

Effects of *Ganoderma lucidum* fermentation on the main chemical components in mulberry leaves and the inhibition of α -glucosidase

Ruiqi Wan^{1,a*}, Tianyi Wu^{2,b}, Yuzhuo Zhang^{3,c}

¹Shihezi University, Shihezi City, Xinjiang Province, China

²Nanjing University of Finance & Economics, Nanjing City, Jiangsu Province, China

³Deqing County No. 1 Middle School, Huzhou City, Zhejiang Province, China

^awrq997400122@163.com, ^b1123959633@qq.com, ^c3442481347@qq.com

*Corresponding author: wrq997400122@163.com

These authors contributed equally to this work

Abstract: Mulberry leaves have been used in the clinical treatment of diabetes since ancient times. A variety of bioactive components in mulberry leaf extract are effective α -glucosidase inhibitors. Though much research has been done on dry mulberry leaves, few have been done on the fresh mulberry leaves. In this study, *Ganoderma lucidum* was used to ferment fresh mulberry leaves, the effects of microbial fermentation on the content of active components and α -glucosidase inhibitory activity in dry and fresh mulberry leaves were measured. The results showed that, compared with dry mulberry leaves, fresh mulberry leaves were more suitable for the growth of *Ganoderma lucidum*. The nutrition needed for the growth of *Ganoderma lucidum* was mainly from the degradation of polysaccharides in fresh mulberry leaves. After fermentation, the polysaccharides content of fresh mulberry leaves decreased from 224.18 mg/g to 22.08 mg/g. The enrichment of bioactive components in fresh mulberry leaves was higher than that in dry mulberry leaves. The contents of flavonoids and alkaloids in fresh mulberry leaves were increased by 34% and 35% respectively compared with nonfermented mulberry leaves. The inhibitory effect of flavonoids and alkaloids on α -glucosidase of fresh mulberry leaves was significantly enhanced by fermentation. In conclusion, the fermentation of fresh mulberry leaves by *Ganoderma lucidum* fungus can not only enrich the content of α -glucosidase inhibitors in mulberry leaves, but also significantly improve their inhibitory activity.

Keywords: Mulberry Leaf ; polysaccharide; alkaloid; flavonoid; α -glucosidase inhibitor

1. Introduction

In recent years, the incidence rate of diabetes has increased rapidly, and it has become the third serious threat to human health after cancer and cardiovascular and cerebrovascular diseases. Diabetes is a series of metabolic disorders caused by genetic and environmental factors leading to absolute or relative insufficiencies of insulin secretion or decreased biological effects of insulin [1]. Diabetes is divided into type I diabetes (insulin dependent) and type II diabetes (non insulin dependent). Traditional hypoglycemic drugs include insulin, biguanides, sulfonylureas, thiazolidinediones, and α -glucosidase inhibitors[2]. Although most traditional hypoglycemic drugs can reduce blood glucose for a short time, they often have some side effects on gastrointestinal function, and may make the body dependent [3]. In contrast, the hypoglycemic substances obtained from natural plants usually have the characteristics of mild and long-lasting effects and small side effects [4].

Mulberry leaf is a traditional Chinese medicine in China. In the field of traditional Chinese medicine, it is used in clinic as a traditional Chinese medicine to control diabetes. Many studies have shown that mulberry leaf contains a variety of hypoglycemic active ingredients such as flavonoids, alkaloids and polysaccharides. The mechanism of hypoglycemic effect is characterized by multi-component, multi-target, multi-channel and multi effect, And its hypoglycemic mechanism may be related to the inhibition of glucosidase[5-8]. α - glucosidase exists in small intestinal mucosa and can decompose disaccharide and polysaccharide into monosaccharide, so α - glucosidase inhibitors can make α - glucosidase degrade carbohydrates more slowly, thus reducing blood glucose by lowering glucose intake [9].

