

Effect of *Eurotium cristatum* fermentation on the α -glucosidase inhibitory activity of mulberry leaves flavonoid extract

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Abstract: Diabetes has become a global chronic disease and alpha-glucosidase inhibitors is an effective ways of treating it. Flavonoids are a group of substances in mulberry leaves that have α -glucosidase inhibitory properties and can reduce blood glucose concentrations effectively. In this paper, flavonoids from mulberry leaves fermented by *Eurotium cristatum* CY-1 will be used to investigate their inhibitory properties on α -glucosidase. The results indicated that the fermentation would increase the total flavonoid content of mulberry leaves by 196.7%. Meanwhile, the α -glucosidase inhibition efficiency of flavonoids extracted from the fermented mulberry leaves was also enhanced under the same concentration. The IC₅₀ values of the flavonoids extracted from 8 d fermented mulberry leaves and the unfermented mulberry leaf was 4.14 μ g/mL and 10.26 μ g/mL, respectively. This article will provide a reference for using microbial fermentation to enrich active ingredients of functional Chinese medicine herbs.

Keywords: Mulberry leaves, Flavonoid, α -Glucosidase Inhibitory Activity, *Eurotium cristatum*, Microbial fermentation, Diabetes

1. Introduction

In recent years, diabetes has become a global chronic disease. With the global incidence increased annually, diabetes has become the third disease that seriously threatens human health after cancer and heart and cerebrovascular diseases [1, 2]. According to the ninth edition of the Global Diabetes Map that released by International Diabetes Federation (IDF) at 2019, there are currently 463 million diabetic patients in the world. It also predicted that by 2045, the worldwide diabetic patients will rise upto 700 million [3]. IDF report the latest data that the number of diabetic patients in China was currently as high as 116.4 million, ranking first in the world, and it is also expected to reach 147.2 million by 2045, among which 90%-95% are type II diabetic patients [4, 5]. Diabetes has become the one of the chronic diseases which seriously threat people's health.

Alpha-glucosidase inhibitors can reduce carbohydrate hydrolysis rate in the intestine by inhibiting glycosidase activity, which can reduce and delay the increment in blood sugar [6]. Therefore, its effect in lowering blood glucose is better than other drugs, and it can also effectively inhibit the development of pre-diabetic patients to type II diabetes. Therefore, α -glucosidase inhibitors are currently relatively mature clinical drugs for the treatment of diabetes. At present, α -glucosidase inhibitor drugs mainly include acarbose, voglibose, miglitol and so on [7]. However, these drugs often have some side-effects on the stomach and intestines, and can also cause a certain degree of dependence [8]. Therefore, the development of new, effective, multi-targeted, safe and inexpensive α -glucosidase inhibitors from natural Chinese herbal medicines has become a research hotspot.

Mulberry leaf (*Mori Folium*) contains a variety of chemical components such as flavonoids, alkaloids, polysaccharides and amino acids, so it has a variety of pharmacological effects such as antioxidant,

hypolipidemic, hypoglycemic, antitumor and immune enhancement [9]. In recent years, the development of mulberry leaves and its efficacious components as special drugs or functional foods for treating chronic diseases and freeing people from sub-healthy state has a broad market prospect. A variety of flavonoids isolated from mulberry leaves have been found to have hypoglycemic functions, and can restore liver glycogen from a fragile state to a stable state by controlling the type of flora in the intestine [10-12]. However, the content of active ingredients in plants is generally low, and the enrichment of active ingredients is very important to increase the content of active ingredients and reduce their preparation cost. Microbial fermentation of herbal medicines has a long history, and it has been extensively used for herbal medicines prepared from the ancient Chinese. In recent years, the modernization of herbal medicine has become a hot topic. Nowadays, modern biotechnology is used to monitor the microbial fermentation process of herbal medicines and its parameter changes, which greatly enhance the efficacy of modern fermented herbal medicines, promote the generation of new active substances and reduce their own toxic side effects [13, 14].

In this article, the changes of flavonoid content in mulberry leaves during the fermentation of *Eurotium cristatum* CY-1 and the effect of flavonoids on the inhibitory activity of α -glucosidase were investigated. The purpose of this paper was to enrich flavonoids in mulberry leaves through microbial fermentation and improve their anti-glycaemic efficacy.

2. Materials and Methods

2.1. Materials and Chemicals

Mulberry leaves were purchased from the market of Shangluo (Shanxi, China). 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 purchased from Yuanye Biotechnology Co., LTD (Shanghai, China). Acarbose was purchased from Aladdin Bio-Chem Technology Co., LTD (China, Shanghai).

2.2. Solid-state fermentation of *E. cristatum* CY-1 using mulberry leaves as substrate

The *E. cristatum* CY-1 was self-isolated from black tea. The preparation of *E. cristatum* CY-1 inoculum was conducted as follows: Inoculated *E. cristatum* CY-1 in potato dextrose broth medium and then cultivated at 28-30 °C with 180-200 rpm for 3-5 days.

