforementioned extract was then corrected to 2.5 using 6 M HCl,and the precipitate was collected by centrifugation at 8000 rpm for 10 minutes.Finally,the mulberry leaf flavonoid extract(MLF)was obtained by dissolved the precipitate in 70%ethanol.

### 2.4. Determination of flavonoids

Mix 2 mL MLF with 0.3 mL 5 percent sodium nitrite solution evenly and let aside for 6 minutes at room temperature.Then 0.3 mL of 10%aluminum nitrate solution was added and statically reacted for another 6 minutes at room temperature.The sodium hydroxide solution(4 wt%)was then added

and diluted to 10 ml with 70% ethanol. The absorbance at 510 nm was measured using a 754PC UV-Visible Spectrophotometer (JingHua Technology Co., Ltd, Shanghai, China) with rutin as the standard.

### 2.5. Analysis of Inhibitory Rate of Flavonoids on $\alpha$ -Glucosidase

On a 96-well plate, the inhibitory efficiency of MLF on  $\alpha$ -glucosidase was evaluated using a microplate reader (MB-96B, Chenghuai technology Co., LTD, Suzhou, China) [22,23]. First, different volume of PBS buffer (pH 6.8) was added to the 96-well plate according to Table 1. Each group's absorbance at 405 nm was measured at the end of the procedure. Formula was used to compute the inhibition rate of  $\alpha$ -glucosidase (1). The IC<sub>50</sub> value was established as the inhibitor concentration required to inhibit 50% of enzyme activity.

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

The absorbance at 405 nm of the blank group, control group, sample blank group, and sample group, respectively, is AB, AC, AS, and ASB.

**Table 1.** Measurement and adding order of each reagent

Groups Reagents	Blank group	Control group	Sample blank group	Sample group
PBS solution( $\mu$ l)	80	70	60	50
Inhibitor/Sample( $\mu$ l)	0	0	20	20
$\alpha$ -glucosidase solution( $\mu$ l)	0	10	0	10
Mixed evenly and activated at 37°C for 15 min				
pNPG substrate( $\mu$ l)	20	20	20	20
Mixed evenly and reacted at 37°C for 30 min				
Sodium carbonate solution( $\mu$ l)	100	100	100	100

### 2.6. Kinetic analysis of flavonoids inhibiting $\alpha$ -glucosidase

To investigate the inhibitory effect of flavonoid extracts obtained from unfermented or fermented mulberry leaves on the formation of p-nitrophenol (PNP), the reactions containing 1 U/mL  $\alpha$ -glucosidase and 10 mM pNPG were recorded at different flavonoid concentrations. Record the absorbance change within 30 min. The substrate pNPG concentrations in kinetic tests were 2.5 mM, 5 mM, 7.5 mM, and 10 mM, whereas the inhibitor concentrations were 2 g/ml, 6 g/ml, and 10 g/ml. The kind of inhibition was determined by graphing the enzyme reaction rate versus substrate concentration at various concentrations of mulberry leaf flavonoid extracts in a double-reciprocal plot (inhibitors).

The competitive inhibition mechanism and mixed-type inhibitory mechanism of mulberry leaf flavonoids on  $\alpha$ -glucosidase were studied using double reciprocal formula (2) and (3), respectively. [24,25].

$$\frac{1}{V} = \frac{1}{V_{max}} + \frac{K_m}{V_{max}[S]} \left(1 + \frac{[I]}{K_i}\right) \tag{2}$$

$$\frac{1}{V} = \frac{K_m}{V_{max}} \left(1 + \frac{[I]}{K_i}\right) \frac{1}{[S]} + \frac{1}{V_{max}} \left(1 + \frac{[I]}{K_{is}}\right) \tag{3}$$

Where  $K_i$  is the enzyme-substrate complex binding inhibition constant. The Y-intercept vs.  $[I]$  secondary plots are linearly fitted.