Compared with liquid fermentation, solid-state fermentation technology has the advantages of natural environmental protection, simple post-treatment and less pollution. With the characteristics of mild reaction conditions, good selectivity and wide range of reaction types, microbial biotransformation enzyme system can effectively reduce toxicity, remove impurities, change the content of active ingredients and modify the production of new active ingredients [10]. *Ganoderma lucidum* is a kind of precious medicinal fungus which can strengthen the foundation and nourish the strength. Because it contains *Ganoderma lucidum* polysaccharides, triterpenoids, sterols, alkaloids, amino acids and other active ingredients, *Ganoderma lucidum* has the functions of hypoglycemic, antioxidant, antitumor, hypolipidemic, anti-inflammatory and analgesic, antithrombotic, anti arteriosclerosis and so on [11]. In recent years, *Ganoderma lucidum* has also been used for the fermentation of *Panax notoginseng* residue, licorice residue and other traditional Chinese medicine residues to enrich the active ingredients. However, in the research of mulberry leaves, most of them were transformed by *Eurotium cristatum* and *Bacillus*, and there was no report about the transformation of mulberry leaves by *Ganoderma lucidum*. At present, the research on mulberry leaves is based on dried mulberry leaves, and there is no research on the changes of active components and pharmacodynamics of fresh mulberry leaves before and after fermentation. This is due to the lack of understanding of the material structure and chemical composition of fresh mulberry leaves, and the high cost of cultivation, transportation and storage of fresh mulberry leaves in terms of current science and technology. Also the application method of fresh medicine and the preservation technology of medicinal materials are backward, and it is easy to deteriorate and fail.

In this paper, *Ganoderma lucidum* was used to produce dry and fresh mulberry leaves by solid-state fermentation. Firstly, the growth of *Ganoderma lucidum* on the culture medium of dry and fresh mulberry leaves was studied, and then the content of active components of α -glucosidase inhibitors of extract of mulberry leaves fermented by *Ganoderma lucidum* was investigated. Finally, the effect of *Ganoderma lucidum* fermentation on the active components of mulberry leaves—the inhibition rate of α -glucosidase was also studied.

2. Materials and methods

2.1 Drug screening

Ganoderma lucidum was purchased from China Center of Industrial Culture Collection(No. 14029); Mulberry leaves were purchased in Kuqa region, Xinjiang province; Corn flour and bran (Henan Feitian Agricultural Development Co.Ltd); Rutin standard、DNJ standard、pNPG standard、glucosamine standard solution, acarbose (Yuan Ye Biotechnology Co., Ltd.)、ultrapure water(self-made) and other reagents are analytically pure.

2.2 Solid state fermentation of mulberry leaves

Weigh 10 g fresh mulberry leaves, add corn flour and bran, then add distilled water, and adjust the solid-liquid ratio of medium to 1:3, stir evenly under natural pH and sterilize at 121°C with high pressure steam sterilizer to sterilize for 20min. After cooling, inoculate 10 ml of *Ganoderma lucidum* seed solution to make the final solid-liquid ratio of the medium 1:4. After inoculation, the mice were placed at 30°C. Uninoculated microorganism was used as control group, respectively by day : 0d; 2d; 4d; 6d; 8d, Samples were taken to measure various indexes(including biomass, total flavonoids, total polysaccharides, total alkaloids), and three parallel time points were set at each time point.

2.3 Determination of biomass

The biomass of solid-state fermentation was determined by glucosamine method. After fermentation, the culture was at 60°C. After drying, weigh about 1g (accurate to 0.0001 g), soak in 10 ml concentrated HCl for 24h, then add 40 ml distilled water, and mix it in 121°C. steam for 2 hours, cool and filter, take the supernatant, fix the volume to 50 ml, take 10 ml, neutralize with NaOH, and fix the volume to 25 ml; Take 1 ml of the above glucosamine extract, add 1 ml of acetylacetone reagent, and keep warm at 90°C. After cooling, 6 ml ethanol and 1 ml rhlich reagent were added,and preserve heat at 65°C. After cooling, the color was measured at 530 nm. Then, the standard curve of *Ganoderma lucidum* biomass and glucosamine content ($y = 0.0015x - 0.001$, $r^2 = 0.9919$) to calculate the biomass of bacteria in mulberry leaf fermentation [12].