The Preparation of mulberry leaves medium: Firstly, the fresh mulberry leaves was crushed in a plant crusher. Then, 9 g of crushed mulberry leaves was added with 1 g wheat bran. According to the water content of fresh mulberry leaves distilled water was added to adjust the solid to liquid ratio of the medium to 1:3. The medium was stirred evenly at natural pH and sterilized by autoclave at 121 °C for 20 min. After being cooled, the medium was used for the fermentation of *E. cristatum* CY-1.

The fermentation of *E. cristatum* CY-1 on mulberry leaves medium: Inoculated the *E. cristatum* CY-1 inoculum into the mulberry leaves medium, after being stirred evenly, the medium was incubated at 28~30°C. Samples were taken every other day and stored at 4°C for use. Each experiment was conducted three repetitions.

2.3. Determination of the biomass of *E. cristatum* CY-1

The glucosamine content of the cell wall of *E. cristatum* CY-1 was used to monitor the biomass during the solid state fermentation. The assay procedure was performed as described by Fan et al with a minor modification [15]. The fermented residues were dehydrated at 60 °C (101-1AB, Tester Instrument Co., LTD, Tianjin, China) to a constant weight. Weighed 1 g of the dried fermented residues (accurate to 0.0001 g) and soaked in 10 ml concentrated HCl for 24 h, and then mixed with 40 ml of distilled water. The mixture was hydrolyzed at 121 °C for 2 h. The supernatant was collected by centrifuged at 8000 rpm for 10 min (H2050R-1, Xiangyi Centrifuge Instrument Co., LTD, Shanghai, China) and then fixed to 50 ml with distilled water. Ten milliliter of the supernatant was neutralized with NaOH and fixed to 25 ml. Taken 1 ml of the above glucosamine extract, added with 1 ml of Ehrlich's reagent, and reacted at 90°C for 1 h. After cooling, 6 ml of ethanol were added and incubated at 65 °C for 10 min. Then, the colorimetric analysis of the solution was performed at 530 nm on a UV-Visible Spectrophotometer (754PC, Jinghua Technology Co., LTD, Shanghai, China). The biomass in the fermented residues could

be calculated according to the standard curve between the cell dry weight of *E. cristatum* CY-1 and glucosamine content.

2.4. Extraction of mulberry leaf flavonoids and determination of their content

The extraction and determination of total flavonoids in mulberry leaves were carried out as described by Cao et al [16] with slight modification.

Extraction of flavonoids in mulberry leaves: Taken 2 g (accurate to 0.001 g) of sample to mix with 20 ml of 70% ethanol. Stirred evenly and ultrasonically extracted at 50 °C for 20 min and collected the supernatant by filtration. Extracted the filtered residue again according to the above steps. Then, combined with the supernatant, the total flavonoids extract of mulberry leaves was obtained.

Determination of flavonoid content: Two milliliter of flavonoids extract of mulberry leaves was mixed with 0.3 ml of 5% NaNO₂ solution and reacted at room temperature for 6 min. Then 0.3 ml of 10% Al(NO₃)₃ solution was added and also reacted at room temperature for 6 min. After that, 4 ml of 4% NaOH solution was added and then diluted with 70% ethanol. The mixture was intensively mixed and then stand at room temperature for 15 min. With rutin as the standard and 70% ethanol solution as the blank, the absorbance was measured at 510 nm.

2.5. Analysis of α -glucosidase inhibitory activity

The determining of the α -glucosidase inhibitory efficiency of flavonoids extract from mulberry leaves was conducted by the steps described following [17, 18]. Four experimental groups, Blank group (B), Control group (C), Sample Blank group (SB), and Sample group (S), were set and the conducted in a 96-well microplate. Added phosphate buffer (pH 6.9), mulberry leaf flavonoid extract, α -glucosidase and pNPG according to the volume and order as described in Table 1. The mixture was incubated at 25 °C for 30 min. Then 100 μ L of 1 M Na₂CO₃ solution was added to terminate the reaction. The absorbance was 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 IC₅₀.

Table 1 Measurement and sequence of adding of each reagent

Reagent	Blank group (B)	Control group (C)	Sample Blank group (SB)	Sample group (S)
PBS buffer (μ l)	80	70	60	50
Flavonoid extract from mulberry leaves (μ l)	0	0	20	20
α -glucosidase(μ l)	0	10	0	10
Mix up completely, activate under 37°C for 15 min				
pNPG (μ l)	20	20	20	20
Mix up completely, react under 37°C for 30 min				
Na ₂ CO ₃ (μ l)	100	100	100	100

The inhibitory rate on α -glucosidase activity of mulberry leaves flavonoid extract is calculated with formula.

$$\alpha - \text{glucosidase inhibition rate (\%)} = \frac{(A_C - A_B) - (A_S - A_{SB})}{(A_C - A_B)} \times 100\%$$

Where, AB is the absorbance owing to the auto-decomposition of PNPG; AC is the sum of absorbance owing to the auto-decomposition of PNPG and the decomposition activity of enzyme; ASB is the sum of the absorbance of sample and pNPG auto-decomposition; AS is the sum of absorbance of pNPG auto-decomposition, the decomposition activity of enzyme, the inhibitor, and the absorbance of the sample.