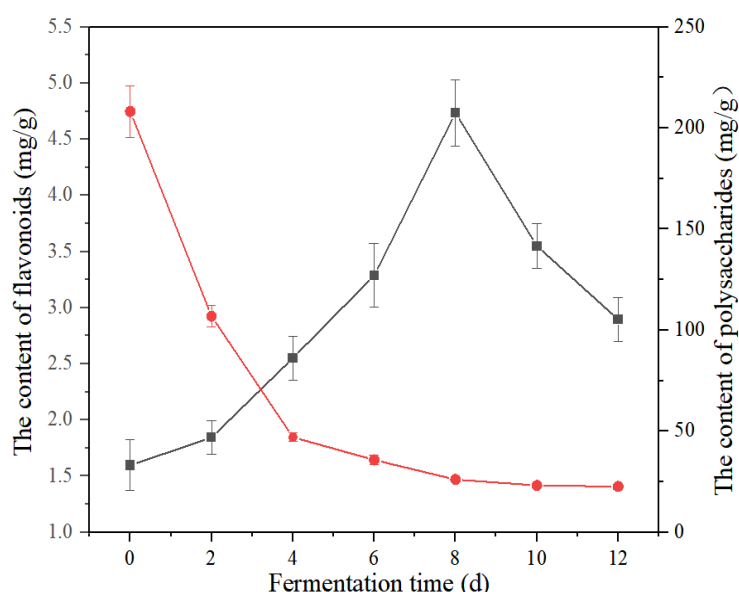
## 3. Result and discussion

### 3.1. Effect of fermentation time on flavonoid content

The variation of the content and biological activity of flavonoids in mulberry leaves was investigated in this paper. Previous studies have shown that the selected *Ganoderma lucidum*

fermentation has the best promotion effect on the production of DNJ(an alkaloid)in mulberry leaves[26].Of course,microbial fermentation experiments must also be carried out under the optimal culture time and conditions[27].In a study on microbial fermentation of Rhizoma Paridis,it was found that microbial fermentation significantly increased the total flavonoid content in Rhizoma Paridis[28].So some scholars use three kinds of Bacillus to ferment mulberry leaves and measure the content of flavonoids[29].The results showed that with the prolongation of fermentation time,the content of total flavonoids also tended to accumulate gradually.However,different microbial fermentations have different effects on the promotion of active substances in plants[13].

The concentrations of flavonoids and polysaccharides in mulberry leaves were determined at different fermentation time periods in this study,as shown in Fig 1.The results showed that the quantity of flavonoids in mulberry leaves increased and then decreased as the fermentation progressed,peaking at 8 days.The fermentation reduced the amount of polysaccharides in mulberry leaves.It could be inferred that this situation was caused by the full utilization of polysaccharides in mulberry leaves by microorganisms.

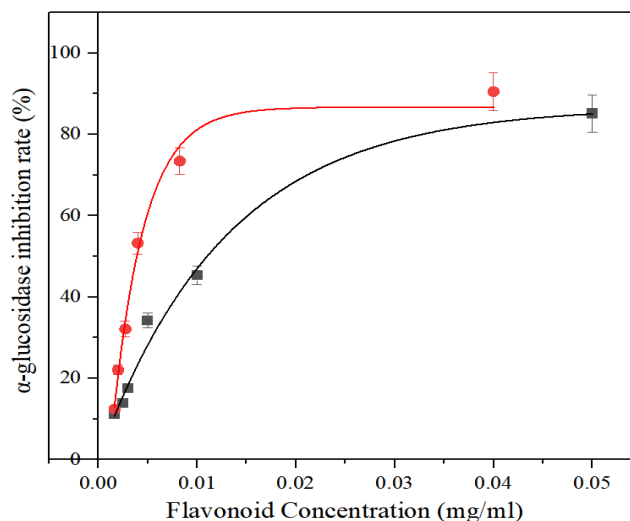


**Figure 1.** The variation of flavonoids and polysaccharides in mulberry leaves changes during *G.lucidum* fermentation.(■The content of flavonoids;●The content of polysaccharides)