2.4 Determination of flavonoids

Accurately weigh 4.0 g of mulberry leaves to be tested and put them into a 50 ml centrifuge tube, add 40 ml of 70% ethanol solution, 50°C water bath ultrasonic extraction for 40min, filter, collect the supernatant and sediment, and then repeat the above steps to extract once again, after being combined with supernatant, the sample extract was obtained. Accurately measure 2 ml of mulberry leaf extract, put it into 10 ml volumetric flask, add 0.3 ml of 5% sodium nitrite solution, shake well, and stand for 6 min; Add 0.3 ml of 10% aluminum nitrate solution, shake well, stand for 6min, then add 4 ml of 4% sodium hydroxide solution, dilute with 60% ethanol to constant volume scale, shake well, stand for 15min, take 60% ethanol solution as blank, and measure the absorbance at 510 nm. Then, rutin was used as the standard substance to determine the standard curve of flavonoids ($y = 13.9x - 0.0577$, $r^2 = 0.9913$), and the content of total flavonoids in mulberry leaf extract was calculated according to the standard curve [13].

2.5 Determination of polysaccharide content

Accurately weigh 4.0 g mulberry leaves to be tested and put them into a 50 ml centrifuge tube, add 30 ml distilled water, mix well and water bath ultrasound 20min at 80°C, filter, collect supernatant and sediment and then repeat the above steps to extract once again, after being combined with supernatant, the sample extract was obtained. 4 ml of mulberry leaf sample solution was precisely removed, put into a triangular conical flask, add 2 ml of 6 mol/l HCl solution (currently prepared), seal it, heat it on a boiling water bath for 30min, take it out and cool it to room temperature, adjust the pH value to 8.0 with 6 mol/l NaOH solution, fix the volume to 20 ml with distilled water, and shake well to obtain mulberry leaf polysaccharide hydrolysate sample. Precisely take 1.0 ml of the above mulberry leaf polysaccharide hydrolysate sample, put it into a tube with plug, add water to 2.0 ml, add 1.5 ml DNS reagent, shake it well, heat it in boiling water bath for 5min, take it out and cool it quickly, then use 2.0 ml distilled water as blank, and measure the absorbance at 550 nm with ultraviolet visible spectrophotometer. Then the standard curve was established with glucose standard solution ($y = 0.7251x - 0.0326$, $r^2 = 0.9956$), and the polysaccharide content in mulberry leaf extract was calculated according to the standard curve [14].

2.6 Determination of alkaloid content

Alkaloids were determined by the method of rheostatin colorimetry. 5 g of mulberry leaves to be measured were accurately weighed and added into 50 ml centrifugal tube, and 50 ml 25% ethanol and 0.05 mol/l hydrochloric acid solution were added. After mixing, keep warm at 30°C by ultrasonic wave in water bath for 20 minutes, and the supernatant was collected by suction filtration. The sample extraction solution was concentrated and evaporated by rotary evaporation instrument, dissolved with 0.05mol/l hydrochloric acid and was fixed to 4 ml. The total alkaloid of mulberry leaves was obtained. 2 ml of the solution to be measured was centrifuged for 10 minutes, the supernatant was taken into 10 ml centrifuge tube, and 0.02 g/ml of leischner salt solution 3 ml was added for 2 hours. Centrifugate for 10 minutes at 8000 rpm/min, skimming the supernatant, adding ethyl acetate after ice bath in the centrifugal tube, centrifuging for 10 minutes at 8000rpm/min, keeping precipitation, dissolving with 10ml 70% acetone solution, and measuring the absorbance value of the solution at 520 nm. The absorbance was determined at 520 nm with 4-hydroxypiperidinol standard. Draw the standard curve ($y = 22.4555x - 0.0196$, $r^2 = 0.9973$). The total alkaloid content in mulberry leaf extract was calculated according to the standard curve [15].

2.7 Analysis of inhibition rate of α -glucosidase inhibitors

The experiment was carried out on 96 well plate. There were four groups in the experiment: blank group (substrate), control group (substrate+enzyme solution), sample blank group (substrate+sample), sample group (substrate + sample + enzyme solution). First of all, respectively add 80 μ L (blank group, B), 70 μ L (control group C), 60 μ L (sample blank group, SB), 80 μ L (sample group, S) into 96 well plate. PBS solution with pH 6.8; Then, 20 μ L tested inhibitor (the sample is each extract, and the control substance is acarbose, DNJ and rutin) were added to the sample group and the sample blank group (SB); Then 10 μ L 1 U/ml α -glucosidase were added to the sample group (S) and the control group (B); Mix well, 37°C oven activation for 15min; Add another 20 μ L 10 mmol/l pNPG; After mixing and 37°C oven reaction for 30min; Finally, add 100 μ L 1mol / l Na₂CO₃ solution and determine at 405 nm.