2.6. Data analysis

Experiments were conducted in three replicates and results were presented as the mean \pm SD. The data

was 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 Discussion

3.1. Fermentation process of *E. cristatum* CY-1 with mulberry leaves as substrate

The fermentation process of *E. cristatum* CY-1 when grown on mulberry leaf medium was shown in Fig.1. The results indicated that the cell dry weight of *E. cristatum* CY-1 kept growing in the range of 1~8 d and tend to be stable after the 8 d, which was similar to the results as describe by Yu [19]. The maximum cell dry weight of *E. cristatum* CY-1 (375 mg) was obtained on the 8 d.

As the grown of *E. cristatum* CY-1, the weight loss of mulberry leaf medium increased as well. At the end of fermentation, the maximum weight loss was nearly 45%. It was inferred that the cellulose, hemicellulose and other fermentable substance in mulberry leaves could be degraded by the enzymes secreted by *E. cristatum*. Then, the degraded monosaccharides can be used as carbon source to support the grown and metabolism of *E. cristatum* [20].

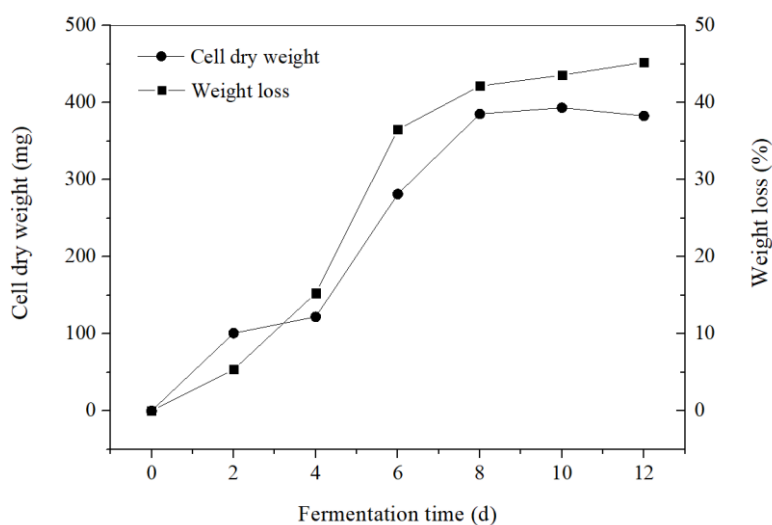


Fig.1 The cell dry weight of *E. cristatum* CY-1 and weight loss of mulberry leaf medium during the fermentation process

3.2. Changes of total flavonoids in mulberry leaves during fermentation

It was found that mulberry leaf flavonoids are the main ingredients for mulberry leaf to exert anti-oxidation, anti-aging, lowering blood lipid and blood pressure [21]. Many researches show that fungal solid-state fermentation can convert flavonoid glycosides into flavonoid aglycones, which is conducive to the absorption and utilization of flavonoids [9]. Therefore, this paper studied the changes of flavonoid content in mulberry leaves fermented with *E. cristatum* CY-1.

The changing process of total flavonoids in the mulberry leaf medium with fermentation of *E. cristatum* CY-1 was shown in the Fig.2. The results indicated that the amount of flavonoids increased slowly at the beginning, but rose rapidly as the fermentation went on. The total amount of flavonoids reached the peak at 8 d (4.736 mg), after then decreased significantly. This results indicated that the fermentation of *E. cristatum* CY-1 could increase the total content of flavonoids in the fermented mulberry leaves. According to the results reported by other researchers, *E. cristatum* could discrete various enzymes, such as cellulase, protease and polyphenol oxidase. These enzymes are able to consume some of the substances in mulberry leaves as nutrition and energy and can also increase the relative concentration of flavonoids in the fermented residues increased [22]. On the other hand, as one of the metabolites of *E. cristatum*, the secreted flavonoid of *E. cristatum* CY-1 might also cause the increase of the final total flavonoid in the fermented residues.