### 3.2. The effect of fermented mulberry leaf flavonoids on the inhibition rate of $\alpha$ -glucosidase

$\alpha$ -glucosidase is a key enzyme that affects blood sugar levels in the body and is one of the most important carbohydrate hydrolases[30].Flavonoids are suitable materials for studying the specificity of  $\alpha$ -glucosidase inhibition because they have a tricyclic structure and chemical structural changes[31].The quantity of flavonoids in mulberry leaves and the inhibition rate of  $\alpha$ -glucosidase before and after 8 days of *G.lucidum* fermentation were measured in this study.The results indicated that the flavonoids obtained from the mulberry leaves fermented for 8d gave the highest inhibitory rate on  $\alpha$ -glucosidase activity(Fig.2).Flavonoids isolated from unfermented and fermented mulberry leaves have IC<sub>50</sub> values of 0.011 and 0.004,respectively.The higher the inhibitory action against  $\alpha$ -glucosidase,the lower the IC<sub>50</sub> value[32].It is well known that mulberry leaf is known for its anti-diabetic activity and it has been used as a tea and drink.The results of this experiment show that the inhibitory activity of flavonoids from fermented mulberry leaves on  $\alpha$ -glucosidase is better than that of unfermented mulberry leaves,it shows that the flavonoids extracted from mulberry leaves after fermentation have good regulating and therapeutic effects on obesity and diabetes caused by hyperglycemia.There have also been research comparing the inhibitory effect of flavonoids and acarbose on  $\alpha$ -glucosidase,with the results showing that flavonoids had higher inhibitory activity than acarbose[33].

Scholars studied the effect of extracts from mulberry leaves fermented by microorganisms such as lactic acid bacteria on the inhibition rate of  $\alpha$ -glucosidase in a study. Its 1mg/ml mulberry leaf mixed extract can achieve a maximum inhibition rate of 75% [34], and this experiment can achieve an inhibition rate of more than 80% at a lower concentration of brass. In another study, scholars have concluded that quercetin, myricetin, fisetin and quercetin have strong inhibitory effects on  $\alpha$ -glucosidase at 0.1 mM. IC<sub>50</sub> was 4, 8, 8 and 20  $\mu$ M, respectively. It can be seen that if different kinds of flavonoids are studied separately, their inhibition rates of  $\alpha$ -glucosidase are also different [35].



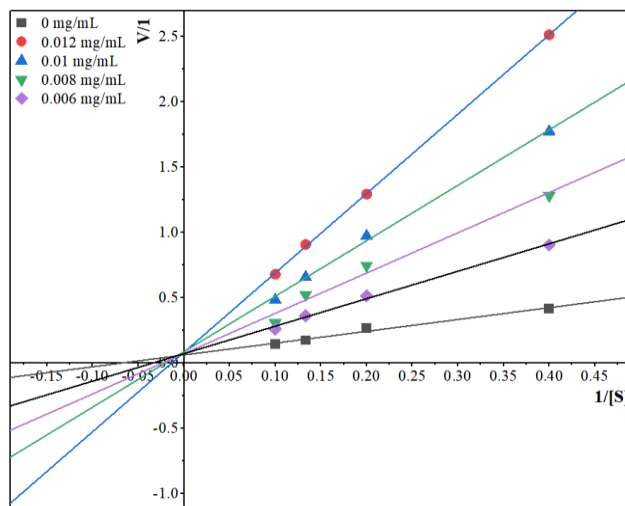
**Figure 2.** The inhibition of  $\alpha$ -glucosidase in mulberry leaves flavonoid isolated from various mulberry leaves (■ Unfermented mulberry leaves; ● 8d-fermented mulberry leaves)