Inhibitors for the inhibition rate of α -glucosidase were calculated according to the following formula

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

3. Results and analysis

3.1 Determination of biomass

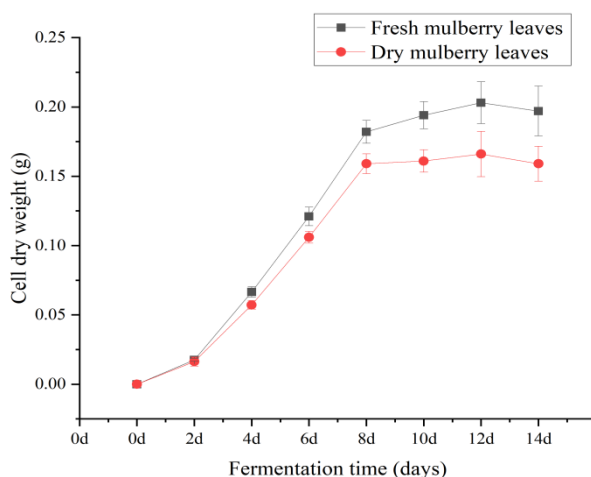


Figure. 1 Growth curve of *Ganoderma lucidum* fermentation on dry and fresh mulberry leaves

According to figure 1, *Ganoderma lucidum* grew rapidly on the 0-8 days of fermentation. On the 8th day, the dry weight of the bacteria was 0.182 g on the fresh mulberry leaves medium and 0.159 g on the dry mulberry leaves; From the 8th day to the 12th day, the growth rate was gradually flat and stable, and reached the peak on the 12th day. The biomass of *Ganoderma lucidum* on fresh mulberry leaves was 1.22 times that of dry mulberry leaves (0.203 g of fresh mulberry leaves and 0.166 g of dry mulberry leaves); The biomass of *Ganoderma lucidum* began to decrease after the 12th day. The biomass of *Ganoderma lucidum* cultivated on fresh mulberry leaves was not significant difference from that of *Ganoderma lucidum* on the 0-2 days; From the second day of fermentation, the biomass and growth rate of *Ganoderma lucidum* cultivated on fresh mulberry leaves were higher than that of *Ganoderma lucidum* cultivated on dry mulberry leaves. After the 12th day, the biomass of both decreased to the same trend.

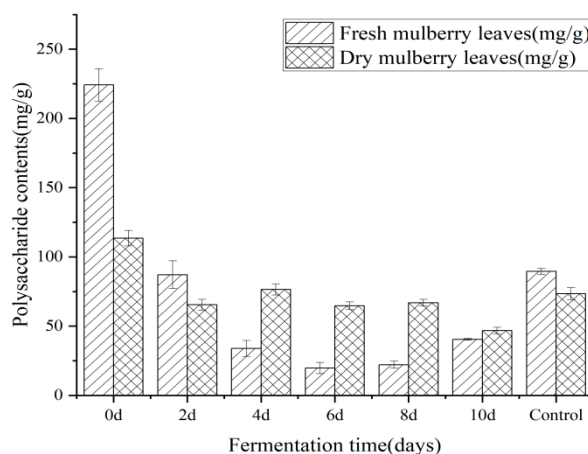


Figure. 2 polysaccharide content of *Ganoderma lucidum* fermented on dry and fresh mulberry leaves

3.2 Determination of polysaccharide content

It can be seen from Fig. 2 that with the fermentation of mulberry leaves by *Ganoderma lucidum*, the content of polysaccharide generally presents a downward trend. On day 0, the polysaccharide content in fresh mulberry leaf medium (224.18 mg/g) was much higher than that in dry mulberry leaf medium (113.48 mg/g), but it decreased to 22.08 mg/g on day 10, while the polysaccharide content in dry mulberry leaf medium decreased to 46.76 mg/g. Maybe with the passage of time, polysaccharides were decomposed by microorganisms, which reduced the content of polysaccharides, and microorganisms may be easier to use polysaccharides in fresh mulberry leaves.