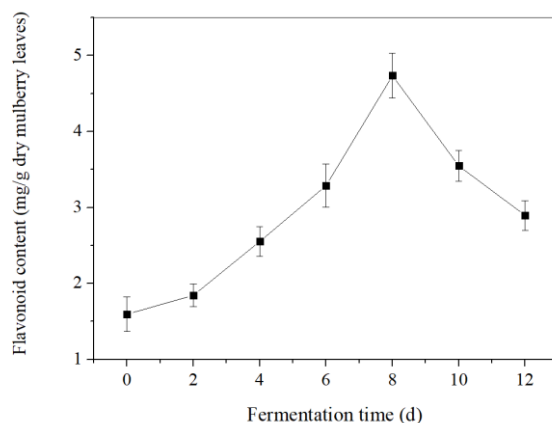


Fig.2 The variation of total content of flavonoids in mulberry leaves during the fermentation of *E. cristatum* CY-1

From the 8d of fermentation, the amount of total flavonoid in the fermented residues was decreased rapidly. At the fermentation of 12 d, the total flavonoids in the fermented residues were 2.896 mg, which was decreased by 35.2% compared with the maximum flavonoids. As reported by Zeng [23], the decrease of total flavonoids might be attributed to the following reasons. The secondary metabolites secreted by *E.cristatum* CY-1 during the fermentation might react with flavonoids and result in the amount declining of flavonoids. Meanwhile, the enzymes produced by *E.cristatum* CY-1 might have the ability to degrade flavonoid.

3.3. Effect of fermentation on the inhibitory activity of mulberry leaf flavonoids

Hyperglycemia can induce diabetes and other chronic complications. Inhibiting the activity of α -glucosidase is a feasible method to reduce the hydrolysis rate of carbohydrate and reduce the concentration of blood glucose [24]. Many studies have confirmed that the hypoglycemic effect of mulberry leaves is closely related to flavonoids, and the flavonoids of mulberry leaves can inhibit the activity of α -glucosidase, thus resulting in a significant hypoglycemic effect [25, 26]. In this paper, figure 3 illustrates the α -glucosidase inhibition of flavonoid from unfermented mulberry leaves and the mulberry leaves after 8 d fermentation. The results indicated that the α -glucosidase inhibition effect of the fermented group was better than the unfermented group. The inhibition rate of the flavonoids obtained after 8 d fermentation at the same concentration was always higher than that of the unfermented group. For example, the α -glucosidase inhibition rate of 0.005 mg/ml unfermented mulberry flavonoids and 0.004 mg/ml 8 d-fermented mulberry leaves were 35.29% and 52.36%, respectively. Therefore, it could be speculated that the fermentation of *E.cristatum* CY-1 would promote the α -glucosidase inhibition effect of mulberry leaves flavonoid. The IC₅₀ values of the 8 d fermented mulberry leaves was 4.14 μ g/mL, while it was 10.26 μ g/mL to unfermented one. In conclusion, the fermentation of *E.cristatum* CY-1 could obviously enhance the α -glucosidase inhibitory efficiency of mulberry leaf flavonoids.

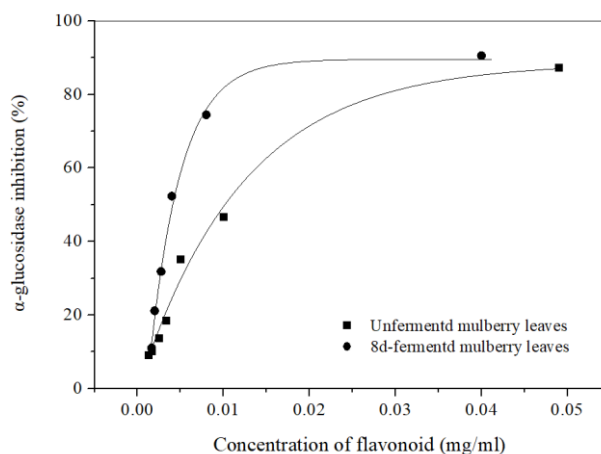


Fig.3 The difference of α -glucosidase inhibition effect of mulberry leaves flavonoid extracted from different mulberry leaves (■ unfermented mulberry leaves; ● 8d-fermented mulberry leaves)

4. Conclusion

The fermentation of *Eurotium cristatum* CY-1 was used to enrich the flavonoid in mulberry leaves, which indicated that after been fermented for 8 d the total flavonoid content was increase to the maximum of 4.736 mg/g dry mulberry leaves. Meanwhile, the α -glucosidase inhibition efficiency of flavonoids extracted from the 8 d fermented mulberry leaves was also enhanced under the same concentration. The IC₅₀ values of the flavonoids extracted from 8 d fermented mulberry leaves and unfermented mulberry leaves were 4.14 μ g/mL and 10.26 μ g/mL, respectively. The results of this paper provide a scientific basis for the subsequent further development of novel drugs for the treatment of diabetes mellitus. In addition, this article will provide a reference for using microbial fermentation to reduce the cost of preparing flavonoids from functional Chinese medicine herbs.

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