### 3.3. Analysis of the inhibition types of mulberry leaf flavonoids on $\alpha$ -glucosidase

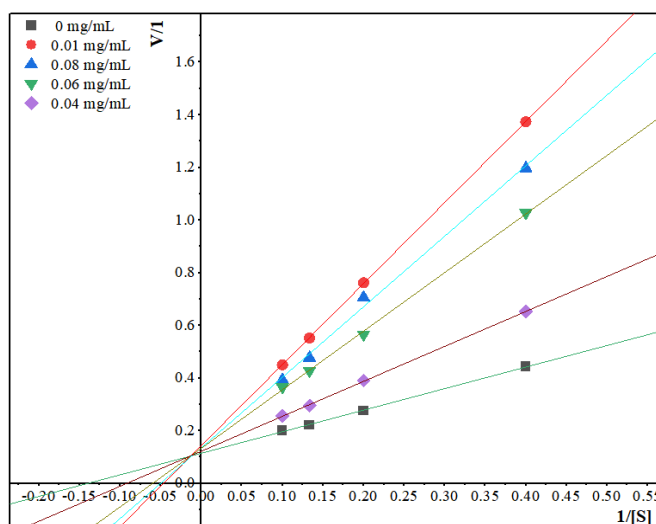
In the existing research conclusions, flavonoids in different fermentation states showed different inhibitory properties, different inhibition constants ( $K_i$ ) and inhibitory mechanisms to  $\alpha$ -glucosidase [36,37]. The inhibition types of flavonoids mainly include competitive inhibition, non-competitive inhibition and mixed inhibition [38,39].

The double-reciprocal graph of the reaction rate of the enzyme and the substrate concentration under different mulberry leaf flavonoid (inhibitor) concentrations were shown in Figure 3 and Figure 4. All lines have a longitudinal intercept of  $1/V_{max}$  and a transverse distance of  $-1/K_m$ . The maximum reaction rate ( $V_{max}$ ) in Figure 3 is unchanged, because the inhibition can be relieved by increasing the substrate concentration, and the Michaelis constant ( $K_m$ ) increases with the increase of the inhibitor concentration, so it can be inferred that the unfermented mulberry leaf flavonoids. Competitive inhibition of  $\alpha$ -glucosidase is the sort of inhibition that occurs. Another study found that 7 of the 9 flavonoid enzyme combinations with a predicted binding point of 1 exhibit a competitive inhibition mechanism [40]. Therefore, the flavonoids in unfermented mulberry leaves should theoretically be competitively inhibited as a whole.

It can be seen from Figure 4 that the  $V_{max}$  of the enzymatic reaction with the inhibitor was decreased, and the  $K_m$  was increased. The cross-sectional distance of the line with the inhibitor decreases in the double-reciprocal graph, while the vertical intercept increases, and all the lines intersect in the second quadrant, indicating that the type of inhibition of  $\alpha$ -glucosidase by mulberry leaf flavonoids after fermentation is mixed. The addition of the inhibitor also reduced  $\alpha$ -glucosidase activity. The researchers discovered that when  $K_i < K_{si}$ , the flavonoids' inhibitory mode on  $\alpha$ -glucosidase was mixed inhibition in several other investigations. [41,42]. For this experiment, the same conclusion may be drawn: the inhibition mode is mixed. This is because the increase in inhibitor concentration reduces the  $V_{max}$  value and increases the  $K_m$  value, resulting in a decrease in  $\alpha$ -glucosidase activity.



**Figure 3.** Inhibition type curve of mulberry leaf flavonoids before fermentation



**Figure 4.** Inhibition type curve of mulberry leaf flavonoids after fermentation

## 4. Conclusion

As the prevalence of diabetes increases, it poses a huge threat to human health. The goal of this study is to increase the -glucosidase inhibition rate of mulberry leaves by enriching hypoglycemic components using solid state fermentation of *Ganoderma lucidum*. The flavonoid concentration of mulberry leaf increased by 197.11 percent when compared to unfermented mulberry leaf, according to the findings. The -glucosidase inhibition rate of mulberry leaf flavonoid isolated from 8d-fermented mulberry leaf and unfermented mulberry leaf was 73.463.28 percent and 45.312.24 percent, respectively, at a concentration of 10 g/ml. Meanwhile, *G. lucidum* fermentation may shift the inhibitory types of mulberry leaf flavonoids on -glucosidase from competitive to mixed-type inhibition. This paper will provide a new method to increase the content of hypoglycemic components in mulberry leaves and improve its hypoglycemic effect.

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