3.3 Determination of alkaloid content

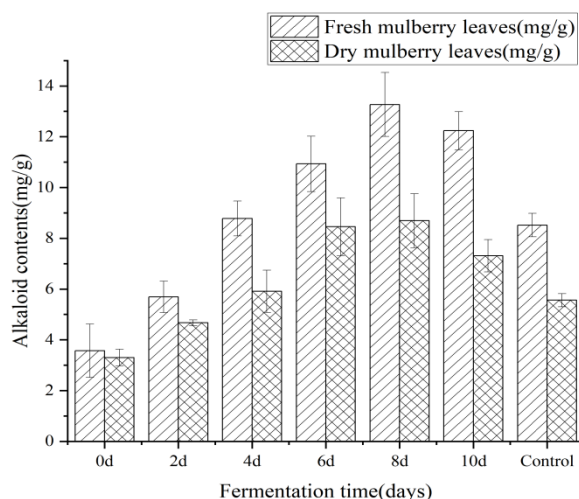


Figure. 3 alkaloid content of *Ganoderma lucidum* fermented on dry and fresh mulberry leaves

It can be seen from Fig. 3 that the alkaloid content in mulberry leaves increases first and then decreases with the increase of fermentation time. The alkaloid content in the fresh mulberry leaves medium was higher than that in the dry mulberry leaves medium. On the 8th day of fermentation, the alkaloid content reached the maximum, fresh mulberry leaves medium (13.27 mg/g), dry mulberry leaf medium (8.69 mg/g).

3.4 Determination of flavonoids

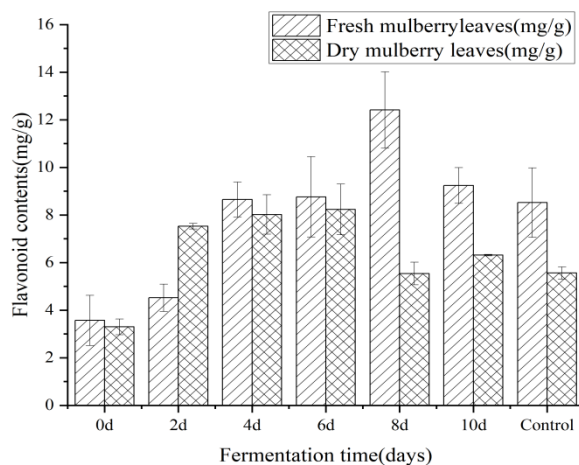


Figure. 4 The flavonoid content of *Ganoderma lucidum* fermented on dry and fresh mulberry leaves

It can be seen from Fig. 4 that with the fermentation time, the flavonoid content in mulberry leaves

increased first and then decreased. The content of flavonoids in fresh mulberry leaves reached the maximum on the 8th day of fermentation (12.4 mg/g), and that in dry mulberry leaves reached the maximum on the 6th day (8.23 mg/g). Among them, the content of flavonoids in fresh mulberry leaf culture medium was generally higher than that in dry mulberry leaf culture medium, but it took less time for the content of flavonoids in dry mulberry leaf culture medium to reach the maximum value.

3.5 The effect of α -glucosidase activity inhibited with active components in mulberry leaves by *Ganoderma lucidum* fermentation

There was a negative correlation between fermentation time and polysaccharide content in mulberry leaf medium, and a positive correlation between fermentation time and flavonoids and alkaloids content at first, and then a negative correlation. Then, 5 $\mu\text{g/ml}$ flavonoids and alkaloids were prepared, and the effects of alkaloids and flavonoids by different fermentation time on the inhibition efficiency of α -glucosidase.

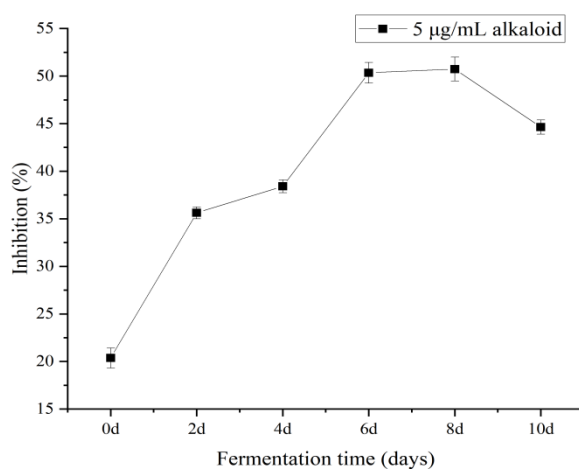


Figure. 5 Inhibition efficiencies of alkaloid (5 $\mu\text{g/ml}$) prepared by different fermentation time on α -glucosidase

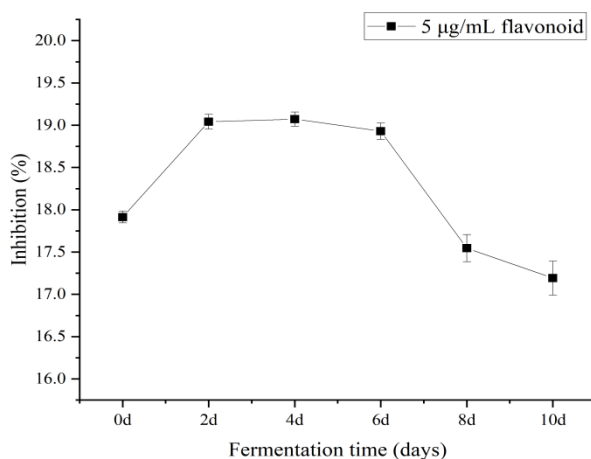


Figure. 6 Inhibition efficiencies of flavonoid (5 $\mu\text{g/ml}$) prepared by different fermentation time on α -glucosidase

The alkaloids (5 $\mu\text{g/ml}$) and flavonoids (5 $\mu\text{g/ml}$) with the same concentration and different fermentation time were used to investigate the effects of fermentation time on the the inhibition rate of α -glucosidase of alkaloids and flavonoids . The results showed that with the increase of fermentation time, the content of alkaloids increased α - glucosidase inhibition first increased and then decreased. The effect of the alkaloids on the fermentation was studied on day 0-6, and the inhibition rate of α -

glucosidase increased rapidly and reached the maximum value of 50.732% on the 8th day, and then decreased. It can be seen from Fig. 6 that the effect of fermentation time on flavonoids in mulberry leaves. The inhibition rate of α -glucosidase increased slowly at first and then decreased with the increase of fermentation time. The content of flavonoids (5 ug/ml) in mulberry leaves fermented by *Ganoderma lucidum* for 4 days was determined the highest inhibition rate of α -glucosidase was 19.072%. Therefore, the effect of *Ganoderma lucidum* fermentation on alkaloids and flavonoids in mulberry leaves was obvious that the inhibition rate of α -glucosidase was promoted by fermentation time.

4. Conclusion

The biomass changes of fresh and dry mulberry leaves, the content of polysaccharides, flavonoids, alkaloids and other active substances were measured in different fermentation time. It was found that the fresh mulberry leaves medium was more suitable for the growth of *Ganoderma lucidum* than that of dry mulberry leaves. The content of polysaccharide, flavonoid and alkaloid in the culture medium of fresh mulberry leaves is higher than that of the dry mulberry leaves medium. The content of polysaccharide in the culture medium of fresh mulberry leaves is 1.97 times that of the dry mulberry leaf medium, the flavonoid content is 1.51 times of that of the dry mulberry leaf medium, and the alkaloid content is 1.53 times of that of the dry mulberry leaf medium. With the fermentation, the content of polysaccharide in fresh and dry mulberry leaves decreased with the growth and metabolism of microorganisms, but the content of flavonoids and alkaloids both rose first and then decreased. It may be because polysaccharide is used as carbon source of microorganism, which makes the content of flavonoids and alkaloids enriched. The alkaloids and flavonoids in mulberry leaves by *Ganoderma lucidum* fermentation and the inhibition rate of α -glucosidase was promoted, and alkaloids were used to promote the inhibition of α -glucosidase, and the effect of glucosidase was greatly influenced by fermentation time.

Through the research of this paper, it is indicated that the material transformation and enzyme catalysis in the process of microbial growth and metabolism are helpful to realize the transformation of mulberry leaves to enrich α -glucosidase inhibitors and make their inhibition efficient. This will help to broaden the application prospects of mulberry leaf in clinical trials and laboratory research of traditional Chinese medicine, research and development of functional food, prevention and treatment of diabetes, hyperglycemia and hyperlipidemia.

References

- [1] WANG L. *Isolation, purification and hypoglycemic activity of polysaccharides from Rosa roxburghii Tratt fruit and their effect on gut microflora.* [D]. Guangzhou: South China University of Technology, 2019.
- [2] ZHU Fangying, DONG Zhengwei, ZHU Wenyuan, LIU Zhiqiang. *Advances in anti-diabetes drugs and their synthesis.*[J].*Bulletin of Fermentation Science and Technology*, 2016, 45(03):175-181.
- [3] Eichler H G et al. *The effect of a new specific alpha-amylase inhibitor on post-prandial glucose and insulin excursions in normal subjects and Type 2 (non-insulin-dependent) diabetic patients.*[J]. *Diabetologia*, 1984, 26(4) : 278-81.
- [4] JI Tao, SU Shu-lan, GUO Sheng, OUYANG Zhen, QIAN Da-wei, TANG Yu-ping, DUAN Jin-ao. *Research progress on bioactive component groups and their action mechanisms of Mori Folium for prevention and treatment of diabetes.*[J]. *Chinese Traditional and Herbal Drugs*, 2015, 46(5): 778-784.
- [5] Kenichi Tanabe et al. *Repeated ingestion of the leaf extract from Morus alba reduces insulin resistance in KK-Ay mice*[J]. *Nutrition Research*, 2011, 31(11) : 848-854.
- [6] Werner A Scherbaum et al. *Cost-effectiveness of pioglitazone in type 2 diabetes patients with a history of macrovascular disease: a German perspective*[J]. *Cost Effectiveness and Resource Allocation*, 2009, 7(1) : 708-714.
- [7] Nakagawa K. *Studies targeting α -glucosidase inhibition, antiangiogenic effects, and lipid modification regulation: background, evaluation, and challenges in the development of food ingredients for therapeutic purposes*[J]. *Biosci Biotechnol Biochem*, 2013, 77(5):900.
- [8] Yuan A H , Ma J , Jiang X F , et al. *Screening of glucosidase inhibitors from various fractions of Mulberry leaves.*[J]. *China Journal of Chinese Materia Medica*, 2006 (03):223-227.
- [9] Patel S S. *Cerebrovascular Complications of Diabetes: Alpha Glucosidase Inhibitor as Potential*

Therapy.[J]. Hormone and metabolic research = Hormon- und Stoffwechselforschung = Hormones et métabolisme, 2016, 48(2) : 83-91.

[10] GUAN Song-Lei, WU Ya-Xin, SUN-He, ZHANG Ya-Chao, ZHAO Rong-Qi, WANG Hong , SUN Li-Jing, SU Ling. Application of microbial transformation technology in the development of Chinese medicine.[J]. *Microbiology China, 2018, 45(4): 900-906.*

[11] ZHANG Rui-ting, ZHOU Tao, SONG Xiao-xiao et al. Research Progress on Active Ingredients and Pharmacological Action of *Ganoderma lucidum*. [J]. *J. Anhui Agric. Sci, 2018, 46(3) : 18-19+22.*

[12] Lu Xiuling Zhao Shuxin Liu Zhonghua. Biomass Estimation of *Monascus* in Solid-state Fermentation.[J].*Food and Fermentation Industries,2001(06):45-49.*

[13] Pian Zhang. Study on extraction and purification of flavonoids from mulberry leaves.[D]. Huazhong University, 2019.

[14] ZHANG Xiaobei, ZHU Guopeng, ZHU Zhixin, NAN Wenzhuo, WU Zhipeng. Determination of the Polysaccharide Content of Sweet Potato Leave by Using 3'5'-Dinitrosalicylic Acid (DNS).[J]. *Journal of Tropical Biology, 2017,8(03):359-363+377.*

[15] Pu Junsong. Determination, Extraction and Concentration Analysis of Alkaloid in Mulberry Leaves.[D]. Southwest University, 2016.